Living on a knife-edge

Unravelling harbour porpoise health through multidisciplinary and cross-border approaches

Lonneke Liza IJsseldijk

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Op het scherpst van de snede

De gezondheid van bruinvissen ontrafelen door multidisciplinaire en grensoverschrijdende benaderingen
(met een samenvatting in het Nederlands)

Proefschrift

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Promotoren:

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"You can never cross the ocean unless you have the courage to lose sight of the shore"

~ *Christopher Columbus* ~

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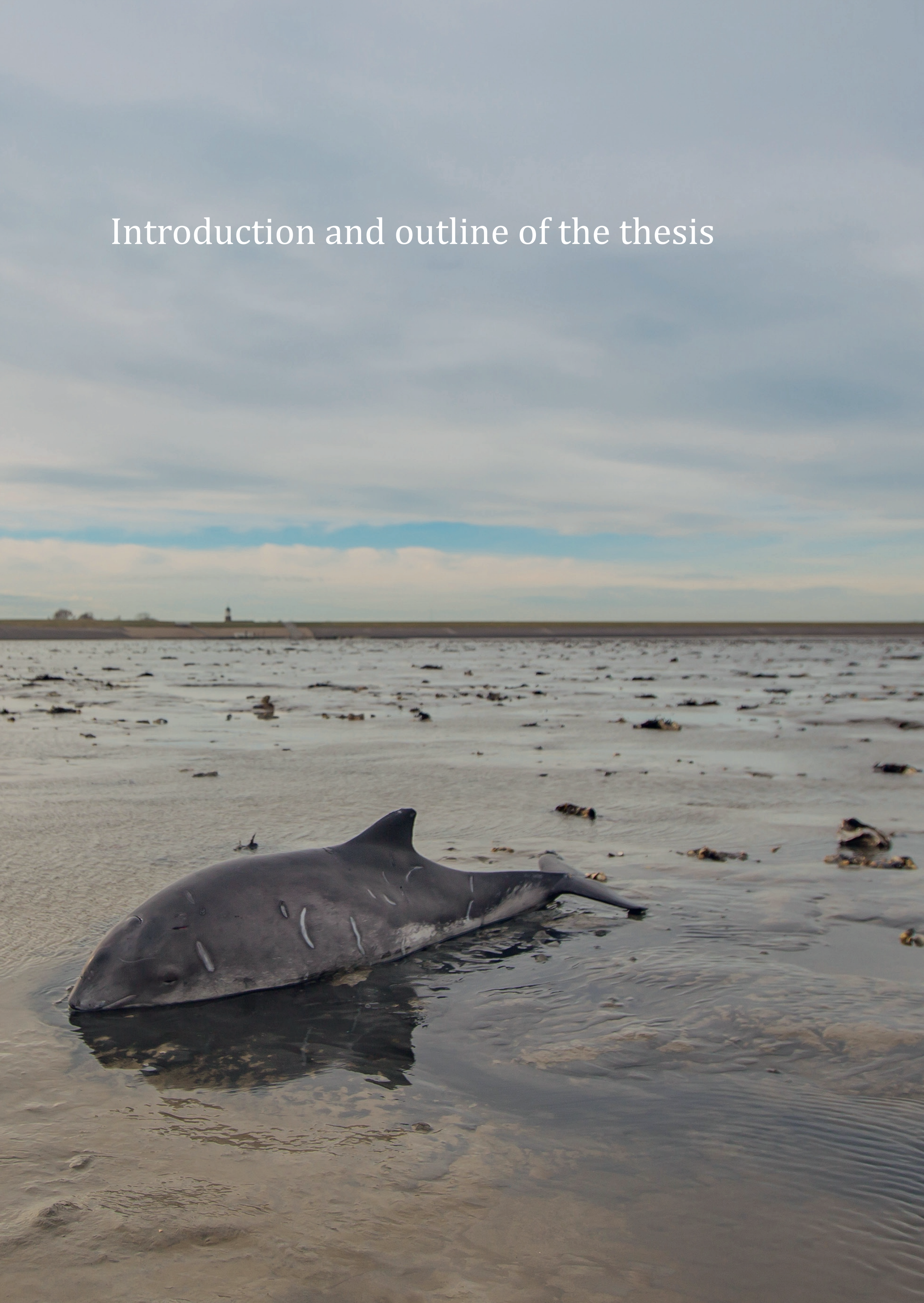
Introduction and outline of the thesis



Introduction

Harbour porpoises (*Phocoena phocoena*) are charismatic and protected animals, that elicit a broad societal, scientific and political interest into their health and wellbeing. They are an integral component of marine food webs and together with their wide-ranging occurrence (Bjørge & Tolley 2018; Hammond et al. 2008) they are key elements in many environmental monitoring programmes (ASCOBANS 1992; Camphuysen & Siemensma 2011; Evans 2019; Evans & Hammond 2004; LNV 2020). These programmes generally aim to increase knowledge on human-related activities impacting the environment, and ultimately to protect and conserve species and habitats.

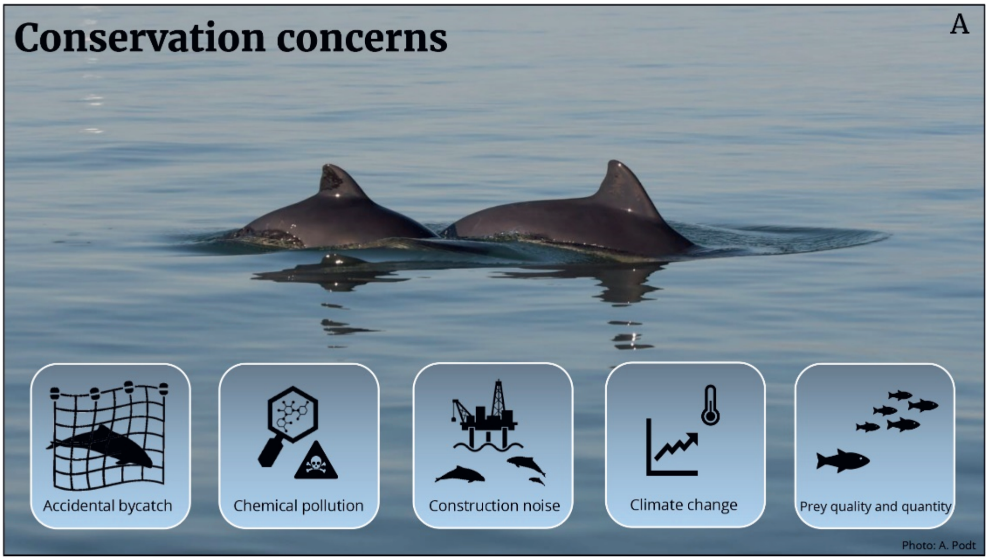
Harbour porpoises are relatively abundant, and their habitat includes both offshore and nearshore waters of the Northern Hemisphere. They are one of the most frequently sighted marine mammals in the North Atlantic Ocean and the adjacent shelf seas (Figure 1A) (Evans & Hammond 2004; Hammond et al. 2017; 2018; Nielsen et al. 2018). Given their large global population size, which is likely well over a million individuals, the harbour porpoise is currently listed as 'least concern' by the International Union for Conservation of Nature (Braulik et al. 2020). Nevertheless, for most geographical areas, population trends are unclear and there are numerous threats posing conservation concerns (Hammond et al. 2008). These concerns result from the frequent and emerging exposure of marine ecosystems and species to human activities (Aguirre & Tabor 2004; Evans & Hammond 2004; Halpern et al. 2008; Tyne et al. 2016; Wisniewska et al. 2018).

The North Sea is one of the regions in the world with the highest level of anthropogenic activities, and co-occurring anthropogenic stressors (Halpern et al. 2008, 2015; Nachtsheim et al. 2021). Harbour porpoises are the most abundant cetacean in this region, with numbers in the Greater North Sea estimated at around 350,000 individuals (Hammond et al. 2002, 2013, 2017). Porpoises are impacted by fishery activities through accidental bycatch, with underwater entanglement hampering them from air breathing, and competition for food (Dolman et al. 2016; Kirkwood et al. 1997; Leeney et al. 2008). Also, chemical pollution from persistent organic pollutants (Jepson et al. 2016; Pierce et al. 2008; Weijs et al. 2009), and underwater noise pollution from shipping (Wisniewska et al. 2018), seismic surveys and unexploded ordnance detonations (Aarts et al. 2016; von Benda-Beckmann et al. 2015). More recent concerns and emerging threats include habitat loss due to the rapid growth of offshore activities related to the construction of wind farms (Gilles et al. 2009; Madsen et al. 2006; Teilmann & Carstensen 2012) and the broadscale effects of climate change, including habitat degradation and changes in food quality and quantity (see infographic A) (Burge et al. 2014; Simmonds & Isaac 2007). These single and cumulative stressors threaten the direct survival of individual porpoises but may also induce nonlethal effects impacting population viability (Aguirre & Tabor 2004; Evans & Hammond 2004; Halpern et al. 2008; Tyne et al. 2016; Wisniewska et al. 2018).

Figure 1. *The harbour porpoise: sightings (A) and strandings (B) nowadays frequently occur in the North Atlantic Ocean and adjacent shelf seas, including the North Sea.*

Large at-sea sighting numbers correspond to a high stranding frequency in North Sea bordering countries, including The Netherlands (Figure 1B) (Camphuysen 2004, 2011; Camphuysen & Peet 2006; Camphuysen & Smeenk 2008; Keijl et al. 2016). Due to the statutory requirements of international legislation, stranding networks that record harbour porpoises and other cetaceans found ashore have been implemented decades ago, and post-mortem programmes in order to sample stranded animals and monitor their causes of mortality and health status were initiated (Camphuysen & Siemensma 2011; Peltier et al. 2013; ten Doeschate et al. 2018).

A successful monitoring program should detect changes in population parameters, which can be used to define and assess the conservation objectives. Using population indices or indicators, particularly those that can inform on population health or even wider environmental health, are increasingly supported and included in the implementation of legislation (Bossart 2011; Carignan & Villard 2002; Moore 2008; Peltier et al. 2012, 2014). Yet, the available knowledge on threats and pressures and their effects on harbour porpoises is far from complete. Most research and management efforts are organised at national levels. Cross-border as well as multidisciplinary approaches would, however, allow a more justifiable assessment of health status and threats, both at individual and population levels and at a scale relevant to the ecosystem. This is where this thesis comes in. The primary **research aims** were to establish methods and metrics of health which aid in the assessment of the most relevant natural and anthropogenic threats to harbour porpoises in the North Sea overtime. This thesis comprises eight studies, addressing fundamental population biology and demography, as well as targeting specific threats to the harbour porpoise. All chapters combine multiple disciplines, including biology, ecology, toxicology, epidemiology and pathology, aimed at unravelling individual, population and ecosystem health for this charismatic marine mammal.



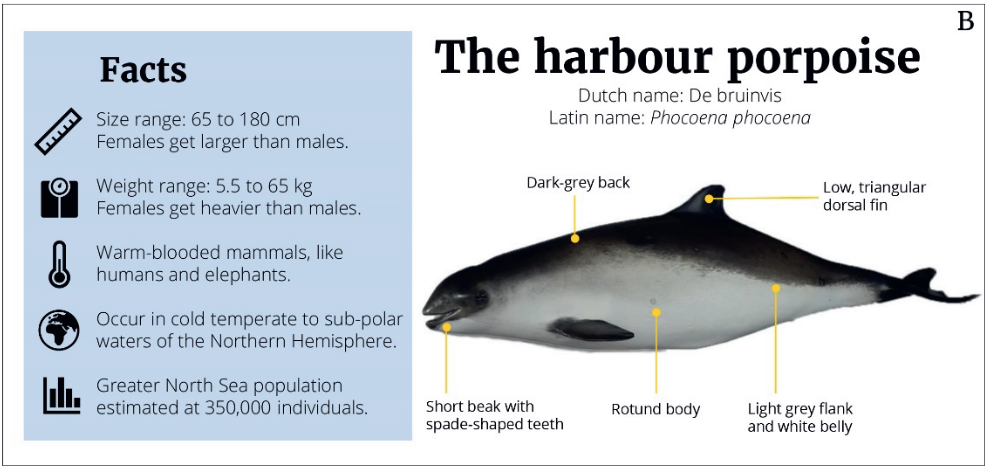
Introducing the harbour porpoise

Porpoises are among the smallest of the toothed-whales, or odontocetes. Along with dolphins and the large whales, they form the order of *Cetacea* (Bjørge & Tolley 2018; Read 1999). Of the six species of true porpoises (*Phocoenidae*), the harbour porpoise is by far the most abundant (Bjørge & Tolley 2018). Harbour porpoises have rounded heads and rotund bodies, with a characteristic low, triangular dorsal fin which makes them easily recognizable at sea (Bjørge & Tolley 2018; Camphuysen & Peet 2006). Females are slightly larger than males, with maximum lengths of around 1.8 meters, weighing approximately 65 kg. The smaller males may reach body lengths up to 1.65 meters and a body mass of 50 kg at most (Lockyer 2003; see infographic B).

Maximum lifespan of harbour porpoises is circa 24 years. Females reach sexual maturity at 4 to 5 years of age; males slightly earlier, at 3 to 4 years (Addink et al. 1995a; Lockyer 2003). Porpoises are income breeders who can produce one large offspring a year when conditions are favourable, with gestation lasting 10-11 months and size at birth between 60-75 cm (Addink et al. 1995a,b; Lockyer 2003; Sørensen & Kinze 1994). Neonates drink milk for at least several months (Lockyer 2003) and start taking solid food while still nursing, with their initial prey being small, in the south-eastern North Sea mostly gobies (Leopold 2015). Juvenile and adult porpoises predate upon numerous schooling fish species, although small squid and crustaceans are also on their menu (Leopold 2015). Harbour porpoises are believed to be opportunistic predators, with limited capacity to store energy. They are therefore dependent on continuous foraging (Kastelein et al. 1997; Koopman et al. 2002; Leopold 2015; Lockyer

2007). They need up to 10% of their own body mass in prey per day to support their metabolic requirements and this makes them live on an energetic knife-edge (Kastelein et al. 1997; Lockyer 2007; Wisniewska et al. 2018).

Like all cetaceans, porpoises are mammals that are fully adapted to life in water and evolved with numerous adaptations equipping them for survival in this environment. Among these adaptations is the well-developed auditory organ which is the basis of their echolocation system (Cozzi et al. 2016). Echolocation in toothed whales is used for the detection of prey and predators, orientation, and communication and is, therefore, their primary sense (Mooney et al. 2012; Morell et al. 2015). Hearing is thus key for survival underwater, making porpoises and other cetaceans highly vulnerable to man-made noise in the marine environment (Morell et al. 2020; von Benda-Beckmann et al. 2015; Wisniewska et al. 2018).



Outline of this thesis

To adequately assess the health status of harbour porpoise populations, the first study aim was to identify current knowledge gaps, predict pressures and threats, and define conservation indicators to facilitate future research (**chapter 2**). This resulted in an extensive review of recent formal and informal knowledge, using the Delphi method, from experts working on harbour porpoises in a range of disciplines. The results of this study provided the themes for the subsequent research chapters of this thesis. A large-scale and international research effort followed, investigating spatiotemporal patterns in population dynamics of harbour porpoises across the North Sea. Data from national stranding networks of five countries bordering the North Sea were collated leading to a dataset of more than 16,000 records, covering 28 years (**chapter 3**). From this, it became apparent that the annual incidence of strandings, which increased throughout the region since 1990, was most notable and steepest in the southern North Sea. This highlights the need to focus research specifically on this region.

Bycatch was judged to be the largest concern for porpoises in the North Sea (**chapter 2**), and for this reason became the focus of the next study (**chapter 4**). There are uncertainties when it comes to the assessment of bycatch in stranded animals. To address this knowledge gap, a literature review was conducted to collect criteria used in the assessment of bycatch in small cetaceans and I subsequently tested which of these criteria applied to harbour porpoises retrieved from gillnets in The Netherlands, i.e., certain bycatch cases. Bycatch-specific and a-specific post-mortem findings were evaluated, which could later be used to assess bycatch among stranded harbour porpoises with greater certainty. The next study (**chapter 5**) focused on chemical pollution, specifically the generational transfer of contaminants, which was additionally considered one of the most important threats affecting porpoises (**chapter 2**). In a large body of literature, reproductive impairment in small cetaceans is put forward as predominantly driven by polychlorinated biphenyls (PCBs) (Jepson et al. 2016; Law et al. 2010; Murphy et al. 2009, 2015; Pierce et al. 2008; Reijnders et al. 2018). Chemical pollution from PCBs was indeed apparent at large in the porpoises stranded in the southern North Sea. However, we observed reproduction failure only in a minority of adult females. This led to a study of harbour porpoise reproduction, focusing on life history characteristics such as age at sexual maturity, pregnancy rates and foetal growth (**chapter 6**). It was investigated whether reproductively active females abandon investment in their foetus when they are in poor physical condition or when they experience environmental harshness, using data on disease, diet, fat reserves and reproductive status obtained from necropsies. In order to place the life history characteristics of porpoises in the southern North Sea in a wider context, we compared these to pregnancy rates and age at sexual maturity reported for porpoises in other geographical areas across their distributional range. This was correlated to parameters reflecting environmental condition: local prey quality, cumulative human impact and chemical contamination by PCBs, in order to assess the relative importance of these pressure and their associations with reproduction rates. Local prey quality was found to govern reproductive success of harbour porpoises.

In addition to anthropogenic threats, also natural pressures may affect porpoise health and survival. In the North Sea, the abundant grey seal (*Halichoerus grypus*) predates upon harbour porpoises (Leopold et al. 2015a). Through forensic microbiological approaches, we show that grey seals also cause chronic and eventually fatal infections. Bite lesions from failed predator attempts were shown to lead to fatal disseminated infections with the zoonotic bacteria *Neisseria animaloris* (**chapter 7**). This study helped in recognising harbour porpoises that initially survive predator attacks but later succumb as a result of the induced trauma or due to infections. This information was needed to further assess the prevalence of acute as well as non-acute grey seal-related mortality for harbour porpoises (**chapters 8 and 9**). Anthropogenic and other causes of death assessed in fresh to moderately fresh stranded harbour porpoises from 2008-2019 were described and analysed (**chapter 8**), with a special focus on human-related threats, such as accidental bycatch in fisheries (with help of the results from **chapter 4**), hearing loss due to anthropogenic activities and marine debris ingestion. An analysis of age- and sex-specific causes of mortality and spatiotemporal trends of causes of death provided estimates of the occurrence of anthropogenic and natural threats overtime. Finally, all biological, ecological and pathological parameters collected as part of the post-mortem investigations were categorised and subsequently analysed using different methods of supervised and unsupervised classification (**chapter 9**). This final chapter serves as a detailed example demonstrating the potential and usefulness of different analytical approaches to health and pathology data of a long-studied small cetacean species, translatable to different taxa, disciplines and geographical areas.

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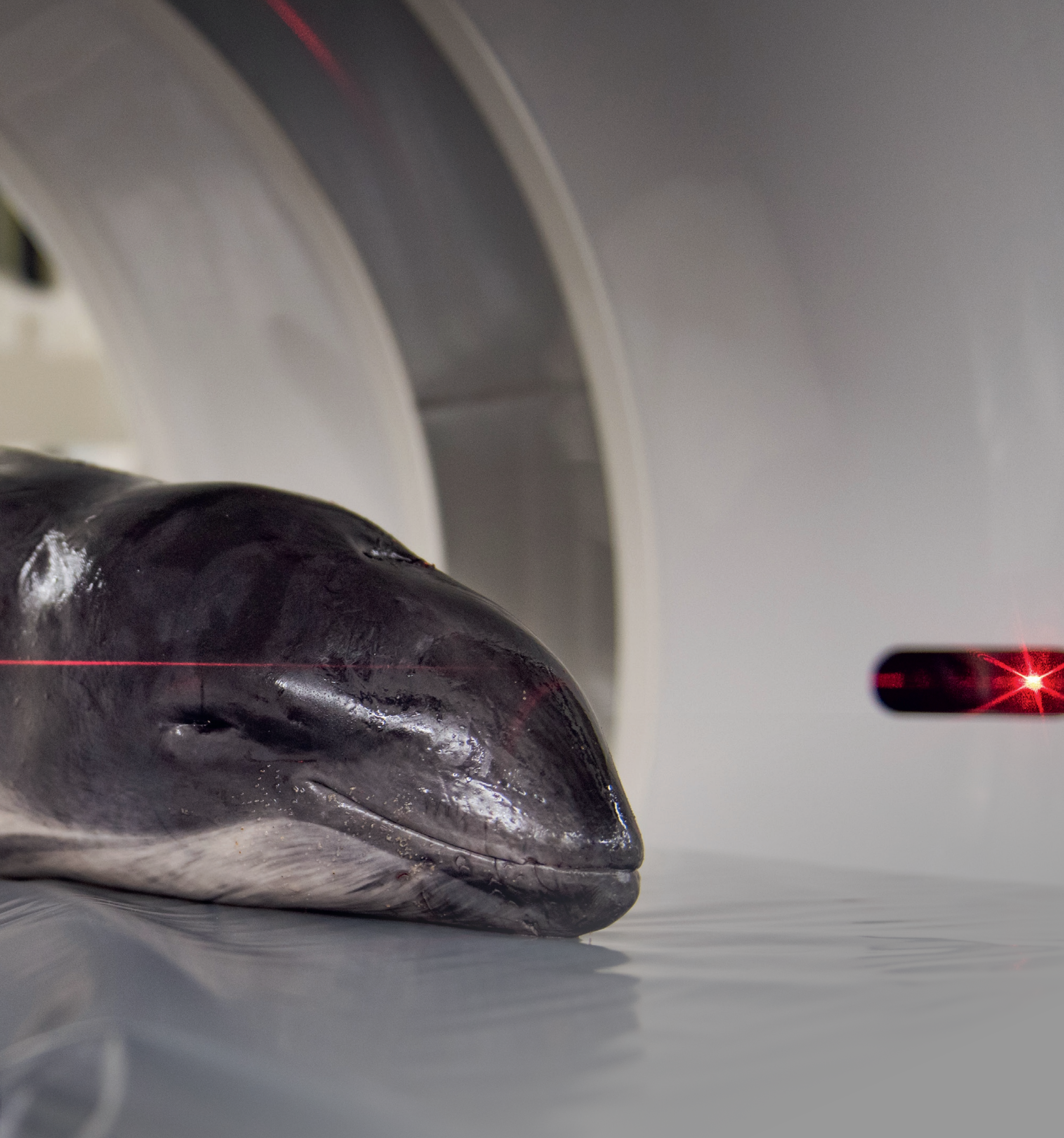

Crossing boundaries for cetacean conservation: Setting research priorities to guide management of harbour porpoises

Lonneke L. IJsseldijk, Mariel T.I. ten Doeschate, Nicholas J. Davison, Andrea Gröne & Andrew C. Brownlow

Abstract

Effective management of natural resources involves a multidisciplinary perspective to address complex issues in data poor-environments. With mobile species that do not conform to human-defined borders a cross-boundary approach is essential. There is a continuing concern of ecological sustainability of marine environments, which demands monitoring of ecosystem indicators. Such indicators are increasingly derived from monitoring sentinel species. Harbour porpoises (*Phocoena phocoena*) are included as indicator species in several national and international agreements. Increasing exposure to anthropogenic stressors may impact harbour porpoise populations. To investigate these risks, a better understanding of threats and their effect is required. This study aimed to identify current knowledge gaps, to predict future pressures or threats, and to define useful conservation indicators to facilitate future research on harbour porpoises in the North Sea, through expert elicitation gained in a two-round Delphi approach. The three most important knowledge gaps addressed were bycatch, population dynamics, and the cumulative effects of multiple stressors. Bycatch was predicted as the highest concern for porpoises in the next 20 years, followed by chemical and noise pollution, respectively. A list of essential indicators aiming to increase understanding of harbour porpoises' health status was established and studying causes of death, distribution, abundance, habitat use, and diet composition were scored as most relevant. These results should guide research focus and management objectives of harbour porpoise populations and the study design could be translated to serve managers in other geographical areas aiming to identify knowledge gaps and defining research priorities for other wildlife species.

Introduction

Human impact has transformed the world's oceans, by direct and indirect means, to such an extent that there is a rising concern on the ecological sustainability of most marine ecosystems (Aguirre & Tabor 2004; Halpern et al. 2008; Ramirez-Llodra et al. 2011). Increased sea surface temperatures, coastal development, removal of prey species, habitat degradation, and chemical or noise pollution all can result in ecosystem changes, influencing population numbers and species composition (Aguirre & Tabor 2004; Moore 2008; Wassmann et al. 2011). The management of the marine environment often involves complex decisions at an international scale where managers need to deal with data-poor environments and lack of ecological understanding (MacMillan & Marshall 2006).

Marine mammals are used as sentinels for monitoring of aquatic ecosystems, as they are relatively long-lived, highly mobile species which feed at or near the top of the food chain (Aguirre & Tabor 2004; Bossart 2011; Moore 2008). For example, studies on arctic ecosystems revealed that increases in water temperature accompanied by a decline in prey availability resulted in spatial and temporal shifts of sea-ice dependent species (Derocher et al. 2004; Stirling 2002; Wassmann et al. 2011) and decreases in abundance and migration changes of mysticetes (Moore 2008). The use of marine mammals as ecosystem sentinels, however, goes beyond investigating changes in distribution and abundance. Their overall health status can reflect the health of the ecosystem in which they live, making monitoring of cetacean population health a useful endeavour that can provide crucial information far beyond the individual populations themselves (Burek et al. 2008). One key example is the investigation of bioaccumulation of persistent organic pollutants (POPs) and heavy metals in cetacean tissue. From the presence and concentrations of such pollutants one can infer contamination levels in the marine ecosystem, and this may provide an early warning system for potential human health hazards (e.g., Borrell et al. 2014; Dorneles et al. 2013; Jepson et al. 2016; Jepson & Law 2016; Schwacke et al. 2011, 2013).

Harbour porpoises (*Phocoena phocoena*) are protected and included under several international, European and national conventions. International protection is provided by e.g., the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) (Green et al. 2012; IAMMWG et al. 2015; Reijnders et al. 2009). On a European scale, the European Union Habitats and Species Directive 1992 recognise the harbour porpoise as a 'species of community interest which is in need of strict protection' and porpoises are listed under Annex II and IV that aim to establish a network of Special Areas of Conservation (SACs) and requires establishment of distinct conservation and management needs, respectively. In 2008, the Marine Strategy Framework Directive (MSFD) was formally adopted by the EU and the first EU legislative instrument related to the protection of marine biodiversity. It aims 'to achieve or maintain good environmental status within the marine environment by the year 2020 at the latest' and determined 'good environmental status descriptors', among which several relate to the harbour porpoise (Green et al. 2012; IAMMWG et al. 2015; Reijnders et al. 2009). On regional scales, the Agreement on the Conservation of Small Cetaceans in the Baltic, North-East Atlantic, Irish and North Seas (ASCOBANS) aims to achieve and maintain a favourable conservation status for small cetaceans within their agreement area. Here, the harbour porpoise is an abundant and widespread species with a population size of around 350,000 individuals (Hammond et al. 2002).

Frequent exposure of harbour porpoises to human activities has raised concerns among conservationists, both in terms of direct impacts as well as nonlethal effects impacting population viability. The available knowledge on pressures affecting this species varies widely, and specific topics, such as climate change, urgently require further attention. Protection measures for harbour porpoises need to be implemented at a scale relevant to their ecosystem. To be effective and as encouraged by existing conventions this requires international co-ordination, yet most current research and management efforts are implemented at a national level (Camphuysen & Siemensma 2011; IAMMWG et al. 2015). To meet the requirements of both the EU Habitats Directive and MSFD, a broad approach identifying current knowledge gaps and predicting future threats is necessary to adequately assess the health status of harbour porpoise populations. This involves a clear understanding of the risks associated with increasing exposure to anthropogenic pressures (Hanisch et al. 2012; Patyk et al. 2015; Stephen 2014).

In this study expert opinions were exploited through a two-round Delphi approach that aimed to identify current knowledge gaps, predict future pressures or threats and suggest useful conservation indicators to guide research and monitoring, at an international and interdisciplinary level. The outcomes of this study subsequently identified and ranked research priorities as defined by the panel of marine mammal experts, with the aim that this could inform future conservation management and mitigate threats to harbour porpoises.

Materials and methods

The Delphi method

The Delphi method is a survey-based research approach that enables experts to collectively address complex problems through structured group communication. It comprises two- or more rounds of questionnaires, each followed by a feedback round that enables participants to clarify and revise responses from previous rounds to ensure accurate judgements (Mukherjee et al. 2015; Patyk et al. 2015). The Delphi method has been used previously in a range of disciplines, such as nursing, tourism and medicine, and is particularly powerful assessing complex issues in data poor environments. One of its major strengths is the possibility for people to respond anonymously, reducing the influence of social pressure and 'group-thinking', yet allowing collation of both formal and informal knowledge in a transparent and robust way (MacMillan & Marshall 2006; Mukherjee et al. 2015). Use of the Delphi method in conservation and ecological management is still uncommon (Mukherjee et al. 2015).

Study area and expert panel

The North Sea was used as the study area, due to the increasing anthropogenic activities in this area (e.g., expansion of offshore windfarm industry) and as it borders multiple countries, all with their own national conservation focus. Experts working in different sectors and geographic areas were selected to generate a complete and robust judgement on the issues addressed. Experts were individuals knowledgeable in harbour porpoise conservation, and particularly practitioners and policymakers working in this field, which was ensured by their affiliation to two conservation bodies:

1. Members of the Advisory Committee (AC) of ASCOBANS, with a sub-selection on those affiliated to North Sea bordering countries. The AC provides advice and information to the Secretariat and the involved Parties on the conservation and management of small cetaceans and on other matters related to the running of the Agreement on an annual base. Each Party is entitled to appoint at least one member and additional advisors to the AC, and therefore consists of experts with different backgrounds; both researchers, as well as conservationist and policy-makers. It annually discusses current knowledge (both published and unpublished) and conservation issues. This selection resulted in 81 contacts.

2. Members of the North Sea Group (NSG), which is the steering group for the ASCOBANS conservation plan for harbour porpoises in the North Sea. This overlapped with the AC selection with 72%, however, an additional eleven contacts were identified through the NSG.

Invited experts suggested other colleagues for participation, resulting in 14 additional participants. 106 people were invited to the first round of the survey. All survey participants voluntarily contributed to this study and contribution was kept anonymous among the expert panel and within the research team, except for the facilitator.

The Delphi design and process

A two-round Delphi exercise was electronically conducted between January and March 2017. The first round of the questionnaire was unstructured, allowing participants to give open answers and comment on the issues raised. The second round summarised responses obtained in the first round, which was provided as feedback to the panel and allowed them to revaluate the topics addressed. The questionnaire of the second round involved evaluation and rating of the answers gained in round one. Both questionnaires can be found in the online Supporting Information. Round one was accessible for 17 workdays, during which reminders were sent to encourage the experts to participate. Round two was made available 15 workdays later and was accessible for an additional 24 workdays. During all stages of both surveys, the expert panel was given the opportunity to provide suggestions or additional information, if desired.

Demographic information on the participants, current country of work, main field of expertise (open-question) and main field of work (closed-question with options: research, welfare, advocacy, government, monitoring or other) was requested to assess the spread in sectors of expertise and geographical areas of the panel. Collation and reviewing of responses were done by three members of the research team. The first task in the process was to encourage the panel to think about what 'harbour porpoise health' means and involves. The survey focus was on three major topics: identifying knowledge gaps, predicting future pressures or threats, and defining conservation indicators.

Defining 'health'

At the beginning of the survey, experts were given the summary statement of a previous Delphi study on polar bears (*Ursus maritimus*) (Patyk et al. 2015) on the definition of health: "Polar bear health is a multidisciplinary concept and is concerned with multiple factors that affect polar bears. Polar bear health can be applied at the individual, species, and ecosystem levels, but its most important defining characteristics are whether a population can respond to factors in its environment and sustain itself long term.". With the aim of encouraging the panel to think about what assessing 'health status' involves, they were asked whether they found the definition by Patyk et al. (2015) sufficient to apply for harbour porpoise health, with a yes/no answering option and, if improvement was necessary, what changes they would suggest. The proposed changes to the definition which were made by two or more participants were presented to the panel in round two and participants were asked to rate whether each change would "improve", "decrease", or "neither" improve or decrease the suitability of the definition according to their opinion. Changes were adopted when >50% of the expert panel responded that these improved the definition.

Current knowledge gaps

In round one the panel was asked to rate currently available information on harbour porpoises regarding its usefulness for assessing their health status in the North Sea on an integer scale from 1 (no relevant information available) to 10 (all necessary information available). This was followed by an open question asking for their opinion of the most significant knowledge gap that, if addressed, would best improve the ability to assess the health status of harbour porpoises in the North Sea. Responses were collated and sixteen statements composed. These were presented to the panel in round two, accompanied by the request to give their percent agreement to each statement from 0% (I do not agree with this statement/I do not think this improves our ability to assess porpoise health status) to 100% (I fully agree with this statement/I think this highly improves our ability to assess porpoise health status).

Future threats or pressures

With an open question the expert panel was asked to predict the most significant threats and pressures affecting harbour porpoise health in the North Sea over the next 20 years. The research team pooled these responses thematically. In the second round, the expert panel was asked to rate each threat for: 1. Index of concern: on a 10-point scale, where 1='not a concern', 5='somewhat a concern', and 10='a major concern' for harbour porpoise populations in the North Sea; 2. Index of the sufficiency of knowledge: on a 10-point scale, where 1='currently, knowledge is insufficient', 5='currently, an average amount of knowledge is present', and 10='currently, knowledge is complete and sufficient'. Participants could rate a 0 in round 2 if they were unsure about specific threats. Subthemes (e.g., specific noise sources like seismic) were only included in round two when two or more experts specifically had addressed this in round one. Density plots were created (using R version 3.3.1) visualising the distribution of scores obtained from the expert panel, providing an overview of the index of concern and index of sufficient knowledge per identified threat.

Conservation indicators

With an open question, the expert panel was asked to list indicators that would be most useful to understand harbour porpoise health status in the future, while keeping in mind the future threats and pressures mentioned in the previous section. To assess the value of each indicator for specific threats, the nine identified threats from round one were presented and the panel was asked to assess each indicator in connection to the different threats on a three-point scoring system: "little to no value", "significant value" and "essential". Any missing indicators could be provided as 'other' and specified in a text box. For this topic, experts could leave sections blank if the addressed threat did not fit within their field of expertise. For the purposes of this study, the definition of indicators according to MSFD was followed: 'indicators are distinctive technical features, which help make the descriptors more concrete and quantifiable'.

Each indicator in relation to each threat was evaluated for its use by translating the three-point scoring system into values, with: "little to no value" =0, "significant value" =1 and "essential" =2. Research priorities were identified through the calculation of the average total score for each indicator per threat, corrected for the number of experts that completed each subsection. All indicators that scored an average value of >1.5 for all threats combined, were appointed as the highest priority.

Results

Composition of the Delphi expert panel

Forty-four experts participated in round one, with 32 completing round two of this Delphi exercise. Experts from all countries surrounding the North Sea were involved, with most participants from the larger North Sea bordering countries: United Kingdom (15 and 10 respectively) and Germany (13 and 8 respectively). Most experts worked in the field of 'research' (18 and 12, respectively), followed by 'government' (10 and 5 respectively) (Table 1). Experts that completed both rounds (n=32) mainly worked on anthropogenic interactions and impacts (n=15), conservation and management (n=10), ecology (including diet studies) (n=9) and population dynamics (including abundance and distribution) (n=9) (Table 1).

Table 1. *Invited and participated expert numbers in both survey rounds, their country, work field and expertise.*

Characteristic		Round one N=	Round two N=
Invited/participated		106/44	44/32
Country	*Belgium*	2	2
	Denmark	2	2
	France	3	3
	Ireland	1	0
	Germany	13	8
	The Netherlands	7	6
	Norway	1	1
	United Kingdom	15	10
Work field	*Advocacy*	5	4
	Government	10	5
	Monitoring	4	4
	Research	18	12
	Welfare	2	2
	Other	5	5
Expertise	*Anthropogenic interaction/impact*		15
	Conservation and management		10
	Population dynamics		9
	Marine policy		7
	Acoustics		5
	Stranding and pathology		5
	Oceanography		1
	Cetacean rescue		1

2

Defining 'health'

Participants were asked if they found the definition by Patyk et al. (2015) sufficient to apply for harbour porpoise health or provide improvements to this definition. Most experts (66%) agreed with the sufficiency of the definition, yet several suggestions were made in round one which were integrated as described in the methods for round two for the whole panel to assess (Table 2). This led to the slightly adapted definition of harbour porpoise health as: "Harbour porpoise health is a multidisciplinary concept and is concerned with multiple factors that affect harbour porpoises. Harbour porpoise health can be applied at the individual, species, and ecosystem levels, but its most important defining characteristics are whether a population or subpopulation is resilient to factors in its environment and can sustain itself long term."

Table 2. *Condensed list of proposed changes to the definition of harbour porpoise health and the average percentage judged by the panel in round two (n=32). The number of experts that identified each change in round one is given in the parentheses.*

Proposed change	Increases %	Decrease %	Neither %
Add [...] its most important defining characteristics are whether an individual or population can [...] (7)	45.2	35.5	22.6
Add [...] its most important defining characteristics are whether a population or subpopulation can [...] (3)	**64.5**	9.7	29.0
Change "in its environment and sustain itself long term" to "in its environment to sustain itself long term" (4)	48.4	19.4	35.5
Change "a population can respond to factors" to "a population is resilient to factors" (9)	**64.5**	19.4	19.4

Knowledge gaps

Experts rated the usefulness of currently available information on harbour porpoises slightly above average for assessing health status (rating of 5.6/10, with minimum 3; maximum 9; mode 7). The four most significant knowledge gaps, that, if addressed, would most improve the ability to assess health status of harbour porpoises in the North Sea were population size and structure (n=12), cumulative impact of stressors (n=11), reproduction parameters (n=9) and the impact and effects of contaminants (n=8). The knowledge gaps identified, as well as the composed statements based on these knowledge gaps with the average percentage agreement of the panel can be found in Table 3.

Table 3. *Collated list of knowledge gaps from the first round in response to the question what currently are the most significant knowledge gaps, which, if addressed, would most improve the ability to assess the health status of harbour porpoises in the North Sea, with the number of experts that identified each knowledge gap provided in the parentheses. Sixteen statements were composed based on these knowledge gaps and presented to the expert panel in round 2, requesting percent agreement to each statement.*

Knowledge gaps	Statements *It is important to…*	Agreement with statement (%)
Bycatch (7)	quantify bycatch numbers, both lethal and non-lethal	92.3
Population size and structure (13)	understand population size and structure	90.0
Effective reduction of impacts (1)	mitigate threats and pressures instead of only characterising them	86.9
Cumulative impacts (11)	understand cumulative impact and combined stressors	86.8
Contaminants (8)	understand the influence of contaminants	85.7
Noise (7)	understand the effects of impulsive and continuous noise	84.5
Reproduction parameters (9)	understand the factors affecting reproduction parameters	82.3
Standardization and research scales (6)	establish more international collaborations to conduct research on appropriate scales	82.0
Feeding ecology and behaviour (3)	understand changes in prey availability and their impacts	80.9
Seasonal and inter-annual movement (6)	understand spatial and temporal aspects of habitat use	77.4
Health of free-ranging individuals (6)	know the health status of free-ranging individuals	73.7
Energetic needs and nutritional status (6)	understand energetic needs and what affects these	71.7
Population boundaries (4)	understand population boundaries, including defining subpopulations	71.5
Representativeness of data from stranded animals (6)	better quantify the biases associated with strandings data	69.7
Climate change (1)	understand the effects of climate change at individual, population and ecosystem levels	65.9
Competition (2)	understand the impact of competition for prey between porpoises and sympatric species	57.9

Future threats and pressures

The panel was asked to predict future threats and pressures. Bycatch (n=31), noise pollution (n=23), decrease in prey quality and quantity (n=21) and chemical pollution (n=20) were most frequently mentioned (Table 4). Within the themes noise- and chemical pollution, some experts identified specific threats. For noise pollution, this included (de)construction of energy platforms (n=11), shipping (n=6), ordnance detonation (n=2), sonar (n=1), wind farm operation (n=1), seismic (n=1) and oil extraction (n=1). For chemical pollution, this included persistent organic pollutants (including polychlorinated biphenyls) (n=4) and eutrophication (n=1). Specific threats within the topics noise- and chemical pollution are included in Table 4 when mentioned by two or more experts.

Threats were rated an index of concern and an index of sufficiency of knowledge in round two (Table 4). The concern was rated consistently higher for almost all threats than the available knowledge. The threat with the highest index of concern was bycatch (8.1/10), followed by chemical pollution (7.5/10) and noise pollution (7.1/10). Competition with sympatric species and shipping interactions (e.g., ship collision) were rated the lowest concern (3.3/10 and 4.2/10, respectively). For knowledge, bycatch and chemical pollution scored the highest indexes (5.5/10 and 5.4/10, respectively), whilst the knowledge on climate change and prey quality and quantity rated as currently most insufficient (3.6/10 and 3.9/10, respectively) (Fig. 1).

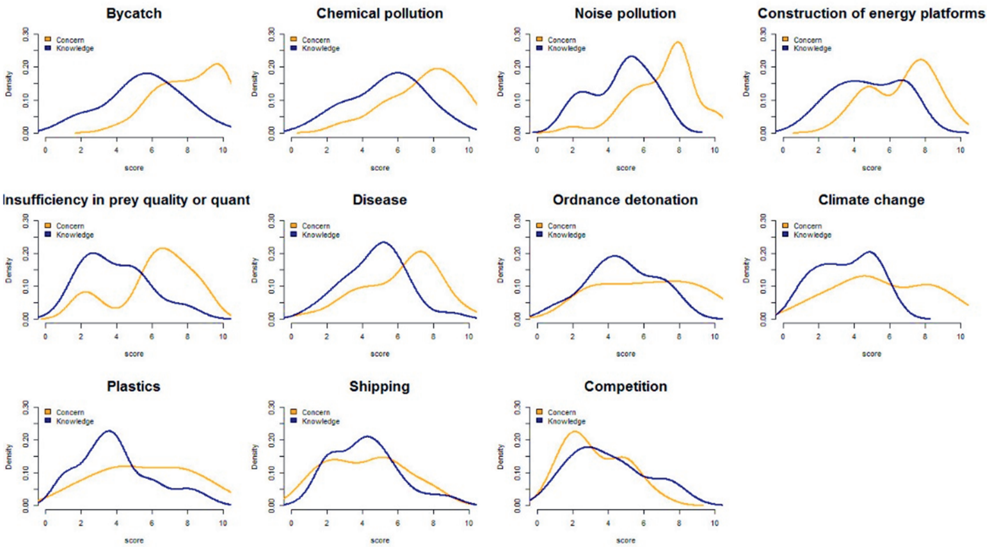

Figure 1. *Density plots showing the distribution of answers obtained by the expert panel, providing an overview of the index of concern and index of sufficiency of knowledge per identified threat. Experts (n=32) rated each subject an index of concern (yellow) and an index of sufficiency of knowledge (blue).*

Table 4. *Condensed list of threats and pressures affecting harbour porpoise health in the North Sea in the next 20 years: experts (n=32) rated each subject an index of concern (top) and an index of sufficiency of knowledge (bottom) on a ten-point scale, with the mean and most rated (mode) indexes presented.*

Index of concern	n	mean	mode
Bycatch (31)	32	8.1	10
Chemical pollution (20)	31	7.5	8
Noise pollution (in general) (23)	32	7.1	8
Construction of energy platforms (11)	31	6.7	8
Insufficiency in prey quality or quantity (21)	32	6.2	6
Disease (3)	29	6.2	7
Ordnance detonation (2)	29	6.1	8
Climate change (9)	29	5.6	5
Plastics (micro and macro) (2)	29	5.6	4
Shipping interactions e.g., ship strikes (6)	32	4.2	5
Competition with sympatric species (1)	30	3.3	2
Index of sufficiency of knowledge	**n**	**mean**	**mode**
Bycatch (31)	32	5.5	6
Chemical pollution (20)	31	5.4	6
Construction of energy platforms (11)	30	4.8	7
Ordnance detonation (2)	28	4.8	4
Disease (3)	28	4.7	5
Noise pollution (in general) (23)	32	4.7	5
Shipping interactions e.g., ship strikes (6)	30	4.2	4
Competition with sympatric species (1)	30	4.0	3
Plastics (micro and macro) (2)	31	4.0	4
Insufficiency in prey quality or quantity (21)	30	3.9	5
Climate change (9)	29	3.6	5

Conservation indicators

The participants were asked to define indicators useful for understanding harbour porpoise health and nine indicators were assigned (left column of Table 5). All indicators were evaluated for their use to assess the defined threats so research priorities could be identified through the calculation of the average total score for each indicator per threat. This shows that certain indicators, or research into these topics, can be of use when assessing a particular threat, whilst other indicators are of value in the assessment of a range of threats. As an example, the indicator to ultimately assess the extent and effects of bycatch was unanimously and logically assigned by the panel to be 'assessment of interactions with fisheries'. However, also investigating causes of death and temporal and spatial distribution were rated as essential research topics to be able to assess bycatch. For assessing the extent and effects of chemical pollution, measurements of contaminant levels in tissues and investigation of causes of death, and to a lesser extent also the assessment of nutritional body condition, were assigned by the panel as essential research topics. To increase knowledge on threats assigned the lowest index of the sufficiency of current knowledge (climate change and prey quality and quantity), research on certain indicators was singled out to encourage progress. For climate change, this included research into spatial and temporal distribution and abundance. To gain knowledge on prey quality and quantity, research into prey availability, diet composition and nutritional body condition needs to be intensified.

The overall value of each indicator was also calculated for all threats combined, to establish a shortlist of indicator research areas that will contribute to the assessment of the full range of threats and could therefore be seen as priority research to assess porpoise health status in the future. The five indicator research areas that were assigned with the highest priority were cause of death, distribution, abundance, habitat use and diet composition (right column Table 5).

Table 5. *List of indicators which would be most necessary to understand harbour porpoise health status in the future, scored for effectiveness in assessing the nine identified main threats on a three-point scoring system: 0="little to no value", 1="significant value" and 2="essential". The numbers in the table represent the calculation of the average total score for each indicator per threat, corrected for the number of experts that completed each subsection and the final column represents the calculation of the average score of each indicator for each threat. Green indicates the 'essential' rated indicators per threat, orange indicates the 'significant' rated indicators per threat.*

(see next page)

Indicators	Bycatch	Pollution	Climate	Prey	Disease	Noise	Competition	Plastic	Shipping	Average score	Number of threats for which indicator is essential	Number of threats for which indicator is useful	Index of usefulness
Causes of death	1.84	1.79	0.93	1.54	1.89	1.34	1.08	1.71	1.74	1.54	7	2	9
Abundance incl. repro. and mortality	1.61	1.33	1.52	1.52	1.43	1.28	1.16	0.88	1.23	1.33	3	6	9
Habitat use	1.33	0.81	1.41	1.15	0.73	1.59	1.62	1.12	1.5	1.25	2	6	8
Temporal and spatial distribution	1.61	0.74	1.86	1.54	0.74	1.66	1.58	0.92	1.58	1.36	6	1	7
Health status of free-ranging population	0.7	1.44	1.14	1.46	1.7	0.96	1.13	1.12	0.38	1.12	1	6	7
Body condition	0.41	1.52	1	1.68	1.68	0.58	1.12	1.23	0.58	1.09	3	4	7
Stranding numbers	1.2	1.11	0.93	1	1.33	0.93	0.8	1	1.23	1.06	0	7	7
Diet composition	0.97	1.41	1.39	1.79	0.96	0.36	1.81	1.27	0.22	1.13	2	4	6
Prey availability	1.2	0.78	1.43	1.89	0.74	0.71	1.88	0.69	0.39	1.08	2	3	5
Contaminant levels in tissue	0.37	1.93	0.26	0.85	1.56	0.25	0.2	1.35	0.08	0.76	2	1	3
Physiological char. e.g. stress indicators	0.55	0.93	0.92	0.89	1.19	1.5	0.68	0.72	0.48	0.87	0	2	2
Reproduction parameters	0.79	1.36	0.96	0.96	1.11	0.75	0.64	0.48	0.17	0.8	0	2	2
Disease surveillance	0.33	1.48	0.77	0.78	1.86	0.54	0.29	0.72	0.22	0.78	1	1	2
Interaction with fisheries/fishing effort	2	0.19	0.43	1.15	0.15	0.32	0.6	0.32	0.52	0.63	1	1	2
Water quality	0.24	1.41	0.59	0.59	0.88	0.15	0.2	1.12	0.09	0.58	0	2	2
Shipping activity	0.47	0.07	0.07	0.11	0.15	1.55	0.16	0.2	1.89	0.52	2	0	2
Age structure	0.9	0.96	0.73	0.81	1.32	0.64	0.75	0.56	0.61	0.81	0	1	1
Occurrence of predators	0.33	0.07	0.68	0.44	0.26	0.14	1.08	0.04	0.17	0.36	0	1	1
Noise levels in the environment	0.5	0.15	0.07	0.26	0.41	1.9	0.28	0.16	0.96	0.52	1	0	1
Genetic variability	0.5	0.4	0.75	0.31	0.65	0.26	0.29	0.21	0.09	0.38	0	0	0

Discussion

In this study current knowledge gaps, future threats and useful conservation indicators for assessing the health status of harbour porpoises were collectively appointed by an international and interdisciplinary panel of marine mammal conservation experts. The expert panel subsequently identified and ranked research priorities that can inform future conservation management aiming to mitigate threats. Through the combination of the involvement of experts from different geographic locations and expertise, and the Delphi method's strength in efficiently collating formal and informal knowledge in a transparent and robust way, this study appoints research priorities that otherwise may not have been collectively reached.

Assessing the cumulative effects of multiple stressors is stated a top-priority problem in marine ecology (NAS 2017) and this also stood out in our study as a major knowledge gap. Cumulative risks derive from a combination of anthropogenic threats (such as bycatch, noise and chemical pollution, marine debris etc.), as well as natural stressors, such as increased presence of predators, decreased presence of prey and increases of pathogens and parasites. Stressors can be intrinsic; a result of internal changes (e.g., fasting), or extrinsic; a factor of the external environment (e.g., noise). A way of quantitatively assessing cumulative effects is however yet to be established but conceptual frameworks have been suggested (NAS 2017). Appointing data gaps is key in this process. The usefulness of currently available information on harbour porpoise health status was scored just above average in our study (rating of 5.6/10) and by large the mean consensual opinion among the experts was that currently the most important knowledge gaps for the North Sea area were the extent and effects of bycatch, and population size and structure. The most significant pressures for harbour porpoise populations in the North Sea were attributed to bycatch, chemical pollution and noise pollution, respectively. Interestingly, the concern for almost all pressures was rated consistently higher than the available knowledge for these pressures; indicating that there was consensus among the expert panel on the need to improve our knowledge concerning harbour porpoise populations and their viability.

The top five indicators as appointed by the expert panel as the most essential to assess harbour porpoise population health in the future were the research into causes of death, identifying the spatial and temporal distribution, establishing abundance estimates, and revealing small scale habitat use and diet composition. Prioritising research and investing resources in these indicators, which are of relevance to the assessment of multiple threats, could be seen as essential to help the research community to better evaluate and predict the health status of harbour porpoises in the future. Therefore, these indicator researches can be seen as 'the best value for money' for conservation managers to focus upon. In addition experts agreed that the standardisation and connection of databases from different research fields to conduct studies on appropriate ecological scales is needed. Moving from only characterising knowledge gaps towards the establishment of an effective way to reduce pressure and mitigate will become necessary. Efforts into the development of methodological approaches to address the integration of complexity of relationships between threats, indicators and knowledge gaps should also be further developed.

The Delphi method proves to be adaptable and is widely used but its scientific merit and outcomes have been reviewed and criticised (Powell 2003). Strengths of the methods lie within the ability to bring together a range of knowledge and experience without geographical limitations, making it quick and relatively efficient. This was demonstrated here; experts from all countries surrounding the North Sea were involved and two questionnaires were conducted in a short period of time, without the use of any funds. The Delphi method allows the best use of current available formal and informal information in a transparent and robust matter, though, its reliability is strongly influenced by the complexity of the topic involved and could well be heuristics-driven. Additionally, the number of questions, rounds and participants could strongly influence the outcomes (MacMillan & Marshall 2006; Powell 2003). This study aimed to generate a broad yet complete view of harbour porpoise health status, a highly complex issue to address, to assess current knowledge gaps and predict future threats and pressures. This did not allow in-depth focus on specific topics, which could be necessary for certain topics, e.g., when geographical differences occur or threats can be subdivided into more specific sources or topics. One example in this study was noise pollution; when an in-depth evaluation for a specific noise source is required, a Delphi exercise (or other approach) with a more specific focus is needed. An example is the study by McWinnie et al. (2017) who investigated marine noise pollution from vessels in Canada and identified priority information needs to inform new research and address policy needs through an iterative Delphi style process and workshop.

The results of any Delphi exercise should be considered as an opinion of the included experts (Powell 2003). This study showed that the most significant pressures for harbour porpoise populations in the North Sea were attributed to bycatch, chemical pollution and noise pollution. These threats are all tangible and well-established threats which form current priorities within ASCOBANS as well as other EU bodies, making these historically and politically ranked as important. It is likely that these widespread and 'familiar' threats have a higher profile amongst the participants than more emergent or nebulous risks, e.g., climate change, which could have resulted in a higher rank. This might also explain the lack of consensus within the expert panel when rating the concern and available knowledge of those more nebulous threats. A low scoring could however also be based on either a comprehensive awareness of the topic, a balanced appraisal that it is of less significance than other topics, or an ignorance of the topic, and hence falsely attributed as less important. The success of a Delphi exercise is thus strongly dependent upon the experience of the panel with the range of topics addressed. The panel in this study consisted of individuals working in governmental and non-governmental organisations. They had a very differing range in experience with varying backgrounds in conservation management and knowledge on harbour porpoise populations. Feedback rounds within a Delphi exercise therefore seem crucial; aiming to ensure accurate understanding of expert knowledge and their given answers. The selection of experts affiliated to ASCOBANS may have eliminated knowledgeable other experts, e.g., those working in research without participating in conservation bodies. Although participants could be assigned by the invited experts and were subsequently included in this study it is likely that valuable individuals were missed with unknown influence on the study outcomes presented here. Yet, the results of the Delphi exercise may be exploited to become a valuable communication tool to generate debate on the topic addressed (Powell 2003) and this exercise presents the first attempt to define knowledge gaps and research priorities for studying harbour porpoise health, at an international and interdisciplinary level.

Where relevant ecological knowledge and experience is lacking and management decisions need to be made, the Delphi approach could bridge the gap between science and policy (MacMillan & Marshall 2006; Mukherjee et al. 2015). Conservation progress may be increased when communication between researchers and wildlife managers is improved, and clear management needs are identified (Greggor et al. 2016; Mukherjee et al. 2015). Current research is often directed to a single or similar set of threats, due to isolated funding sources and managers having less funds to support research directly. The study results presented here suggests that the expert panel understands the multifactorial and non-linear link between individual threats and general ecosystem health. Whilst such specialisations may be useful in gaining details on specific topics, only integrating these research findings in a wider context will significantly increase our understanding of health and a paradigm shift from research into specific threats towards a broader approach seems to be required. The results of our study can be used in conservation management for prioritising research to eventually mitigate threats for harbour porpoises in the North Sea and in order to meet the requirement of several international conventions, aiming for a more favourable conservation status of this species. Additionally, this study presents a study design that could be adapted to function as a technique usable in other geographic areas where managers are in need of defining knowledge gaps and research priorities for other (wildlife) species.

Conclusion

Harbour porpoises are protected and included under several international, European and national conventions aiming for a favourable conservation status of this species. To meet the requirements of such conventions this study used an international and interdisciplinary approach aiming to identify current knowledge gaps and predict future threats to harbour porpoise populations in the North Sea. The three most important knowledge gaps addressed were bycatch, population dynamics, and the cumulative effects of multiple stressors. Bycatch was predicted as the highest concern for porpoises in the next 20 years followed by chemical and noise pollution respectively. In order to affectively assess harbour porpoise populations and to guide research focus and management objectives in the future, a list of essential indicators was established. Studying causes of death, distribution, abundance, habitat use and diet composition were scored as most relevant to assess and understand the health status of harbour porpoises in the future.

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Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.marpol.2018.07.006.

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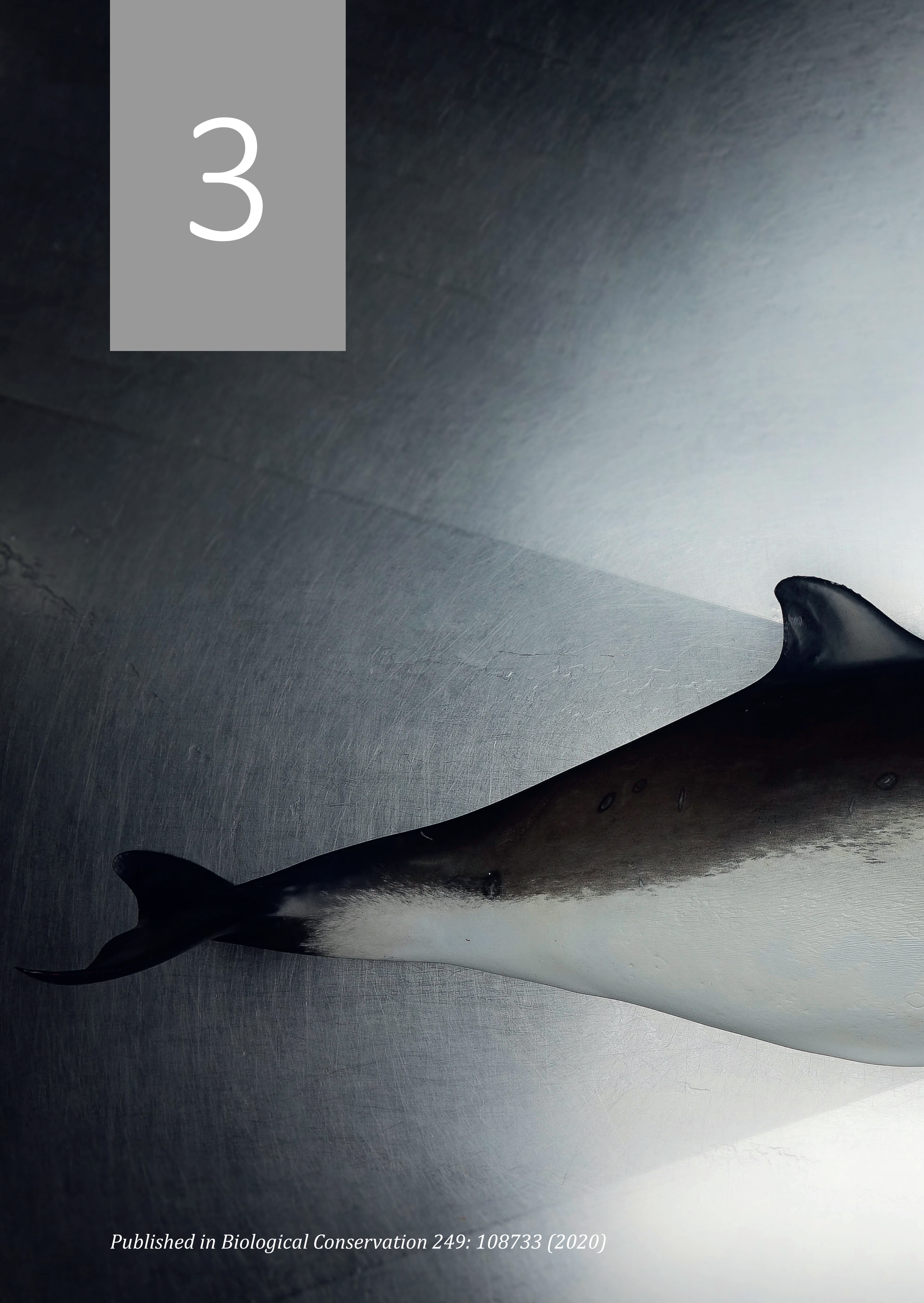

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Spatiotemporal mortality and demographic trends in a small cetacean: Strandings to inform conservation management

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Abstract

With global increases in anthropogenic pressures on wildlife populations comes a responsibility to manage them effectively. The assessment of marine ecosystem health is challenging and often relies on monitoring indicator species, such as cetaceans. Most cetaceans are however highly mobile and spend the majority of their time hidden from direct view, resulting in uncertainty on even the most basic population metrics. Here, we discuss the value of long-term and internationally combined stranding records as a valuable source of information on the demographic and mortality trends of the harbour porpoise (*Phocoena phocoena*) in the North Sea. We analysed stranding records (n=16,181) from 1990-2017 and demonstrate a strong heterogeneous seasonal pattern of strandings throughout the North Sea, indicative of season-specific distribution or habitat use, and season-specific mortality. The annual incidence of strandings has increased since 1990, with a notable steeper rise particularly in the southern North Sea since 2005. A high density of neonatal strandings occurred specifically in the eastern North Sea, indicative of areas important for calving, and large numbers of juvenile males stranded in the southern parts, indicative of a population sink or reflecting higher male dispersion. These findings highlight the power of stranding records to detect potentially vulnerable population groups in time and space. This knowledge is vital for managers and can guide, for example, conservation measures such as the establishment of time-area-specific limits to potentially harmful human activities, aiming to reduce the number and intensity of human-wildlife conflicts.

Introduction

As the pace of environmental change quickens, the need to monitor its impacts on wildlife populations becomes ever more pressing. Growth of the human population and the increase in global anthropogenic activities leads to increasing encroachment on ecosystems and wildlife communities (Nickel et al. 2020; Tyne et al. 2016). Surveillance or monitoring studies designed to quantify these pressures incorporate the ability to collect and analyse (repeated) observations or measurements, aiming at the detection of changes over time and space (Elzinga et al. 2009; Peltier et al. 2012). The efficiency of a monitoring plan relies on its ecological relevance, statistical credibility and cost-effectiveness; three aspects challenging to optimally achieve in wildlife studies (Caughlan and Oakley 2011; Hinds 1984; Tyne et al. 2016). The use of population indices and indicator species, particularly those that can inform on wider environmental health, are increasingly supported and included in the implementation of legislation (Bossart 2011; Carignan & Villard 2002; Moore 2008; Peltier et al. 2012, 2013; Roberge & Angelstam 2006).

The conservation and management of the marine environment is particularly challenging, as these habitats are difficult to monitor and often data-poor (Hiscock et al. 2003). Marine mammals, especially species which are long-lived and feed in the higher tropic levels, can be used as sentinels for monitoring of aquatic ecosystems (Bossart 2011; Moore 2008) but not without its complications. Marine mammals, and cetaceans specifically, are highly mobile animals that spend the majority of their time hidden from direct view. Therefore, even the most basic population metrics, such as abundance and life history parameters, remain mostly unknown for the majority of these species. Anthropogenic activities in the marine environment are increasing and this has raised significant concerns among conservationists (Aguirre & Tabor 2004; Halpern et al. 2015; Moore 2008; Wassmann et al. 2011). Without knowledge on population demographics and distribution, it is almost impossible to understand the severity of anthropogenic impacts on populations, and consequently how to effectively mitigate anthropogenic activities (IJsseldijk et al. 2018a; National Academies of Sciences, Engineering, and Medicine 2017).

There are four fundamental population demographic metrics: reproduction, mortality, immigration and emigration. Declines in reproduction and/or immigration, or increases in emigration and mortality, will prevent population growth or may cause population declines. Monitoring changes in population dynamics is thereby most effective when demographic parameters can be measured at the scale of these four metrics directly, rather than focussing solely upon detecting changes in abundance, which is often the norm (National Academies of Sciences, Engineering, and Medicine 2017). Although demographic parameters for marine mammals can in some cases be estimated from capture-recapture methods such as photo-identification, these methods come with a range of uncertainties and logistical restrictions (Evans & Hammond 2003; Tyne et al. 2016; Urian et al. 2014) and are not applicable for all species. Population diversity, richness, and important metrics like age-specific mortality and age at sexual maturity, can be derived from analysis of stranded individuals, especially in areas where stranded marine mammal carcasses are found in sufficient numbers, and dedicated long-term data series are available (National Academies of Sciences, Engineering, and Medicine 2017; Peltier et al. 2012; Pyenson 2011; Saaveda et al. 2017).

Located in North-western Europe and adjacent to the North Atlantic Ocean, the North Sea basin is bordered by the United Kingdom, Belgium, The Netherlands, Germany and Denmark, in its western, southern and eastern parts, respectively. In these countries, systems for reporting, documenting and retrieving of stranded and bycaught marine mammals are well established and, in most countries, have been in place for decades. These systems were initiated due to the statutory requirement of several national, regional and international agreements and directives aiming at the protection of small cetaceans in these waters (such as ASCOBANS 1992, European Habitat Directive 92/43/EEC, the European Marine Strategy Framework Directive (2008/56/EC), and Regional Sea Conventions, such as the Oslo-Paris Convention (OSPAR) and The Baltic Marine Environment Protection Commission (Helsinki Commission - HELCOM)). In the North Sea, this particularly involves a small, elusive yet abundant whale: the harbour porpoise (*Phocoena phocoena*) (Hammond et al. 2002, 2013; IJsseldijk et al. 2018a).

European stranding schemes have focussed mainly on reporting local stranding frequencies and seasonality within administrative management units (Haelters et al. 2018; Jepson 2005; Keijl et al. 2016; Kinze et al. 2018; Siebert et al. 2006a). However, to effectively investigate population dynamics among stranded individuals, assessments should be conducted at a relevant ecological and biological scale, independent of national borders. The objectives of this study were to assess the spatiotemporal stranding frequencies of harbour porpoises throughout the North Sea coastal area and to determine whether significant differences exist in the biological characteristics of the stranded specimens, using data spanning 28 years (1990-2017). The analysis conducted may serve as an example showing how long-term mortality data of a protected species can be used to assess demographic trends; a similar approach can be useful for wildlife conservation across different environments and taxa.

Methods

Stranding networks and study area

Harbour porpoise strandings data were collated from national stranding networks. These included records from the Dutch and Belgian coastlines, the North Sea coastlines of Denmark and Schleswig-Holstein (SH) in Germany, as well as the east coast of the United Kingdom (UK), starting at Romney Marsh (Kent) in the south, to Skerray (Sutherland) on the north coast of Scotland, including the Orkney Islands but excluding Shetland. Extensive descriptions on the history and procedures of these stranding networks can be found in Peltier et al. (2013).

Data collection and preparation

Stranding records from 1 January 1990 to 31 December 2017 (28 years) were selected, as the majority of the participating national stranding networks were initiated in 1990 (except for the Danish network, where quantitative stranding data was collected since 2008, see Kinze et al. 2018). Data submitted by each stranding network included as a minimum stranding month and year, and spatial location. Harbour porpoises floating or bycaught at sea were excluded. Animals that live stranded and died on the beach or were euthanised, without further rehabilitation or medical treatments, were included. The data comprised of 16,181 stranding records, all of which were single animal stranding events (Supplementary Table 1).

For the purpose of this analysis, the North Sea coastline was partitioned into six regions (A-F) of roughly equal coastal length and water depth. These regions closely follow the borders of the survey blocks as assigned by Hammond et al. (2017) to allow regional comparison with the results of a similar time-series of large-scale cetacean population abundance surveys (Small Cetacean Abundance in the North Sea and Adjacent waters; SCANS). Region A comprised of the Northeast of Scotland from Thurso to St Fergus, including Orkney. Region B followed the UK coastline southwards to Newcastle. Region C followed the UK coastline further south to Great Yarmouth. Region D included the rest of the English coast, the Belgian coastline, and the Delta area of The Netherlands. Region E included the rest of The Netherlands, and region F included the North Sea coastlines of Germany (SH) and Denmark (Fig. 1).

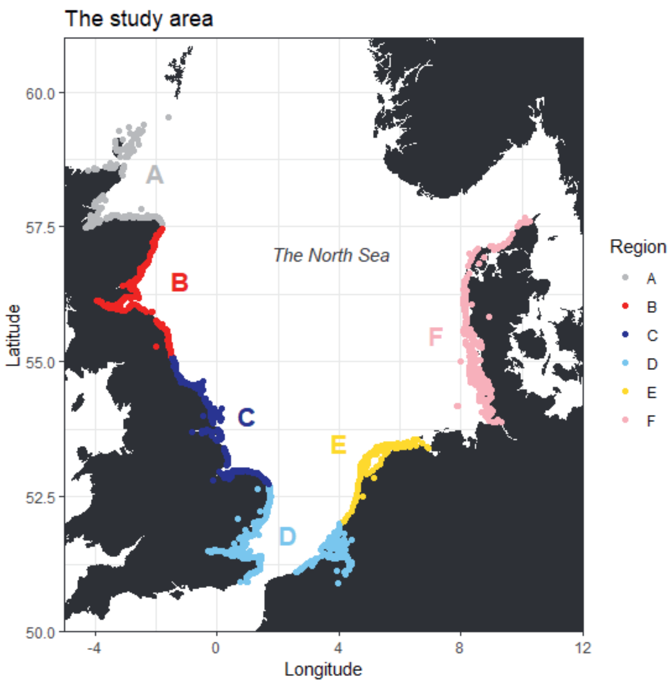


Figure 1: *The study area: the North Sea. The colours represent the six regions as assigned in this study, with: region A (in grey) comprised of the Northeast of Scotland from Thurso to St Fergus, including Orkney; region B (in red) from St Fergus, Scotland to Newcastle, England; region C (in dark-blue) from Newcastle, England to Great Yarmouth, England; region D (in light-blue) the rest of the English coast, the Belgian coastline, and the Delta area of The Netherlands; region E (in yellow) the mainland and Wadden area of The Netherlands; and region F (in pink) the North Sea coastlines of Schleswig-Holstein (Germany) and Denmark.*

All animals were assigned to an age class based on body length, with animals <91 cm classified as neonate, 91 cm to 130 cm as juvenile, and >130 cm as adult (following Lockyer 2003).

Due to differences in operational, logistical and financial capacity between stranding networks, two assumptions had to be adopted to allow for data comparison (also see Discussion). Effort and reporting are assumed to have been improved over time, likely attributable in part to increasing public awareness, technological developments facilitating submission of stranding reports, and an increased role of citizen science in marine conservation. This bias is unquantified for all stranding networks contributing to this study but temporal variation in effort was assumed to be homogenous across the study area. The impact of carcass drift is considered important, although at the scale of this analysis we assume that animals stranded within a particular region, had died in that region (i.e., on that coastline or in the adjacent waters of that region).

Data exploration and analyses

Data were explored prior to analysis following Zuur et al. (2010). Data exploration and analyses were performed using R version 3.4.4 (R Core Team 2017).

Spatiotemporal variation and seasonality

Maps of harbour porpoise strandings were created using the ggplot2 version 3.1.1 (Wickham 2016) and ggmap version 3.0.0 (Kahle & Wickham 2013) libraries. Kernel densities were estimated to visualise point density and potential shifts in distribution across the study area and study period.

A Generalised Additive Mixed Model (GAMM) was implemented using the nlme version 3.1-140 (Pinheiro et al. 2018) and mgcv version 1.8-28 (Wood et al. 2016) packages. The number of strandings was modelled as a function of month to capture a potential seasonal effect, of year to examine long-term trends, and of region. The model was fitted using a Poisson error distribution with a log-link function, and the appropriate level of smoothness was found by utilising the integrated smoothness estimation and cross-validation function available within the mgcv library. As autocorrelation can be expected in timeseries data, this was assessed following each model fit and appropriate correlation structures were fitted where necessary. Model selection was carried out through backwards elimination of variables. Data exploration indicated potential seasonality in all regions but patterns were not identical and interactions between the three variables were therefore considered in the model selection. The model best describing the data was identified by examining the scaled residuals, parameter estimates, and the Akaike Information Criterion (AIC, Akaike 1974). Model validation was done by evaluating diagnostic plots and residual variance using normalised Pearson residuals. The ratio of residual scaled deviance to residual degrees of freedom was calculated to examine over- or under-dispersion.

Additionally, it was investigated whether observed increases in stranding frequencies were related to regions. For this, the number of strandings was modelled as a dependent timeseries per region. A non-homogeneous birth process in discrete time was used following methods described in Van den Broek & Heesterbeek (2007) and Van den Broek (2020), with a negative binomial distribution for the observed strandings. The non-homogeneous birth process is a Markov model that is able to deal with the dependence in the data in time. Using this, we estimate the reproductive power directly from the data using the (log-)likelihood. This is a measure for the occurrence of the event 'stranding', and allowed to depend on age class, region and year. From this model, power odds ratios per region were calculated with region A as baseline.

Biological characteristics

The distribution of the biological characteristics (sex, body length, age class) in the set of stranded animals was examined to gain insight into population structure. Harbour porpoises are born at a length of 65-75 cm and in the North Sea parturition occurs between May and

August (Lockyer 2003; Sørensen & Kinze 1994). Records with an unknown length/age class were excluded. We investigated whether areas important for calving could be identified, therefore neonates with body lengths between 60-80 cm, stranded in the period May to August (hereafter referred to as 'new-borns') were used to assess those areas with a high density of stranded new-borns. Additionally, sex ratios per region were assessed.

Strandings of juvenile and adult porpoises (>90 cm) were analysed using Generalised Linear Models (GLM) fitted with a binomial error distribution and logit link. Sex (indicated as 1 for males and 0 for females) was modelled as a function of body length as a proxy of age, of month to examine seasonal differences, of year to assess potential long-term changes over time, and of region to evaluate potential heterogeneity between regions. Model selection was carried out by backwards elimination of variables and interaction, using the AIC to identify the optimal model. Model validation was done by evaluating calculated dispersion parameters and diagnostic plots using Pearson residuals.

Results

Spatiotemporal analysis

Annual stranding frequency varied greatly over the study period, with annual totals gradually increasing from around 150 in 1990 to almost 500 in 2004. After 2004, the density of strandings changed spatially and strandings started to concentrate more along the southern North Sea, with a steep increase in annual stranding numbers from 2004 until 2013. From 2009, the largest proportion of the total annual strandings was consistently observed in region D (southern England, Belgium and the south of The Netherlands). In contrast, absolute stranding numbers along the coastlines of regions A, B, C and F remained at levels similar to earlier years. Overall, years with the highest number of strandings within the dataset were 2011 and 2013, with 1,313 and 1,374 stranded harbour porpoises respectively (Fig. 2-3, Supplementary Fig. 1).

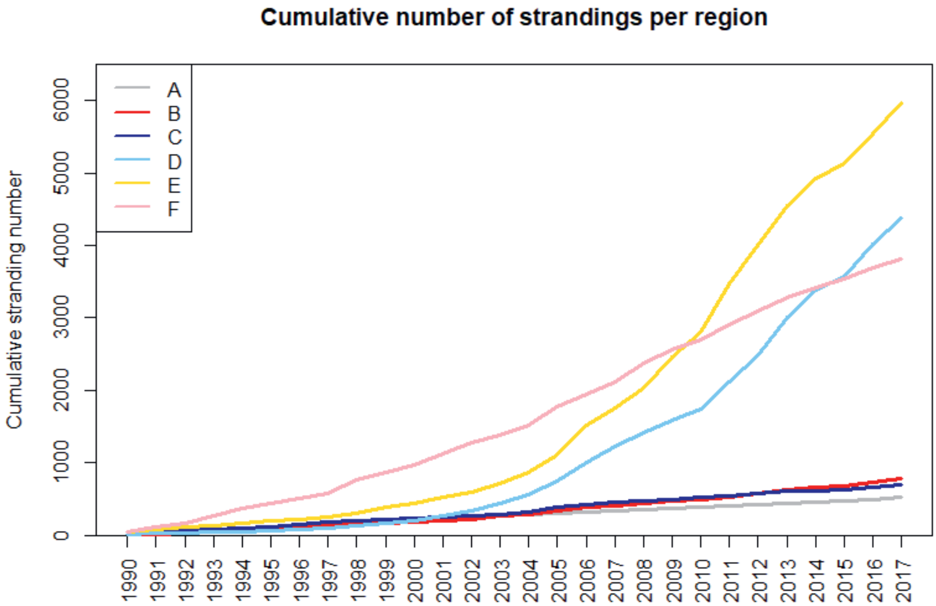


Figure 2*: Cumulative number of recorded stranded harbour porpoises per region over the study period (1990-2017).*

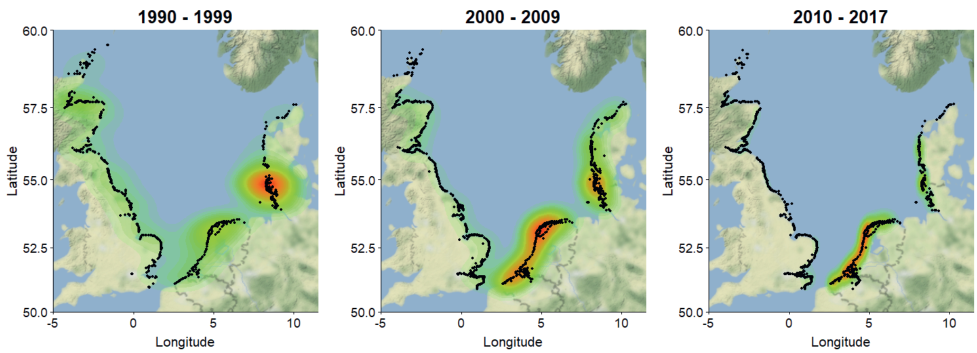


Figure 3*: Study area showing the density of all recorded harbour porpoise strandings over three time periods.*

The spatiotemporal model best describing the data incorporated a smooth seasonal effect per region and a long-term trend per region (Supplementary Table 2-3). There was a significant difference in seasonality in stranding frequency between regions, confirming that the long-term trend in annual stranding frequencies varied across the study area. This model was preferred over a model incorporating an interaction between month and year, suggesting that the observed seasonality in each region has been relatively consistent throughout the study period. The final model was fitted with an AR1 correlation structure, describing the correlation between residuals separated by one month. Plotting model residuals versus fitted values showed patterns indicative of heterogeneity in the variance between regions, with the spread being much larger for regions D, E and F (south-western

North Sea) than for regions A, B and C (northern UK coastline). Adding a variance structure to a GAMM is computationally highly intensive (Zuur et al. 2007), and there were convergence issues with a model allowing for a different variance in each region. Due to spatiotemporal similarities, it was decided to group regions A, B and C (northern-mid UK coastline), and regions D, E and F (south-western North Sea), and to incorporate a variance structure allowing for heterogeneity between the two groups only, which removed the majority of the heterogeneity in the residuals. The ratio of residual scaled deviance to residual degrees of freedom was 0.99, meaning the model was not under- or over-dispersed.

Seasonality in the regions A, B and C showed a similar pattern with a single peak in March (region B and C) and in May and June (region A) (Fig. 4). These single peaks were followed by a slow decrease and low stranding numbers throughout winter. Regions D and E were characterised by a bimodal pattern: one peak in March and April and a second peak in August. Region F was unimodal and only showed a clear peak from June to August with low numbers throughout the rest of the year (Supplementary Fig. 2).

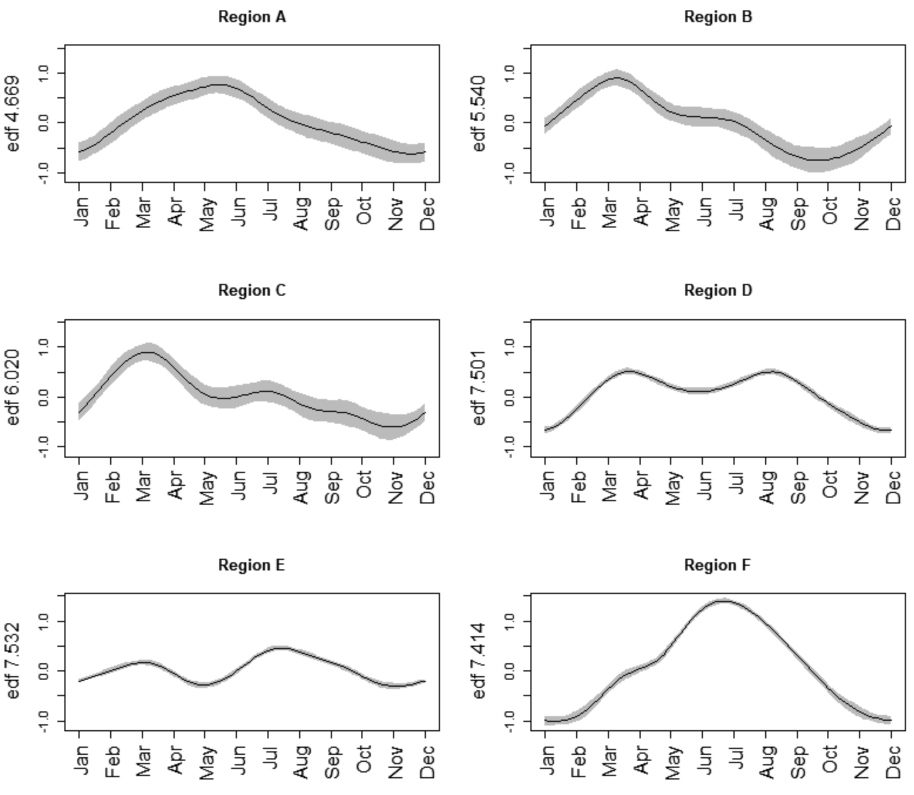


Figure 4: *Estimated smoothing curves showing seasonal patterns in the number of recorded strandings per region for the most parsimonious model, which incorporated a smoother for the month predictor for each region. Grey shaded areas represent 95% confidence intervals.*

The non-homogeneous birth model best describing the data incorporated a two-way interaction with year-region. Figure 5 shows the odds of the regions B-F compared to the models' baseline region (A). Regions B, C and F had limited deviations from region A, but there were large effects in region D around 2004/2005 and again in 2011/2012, with much higher predicted strandings in these years. Additionally, region E presented a markedly high stranding rate in 2011compared to region A.

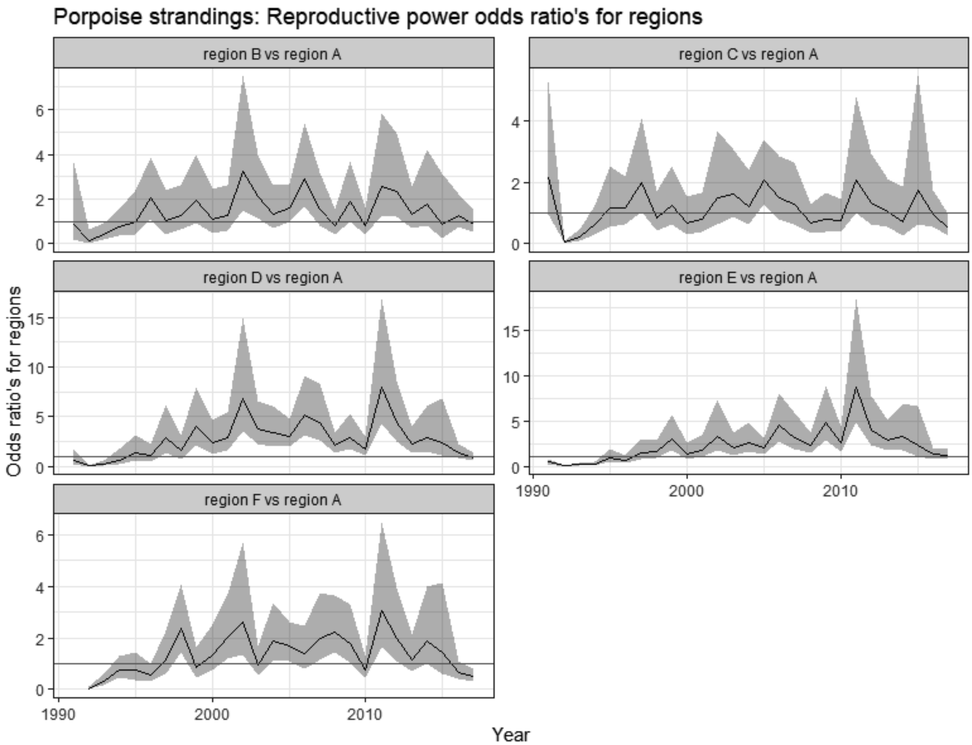


Figure 5: *Harbour porpoise stranding frequencies output from the non-homogeneous birth model presenting the reproductive power odds ratios for region B (2), region C (3), region D (4), region E (5) and region F (6) as a function of the baseline region A. Grey shaded areas represent 95% confidence intervals. The red horizontal line represents the odds ratio of 1.*

Biological characteristics

Sex was recorded for 58.7% of the individuals (n=9,496) and there were 5,292 males and 4,204 females. Body length was measured or estimated for 67.1% of the individuals (n=10,863) and there were 1,438 neonates, 6,310 juveniles and 3,115 adults (male/female-ratios given below). The distribution of lengths varied across the regions. In most regions but especially in regions D and E, the majority of animals were juveniles. Regions A, B and C showed a relatively even distribution of porpoises with lengths of 90-160 cm, with a slightly higher proportion of juveniles in region B. Proportionally fewer neonates were found in these regions. Regions D and E were dominated by juveniles, whilst region F was characterised by a proportionally higher number of neonates. Female harbour porpoises grow larger than males (Lockyer 2003), explaining why the majority of individuals >150 cm were females. Adult males were less commonly found in regions D, E and F compared to regions A, B and C (Supplementary Fig. 3-5).

New-borns (n=963) were found in all regions, however, numbers in regions A, B and C were lowest (n=30, n=27 and n=28, respectively), numbers in regions D and E intermediate (n=122 and n=260, respectively) and numbers in region F highest (n=496) (Supplementary Fig. 6). From the new-borns where sex was known (n=683), 378 were male and 305 were female (Fig. 6). Sex-ratio was skewed towards males in the neonate and juvenile age classes, with a male to female (M:F) ratio of 1:0.81 and 1:0.71, respectively. For adults in the regions D, E and F, the M:F ratios were equal; 1:1.05. Overall, the adult M:F ratio was 1:1.09. The largest difference in M:F ratio was found for juveniles in regions D and E, with a ratio of 1:0.68 (Supplementary Table 4).

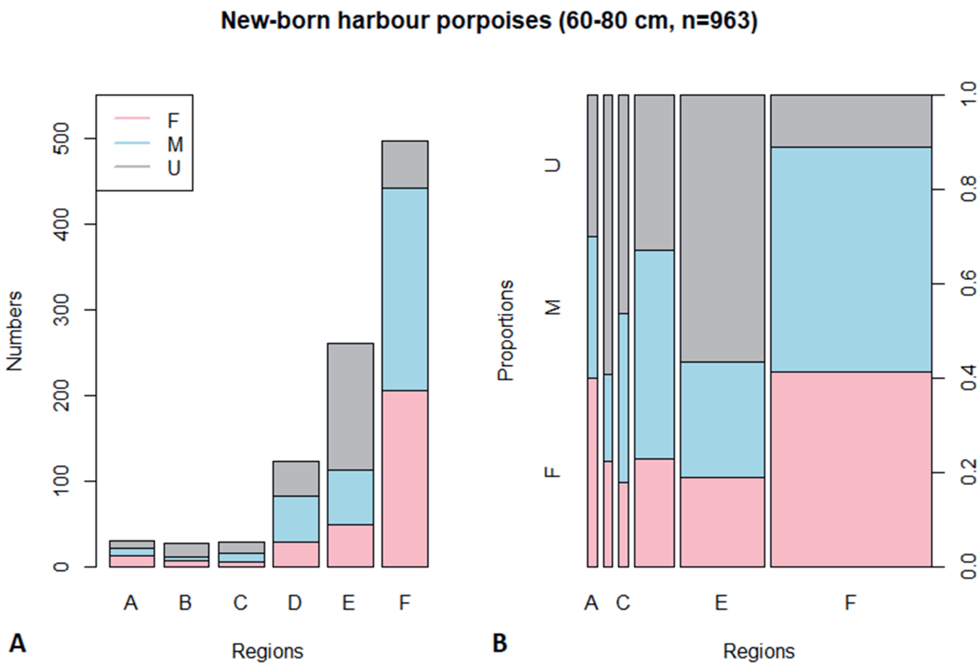


Figure 6: *Stranded new-born porpoises (60-80 cm). A: absolute numbers per region. B: proportions of sex per region, with bar widths reflecting the sample size. Pink blocks are females, blue blocks are males, grey blocks are those records with unknown sex.*

The regional heterogeneity in sex- and age class was analysed for juveniles and adults. The optimal model included body length and an interaction of body length with region, providing further evidence of a significant relationship between sex-ratio and age class, and heterogeneity of this relationship between regions (Supplementary Table 5). Month was not incorporated in the final model, indicating that there was no significant seasonal variation in M:F ratio. Model results showed that the probability of a stranded porpoise being a male in regions A and B was 0.5, and that this was approximately stable with increasing length, suggesting more or less equal distribution of males and females across age classes within these regions. For regions C to F, however, the probability of a stranded porpoise being a male was 0.7, with a clearly decreasing relationship with increasing length (Fig. 7), i.e., in those regions sex ratio seems skewed towards males for shorter animals. Model validation showed no evidence for violation of the underlying model assumptions.

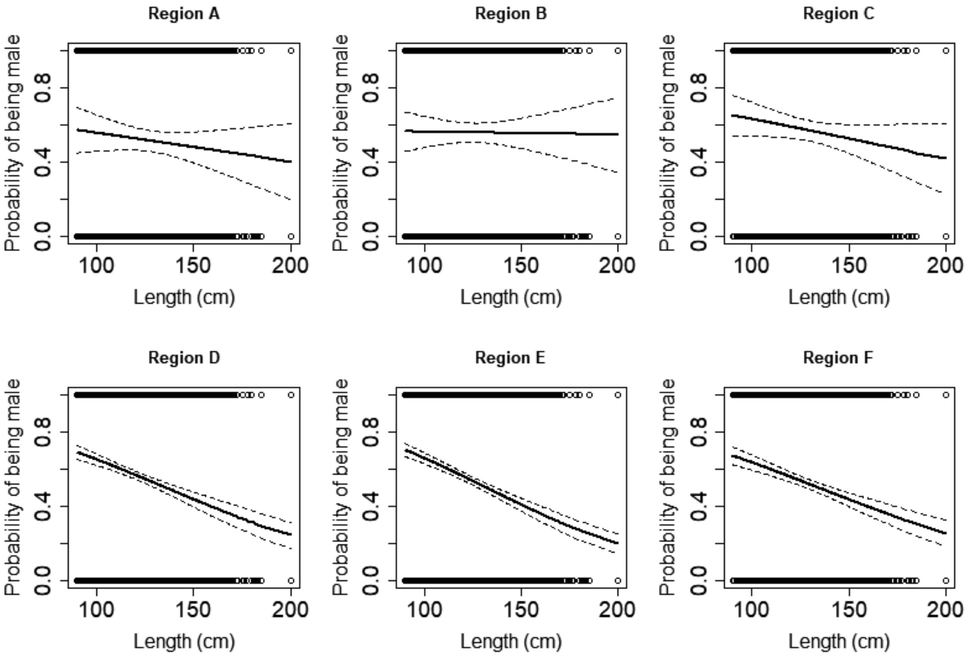


Figure 7: *Output of the optimal model representing the probability of a stranded harbour porpoise being male in relation to total body length (cm) plotted per region. The dots represent the raw data (being either 0 for the females, or 1 for the males). Solid line represent the predicted probability of being a male, with dotted lines representing the 95% confidence intervals.*

Discussion

Our study assessed population demographics and spatiotemporal trends of harbour porpoise based on data derived from an existing and continuous surveillance tool: stranded animals. Analysis demonstrated clear seasonality of strandings throughout the study area, indicative of both season-specific abundance or habitat use, and season-specific or age-specific mortality. The high density of new-born strandings in certain regions is suggestive of areas important for calving, and the higher juvenile male mortality in the southern North Sea could indicate a possible population sink or reflect higher dispersion of males (further discussed below). It is essential to minimise disturbance and negative impacts on protected species when granting permits to proposed anthropogenic activities. To be able to do this, managers need to consider the nature and extent of impacts on vulnerable population groups, and thus information on spatiotemporal variation in population distribution is needed. With marine mammals being difficult to monitor in situ, stranding records provide a unique and cost-effective opportunity for surveillance purposes (IJsseldijk et al. 2018b; Peltier et al. 2012, 2013; ten Doeschate et al. 2017). Our results present key examples of potential vulnerable population groups in time and space, and we therefore provide vital knowledge for managers, who can impose time-area closures as a management tool for specific human activities, aiming to reduce the number and intensity of human-wildlife interactions.

Spatiotemporal variation

Seasonality in strandings occurs as a result of variation in abundance, distribution or mortality of animals, as well as the non-biological components of the stranding process including oceanographic and effort factors (which are further discussed in section 4.3). Understanding baseline variation in stranding rates is an essential first step in detecting unusual stranding events and requires a good knowledge of the existing natural variation at a relevant spatial scale. Clear seasonal patterns in stranding frequency were detected, consistent throughout the study period, with patterns differing between North Sea regions.

High stranding numbers in early spring were apparent in five regions (A to E), matching the high porpoise densities in spring in the southern and south-western region of the North Sea estimated through local abundance surveys and fine-scale model predictions (e.g., Geelhoed & Scheidat 2018; Gilles et al. 2009, 2011, 2016; Siebert et al. 2006a). The majority of stranded animals in March/April were juveniles. Harbour porpoises of <115 cm and a presumable age of up to ten months may be maternally dependent (Lockyer 2003). With calving occurring from May to August, this means that animals should become independent in February to April of the following year. This also coincides with the lowest sea surface temperatures in the North Sea (Høyer & Karagali 2016). In marine mammal species, post-weaning mortality is reported to be highest (Barlow & Boveng 1991). Long-term unsuccessful foraging can lead to dramatic loss of body condition, which is particularly ominous for smaller cetaceans such as harbour porpoises given their large body surface to body volume ratio and concomitant high and constant energetic requirements (Kastelein et al. 1997; Lockyer 2007). Foraging independently for the first time combined with cold water temperatures can rapidly result in nutritional and physiological stress, eventually leading to hypothermia and death. The observed peak in March and April could potentially be explained by high juvenile mortality following emaciation and/or starvation. However, seasonal differences in both abundance and anthropogenic pressures, e.g., following spatiotemporal differences in fishery intensity and risk of bycatch mortality (Leeney et al. 2008), could also contribute to the observed pattern. Such increases in strandings should further be investigated incorporating data collected during post-mortem investigations, including information on causes of death and nutritional condition (further discussed below).

Age- and sex-specific variation

Information on the increases in mortality of particular age groups can act as early warning signals for population declines (National Academies of Sciences, Engineering, and Medicine 2017). Assessing demographic parameters of stranded animals provides knowledge on distributional variation, regions of importance for reproduction, and age-specific mortality. A higher number of new-born animals were found in region F, corresponding to the coastline of Denmark and SH, Germany. The area around the islands of Sylt, Amrum, and southern Rømø, and the region of the Sylt Outer Reef have previously been identified as important calving grounds for the harbour porpoise (Gilles et al. 2009; Siebert et al. 2006a; Sonntag et al. 1999). Our findings are in agreement with this and additionally add the northern Dutch coastal waters as a potentially important area for calving. Other areas important for calving may exist but remain undetected where drift conditions or discovery conditions are less favourable.

A higher proportion of males was observed among the juveniles stranded in regions D, E and F. This was not apparent in regions A and B and only marginally in region C, revealing a difference in the age-specific sex ratio of porpoises across the North Sea. Relatively more adult females compared to adult males stranded in regions D, E and F and given that these latter areas were also suggested to be important calving grounds, this might be explained by mortality following calving and reproductive stress or by a higher density of adult females. It should be emphasised that the M:F ratio of neonates was also skewed towards males (1.24:1) and that a higher M:F ratio at birth was previously suggested by others (Lockyer & Kinze 2003; Ólafsdóttir et al. 2003).

In mammals, it is not uncommon to find higher mortality in juvenile males compared to juvenile females (Clutton-Brock et al. 1985a,b; Clutton-Brock & Isvaran 2007). The region-specific difference in sex ratio warrants further investigation and only allows for speculation at this stage. The most optimal habitat for harbour porpoises is yet to be defined. Due to their relatively small size, limited ability to store energy, and high energetic requirement, harbour porpoises are sensitive to even short-term decreases in food availability. In addition, prey quality rather than quantity is suggested to be an important determinant of foraging strategies (Spitz et al. 2012). Energy-budget population models for pilot whales (*Globicephala melas*) suggest that vulnerability to disturbance increases in areas, or during periods, with lower resource availability and strongly depends on population density (Hin et al. 2019). This is likely also the case for other cetacean species, including the harbour porpoise. Human activities are highly concentrated in the North Sea, where nearly all known anthropogenic stressors to marine mammals occur and overlap. There are indications that the southern North Sea specifically is an area of higher disturbance compared to the more northern areas (Halpern et al. 2008, 2015). Von Benda-Beckmann et al. (2015) reported on the impact of underwater clearance of unexploded ordnance on harbour porpoises in the Dutch sector of the North Sea and indicated that detonation events occurred only in the most southern part. The English Channel is an area with higher marine traffic densities than other North Sea areas (NorthSee 2016; Wisniewska et al. 2016, 2018). This could result in a higher mortality rate in this area or reflect a less optimal habitat compared to other North Sea regions. Consequently, the southern North Sea may be inhabited by the weaker population groups, i.e., juvenile males or compromised individuals, and reflects a possible population sink; a phenomenon described for other species and taxa (Clutton-Brock et al. 1985a,b; Mosser et al. 2009; Pulliam 1988; Swennen 1983). Another hypothesis is that this finding reflects higher dispersion of males. Sex-specific dispersal is known to occur in other odontocetes like the sperm whale (*Physeter macrocephalus*) (Lyrholm et al. 1999) and bottlenose dolphin (*Tursiops truncatus*) (Connor et al. 2000). To assess this further, additional data on causes of mortality and health parameters such as nutritional condition, incorporated with data on prey abundance and anthropogenic activities, would be essential, although causality would be difficult to determine. An assessment of genetic variation of stranded animals along the North Sea coastline, as well as the more southern areas of the harbour porpoise (the French, Spanish and Portuguese coastlines) could additionally provide valuable insights into such hypotheses (Manlik et al. 2019).

Strandings as a surveillance tool

Population abundance surveys are mostly used as a monitoring tool for wildlife species. However, the timely detection of declines in marine mammal abundance requires intense levels of survey effort and currently there is fundamentally no reliable method to detect precipitous declines in most whales, dolphins and porpoise populations (Taylor et al. 2007; Tyne et al. 2016). Increasing the extent and frequency of surveys would improve the chances of detecting declines but surveys are limited by the resources made available for them (Tyne et al. 2016). Three large-scale multinational abundance estimate surveys (SCANS) targeting harbour porpoises among other cetaceans have been conducted in European Atlantic waters, including the North Sea (Hammond et al. 2002, 2013, 2017). The dedicated SCANS surveys were run along particular transects during summer months at decadal intervals. Due to this temporal restriction, seasonal differences in distribution are not captured and precipitous changes would likely go unnoticed. Differences in seasonal distribution of harbour porpoises in the North Sea occur (Geelhoed & Scheidat 2018; Gilles et al. 2011, 2016). Without the ability to quantify these, significant consequences for species conservation can be expected, specifically when assigning protected areas or aiming at minimising disturbance from anthropogenic activities.

Strandings data, which are continuously recorded, have a high temporal resolution and cover areas that cannot easily be surveyed through other monitoring strategies. The strandings data, however, have innate biases, as is the case with many wildlife population monitoring methods. A stranding event is a result of physical, social and biological processes (Peltier et al. 2013; Saavedra et al. 2017; ten Doeschate et al. 2017) and whilst an unusual increase or decrease in strandings could reflect a change in abundance and mortality, it may also be a function of variations in environmental, sea and climatic conditions, observer effort or a combination of these factors. Despite robust signals arising from our analyses, it should be emphasised that there is uncertainty regarding the extent to which strandings are representative of the at-sea populations and their distribution. In our study, the majority of the strandings were recorded on beaches facing west, which could be a result of the prevailing westerly winds that are apparent in the North Sea. Variation in coastal geomorphology also likely has profound impact on the rates of retention on the coast and the detection of carcasses. Additionally, it is possible that more strandings occurred than were recorded in earlier years, with the increase in records being a result of development and growth of coastal communities, increased awareness of the stranding schemes, or easier access to technologies to report and record strandings. Reporting biases could exist, either in protocols or in coverage. For example, in the Danish data a reporting bias is likely due to the absence of a dedicated stranding scheme prior to 2008 (Kinze et al. 2018), with effort likely to be higher in summer during the years where a systematic strandings network was not in place. In other North Sea areas, like Lower Saxony (Germany) or Norway, no long-term, dedicated stranding network exist. Reporting effort is additionally prone to fine-scale variation, for example when local awareness increases following targeted knowledge exchange efforts, or after a particular stranding event resulting in significant publicity and subsequent engagement. This can cause spikes in reporting effort but the potential effect of finer-scale influences is difficult to quantify. Despite these biases affecting the data, the use of long-term large-scale datasets, and analytical treatment of these data as undertaken in this study, results in a robust baseline pattern of spatiotemporal variation, resilient to small or short-term variations in mortality, distribution, effort or oceanographic factors.

When we compare the observed trends in stranding numbers with the outcome of the abundance estimates of harbour porpoises in the North Sea (Hammond et al. 2002, 2013, 2017), they both suggest a population shift from north to south. However, this change in distribution does not fully explain the high stranding numbers in the most southerly part of the North Sea (our regions D and E) since 2004. This may reflect a higher mortality rate, although a higher probability of deceased animals that wash ashore cannot be excluded. It is therefore strongly recommended to consider drift as part of further stranding investigations, as previously demonstrated by others (Peltier et al. 2012, 2013; Saavedra et al. 2017). If the increase in strandings in the southern North Sea reflects a larger per capita mortality at a given population size, changes in mortality due to changes in pressures (e.g., fishing or other anthropogenic activities or predatory presence) should be further investigated. Reducing the uncertainty around the use of information on stranded individuals when making population level inferences, e.g., by increasing the statistical credibility of this data, is a necessary next step. Including metrics collected at post-mortem investigations in future analyses on strandings, like information on causes of death, nutritional condition, parasitism, diseases, and immunology, would allow profiling of health status and spatiotemporal variation within these factors. This would facilitate the assessment of potential region-specific or local issues and possibly, the identification of high-risk areas of human-wildlife conflicts at sea.

Using mortality and citizen-science data to inform conservation management

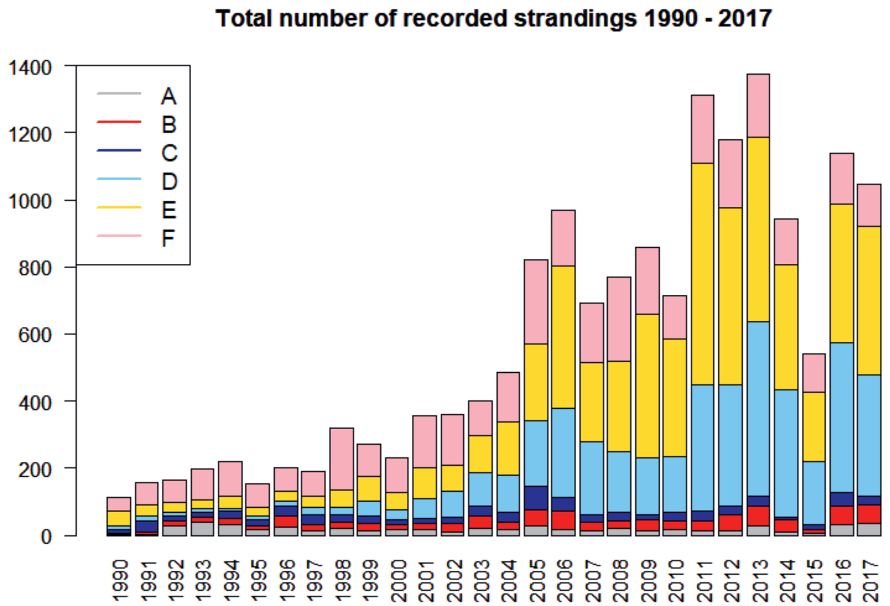
The spatiotemporal scale at which information derived from stranded cetaceans is gathered is not easily recovered by other means of surveillance. When it comes to the conservation of protected species in cryptic habitats or those with low-resource allocation to wildlife monitoring, the assessment of trends in mortality could present a cost-effective alternative or supplement. This is especially true in habitats where animal carcasses are found in sufficient numbers and/or where data exist over a long period, as is the case for harbour porpoises in the North Sea. A similar example can be found in the assessment of the spatial and temporal distribution of three species of ziphiids (beaked whales) in New Zealand, where data and samples collected at stranding events provided new evidence for key habitat use of these otherwise rarely encountered and threatened odontocetes (Thompson et al. 2013). Important population demographic parameters as absolute or relative survival rates can also be estimated based on the recoveries of dead animals, like previously demonstrated in bird banding, where the subsequent recovery of the proportion of dead birds provided information on the survival rates over successive years (Manly 1981). Data collected as part of ecotourism, e.g., by birdwatchers (Horns et al. 2018), or more dedicated citizen-science programmes on a range of taxa, like reptiles and amphibians (Tiago et al. 2017) and terrestrial wildlife (Paul et al. 2014) have been demonstrated to provide robust knowledge on species distribution that successfully informs conservation management. The approach presented in our study provides a model for developing data collection and additional sampling strategies for deceased wildlife and additionally proves useful to provide a wider picture on mortality patterns and trends. Our analysis may therefore serve as a detailed example showing how long-term mortality data of a protected species could be used to assess demographic trends, applicable for wildlife conservation across different environments and taxa.

Acknowledgements

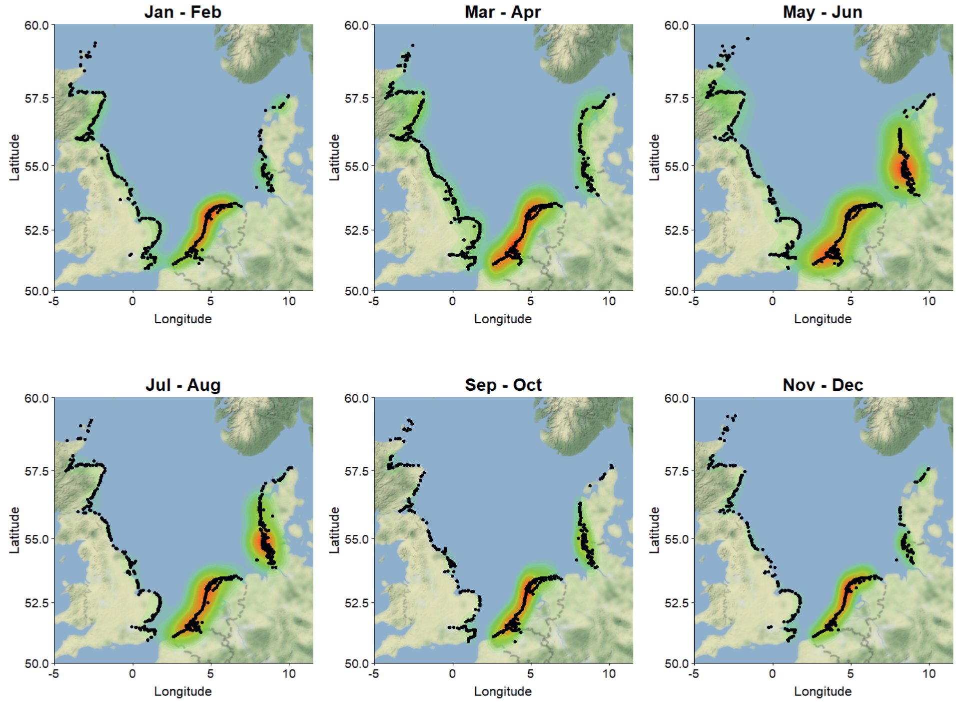
Stranding records were made available by the Royal Belgian Institute of Natural Sciences; the Fisheries and Maritime Museum of Esbjerg and the Natural History Museum of Denmark; the Institute for Terrestrial and Aquatic Wildlife Research, Hannover, Germany; Naturalis Biodiversity Center, Leiden and the Faculty of Veterinary Medicine, Utrecht, The Netherlands; and the UK Cetacean Strandings Investigation Programme (CSIP). We sincerely thank the stranding network volunteers and organisations, whose thousands of hours of reporting and retrieving deceased animals generated the datasets with which we could work. We thank Rijkswaterstaat – Wind op Zee Ecologisch Programma – for funding the analyses (project 31141256), specifically Aylin Erkman and Inger van den Bosch for their valuable help and input. The UK CSIP is co-funded by Defra and the Devolved Governments of Scotland and Wales.

Supporting information

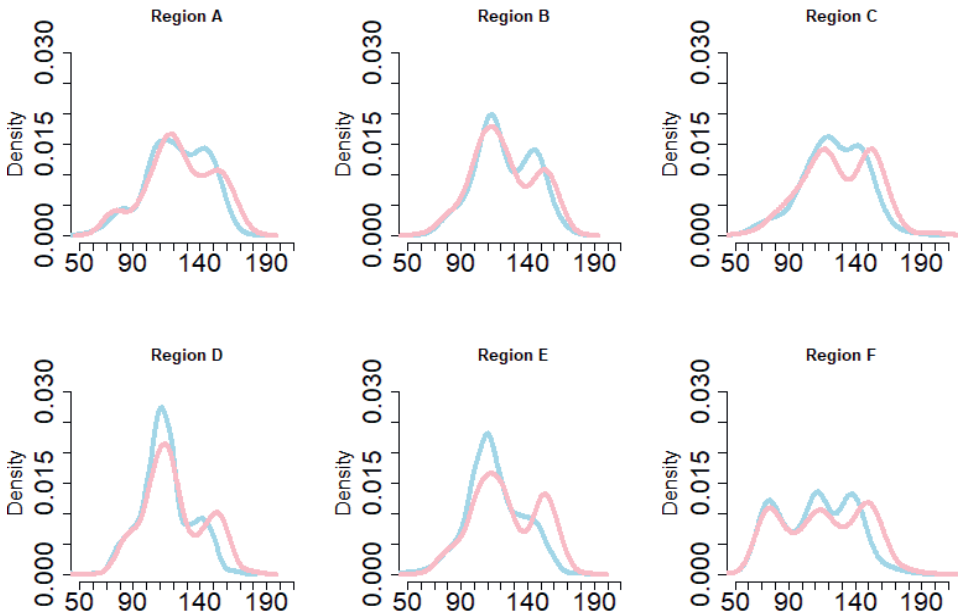
Supplementary data associated with this article can be found in the online version at doi:10.1016/j.biocon.2020.108733.



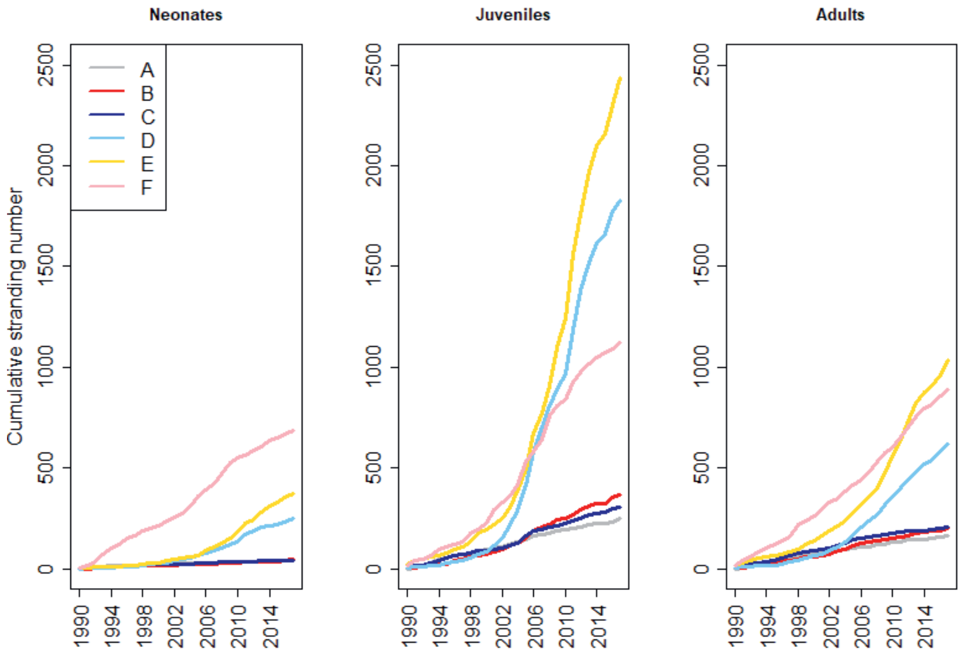
Supplementary Figure 1: *Stacked bar-chart with total numbers of recorded strandings per region (1990-2017)*



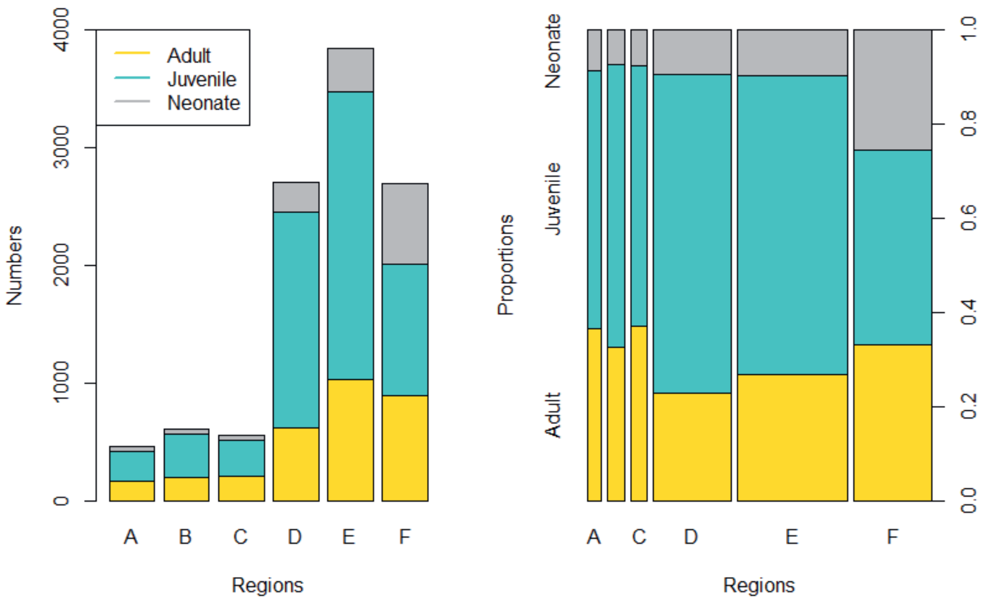
Supplementary Figure 2*: Study area showing the distribution of recorded harbour porpoise strandings per two month interval.*



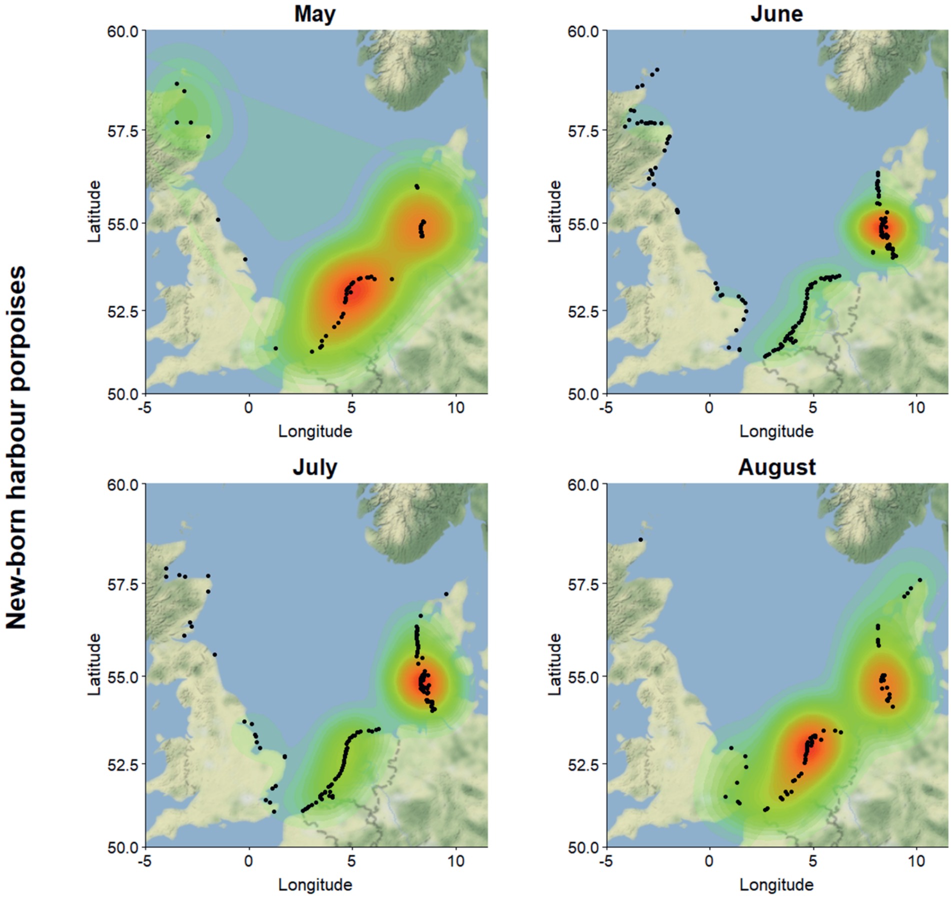
Supplementary Figure 3*: Density graph of total length of recorded stranded harbour porpoises per region. The pink line represents the females and the blue line represents the males.*



Supplementary Figure 4: *Cumulative number of recorded stranded harbour porpoises per region over the study period, presented per age class.*



Supplementary Figure 5: *Age class proportions of stranded harbour porpoises per region. The width of the bars reflect the sample size.*



Supplementary Figure 6: *Harbour porpoise new-borns (with a length of 60-80 cm) stranding frequencies in the study area in May – August (1990-2017).*

4

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Challenges in the assessment of bycatch: Postmortem findings in harbour porpoises (*Phocoena phocoena*) retrieved from gillnets

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Abstract

Bycatch is considered one of the most significant threats affecting cetaceans worldwide. In the North Sea, bottom-set gillnets are a specific risk for harbour porpoises (*Phocoena phocoena*). Methods to estimate bycatch rates include on-board observers, remote electronic monitoring, and fishermen voluntarily reporting; all are not systematically conducted. Additionally, necropsies of stranded animals can provide insights into bycatch occurrence and health status of individuals. There are, however, uncertainties when it comes to the assessment of bycatch in stranded animals, mainly due to the lack of diagnostic tools specific for underwater entrapment. We conducted a literature review to establish criteria that aid in the assessment of bycatch in small cetaceans, and we tested which of these criteria applied upon harbour porpoises retrieved from gillnets in The Netherlands (n=12). 25 criteria were gathered from literature, of which the criteria ‘superficial incisions’, ‘encircling imprints’ and ‘recent feeding’, were observed in the vast majority of our certain bycatch cases. Criteria like ‘pulmonary oedema’, ‘pulmonary emphysema’, and ‘organ congestion’ were also frequently observed, although considered unspecific as an indicator of solely bycatch. Notably, previously mentioned criteria as ‘favourable health status’, ‘absence of disease’ or ‘good nutritional condition’ did not apply to the majority of our certain bycaught porpoises. This may reflect an overall reduced fitness of harbour porpoises inhabiting the southern North Sea or a higher chance of a debilitated porpoise becoming bycaught and could result in an underestimation of bycatch rates when assessing stranded animals.

Introduction

The incidental or unintended capture of cetaceans in fishing nets, hereafter referred to as 'bycatch', is considered one of the most significant threats to marine mammals worldwide (Dolman et al. 2016; Jefferson & Curry 1994; Moore 2014; Reeves et al. 2013). In the North Sea, this problem is particularly related to bottom-set gillnets (Vinther & Larsen 2004). Here, the harbour porpoise (*Phocoena phocoena*) is the most frequently encountered cetacean, both in terms of sightings and strandings (Hammond et al. 2002; IJsseldijk et al. 2020). Harbour porpoises in the North Sea are protected under the EU Habitats Directive and the Agreement on the Conservation of Small Cetaceans of the Baltic and North Seas (ASCOBANS). The latter requirement aims to reduce overall human-induced mortality to below 1.7% of the best population estimate, and specifically to reduce bycatch below 1% (Camphuysen & Siemensma 2011; Scheidat et al. 2018). Assessment of bycatch in commercial bottom-set gillnets in the Greater North Sea indicated that between 1175 and 2126 porpoises are bycaught annually (0.33 – 0.59% of the population), however, the authors highlighted that the underlying effort data was incomplete which likely led to an underestimation of bycatch rates (ICES 2019). Bycatch mortality remains one of the major concerns that may negatively affect harbour porpoise populations (Dolman et al. 2016; IJsseldijk et al. 2018a; Read et al. 2006; Siebert et al. 2001).

There are different methods to estimate bycatch rates and calculate the total number of bycaught marine mammals per fishing metier, area and time. These include on-board observers, remote electronic monitoring (REM) as well as fishermen voluntarily reporting bycatches (Bjørge et al. 2013; Kindt-Larsen et al. 2012; Read et al. 2006; Vinther & Larsen 2004). Collecting representative and reliable data for the relevant fishing fleets has been challenging. While it is known that the primary fishing gear causing bycatch in harbour porpoises and other small cetaceans are bottom-set gillnets (Reeves et al. 2013; Vinther & Larsen 2004), the coverage with observer programs in this (and many other types of) fisheries has been proven inadequate to result in management action (Dolman et al. 2016). Information on bycatch occurrence can alternatively be gained indirectly from post-mortem examinations of stranded animals. Fishermen generally discard bycaught cetaceans (Kuiken 1996). Depending upon environmental factors like wind and tides, a percentage of discarded porpoises will eventually make landfall.

There are uncertainties when it comes to the assessment of bycatch in stranded animals and the biggest challenge is the diagnosis of drowning following underwater entrapment (McEwen & Gerdin 2018). In 2002, drowning was defined by an international expert committee of the World Health Organization as ''*the process of experiencing respiratory impairment from submersion/immersion in liquid*'' with outcomes being ''*death, morbidity and no morbidity*'' (van Beeck et al. 2005). Death by drowning may occur when fluid is aspirated, resulting in respiratory obstruction and asphyxia, and changes secondary to fluid aspiration. However, aspiration of fluid does not always occur, e.g., due to laryngospasm, resulting in death from the combined effects of asphyxia (van Beeck et al. 2005). Drowned cetaceans frequently do not have grossly apparent or voluminous seawater within their lungs, an observation that has been attributed to their diving adaptations (García-Hartmann et al. 1996; Jepson et al. 2000; Knieriem & García-Hartmann 2001). The eventual cause of death in submerged marine mammals is therefore most often hypoxia. Hypoxia and subsequent asphyxiation, causes gross and histological changes to e.g., the heart and lungs (Jepson et al. 2000; Telle & Betbeze 2015). However, the presence of these changes in dead, stranded cetaceans may not solely be caused by drowning due to underwater entanglement. This highlights the need to assess further criteria that aid in the diagnosis of drowning following bycatch.

Throughout the past decades, several studies have established criteria for the diagnosis of drowning following bycatch in cetaceans and the pathogenesis of 'death by submersion' is based on the presence or absence of a number of characteristics (Bernaldo de Quirós et al. 2018; Jepson et al. 2013; Kuiken 1996). However, pathological findings in bycaught individuals may vary depending on local fishery practices and gear used, and also anatomical features of species involved, and therefore differ geographically (García-Hartmann et al. 1996). In this study, we firstly conducted a literature review to establish criteria that aid in diagnosing underwater entrapment in small cetaceans, and secondly, we tested which of these criteria can be applied upon certain bycaught harbour porpoises that were retrieved from gillnets in The Netherlands. The ultimate aim is to appoint which criteria aid in the assessment of bycatch in small cetaceans from the southern North Sea area and should be used to assess the prevalence of bycatch among stranded individuals.

Materials and methods

Review of bycatch criteria

The 'Proceedings of the Second European Cetacean Society Workshop on Cetacean Pathology: Diagnosis of By-catch in Cetaceans' were used to establish the baseline of bycatch criteria for deceased small cetaceans (Kuiken 1996). In addition, a literature search was conducted, and we selected studies post 1996 that presented post-mortem results of bycaught small cetaceans (delphinids or phocoenids), which were directly retrieved from gillnets. These publications were reviewed for descriptions of post-mortem findings in bycaught animals, or for criteria for which these animals were assessed, and these findings were tabulated. Criteria listed in the *Diseases of Aquatic Organisms* theme section on 'Criteria and case definitions for serious injury and death of pinnipeds and cetaceans caused by anthropogenic trauma' were additionally included (Moore et al. 2013a). Publications that solely described post-mortem findings in stranded animals, or those describing post-mortem findings from bycaught animals in other types of fisheries were not included.

Bycaught harbour porpoises

Since 2008, post-mortem examinations on marine mammals are conducted at the Faculty of Veterinary Medicine, Utrecht University in The Netherlands. Harbour porpoises included in this study were all bycaught animals that were directly obtained from fishing nets/fishermen. Between 2008-2019, four harbour porpoises were available for examination following entanglement in gillnets. Additionally, from June 2013 to June 2017, fourteen Dutch commercial bottom-set gillnet fishing vessels were equipped with closed-circuit television cameras to assess bycatch rates (Scheidat et al. 2018). During this project, eight bycaught harbour porpoises were brought ashore for post-mortem examination. All harbour porpoises were caught in bottom-set single-walled gillnets and trammel nets in Dutch coastal waters (Supplementary Figure S1). One of the animals, however, was in an advanced state of decomposition and it was deemed most likely that this animal was caught when already dead (see discussion).

Necropsies of bycaught animals

The necropsies were conducted following internationally standardised guidelines (IJsseldijk, Brownlow & Mazzariol 2019). The following data was collected from all cases: date and location, sex, total body length (measured from the tip of the rostrum to the fluke notch, in a straight line next to the body, in cm), and mass (in kg). All animals were given a decomposition condition code (DCC), from DCC1 representing very fresh carcasses to DCC4 representing severely autolytic carcasses. The nutritional status was visually assessed based on the dorsal musculature, presence of visceral fat and blubber thickness. The latter was measured immediately anterior to the dorsal fin at three locations (dorsal, lateral and ventral, in mm). A nutritional condition code (NCC) was appointed on a six-point scale with 1 representing very fat and muscular animals and 6 emaciated animals. Based on total length, animals were assigned an age class, with animals <91 cm classified as neonates, 91 cm to 130 cm considered juvenile, and >130 cm classified as adults. Age was estimated for animals <120 cm as either 1 or <1 depending on the month it was caught, with May as cut-off month, and determined for cases >120 cm following earlier published methods (Hohn & Lockyer 1995).

The organs of all cases were examined grossly. Parasite presence and severity were scored for four major organs (lung, liver, stomach and middle ear/sinuses) as none, mild, moderate and severe (Siebert et al. 2006b; ten Doeschate et al. 2017). From the animals which were in DCC1-2 at the time of necropsy (n=10), a range of tissue samples were collected for histopathology, fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned at 4–7 µm, stained with haematoxylin and eosin (HE), and microscopically examined. Photographs of external features taken during necropsies, and gross- and histopathological reports were retrospectively assessed for the criteria listed following the literature review. In addition, from cases for which paraffin-embedded tissues were available, skeletal muscle was stained with phosphotungstic acid-haematoxylin (PTAH) (Pernick 2020), to assess striated muscle fibres (n=8) and lung tissue was stained with Gomori silver (Knieriem & García-Hartmann 2001), to reveal any damage to the reticulate fibres of the lungs (n=9). Control tissues of a harbour porpoise which was euthanised following live stranding were included in the PTAH and Gomori silver staining process.

Diet analysis

Prey remains from stomachs were examined following earlier published methods (Leopold et al. 2015b). In short: first and foremost, fish sagittal otoliths were used to identify fish species and to estimate fish length and weight. Prey mass was back calculated (Leopold et al. 2015a). A total of nine different prey species were found and these were subsequently grouped into two prey guilds: demersal and pelagic (Leopold et al. 2015c). The percentage of demersal prey mass (in grams) out of the total reconstructed preys mass was calculated for each porpoise.

Inner ear analysis

The four inner ears of two very fresh individuals (sampled and fixed within 18 hours post-mortem) were collected following earlier published methods (Morell & André 2009). The ears were transported to the University of British Columbia (UBC), Canada for analysis, with appropriate CITES permits (14NL220354/12, 14CA01694/CWHQ-1, 16NL231424/12 and 16CA02279/CHWQ). The inner ears were processed for scanning electron microscopy (SEM) and histopathology in the first individual, and for SEM and immunofluorescence for the second individual, following previously optimised protocols (Morell et al. 2015, 2017a,b, 2020. The sensory cells of the organ of Corti were labelled with anti-prestin (Santa Cruz, USA SC-22692, 1:200) and anti-myosin VI (Proteus Biosciences 25-6791, 1:500) antibodies and type I afferent innervation was labelled with anti-neurofilament 200KD (Sigma-Aldrich N0142, 1:400). Nuclei were counterstained with DAPI (4', 6-diamidino-2'-phenylindole, dihydrochloride; Thermo ScientificTM 62247). The inner ears were observed using a S-4700 SEM and an Olympus FV1000 confocal microscope at the UBC Bioimaging Facility.

Results

Review of bycatch criteria

A total of 25 criteria that may aid in the assessment of bycatch were retrieved from literature, subdivided into four main topics and tabulated (Table 1, references in Supplementary Table 1). These four main topics were: findings related to the drowning process, findings related to contact with or hauling of the net, findings related to disentanglement of bycaught animals, and findings related to the health status of the bycaught individuals.

Table 1. *Bycatch criteria retrieved from literature (for references see Supplementary file 1).*

Criteria	Description
1: Findings related to the drowning process	
Hyphaema	Haemorrhage in the anterior chamber of the eye, with the eye grossly appearing as red, bulging and bloody.
Pulmonary oedema	Blood-tinged or white foam, or fine persistent froth in trachea or elsewhere in the respiratory tract (sometimes protruding from blowhole). Lungs may be heavy and voluminous.
Pulmonary emphysema	Focal or generalised emphysema, e.g., large air cavities in the lung parenchyma.
Pleural petechiae or pulmonary haemorrhage	Pinpoint haemorrhage in the lung pleura following rupture of capillaries or in the lung parenchyma.
Changes in reticulum fibre structures of lung	Histological changes include distention and rupture of reticular fibres (confirmed using Gomori silver stain or comparable)
Regurgitation of food	The presence of partly digested stomach content in the upper alimentary tract.
Epicardial petechiae	Pinpoint haemorrhage in the epicardium following rupture capillaries.
Organ congestion	Organs to assess are: adrenal, brain, heart, kidneys, liver, lung or spleen.
Disseminated congestion	Congestion of two or more organs as listed above.
Presence of foreign material in lungs or bones	E.g., marine flora and fauna (diatoms), microscopically visible in airways.
2: Findings related to contact with or hauling of the net	
Superficial incisions in edges of the mouth, fins or tail(stock) / fluke	Incisions (nicks and notches), mostly superficial penetrating the epidermis, sometimes also the underlying tissue. There could be underlying haemorrhage but this is not inclusive. In lip cuts: tooth can be missing. Morphologically, no signs of chronic tissue response (e.g., inflammation), although histopathological assessment of lesions is needed for confirmation.
Encircling lesions, anywhere on the body	Sharp-edged line imprints, mostly around the rostrum, head or around extremities, sometimes resulting in incisions in the edges (see above).
Subcutaneous haemorrhage/ contusions	Haemorrhage in the subcutaneous tissue, e.g., on the skull (mainly peri-mandibular) or peri-scapular.

Intramuscular haemorrhage, myofiber degeneration or skeletal muscle contusions	Haemorrhage in epaxial and hypaxial muscles, and in thoracic rete mirabile. Histological changes include myofiber degeneration or myopathy (confirmed using PTAH stain or comparable).
Disseminated gas bubbles	Vascular and interstitial gas bubbles as a consequence of post-mortem off gassing from hauling out deceased animals with nitrogen saturated tissues. Diagnosis should be done along with histologic absence of bacteria and autolytic changes.
Pneumothorax	(In)complete collapse of the lung(s), with air- or gas-filled space in the thorax.
(Acute) skull or rib fractures	Likely occurring when the net is hauled, therefore most likely post-mortem.
3: Findings related to disentanglement of bycaught animals	
Amputations	Straight (sharp-edged) cut-off extremities (mostly the tail/fluke), likely post-mortem to release the animals from net.
Presence of fishing material (netting, rope) around body extremities	The actual presence of fishing gear, e.g., rope or monofilament materials on the animal.
Gaff marks	Angular mutilation(s), could be anywhere on the body, likely post-mortem to move carcasses aboard.
Penetrating incision wounds or lacerations into body cavities	Post-mortem cuts (tissue tears) into the body cavities to release gas, possibly induced when releasing an animal from the net and/or to make a carcass sink.
4: General health status of bycaught animals	
Good nutritional condition	Well-developed musculature, thick blubber-layer, there may be visceral fat.
Low incidence of parasitism	Parasitism is common in small cetaceans, including harbour porpoises, in respiratory-, gastrointestinal tracts, liver and ears, often related to health status (Siebert et al. 2001, 2006b; ten Doeschate et al. 2017).
Exclusion of other cause of death	In relatively fresh cases where full pathological examination is conducted, other causes of death or ante-mortem changes that significantly debilitated an animal should be assessed. Bycatch is a peracute cause of death, and in general animals dying as bycatch are in a good health status and lack significant disease (Kirkwood et al. 1997; Siebert et al. 2006b).
Recently ingested gastric content	The presence of partly digested prey items (whole prey, partially digested ingesta, like hard parts as otoliths, squid beaks or skeletons) in the upper alimentary tract or stomach.

Post-mortem results of the bycaught harbour porpoises

Ten bycaught porpoises were juveniles, ranging in size from 93.5-117 cm. These were seven males and three females. The other two harbour porpoises were adults: a ten-year old pregnant female of 141 cm and an eight-year old resting female of 171 cm. All animals were in a (very) fresh decomposition condition (DCC1-2) at the time of retrieval from the net, with the exception of the adult resting female, which had a DCC4. One very fresh animal was temporarily frozen at -20 °C prior to further examination (for full details on all cases, see Supplementary Table 2-3).

Findings related to drowning process

Hyphaema could be assessed grossly in eleven porpoises and was unilaterally present in three and bilaterally in two other cases (in total: 5/11, 45.5%). Pulmonary oedema, where the alveolar lumina are filled with pale-eosinophilic liquid, was both grossly and histologically seen in all cases in which this could be assessed (10/10, 100%), pulmonary emphysema was histologically detected in eight (8/10, 80%), and pleural petechiae or pulmonary haemorrhage in three porpoises (3/10, 30%). Digested food remains in the upper alimentary tract, indicative of regurgitation, were observed in four cases (4/11, 36.4%). Epicardial petechiae were histologically observed in two cases (2/10, 20%). Organ congestion was histologically observed in one or more organs for all cases (10/10, 100%), mostly in the lungs (9/10, 90%), adrenal and kidneys (both 8/10, 80%). Additionally, congestion was seen in brain (6/9, 66.7%), heart and spleen (both 5/10, 50%), and liver (4/10, 40%). One case presented congestion solely in the lung, with all other cases presenting disseminated congestion, ranging from two to all scored organs affected. No foreign material was observed grossly or in histological slides of lung tissue of any case. Changes in reticulum fibre structures of the lungs were deemed unspecific, with the Gomori silver stain not adding information in addition to the HE stain (Supplementary Table 3).

Findings related to contact with or hauling of the net

Incisions were present on the extremities of all cases (12/12, 100%): on the pectoral fins (12/12, 100%), dorsal fin and fluke (10/12, 83.3%), and on the edges of the maxilla and/or mandible (7/12, 58.3%). Grossly, the severity and extensiveness of these incisions varied between animals (Supplementary Table 4; Fig. 1-6). In the DCC4 case (#9), imprints were apparent on the dorsal fin, pectoral fins and fluke, although sloughing of the epidermis as a result of post-mortem changes was also apparent. Histologically, lesions showed focal loss of epidermis in all cases assessed (n=9) with tissue response, mostly mild amount of haemorrhage, in six cases (Supplementary Table 3). Encircling imprints were grossly present on eight cases (8/12, 66.7%), on the rostrum (n=6) or head (n=2). Subcutaneous haemorrhage was observed in four animals (4/12, 33.3%), in each animal at a different location: caudo-dorsally of the skull; on the mandible or submandibular, on the head and on the scapula. Myofiber degeneration was mild and only observed in one of the skeletal muscles (*M. longissimus dorsi*) stained with PTAH, while the control muscle of the live stranded and euthanised harbour porpoise stained positive. One animal had an acute, ante-mortem unilateral mandible fracture (1/12, 9.1%). Grossly, there were no prominent amounts of gas bubbles noted. Retrospective histologic assessment for liver, spleen, heart and lymph nodes, revealed only one animal with very few gas bubbles in the pre scapular lymph node. No animals demonstrated a pneumothorax (Supplementary Table 3).

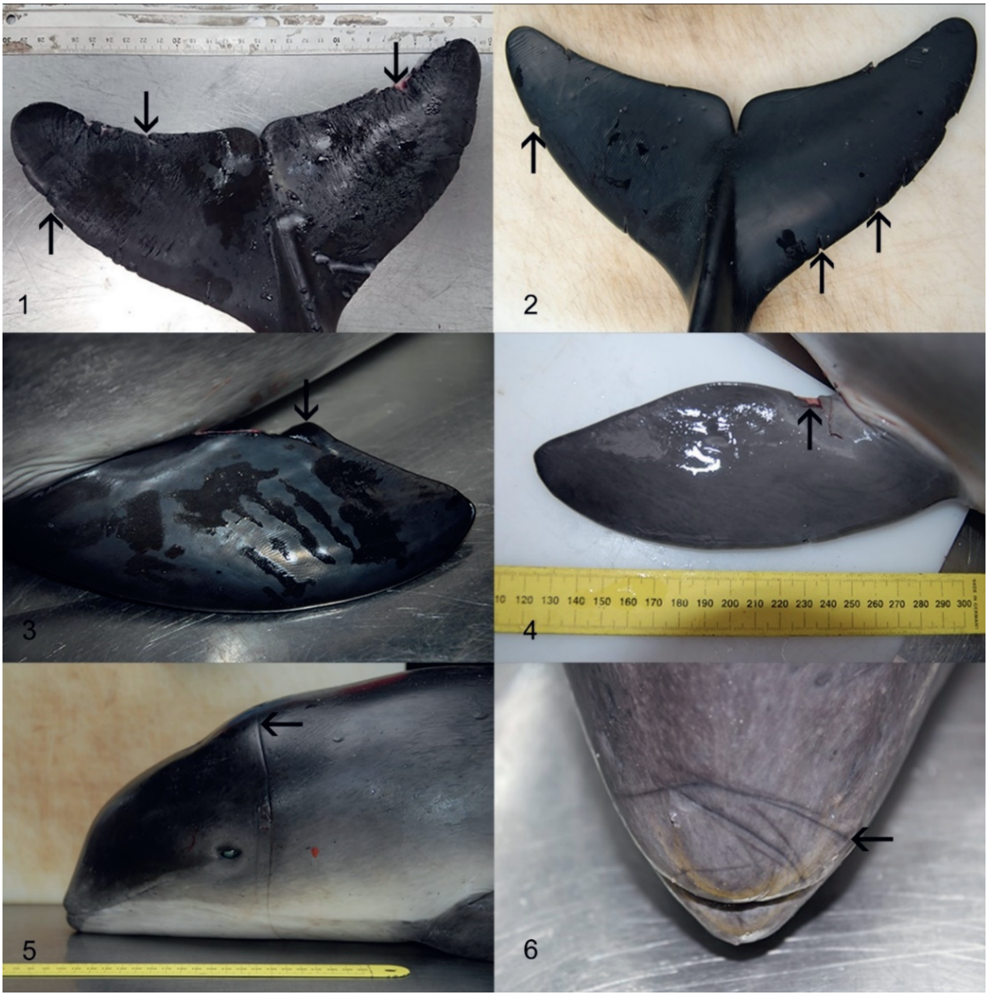

Figure 1. *Fluke, case #12. Incisions in the edges of the fluke (arrows).* ***Figure 2.*** *Fluke, case #2. Incisions in the edges of the fluke (arrows).* ***Figure 3.*** *Pectoral fin, case #11. Loose hanging epidermis (arrow) on the left pectoral fin.* ***Figure 4.*** *Pectoral fin, case #5. Incision and loss of epidermis (arrow) on the right pectoral fin.* ***Figure 5.*** *Head, case #3. Encircling imprints on the head (arrow).* ***Figure 6****. Rostrum, case #8. Encircling imprints on the rostrum (arrow).*

Findings related to disentanglement

In none of the animals, fishing material was still present upon arrival at the faculty. None presented with gaff marks, amputations or penetrating incision wounds or lacerations into the thoracic or abdominal cavity (Supplementary Table 3).

Health status of bycaught individuals

Nutritional conditions varied, with five animals in a good nutritional condition (NCC1-2), five in a moderate nutritional condition (NCC3-4) and two emaciated animals (NCC6) (Supplementary Table 2). The parasitic infestation was higher in lungs and ears compared to stomach and liver, but generally mild to moderate (Table 2). Verminous bronchopneumonia was diagnosed in nine cases, with a suspected bacterial coinfection (based on histology) in four (Fig. 7). Other significant morphological changes included encephalitis in one animal, myocarditis in another and mandibular osteomyelitis following a previous trauma in another porpoise (Fig. 8-9). Additionally, moderate to severe dermatitis was diagnosed in four porpoises and chronic hepatitis in two. No ancillary testing was conducted to establish aetiologies of detected infections. No significant lesions were observed in the one animal which was frozen prior to the necropsy. However it should be noted that freezing artefacts may have hampered the diagnosis of microscopic lesions.

***Table 2**. Parasite infestation in certain bycatch cases in the lungs, stomach, liver, and ears.*

Parasite infestation	None *N=*	Mild *N=*	Moderate *N=*	Severe *N=*
Lungs	3	4	5	0
Stomach	10	0	1	1
Liver	11	1	0	0
Ears	2	2	2	4

The SEM analysis of one of the inner ears revealed unilateral impaired hearing with severe haemorrhage due to parasite migration into the right cochlea (details previously published: Morell et al. 2017a) and an additional focal haemorrhage in one cochlea of the other individual.

The stomach of the DCC4 adult female was empty. The other eleven stomachs were assessed for food remains, revealing an overall percentage of demersal prey of 98%. The number of prey items varied, with a minimum of n=15 prey items and a maximum of n=1679 (prey species composition, total prey numbers and reconstructed masses are given in Supplementary Table 5).

Based on the state of decomposition, it was believed that one of the emaciated animals was bycaught when already dead. When excluding this case, in four out of eleven bycaught harbour porpoises no significant gross or histologic lesions could be detected that could have resulted in debilitation of the animals and therefore these were considered healthy at the time they were bycaught (4/11, 36%). The other seven animals, however, presented signs of disease or debilitation, among which four (36%) suffered from severe, or extensive, significant morphological changes that likely negatively affected these animals.

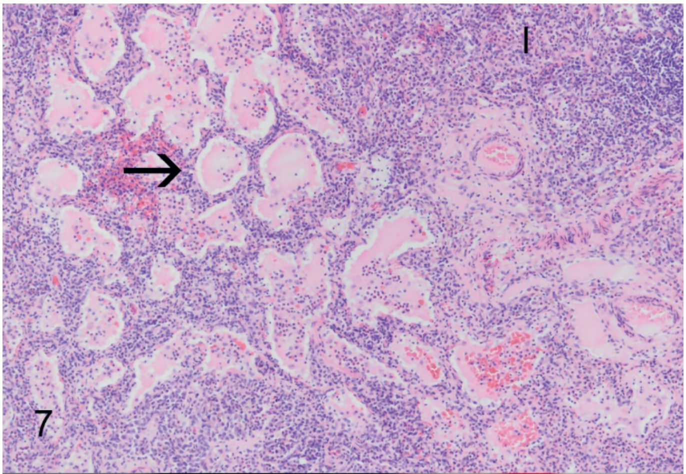

Figure ***7.*** *Pneumonia and alveolar oedema, lung, harbour porpoise, case #6. There is alveolar oedema (arrow) and interstitial infiltrates (I). Haematoxylin and eosin.*

Figure ***8.*** *Mandibular osteomyelitis, harbour porpoise, case #12. Displacement of right mandibular bone.*

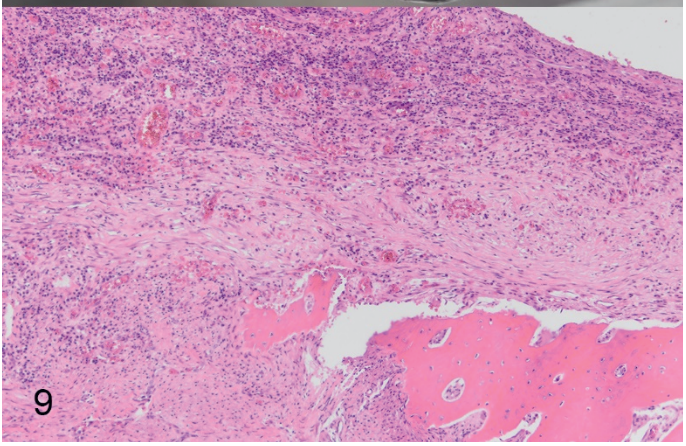

Figure ***9.*** *Mandibular osteomyelitis, harbour porpoise, case #12. Chronic-active osteitis in the right mandibular bone, with loss of bone tissue, fibrosis and mixed inflammatory reaction of lymphocytes and neutrophils. Haematoxylin and eosin.*

Discussion

A total of 25 criteria that aid in the assessment of bycatch in small cetaceans were gained from literature (Table 1, references in Supplementary Table 1) and twelve harbour porpoises which were bycaught in gillnet fisheries in Dutch coastal waters were retrospectively assessed for the presence of these conditions. Eight criteria (pulmonary oedema and emphysema, single organ congestion, disseminated congestion, presence of superficial incisions on extremities, presence of encircling imprints, low incidence of parasites, and the recent uptake of food, with a dominance of demersal prey) were seen in (over) two-third of these certain bycatch cases, with the other 17 criteria diagnosed less frequently. Results are discussed per topic in the subchapters below, in particular in light of the applicability of the assessment of each bycatch criterium in stranded harbour porpoises.

Findings related to the drowning process

Pulmonary changes and congestion of organs were present in the majority of the bycaught porpoises but are solely not indicative of bycatch nor drowning. Lung oedema (where the alveolar lumina are filled with pale-eosinophilic liquid) is also frequently observed in live stranded cetaceans and animals dying of predatory attacks (Haelters et al. 2012), and may also occur post-mortem, following intrinsic contractions of the atrio-ventricular and sino-atrial nodes in the heart (Durlacher et al. 1950; Jepson et al. 2013). Pulmonary emphysema occurs as well in association with pulmonary disease. Pneumonia is one of the most common diagnosis in stranded harbour porpoises (Jepson et al. 2000; Siebert et al. 2001), and was detected in varying severity in nine animals in this study (Supplementary Table 3). Organ congestion may also occur following euthanasia (Jepson et al. 2013), or various physiological processes (Mosier 2017). A study on drowned and non-drowned dogs concluded that pulmonary congestion, oedema, and haemorrhages in the lung were present in both groups and therefore deemed an unreliable indicator of drowning (Piegari et al. 2019). In addition, changes in reticulum fibre structures in lung tissues as histological criteria for death by drowning (Knieriem & Garcíá-Hartmann 2001; Reeves et al. 2013) could not be confirmed in other investigations (Heinen & Dotzauer 1973), nor this study.

Hyphaema was significantly related to drowning of cetaceans by others (Bernaldo de Quirós et al. 2018; Haelters et al. 2012), and was observed in 5/11 of the cases included here. It presumably results following systemic hypertension which may occur as result of hypoxemia during drowning. However, it cannot be ruled out that this was a result of blunt force trauma (Telle & Betbeze 2015), as two of the porpoises with hyphaema also presented subcutaneous haemorrhage on the head (Supplementary Table 3). As severe and prolonged physical struggle while being trapped in gillnets is not expected (see below), there is a possibility that blunt force trauma occurred during e.g., landing of the net onboard the fishing vessels when animals were still alive, making hyphaema also a criterium that fits under “findings related to contact with or hauling of the net”. Subconjunctival haemorrhages have been described in animals stranded in association with acoustic disturbances (Fernández et al. 2005), following canid- (Bernaldo de Quirós et al. 2018) or grey seal attacks (Haelters et al. 2012), or ocular blunt trauma (Ressel et al. 2016), and may occur following altered intra-thoracic or intra-abdominal pressure to veins and capillaries of the head and neck region (e.g., due to vomiting, parturition, coughing) (Dettmeyer 2011). Hyphaema as a single criterium can therefore be considered unspecific in bycatch assessment, although informative upon the diagnosis of a number of causes. Importantly, the absence of this criterium does not rule out drowning due to bycatch, as more than half of our cases did not present hyphaema.

Other criteria under this topic (pleural petechiae or haemorrhage, epicardial petechiae, regurgitation of food and presence of foreign material in lungs) were seen in less than one third to none of the bycaught harbour porpoises. Assessment of these criteria in stranded harbour porpoises is therefore deemed uninformative in order to establish bycatch rates. It is worthy to note, however, that in humane forensic pathology, the diatom test is considered by some as the “golden standard” for the diagnosis of drowning (Piette & De Letter 2006), although the utility and reliability of this method is considered controversial by others (Lunetta et al. 2005). In our study, no sign of foreign material was observed in histological slides of lung, however, no further attempt was made to assess specifically diatom presence. This method has proven to be informative upon the diagnosis of drowning in dogs (Piegari et al. 2019) and terrestrial wildlife (Fucci et al. 2017), but less useful in animals that live in the marine environment and have fish in their diet, like otters (Fucci et al. 2017), cetaceans and sea turtles (Rubini et al. 2018) and its applicability for marine species therefore requires further investigation.

Findings related to contact with or hauling of the net

Superficial incisions in extremities and encircling imprints were present on all of the bycaught porpoises and therefore deemed most informative in the assessment of bycatch. All lesions were of an acute nature. Detection of such lesions is, however, strongly influenced by post-mortem changes. Decomposition results in sloughing of skin, as also observed in the DCC4 case in this study, and additionally scavengers may quickly mask external features. When bycaught marine mammals are discarded and eventually strand, they may do so on mussel beds or rocky shores, that could damage the epidermis and underlying tissue, masking the important external features that reflect entanglement. Additionally, transport of carcasses in body bags and subsequent storage prior to post-mortem examination, e.g., in freezers or chillers, could also leave imprints (Jepson et al. 2013). When assessing bycatch in stranded individuals, the likelihood of the occurrence of nicks and notches from other origins, that might mimic net imprints, should therefore always be considered. It is therefore strongly recommended to take photographs of a carcass and its (stranding) location immediately upon the moment that it is found but at least prior to handling the carcass.

Mild myofiber degeneration with minimal loss of visible cross-striation was observed in skeletal muscle of one case. The myofiber degeneration and necrosis with loss of cross-striation was more extensive in the live stranded and euthanised harbour porpoise (control case). The period between capture and death following submersion is likely in the order of minutes (Soulsbury et al. 2008), leaving limited time for animals to overexert, hence explaining the lack of myofiber degeneration. Haemorrhages in subcutaneous areas or intramuscular were also not frequently detected, indicating the lack of severe physical struggle while trapped (Kastelein et al. 1995).

Other criteria listed under this topic, including fractures, pneumothorax and gas bubbles, were seen infrequently or not at all in the bycaught harbour porpoises. In previous studies, the presence of gas bubbles was significantly higher in bycaught marine mammals compared to stranded animals (Bernaldo de Quirós et al. 2018; Moore et al. 2009). Gas bubbles might be induced following hauling of the net, by bacterial and autolytic changes or could result from dissection artefacts (Jepson et al. 2005a; Moore et al. 2009). Only one of the bycaught harbour porpoises (#2), which was a very fresh carcass (DCC1) at the time of the necropsy, presented a minor amount of gas bubbles in a lymph node which could have been a results of hauling of the net in which it was bycaught. However, the lack of this finding in the majority of our cases is most likely explained by a difference in the depth at which the gillnets were set compared to previous studies (Bernaldo de Quirós et al. 2018; Moore et al. 2009). Bycaught cetaceans with evidence of bubbles in the veins and/or tissues came from gillnets set at a depth of around 80m (Moore et al. 2009), while the porpoises included in this study were retrieved from bottom-set single-walled gillnets and trammel nets set in coastal waters of less than 20m deep. Gas bubbles may therefore only form after hauling of nets from greater depths.

Findings related to disentanglement

Criteria listed under this topic (presence of fishing material, amputations, penetrating wounds and gaff marks) were diagnosed in none or less than a third of the bycaught harbour porpoises, indicating that these criteria may not be useful for detecting fisheries mortality in stranded animals. However, we cannot rule out that since video monitoring was involved in a significant proportion of the sample, this may have altered the behaviour of the fishermen (i.e., more careful handling of the bycaught porpoises). Besides, the presence of findings such as amputations and penetrating wounds or gaff marks could also mimic other types of trauma, including those of other anthropogenic origin like ship of propeller collisions (Moore et al. 2013b), or of a natural cause, following predatory attacks by grey seals (*Halichoerus grypus*) (Leopold et al. 2015a).

Assessment of the general health status

Bycaught cetaceans are considered a better representation of the living population than stranded animals (Siebert et al. 2006b), due to the acute nature of this cause of death. Four porpoises in this study did not present any significant other findings and could have been considered 'healthy' at the time of bycatch. In the other cases, abnormalities were observed, with four porpoises suffering of severe or extensive disease or signs of debilitation that likely negatively affected these animals, making their chances of long-term survival questionable had they not been bycaught. Our study highlights the need to conduct systematic health assessments of incidentally caught cetaceans, as demonstrated previously elsewhere (Kuiken et al. 1994a; Lane et al. 2014).

Bycaught porpoises in German waters presented poorer health status compared to those caught in Norwegian and Icelandic waters but comparable health status to animals caught in Greenlandic waters (Siebert et al. 2006b). Ten percent of bycaught porpoises from the German Baltic Sea were emaciated (Siebert et al. 2001, 2006). Parasite infestations in our cases were in line with previously reported loads, with infestation increasing with body size (ten Doeschate et al. 2017). Associated lesions were mild to moderate and solely not considered to directly have caused health problems, except for the one case where the parasite was found in the inner ear. The overall 'low incidence of parasites' in our study is, however, likely biased by the fact that the majority of animals in this study were juveniles. Other studies describing pathological findings in bycaught harbour porpoises did not state significant pathological changes diagnosed as seen here. The proportion of bycaught porpoises in poor health is of concern and warrants further study of the broader population.

Two animals in this study were emaciated, causes were inflammatory processes in multiple organs in one case but undetermined for the other. The latter was the only case without any stomach content and, additionally, the only case in an advanced state of decomposition. It was deemed most likely that this animal was caught when already dead. Information regarding the fishing effort is vital in order to draw such conclusions. In this case, the gillnet was set for <12h and the necropsy was performed the following morning. The state of decomposition of this animal reflects an animal that was dead for a longer time, at least exceeding 32 hours. Hence, occasional claims of fishermen that porpoises were already dead before they were bycaught (Helmond & Couperus 2012), may sometimes be true.

Food remains in the gastrointestinal tract were observed in all animals which died as a result of bycatch (n=11). This represent a good indicator of an acute cause of death, like bycatch, although recent ingestion of prey is also seen in other acute causes of death, including predatory attacks (Leopold et al. 2015a). Undigested prey species in stomachs of stranded but suspected bycatch animals could be indicative of the location of death, regardless of the quantity, and prey species composition therefore informative in regards to the manner of death (Leopold et al. 2015c). This is particularly true when fisheries in a certain geographic area target solely bottom or pelagic species, like in the southern North Sea. Here, bottom-set gillnets are the primary fishery type and demersal prey in stomachs of bycaught porpoises can therefore be expected. Although the majority of porpoises eat demersal prey in the southern North Sea, this is proportionally higher in the diet of porpoises dying in bottom-set gillnets as demonstrated previously (Leopold et al. 2015c). Additionally, stomach content analysis might also reveal clues about potential risk of bycatch. This was demonstrated in a study on bycaught Atlantic white-sided dolphins (*Lagenorhynchus acutus*) in pelagic trawlers, in which their diet was associated to the discarded fish from the trawlers, suggesting this might attracted the animals in the first place (Couperus et al. 2006). Analysing stomach contents as part of post-mortem investigations would therefore aid in the assessment and understanding of the bycatch problem and is therefore recommended. Besides gastrointestinal tract and prey species assessment, the presence of chyle in lymphatic ducts is also a reliable predictor of recent feeding (Bernaldo de Quirós et al. 2018) and should be scored during future necropsies of stranded animals.

Conclusion and recommendation

The diagnosis of drowning relies on circumstances of discovery or inference as well as the exclusion of other fatal conditions, with the pathognomonic lesions for drowning currently still lacking (Lunetta & Modell 2005). Therefore, it is only through the implementation of a range of tests and assessment that the likelihood of drowning following bycatch as a cause of death can be estimated in deceased, stranded cetaceans. The most common findings in the certain bycaught harbour porpoises available for this study were non-specific lesions such as emphysema, alveolar oedema or organ congestion. More specific findings, like incisions and net imprints, were proven good indicators of bycatch. Recently ingested (demersal) food also proved to be a good indicator, at least pointing towards an acute cause of death close to the sea floor. In contrast to previous studies, a favourable health status, the absence of disease or good nutritional conditions were proven poor indicators of bycatch, at least for porpoises caught in coastal waters of the southern North Sea. When assessing bycatch rates among stranded animals, excluding cases with notable pathological findings, e.g., severe parasitosis, poor nutritional conditions and infectious diseases, as possible bycatch mortalities could result in an underestimation of the proportion of strandings related to fisheries. This demonstrates a need for understanding endemic disease and an appropriate calibration of diagnostic interpretations, taking into account a range of tests and investigations to establish the conclusion on the manner and most likely cause of death of stranded individuals.

Bycatch criteria gained from the literature review should be tested and validated when implemented in other geographical areas or fishery types. We stretch the importance of obtaining more bycaught animals from cooperation with the fisheries sector for validation and further calibration of the results, as the number of landed harbour porpoises by fishermen was low. To understand the scale of the bycatch issue, monitoring programs are required (Scheidat et al. 2018). A good cooperation with the participating fishermen is needed to obtain bycaught marine mammals for post-mortem investigations. Governmental support is required to acquire the necessary permits, and to meet the requirements of several international conventions aiming to reduce human-induced mortality levels. Lastly, based on the necropsy results of the animals included in this study, the health status of porpoises in the southern North Sea can, at a minimum, be considered worrying. Measures that exceed fisheries management may therefore be needed in order to protect harbour porpoises in the (southern) North Sea.

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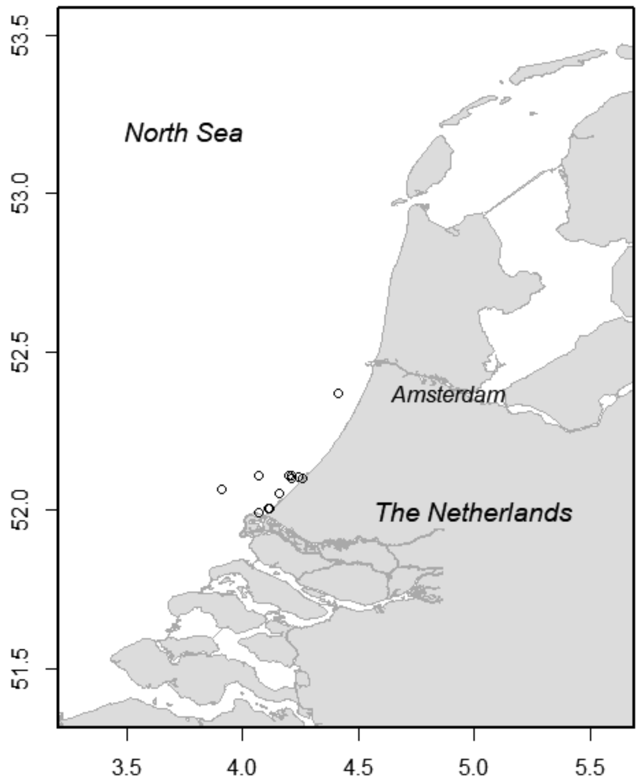
Supporting information

Supplementary data associated with this article can be found in the online version at doi: 10.1177/0300985820972454.



Supplementary Figure 1. *Locations where harbour porpoises were caught*

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A version of this chapter has been published after minor revision in Science of the Total Environment (2021)

Polluted porpoises: Generational transfer of organic contaminants in harbour porpoises from the southern North Sea

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Abstract

Persistent organic pollutants (POPs), including polychlorinated biphenyls (PCBs), polybrominated diphenylethers (PBDEs) and hexachlorobenzene (HCB), bioaccumulate in marine ecosystems. Top predators contain high levels of POPs in their lipid-rich tissues, which may result in adverse effects on their reproductive, immune and endocrine functions. Harbour porpoises (*Phocoena phocoena*) are among the smallest of cetaceans and live under high metabolic demand, making them particularly vulnerable to environmental pressures. Using samples from individuals of all maturity classes and sexes stranded along the southern North Sea (n=121), we show the generational transfer of PCBs, PBDEs and HCB from adults to foetuses. Porpoise placentas contained 1.3-8.2 mg/kg lipid weight (lw) Sum-17PCB), <dl-0.08 mg/kg lw Sum-17PBDE and 0.14-0.16 mg/kg lw HCB, which were similar to concentrations in foetus blubber. Contaminant levels increased significantly after birth through suckling. Milk samples contained 0.20-33.8 mg/kg lw Sum-17PCB, 0.002-0.51 mg/kg lw Sum-17PBDE and 0.03-0.21 mg/kg lw HCB. Especially lower halogenated and more toxic contaminants were transferred to calves, exposing them to high levels of contaminants early in life. Of all animals included in this study, 38.5% had PCB concentrations exceeding a threshold level for negative health effects (>9 mg/kg lw). This was particularly true for adult males (92.3% >9 mg/kg lw), whilst adult females had relatively low PCB levels (10.5% >9 mg/kg lw) due to offloading. Nutritional stress led to higher offloading in the milk, causing a greater potential for toxicity in calves of nutritionally stressed females. No correlation between PCB concentration and parasite infestation was detected, although the probability of a porpoise dying due to infectious disease or debilitation increased with increasing PCB concentrations. Despite current regulations to reduce pollution, these results provide further evidence of potential health effects of POPs on harbour porpoises of the southern North Sea, which may consequently increase their susceptibility to other pressures.

Introduction

Exposure to chemical contaminants is one of the many stressors that may result in adverse effects on the health status of individual animals (Aguirre & Tabor 2008; Harrison et al. 1997; McHuron et al. 2018). Known contaminants of concern to marine wildlife are the lipophilic persistent organic pollutants (POPs), which include polychlorinated biphenyls (PCBs), polybrominated diphenyl ethers (PBDEs), hexachlorobenzene (HCB) and hexachlorobutadiene (HCBD). These POPs are resistant to biological and physical degradation (McHuron et al. 2018), some with half-lives of up to a hundred years, and they bioaccumulate and biomagnify in marine food webs (Jepson et al. 2016; Murphy et al. 2018; Norstrom 2002).

As relatively long-living top predators, marine mammals can accumulate high levels of POPs in their lipid tissue (Desforges et al. 2018; Weijs et al. 2009). As such, they have been the focus of many studies showing associations between POPs and impairment of reproductive, immune and endocrine functions (Desforges et al. 2016, 2018; Jepson et al. 2016; Murphy et al. 2015; Reijnders 1986; Williams et al. 2020, 2021). Exposure of mammalian species to contaminants occurs mainly through dietary intake (McHuron et al. 2018).

The effects of contaminants on the health status of harbour porpoises (*Phocoena phocoena*) in the North Sea have been a particular concern (Hall et al. 2006a; IJsseldijk et al. 2018a; Jepson et al. 2005b; Murphy et al. 2015; Weijs et al. 2009). Harbour porpoises are among the smallest of cetacean species with a relatively thick blubber layer compared to other small cetaceans (Worthy & Edwards 1990). They live on an energetic knife-edge because they have a relatively large body surface to body volume ratio, a high metabolic demand and live in cold-water environments (Murphy et al. 2015; Wisniewska et al. 2016). This makes them vulnerable to environmental pressures impacting their health and reproduction. The Northeast Atlantic houses a continuous population of harbour porpoises ranging from France to northern Norway (Fontaine et al. 2007). At the same time, it is a hotspot of anthropogenic activities, with many coinciding and cumulative stressors (Halpern et al. 2008, 2015), resulting in high disturbance rates for the species living within these waters. High contaminant exposure in harbour porpoises from the North Sea area has previously been associated with high parasitic exposure (Bull et al. 2006; Pierce et al. 2008), increased prevalence of infectious diseases (Hall et al. 2006a; Jepson et al. 2005b, 2016; Mahfouz et al. 2014; Pierce et al. 2008) and reproductive failure (Farré et al. 2010; Jepson et al. 2016; Murphy et al. 2015). More recent studies suggest differences in chemical exposure during the life stages, which potentially negatively affect development and reproduction. Immature harbour porpoises were exposed to more neurotoxic mixtures of PCB congeners than adults (Williams et al. 2020) and testes weights were negatively associated with PCB concentrations and nutritional stress (Williams et al. 2021).

In the southern North Sea, harbour porpoise stranding rates have significantly increased since 2005. This increase was most prominent along the coastline of The Netherlands (IJsseldijk & ten Doeschate et al. 2020). A shift in distribution of harbour porpoises from the northern to the southern North Sea was reported based on two large-scale international abundance surveys in 1994 and 2005 (Hammond et al. 2002, 2013). Although a range shift likely explains the majority of the increase in stranding numbers in the southern North Sea since the 1990s, stranding numbers have been excessively high since 2005, with several periods of temporal elevated mortality levels thereafter (IJsseldijk & ten Doeschate et al. 2020; IJsseldijk et al. 2021a). As the cause(s) of the increase in strandings and mortalities are yet to be determined, there is a need to assess the overall health status of harbour porpoises and their threats in this region.

The aims of this study were threefold: 1) to assess levels of exposure of PCBs, PBDEs, HCB and HCBD in stranded harbour porpoises from the southern North Sea across the different maturity classes and sexes, 2) to assess the importance of placental and lactational transfer of these compounds through the analyses of umbilical cord, placenta, milk and foetus samples and the influence of nutritional status on offloading via lactation, and 3) to investigate the relationship between exposure levels and health status using data on nutritional condition, health status, causes of death and parasite infestation gained from necropsies. This study represents the most recent and comprehensive investigation into generational cycling of persistent organic pollutants in an abundantly stranded small cetacean of the southern North Sea. We provide novel insights into the understanding of generational transfer of contaminants in cetaceans, the health status of harbour porpoises in the southern North Sea, and the threats that they face.

Material & Methods

Collection

Post-mortem investigations

Between January 2006 and September 2019, more than 1800 stranded or bycaught harbour porpoises were collected in The Netherlands for post-mortem investigations, that took place at the Dutch Royal Institute of Sea Research on Texel in 2006 and 2007, and at the Faculty of Veterinary Medicine, Utrecht University, since 2008. Necropsies and tissue sampling were conducted following internationally standardised guidelines (IJsseldijk et al. 2019). For each case, stranding date, location, sex, total length (cm), weight (kg) and blubber thickness (mm) were recorded. Blubber thickness was measured immediately anterior to the dorsal fin at three locations (dorsal, lateral and ventral, in mm). Each case was assigned a nutritional condition code (NCC) which was visually assessed based on the dorsal musculature, presence/absence of visceral fat and through quantitative assessment of blubber thickness.

NCC was appointed on a six-point scale with NCC1 representing very fat and muscular animals and NCC6 emaciated animals. These scores were later grouped with NCC1-2=good condition, NCC3-4=moderate condition and NCC5-6=poor condition.

Sample selection

A total of 112 harbour porpoises that were stranded along the Dutch coast and 9 foetuses were analysed for contaminants, with the samples comprising both sexes and all maturity classes (Supplementary Table (STab) 1 and Supplementary Figure (SFig) 1). These cases were selected based on carcass freshness and, subsequently, the availability of samples for toxicological analyses. During necropsies, blubber samples were collected and stored in aluminium foil at -20°C. Additionally, milk from 14 adult females was collected and stored in glass containers at -20°C, and placenta (n=3) and umbilical cord tissues (n=2) of mother-foetus pairs were collected and stored at -20°C.

Assessment of reproductive status

Following Murphy et al. (2010, 2015, 2018), ovarian samples of adult females in the study were assessed for the presence of ovarian corpora scars (corpus albicans or corpus luteum, CA and CL respectively). These samples were available for 20 of the 38 adult females. After macroscopic assessment, the ovaries were hand-sectioned into 0.5-2 mm slices and examined under a binocular microscope for the presence of additional ovarian corpora scars. Total numbers of ovarian corpora scars were counted, which is taken to represent the number of ovulations. Females were classified as sexually mature if they had one or more ovarian corpora scars. During the necropsies, pregnancy was established by the presence of a foetus. Lactation was assessed via gross examination of the mammary glands. Females were classified into six reproductive states: (1) sexually immature, (2) pregnant (foetus present), (3) pregnant and lactating, (4) sexually mature and lactating, (5) resting mature (not pregnant or lactating), and (6) suspected recently aborted (dilation of uterus outside the calving season).

Preparation

Health status categorisation

For harbour porpoises in a very fresh to moderately putrefied condition, the most likely cause of death was determined based on macroscopic and microscopic examination. Additionally, for all cases the parasite presence and severity of parasite infestation were scored for four major organs (lung, liver, stomach and middle ear/sinuses): 0=none, 1=mild, 2=moderate and 3=severe (ten Doeschate et al. 2017). The porpoises included in this study were subdivided into two categories based on the most significant necropsy findings. The first category includes all individuals that most likely died as a result of bycatch (diagnosed following IJsseldijk et al. 2021b), due to a predatory attack (diagnosed following Leopold et al. 2015a), due to sharp or blunt-force trauma, and due to dystocia (birth problems of full-term foetus) and intestinal volvulus where no signs of significant disease were detected to explain the dystocia or intestinal volvulus. The second category included all individuals that showed signs of significant infectious disease, high parasite loads and/or (very) poor nutritional conditions, and lacked signs of aforementioned 'acute' causes of death (STab 1). Foetuses and neonates were excluded from analyses where cause of death and parasitism were used as explanatory variables.

Maturity class determination
All cases were categorised into a maturity class based on their total length. Individuals <91 cm in length, and which stranded during the peak calving period for the species (May-August), were classified as neonates or immatures. Individuals with total lengths between 91 and 130 cm were considered juveniles or immatures, and individuals with total lengths of over 130 cm were classified as adults or matures. For those individuals at around 130 cm, visual inspection of the reproduction organs was conducted to properly subdivide them into juvenile or adult age classes (Lockyer 2007; Murphy et al. 2020). Further subdivision of mature females into reproductive status categories was done as described under 2.1.3. The *in-utero* unborn calves are referred to as foetuses. The gestation period in the species is estimated to last around 10 to 11 months (Learmonth et al. 2014) and the lactation period may be up to 10 months (Lockyer 2003).

Sample analyses

Sample homogenisation
The epidermis and 'air-exposed' parts of the blubber samples were removed, and the remaining blubber sample was dissected into small pieces. The placenta and umbilical cord samples were homogenised using a blender prior to analysis.

Lipid content
Total extractable lipid (triglycerides and phospholipids) levels were determined in the blubber samples following the Bligh and Dyer (B&D) method, modified by De Boer (1988). In short, samples were extracted three times with a mix of chloroform, methanol and demineralised water. Lipid level was determined by weighing the residue after evaporation of the solvent.

Chemical analyses
Samples were analysed using accelerated solvent extraction (ASE) and gas chromatography coupled to a mass spectrometry (GC-MS) method to quantify PCBs, PBDEs, HCB and HCBD. This method (SOP 2.10.3.050 Biota and environmental matrices: Determination of micro pollutants after ASE extraction and GC-MS detection) was validated for biota according to ISO 17025. Solvents and Florisil were obtained from LGC Standards Promochem. The samples were mixed with sodium sulphate and transferred to an ASE cell containing 25 g Florisil. The ASE cell was extracted three times using pentane/dichloromethane (85/15). After addition of 1 ml of iso-octane as a keeper, the extract was concentrated to 1 ml in a rotary evaporator.

PCBs, PBDEs, HCB and HCBD were then determined by a GC-MS detector. PCBs, HCB and HCBD were measured using a Shimadzu 2010GC coupled with a Shimadzu QP2010 Ultra MS using an Electron Impact (EI) source. Separation was performed over 60m (0.25x0.25) J&W HT-8 column (DaVinci Europe, The Netherlands, manufactured by SGE Analytical Science). Source and transfer line temperature were set to 300°C. 5 µl sample was injected by Large Volume Injection (LVI). The oven program was as follows: start at 95°C, hold for 3 min, 25°C/min to 170°C, 2.5°C/min to 255°C, hold for 10 min, 45°C/min to 325°C. m/z 256/258, 290/292, 326/324, 360/362, 394/396 and 428/430 were used as quantifier and qualifier ions for PCBs while 227/225 was used for HCBD and 286/284 were used for HCB.

PBDEs were measured using an Agilent 6890GC coupled with a 5973MS with NCI source and a 50m (0.25x0.25) J&W CPsil8 column (DaVinci Europe, The Netherlands) for separation. Source and transfer line temperature were set to 200 and 290°C respectively. 1 µl sample was injected by split/splitless injection at 275°C. The oven program was: start at 90°C, hold for 3 min, 30°C/min to 210°C, hold for 20 min, 5°C/min to 290°C, hold for 13 min, 30°C/min to 325°C and hold for 15 min. m/z 81 and 79 were used as quantifier and qualifier ions.

Chemical analysis was carried out according to ISO17025 accredited methods with full quality control procedures such as a blank and reference sample (eel filet). For compounds that were detected in a blank sample the limit of quantification was set to 5x blank value. Compounds in the control sample were plotted in Sheward quality charts and results were required to be within the 2s. Also, the lab participates yearly in proficiency testing with satisfactory scores in the QUASIMEME (Quality Assurance of Information for Marine Environmental Monitoring in Europe) proficiency test scheme. MDL and precision for each compound is available in the supplementary information (STab2). Calibration was performed using certified standards (AccuStandard, The Netherlands) and at least a six-point calibration curve with points between 0.5-1000 ng/ml and $r^2>0.99$. Concentrations below the detection limit were reported as <dl = <detection limit.

A maximum of 28 PCB congeners could be quantified based on the calibration standards, whereas 20-24 PCB congeners were actually detected in the samples. For PBDEs 17 congeners could be quantified based on the calibration standards, and up to 12 congeners were detected in the samples. For the comparison of PCB, PBDE, HCBD and HCB concentrations between harbour porpoises and between different sample types, concentrations were calculated to 100% lipid per sample (mg/kg lipid weight (lw)) using the lipid content of each sample.

Analyses with respect to threshold levels

A Sum-17PCB, based on the most relevant PCB congeners, consisted of congener # 47, 49, 52, 101, 105, 118, 128, 138, 149, 151, 153, 156, 170, 180, 187, 194, and 202. To enable a comparison with the International Council for the Exploration of the Sea (ICES) Sum-PCB, a Sum-7PCB was calculated using congener # 28, 52, 101, 118, 138, 153, and 180.

A Sum-17PBDE, based on the predominant PBDE congeners in the samples, consisted of congener # 28, 47, 49, 66, 71, 75, 77, 85, 99, 100, 119, 138, 153, 154(+153), 183, 190, and 209. To allow comparison with the Environmental Quality Standards (EQS) within the Water Framework Directive (WFD; European Commission, 2013), an additional sum-parameter was selected, and sample concentrations were based on wet weight. This Sum-6PBDE consisted of congeners 28, 47, 99, 100, 153 and 154.

A first risk assessment for PCB exposure in cetaceans was carried out using earlier developed threshold levels for summed PCB concentrations (Jepson et al. 2016; Kannan et al. 2000; Murphy et al. 2015). These threshold levels were based on laboratory results and field studies of a variety of mammalians (for instance Helle et al. 1976; Murphy et al. 2015). The first one consisted of a threshold concentration of 9 mg/kg lw Sum-23PCB (congener # 95, 101, 110, 118, 128, 136, 138, 141, 144, 149, 151, 153, 170, 171, 174, 177, 180, 183, 187, 195, 201, 202, and 203) for the onset of physiological effects in marine mammals in general, based on studies assessing immunological and reproductive effects in seals, otters, and mink (Jepson et al. 2016; Kannan et al. 2000). A second threshold level consisted of a concentration of 11 mg/kg lw Sum-25PCB (congener # 18, 28, 31, 44, 47 ,49, 52, 66, 101, 105, 110, 118, 128, 138, 141, 149, 151, 153, 156, 158, 170, 180, 183, 187, 194) for potential reproduction failure in resting adult female harbour porpoises (Murphy et al. 2015). Although female harbour porpoises with concentrations above this threshold may still become gravid, they probably did not offload their contaminant burdens due to either foetal or new-born mortality (Murphy et al. 2015). A third threshold of 41 mg/kg lw Sum-23PCB (congener # 95, 101, 110, 118, 128, 136, 138, 141, 144, 149, 151, 153, 170, 171, 174, 177, 180, 183, 187, 195, 201, 202, and 203) was used based on profound reproductive impairment in Baltic ringed seals (*Pusa hispida*) (Helle et al. 1976; Jepson et al. 2016).

Summed PCB concentrations, however, do not take into account individual toxicity of PCB congeners and chemical profiles may differ between e.g., age classes and sample types (Williams et al. 2020). Therefore, the chemical profiles of the analysed congeners were also assessed from another perspective, grouping the PCB congeners in two ways. Grouping per degree of chlorination (tri-PCBs, tetra-PCBs, etcetera) provided information on the lipophilicity of a congener, which increases with a higher number of chlorine atoms attached (Safe and Hutzinger 1984). As metabolism is structure dependant, PCBs were also grouped into six Structure Activity Groups (SAGs) based on their capacity to be biotransformed (Boon et al. 1994, 1998; Cullon et al. 2012). SAGs I-II-V are considered highly persistent in cetaceans, whereas SAGs III-IV-VI may be metabolised to a certain extent dependant on the species (Boon et al. 1994; Cullon et al. 2012) (STab 3).

For PBDEs, HCB and HCBD no thresholds for effects on marine mammals have been established. The WFD uses the following EQS for these compounds in biota: 0.0085 µg/kg wet weight (ww) Sum-6PBDE (see above), 10 µg/kg ww HCB, and 55 µg/kg ww HCBD

(European Commission 2013). However, the EQS for these contaminants were defined for fish and the relevance of the EQS for higher trophic levels, such as harbour porpoises remains unexplored.

Data analyses

Data exploration was applied for PCB and PBDE data following the method described by Zuur et al. (2009) prior to analysis. As for HCBs only 47 individuals (6 foetuses, 6 neonates, no juveniles, 22 adult females, 13 adult males) were analysed, this sample size was considered too small for an extensive data analysis. Data exploration, visualization and analyses were performed using R version 3.6.3 (R Core Team, 2017), with package ggplot2 (version 3.3.2), grid (version 3.6.3) and gridExtra (version 2.3). An overview of statistical models is given in STab 4, with descriptions in the following subsections.

5

Generational transfer of PCB and PBDE

A Generalized Linear Model (GLM) was used to assess the log-Sum17PCB exposure in male and female harbour porpoises of different maturity classes (Model 1). The log-Sum17PCB was analysed in relation to the independent indicator variables: sex, maturity class, the interaction between sex and maturity class, and the log-Sum17PBDE. Seven individuals had missing data and were excluded from this analysis. Model selection was undertaken by a stepwise backward selection using Akaike Information Criterion (AIC), where a value difference of >2 was considered an improved model. Residuals were checked for normality by normal probability (QQ) plots. For the variables in the final model, 95% (log-)likelihood profile confidence intervals were calculated.

The average proportion of highly persistent (SAGs I-II-V; STab 3) and less persistent (SAGs III-IV-VI; STab 3) PCBs in blubber was assessed over total length of the harbour porpoises using a linear model (Model 2) and 95% confidence intervals of the model as well as the correlation coefficient (R-squared) were calculated. Additionally, the mean proportion per maturity class was calculated to visualize the PCB and PBDE profiles.

Partial correlation analysis, corrected for maturity class, sex and the interaction between maturity class and sex, was performed for log-Sum-17PCB and log-Sum-17PBDE (Model 3).

Contaminant concentration in adult females

To assess the relation between ovarian corpora scars, porpoise total length, log-Sum17PCB and log-Sum17PBDE concentrations in adult females, a GLM with a Poisson distribution was used with the number of ovarian scars as the dependent variable, and the reproductive status of the female as independent indicator variable (five categories: lactating, pregnant, pregnant and lactating, resting and suspect aborted), total length, log-Sum17PCB in blubber, log-Sum17PBDE in blubber, and an interaction between the reproductive status and total length (Model 4). Lastly, two linear models were used to assess the log-Sum17PCB and log-Sum17PBDE concentrations in milk in relation to total length (Model 5 and 6, respectively).

PCB and PBDE exposure in relation to health parameters
To assess relationships between log-Sum17PCB and necropsy findings as proxies of the health status of the harbour porpoises, a GLM was used (Model 7). The log-Sum17PCB was analysed in relation to the independent indicator variables: maturity class, sex, log-Sum17PBDE, cause of death (two categories: physical trauma and other acute causes versus infectious disease and/or debilitated animals), nutritional condition (NCC, three categories: good, moderate or poor), the four organs scored for parasite infestation (lung, liver, stomach, ear), and an interaction between maturity class and sex, and an interaction between cause of death categories and NCC. Model selection was done by a stepwise backward selection using AIC, where a value difference of >2 was considered an improved model. Residuals were checked for normality by normal probability (QQ) plots. For the variables in the final model, 95% (log-)likelihood profile confidence intervals were calculated.

A binomial logistic regression (Model 8) was conducted to assess the relationship between the Sum17PCB and the cause of death categories of the harbour porpoises. All animals that mostly likely died as a result of infectious disease and/or debilitation were given a score 1 and all animals that most likely died due to physical trauma or other acute causes were given a score 0. Log odds ratios given, and 95% (log-)likelihood profile confidence intervals were calculated.

Results

Contaminant concentrations and profiles in maturity classes

Generational transfer of contaminants
Contaminant concentrations in blubber samples of harbour porpoises ranged from 0.21-90.15 mg/kg lw for Sum-17PCB, <dl-3.24 mg/kg lw for Sum-17PBDE and <dl-0.42 mg/kg lw for HCB (STab 1, STab 5). Concentrations of HCBD were below the detection limit (<0.1 µg/kg) in all analysed samples, except when specifically mentioned. Lowest mean contaminant concentrations were detected in blubber samples of foetuses and adult females (Figure 1). No clear differences in mean concentrations of PCBs and PBDEs were observed between neonates and juveniles. No comparison could be made for HCB as no chemical analysis was performed on samples of juvenile harbour porpoises in this study.

Sum-17PCB concentrations were significantly higher in adult males compared with adult females. All independent indicator variables remained in the final model; maturity class, sex, an interaction between maturity class and sex, and the log-Sum17PBDE (Model 1, AIC= 253.6). 95% (log-) likelihood profile confidence intervals for Sum-17PBDE were 0.60;0.88, for neonates were 0.21;1.61 and for adult males were 0.32;1.70: indicating strong, positive relationships. All 95% (log-) likelihood profile confidence intervals can be found in STab 6.

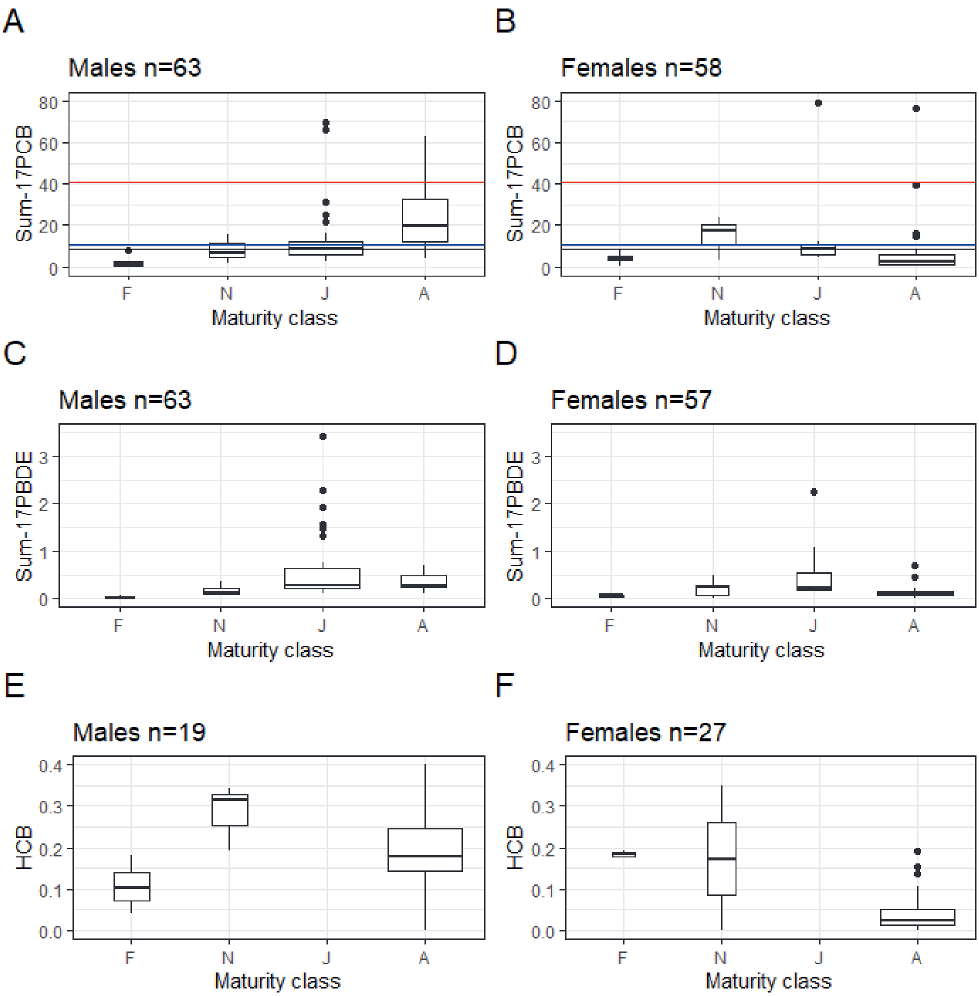


Figure 1. *Average contaminant concentrations (mg/kg lw) in blubber samples of harbour porpoises stranded along the Dutch coast in 2006-2019 per maturity class. In the left panels the males and in the right panels the females. F= foetus, N= neonate, J= juvenile and A= adult. Upper panels (A and B): Sum-17PCB, including threshold levels of 9 mg/kg lw (horizontal black line), 11 mg/kg lw (horizontal blue line) and 41 mg/kg lw (horizontal red line). Middle panels (C and D): Sum-17PBDE. Lower panels (E and F): HCB (no data available for juveniles). The black dots represent the outliers in the dataset. The width of the bars represents the sample size, the horizontal lines the medians, the boxes the first to third quartile, and the tails the minimum and maximum.*

<u>*PCB and PBDE profiles*</u>

The dominant PCB congener groups in the blubber samples of harbour porpoises were the hexa PCBs followed by the penta and/or hepta PCBs (Figure 2, SFig 2). Foetuses, neonates and juveniles contained a higher proportion of lower chlorinated PCBs (tri, tetra and penta) than adult porpoises. Adult males had the highest proportion of hexa and hepta PCBs compared to the other maturity classes, whereas adult females had the highest proportion of octa PCBs compared to the other maturity classes.

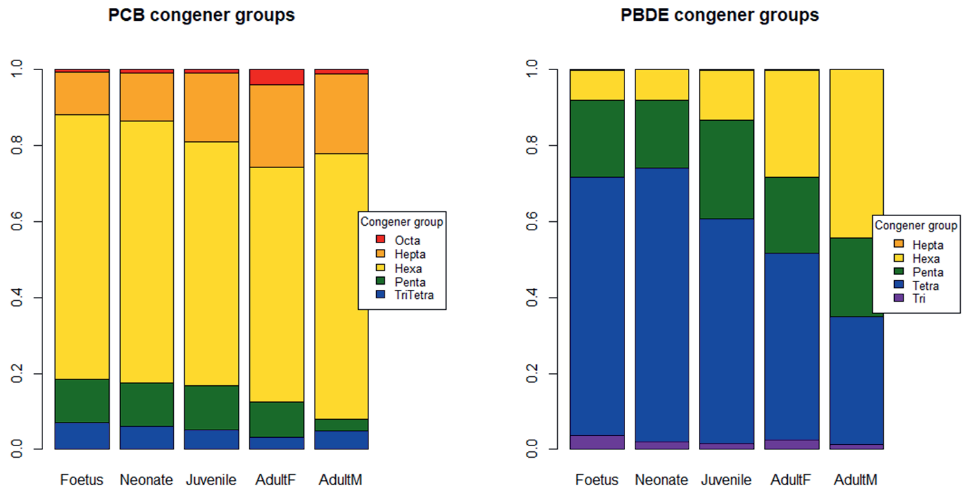


Figure 2. *Average proportion of PCB (left panel) and PBDE (right panel) concentrations per congener group in blubber of all maturity classes of harbour porpoises stranded along the Dutch coast in 2006-2019 (n= 120). AdultF= adult females, AdultM= adult males.*

Based on Structure Activity Groups (SAGs), the highly persistent PCBs (SAGs I-II-V) showed a significant increase in proportion per total length (95%CI 113.33;277.35), whereas for the less persistent PCBs (SAGs III-IV-VI) a significant decrease in proportion was observed with length (95% CI -277.35;-113.33) (Figure 3). The increase of highly persistent PCBs was mainly based on a build-up of SAG I PCBs in the adult porpoises, together with a decrease of the less persistent PCBs in the adult porpoises (SFig 3).

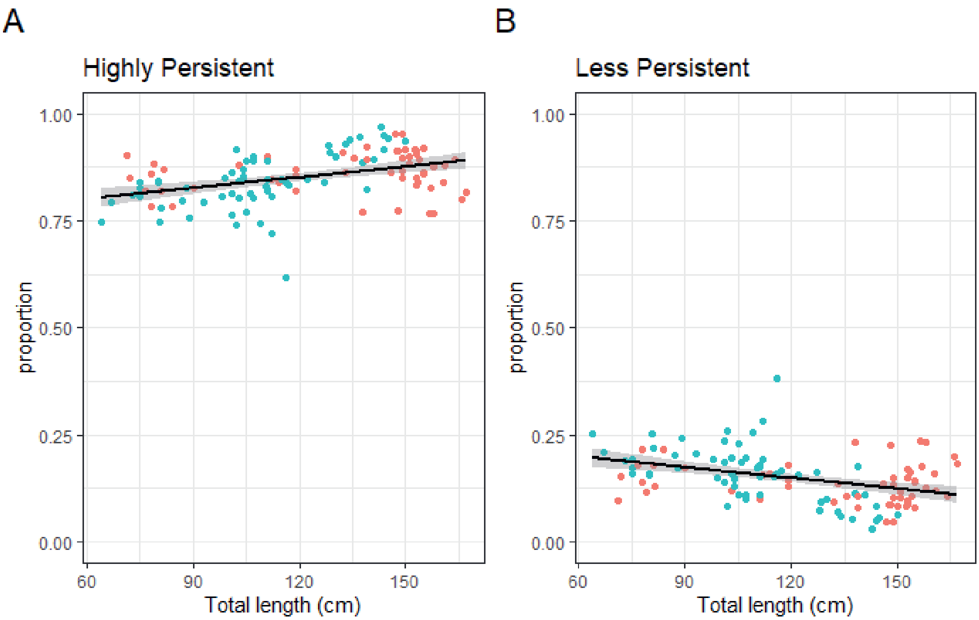


Figure 3. *Average proportion of highly persistent (SAG groups I-II-V; left panel) and less persistent (SAG groups III-IV-VI; right panel) PCBs in blubber of all maturity classes (here expressed as total length) of harbour porpoises stranded along the Dutch coast in 2006-2019 (n= 120) (Multiple R-squared: 0.1646). Females plotted in pink, males in blue.*

The most dominant PCB congeners in both adult females and foetuses were the hexa PCBs (predominantly CB-138, CB-149 and CB-153) and hepta PCBs (CB-187). The relative PCB congener profiles of the foetus differed from its mother, as a higher proportion of lower chlorinated PCBs was observed in the foetus compared to its mother (SFig 4). Hexa PCB levels in the foetus and its mother were similar (relative value of 1). Tri PCBs were often below detection limit and therefore excluded from this comparison.

Dominant PBDE congener groups were tetra PBDEs, followed by penta and/or hexa PBDEs (Figure 2, SFig 6). Foetuses and neonates had the highest proportion of tetra PBDEs, whereas the proportion of tetra PBDEs decreased in juveniles and adults which in turn showed a higher proportion of hexa PBDEs (Figure 2). Adult males had the highest proportion of hexa PBDEs compared to the other maturity classes, whereas the proportion of penta PBDEs was relatively similar in all classes.

PBDE patterns in all maturity classes showed a large variation (SFig 6). The relative proportion, however, showed a similar relation for mother and foetus as for PCBs, with a decreasing transfer of PBDEs to the foetus with increasing bromination (SFig 7). Foetuses contained a higher proportion of tetra PBDEs, whereas hexa PBDEs were more retained in the mother (SFig 7).

Partial correlation analysis Sum17PCB and Sum17PBDE

Sum-17PCB and Sum-17PBDE concentrations in stranded harbour porpoises were strongly correlated (Model 3, correlation coefficient (R)= 0.701) (n=114) (Figure 4).

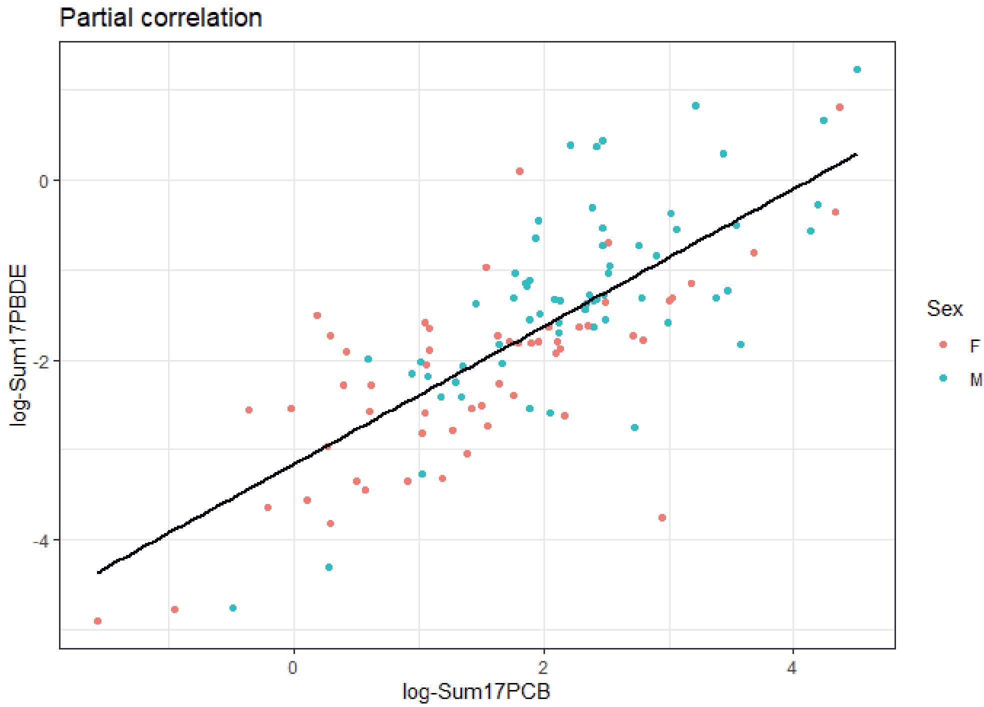


Figure 4. *Correlation between log-Sum17PCB and log-Sum17PBDE concentrations in blubber samples of harbour porpoises stranded along the Dutch coast in 2006-2019 (mg/kg lw, n=114), with a correlation coefficient of 0.70. F= females (pink), M= males (blue).*

Contaminant concentrations in milk, placenta and umbilical cord

Concentrations of Sum-17PCB in milk samples ranged between 0.20-33.8 mg/kg lw with a mean concentration of 8.6 mg/kg lw (n=14) (Figure 5A). No linear clear relation between PCB concentration in milk and total length was found, although smaller females tended to have higher PCB levels in their milk than larger females (Model 4, 95% profile likelihood interval slope of -0.142-0.046). The main PCB congener group in milk was formed by the hexa PCBs followed by the hepta PCBs (SFig 9). PCB profiles in milk resembled the PCB profiles in the blubber of the adult females (SFig 9). The proportion of Hepta and Octa PCBs in milk was slightly higher compared with the foetus, and the proportion of Tri/Tetra, Penta and Hexa PCBs in milk slightly lower (SFig 5).

Concentrations of Sum-17PBDE in milk samples varied between 0.002-0.51 mg/kg lw with an average concentration of 0.18 mg/kg lw (n=14) (Figure 5B). No relation between PBDE concentrations in milk and total length was found, although smaller-sized females also seemed to have higher PBDE concentrations (Model 5, 95% profile likelihood interval slope of -0.173-0.057). Main PBDEs in milk consisted of Tetra PBDEs followed by Penta and Hexa PBDEs (SFig 9). The proportion of hexa PBDEs in milk was slightly higher compared with the foetus, whereas the proportion of Tri/Tetra PBDEs was a little lower in milk (SFig 8).

For both Sum-17PCB and Sum-17PBDE a higher concentration in blubber was observed in combination with a higher concentration in milk (Figures 5C and 5D).

HCB concentrations in milk samples ranged between 0.03 - 0.21 mg/kg lw (STab 1). Where analysed, HCBD concentration in milk samples were below detection limit.

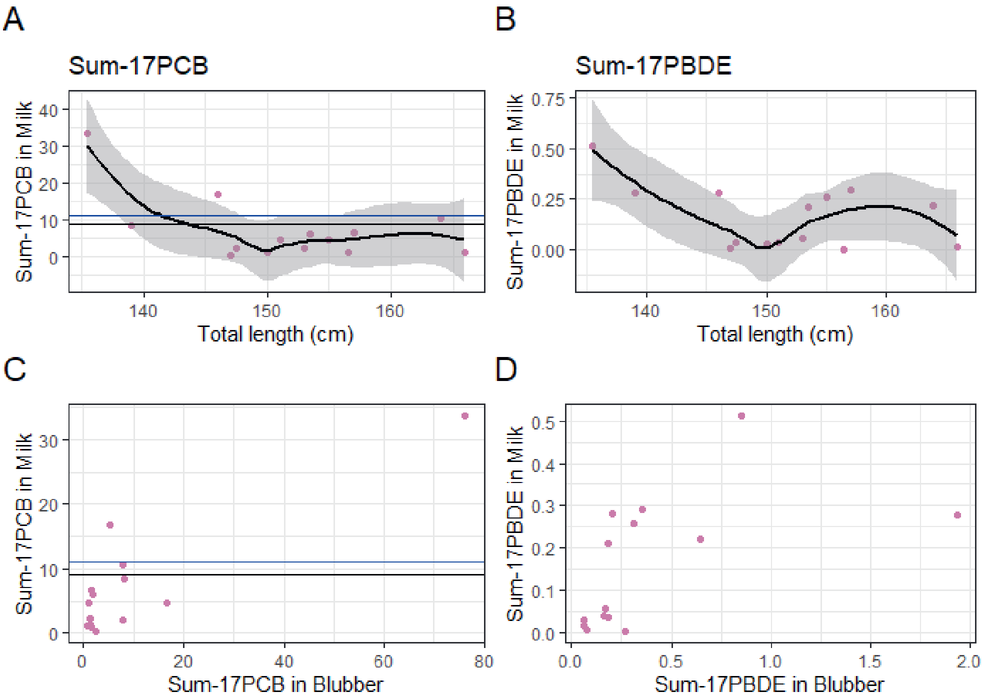


Figure 5. *A. Sum-17PCB concentrations in milk per total length (in cm) in adult females (mg/kg lw). B. Sum-17PBDE concentrations in milk per total length (in cm) in adult females. C. Sum-17PCB concentration in milk per Sum-17PCB in blubber of the same adult females. D. Sum-17PBDE concentration in milk per Sum-17PBDE concentration in blubber of the same adult females. For PCB graphs (A,C), threshold levels are indicated at 9 mg/kg lw (horizontal black line) and 11 mg/kg lw (horizontal blue line).*

Concentrations in placenta (n=3) were 1.3-8.2 mg/kg lw for Sum-17PCB, <dl-0.08 mg/kg lw for Sum-17PBDE, 0.14-0.16 mg/kg lw for HCB and <dl for HCBD (lipid % of 0.7-1.1). Concentrations in samples of the umbilical cord (n=2) were 0.1-1.4 mg/kg lw for Sum-17PCB, 0.11-0.26 mg/kg lw for Sum-17PBDE, and <dl for both HCB and HCBD (lipid % of 0.5-0.7%). PCB, PBDE and HCB concentrations in the placenta samples were comparable to concentrations found in the blubber of the foetuses when based on lipid weight (although the lipid content of the placenta was low: 0.9% ± 0.2% lipid).

Individuals with a good nutritional condition (NCC 1-2) appeared to have higher milk lipid % (40.2-67.5) (n=3) than individuals with a moderate and poor nutritional condition (NCC 3-6; milk lipid % of 7.6-50.6%; n=11) (SFig 10A, STab 1 and STab 7). As the total number of milk samples was relatively low (n=14), differences could not be tested on significance.

Sum-17PCB concentrations in the milk of adult females were lower in individuals with a good and moderate nutritional condition compared to that in individuals with a poor nutritional condition (SFig 10B). Adult females in good nutritional condition had on average 3.3 mg/kg lw Sum-17PCB in their milk (n=3, range: 0.2-8.5 mg/kg lw), whereas individuals in moderate nutritional condition had an average concentration of 5.6 mg/kg 17PCB lw (n=5, range: 1-17.6 mg/kg lw), and emaciated adult females had on average 13.7 mg/kg lw 17PCB in their milk (n=6, range 4.7-33.7 mg/kg lw).

Sum-17PBDE concentrations in milk of adult females in good and moderate nutritional condition were lower than those in poor nutritional condition (SFig 10C). Sum-17PBDE concentrations in milk of adult female in good nutritional condition were on average 0.11 mg/kg lw (n=3; range 0.006-0.28 mg/kg lw). Concentrations in animals in moderate condition were on average 0.08 mg/kg lw (n=5, range 0.002-0.21 mg/kg lw). Sum-17PBDE concentrations in the milk of emaciated adult females contained an average concentration of 0.29 mg/kg lw (n=6, range 0.18-0.51 mg/kg lw).

There was no clear relationship between early and late lactation and percentage of lipid of the milk samples when assessed over time; the percentage of lipid in milk was highly variable and ranged from 7.6-67.5% (SFig 11A). PCB and PBDE concentrations in milk varied throughout lactation but on average were highest during early lactation (May-August) compared to late lactation (September-February) (SFig 11B-C). During early lactation, the median PCB concentration in milk was 9.89 mg/kg lw (range of 4.67-17.55 mg/kg lw), and the median PBDE concentration was 0.26 mg/kw lw (range of 0.13-0.29 mg/kw lw) (n=7). During late lactation, the median PCB concentration in milk was 1.29 mg/kg lw (range of 0.20-33.76 mg/kg lw), and the median PBDE concentration 0.03 mg/kg lw (range of <0.01-0.51 mg/kg lw) (n=7).

Potential effects of contaminants on health and reproduction

Threshold levels for negative effects

Of all harbour porpoises, 38.8% (47 out of 121) had PCB concentrations exceeding the 9.0 mg/kg lw Sum-PCB threshold for the onset of physiological endpoints in marine mammals (STab 8). While concentrations in foetuses were all below this threshold, 56.3% of the neonates and 48.9% of the juveniles had PCB concentrations exceeding this threshold. Whereas relatively few adult female harbour porpoises exceeded this threshold level (10.5%), almost all adult males did (92.3%).

Of all analysed porpoises, 33.1% had PCB concentrations above the threshold for potential reproduction failure in resting adult female harbour porpoises of 11 mg/kg lw (STab 8). As this threshold was mainly derived for resting adult females, a comparison is best made with this maturity class. In 10.5 % of the adult females PCB concentrations were above the threshold level.

When applying the highest threshold level for profound reproductive impairment as derived from Baltic ringed seals (41 mg/kg lw Sum-PCB), 6.6% of the harbour porpoise sample exceeded this threshold level, which comprised mainly of adult males (15.4%), juveniles (both sexes), and, to a lesser extent, adult females (STab 8).

Most individuals had Sum-6PBDE concentrations well above the EQS for PBDEs of the WFD in all sample types, apart from a few where PBDE congener levels were all below the detection limit (STab 9).

Health parameters

The final model (Model 6), including the two categories reflecting the most likely cause of death (physical trauma and other acute causes versus infectious disease and/or debilitated animals), NCC and parasite load in the four scored organs showed a significant relationship between log-Sum17PCB, cause of death and NCC but no correlations between the parasite load in each of the four organs and PCB concentrations in blubber. The difference in the AIC of the model including or excluding the interaction between cause of death and NCC was <2 (AIC=198.84 versus AIC=197.61). The optimal model therefore included: sex, maturity class, log-SumPBDE, cause of death, NCC, the interaction between sex and maturity class, and the interaction between cause of death and NCC (for model selection, see STab 10).

The binomial logistic regression model (Model 7, Figure 6) showed that the probability of dying from infectious disease and/or debilitation increased with higher PCB concentrations in the blubber. Log odds ratios were -0.004;0.055, indicating that our data could not confirm a clear relationship. It is apparent that outliers in the dataset (those animals with Sum-17PCB in blubber of >40 mg/kg lw) strongly influenced these results (Figure 6).

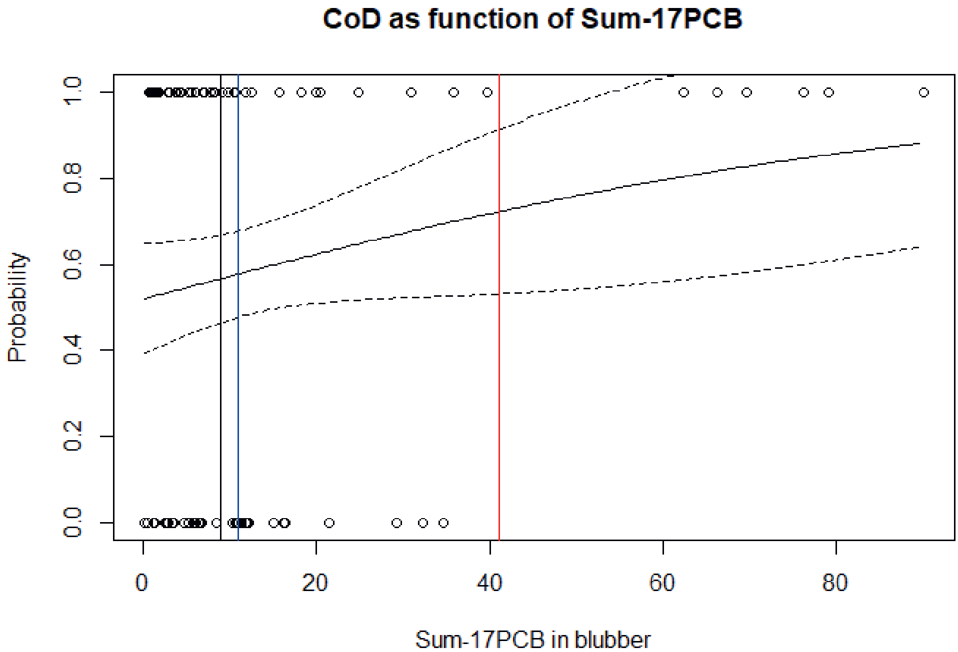


Figure 6. *Sum-17PCB concentrations in blubber as a function of 'cause of death', which is categorised as 'physical trauma and other acute causes' (plotted on the y-axis as 0) or 'infectious disease and/or debilitated' (plotted on the y-axis at 1). The probability of dying from physical trauma or other acute causes is the same as the probability of dying due to infectious disease and/or debilitation at low levels of Sum-17PCB (probability of 0.5). With increasing PCB, a positive correlation can be observed, meaning that the probability of dying due to infectious disease and/or debilitation is increasing. Note the wide 95% confidence interval range (dashed lines) as a result of small number of cases with Sum-17PCB concentrations >20. Threshold levels are indicated at 9 mg/kg lw (vertical black line), 11 mg/kg lw (vertical blue line) and 41 mg/kg lw (vertical red line).*

<u>Reproductive organs and contaminant concentrations in adult females</u>
The number of ovarian corpora scars in adult females increased with total length (Figure 7). The smallest adult females (length between 130-140 cm) had 1-3 corpora scars (n=3), while individuals with a total length of >140 cm had 4-17 corpora scars (n=17). Pregnant females had 1-7 ovarian corpora scars (n=6), (pregnant and) lactating females had 4-15 corpora scars (n=5), females with suspected abortions had 9 and 12 corpora scars (n=2), and resting females had more than 14 corpora scars (n=4).

Pregnant females were in a better nutritional status than lactating females. Pregnant females had, on average, an NCC of 2.8 (n=9), compared to 4.3 (n=11, excluding pregnant females) in lactating females, and 5.7 (n=3) in resting females.

The optimal model with the number of corpora scars as dependent variable, included the reproductive status, log-Sum17PCB in blubber, log-Sum17PBDE in blubber and total length, and not the interaction between reproductive status and total length (Model 8, AIC=101.26).

However, evaluation of the 95% CI of the most optimal model showed that only the reproductive status 'pregnant' was negatively correlated with increasing ovary scars (95% CI: -1.26- -0.08), whilst no significant relationship between the other parameters and number of corpora scars could be identified.

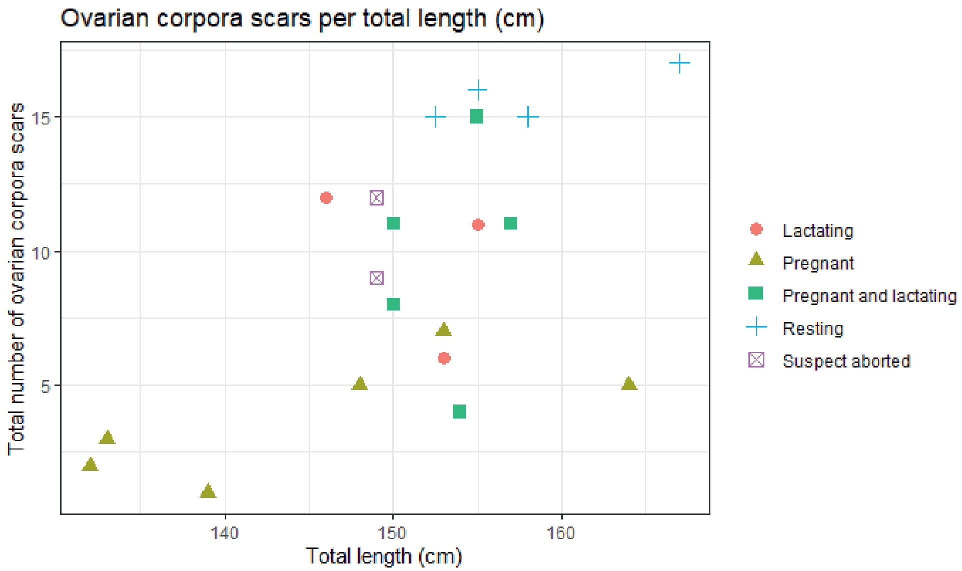


Figure 7. *The number of ovarian corpora scars (y-axis) per total length (x-axis) of adult female harbour porpoises that stranded along the Dutch coast in 2006-2019, grouped per reproductive class (n=20).*

The number of ovarian corpora scars in adult females showed no correlation with either Sum-17PCB and Sum-17PBDE concentration in blubber samples (Figure 8). There were two individuals, for which ovarian assessment was conducted, with a Sum-17PCB concentration above the thresholds of 9 mg/kg lw Sum-23PCB and 11 mg/kg lw Sum-25PCB (Figure 8). The first individual was pregnant, had one ovary scar and a Sum-17PCB of 15 mg/kg lw in the blubber sample (See SText: case id UT1581). It had thus not yet been offloading. The second female was resting and had 15 ovary scars and a Sum-17PCB of 39.7 mg/kg lw in her blubber. This strongly indicates that this female was unsuccessful in previously offloading, which based on Murphy et al. (2015) may be due to not successfully carrying a foetus to term, and/or the neonate dying soon after birth (See SText: case id UT1470).

Recent abortion was suspected for two other adult females (Figure 7-8). One had a severely dilated cervix, large asymmetry of the uterine horns, haemorrhage of the cervical wall and an endometritis. This individual stranded in April (2017) and did not show signs of lactation. The second female also had a severely dilated cervix, large asymmetry of the uterine horns and histologically oedema. This female live-stranded in May (2017), did not show signs of lactation, and no calf was observed at the time of stranding. Both animals had a CL on the left ovary and with a Sum-17PCB concentration <9 mg/kg lw they likely previously offloaded successfully. The other sixteen adult females for which ovaria were assessed had a Sum-17PCB <9 mg/kw lw. No signs of reproduction failure were detected in these individuals, indicating successful offloading through pregnancy and (previous) lactation.

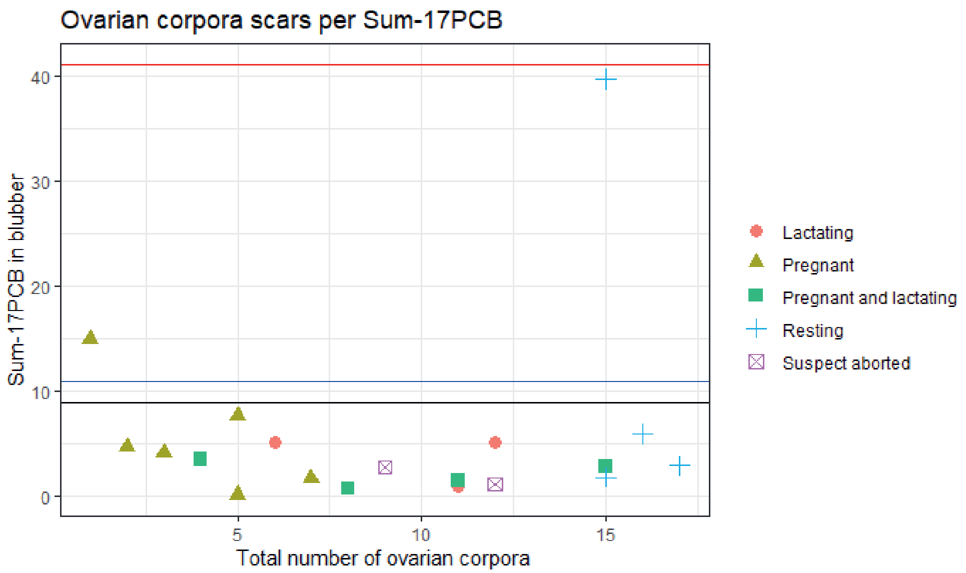


Figure 8. *Sum-17PCB per total number of ovarian corpora scars among the adult females that stranded along the Dutch coast in 2006-2019, grouped per reproductive class (n=20; two green squares overlap at 11 ovarian corpora, cf. Figure 7)), including threshold levels of 9 mg/kg lw (horizontal black line), 11 mg/kg lw (horizontal blue line) and 41 mg/kg lw (horizontal red line).*

Among the adult females of which no ovarian assessment was conducted, another two individuals had PCB levels above the thresholds of 9 mg/kg lw Sum-23PCB and 11 mg/kg lw Sum-25PCB. One pregnant and lactating female had a Sum-17PCB concentrations of 16.4 mg/kg lw in her blubber and 17.6 mg/kg lw in her milk. The individual died due to dystocia (problems during birth) and was actively lactating for the impending birth. PCB mobilisation in the milk was apparent but offloading had not yet occurred (See SText: case id UT1756). The other individual, an emaciated but lactating female, had 76.2 mg/kg lw in her blubber and 33.8 mg/kg lw in her milk, also suggesting that it had not yet successfully offloaded (See SText: case id UT529).

Discussion

Here we present the levels of exposure of PCBs, PBDEs, HCB and HCBD of 121 harbour porpoises of all maturity classes that were stranded along the Dutch coast (2008-2019), including nine foetuses. This study represents the most recent and comprehensive investigation into generational cycling of persistent organic pollutant in an abundantly stranded small cetacean in the southern North Sea. We included samples of infrequently accessible foetuses, placenta, umbilical cord and milk and combined this with data on causes of death, nutritional condition and parasitology from necropsies.

Uptake and elimination in maturity classes

Concentrations of organic contaminants in blubber samples of harbour porpoises differed between sex and maturity classes (Figure 1, STab 5). These differences in contaminant concentrations can be explained by maternal transfer, specifically offloading of contaminant by mature females to their offspring, as well as differences in the uptake via diet (Jepson et al. 1999; Sørmo et al. 2003; Williams et al. 2020a).

In general, contaminant concentrations were lowest in foetuses. Foetuses obtain organic contaminants from their mother via transplacental transfer during gestation (Desforges et al. 2012). The transfer of contaminants is influenced by the chemical characteristics of the contaminants. PCB congeners with a low molecular weight can move easier across the placenta than larger congeners (Borrell & Aguilar 2005; Salata et al. 1995). This was also observed in this study, with a predominant transfer of Tetra and Penta PCBs and Tetra PBDEs from the mother to the foetus. Duinker & Hillebrand (1979) estimated that 15% of the organochlorine contaminants in harbour porpoise adult females were transferred to their foetuses, with foetuses having an average weight of 17% of the mass of their mother. This is a higher percentage than in other (larger) odontocete species, in which adult females transferred an estimated 4-10% of total body burdens of organochlorine compounds to their foetus during gestation (Borrell et al. 1995; Fukushima & Kawai 1981, in: Yordy et al. 2010).

Neonate harbour porpoises contained higher contaminant concentrations than foetuses. Concentrations of organic contaminants in neonates are a result of both transplacental transfer and lactational transfer. Transfer rates during lactation are considered higher than during transplacental transfer (Mongillo et al. 2016). Our results showed that milk resembled PCB and PBDE profiles of the blubber samples of the adult females. This means that after birth neonates receive a higher proportion of higher halogenated congeners (Hepta/Octa PCBs, Hexa PBDEs). Hence their profiles become more similar to those of older animals.

First born calves are thought to receive higher contaminant loads than subsequent calves (Cockcroft et al. 1989; Lundin et al. 2016; Mongillo et al. 2016; Yordy et al. 2010), which may result in up to four times higher concentrations in first-born calves, as was suggested for striped dolphins (*Stenella coeruleoalba*) (Fukushima & Kawai, 1981 in: Yordy et al. 2010), bottlenose dolphins (*Tursiops truncatus*) (Cockcroft et al. 1989) and long-finned pilot whales (*Globicephala melas*) (Borrell et al. 1995). Our results suggest the same, with adult females with a smaller total length having higher PCB levels in milk. A decrease in PCB levels in milk of adult females with a larger size can be explained with a high offloading in firstborn calves. A linear relation between total length and PCB/PBDE concentration in milk was therefore not expected.

Foetuses and neonates are more susceptible to toxic effects of POPs than older, more developed individuals. Effects of the contaminants can be exerted directly on the foetus and neonate, or indirectly via the mother and/or the placenta, or both (Rogers & Kavlock 2001). Young individuals may also have a lower capability to metabolise and eliminate compounds (Weijs et al. 2010a). The high developmental rate of foetuses and neonates enhances the potential for toxicological effects. Williams et al. (2020a) concluded that calves were likely to be exposed to more neurotoxic PCB congeners (Tri-Penta PCBs) than adult harbour porpoises. These effects may eventually result in a lower survival of exposed individuals (Borrell & Aguilar 2005).

Juvenile harbour porpoises contained slightly higher contaminant concentrations than neonates and these levels were similar for both sexes. In juveniles the diet shifts gradually from milk to prey, resulting in new sources for uptake of organic contaminants (Boon et al. 2002; Yordy et al. 2010). Juvenile and young adult odontocetes experience high growth rates during this life stage requesting higher metabolic rates and/or increased feeding rates, as was also observed in bottlenose dolphins (Yordy et al. 2010). Rapid growth may enhance the bioaccumulation of organic contaminants through increased intake of food. PCB congener profiles of juveniles were similar to those of the neonates with a slightly higher proportion of Hepta PCBs. Compared with adults, juveniles still had higher proportions of Tri to Penta PCBs, which was also observed in earlier studies in Dutch waters (Weijs et al. 2007, 2009).

The highest PCB and PBDE concentrations were found in adult male harbour porpoises. An average PCB accumulation rate of 1.1 mg/kg lw per year through dietary intake was estimated for male harbour porpoises based on levels in stranded individuals (Murphy et al. 2015). Adult males are only able to eliminate some of the congeners via metabolism and consequent elimination. Of the Sum-17PCB, PCB congeners 28, 105, 118, 156 were described to be (partly) metabolised by CYP1A1 in cetaceans (Boon et al. 1997a; Cullon et al. 2012). Both adult males and adult females showed concentrations of these congeners that were a factor 2-3 lower than in foetuses, neonates and juveniles. This further supports the hypothesis that younger age classes have a lower ability to eliminate compounds than adults. Adult males in the current study showed a higher proportion of Hexa, Hepta and Octa PCBs, compared with the other maturity classes, pointing at retainment of mainly highly persistent PCBs in adult males. In male bottlenose dolphins, the concentrations of persistent (non-degradable) PCBs increased with age (Yordy et al. 2010). Larger PBDE congeners have a much lower potential to be taken up by marine mammals through food web transfer than PCBs (De Boer et al. 1998; Boon et al. 1997b), which was supported with our findings that the dominant PBDE group in adult males was the Hexa PBDEs, whereas Hepta to Deca BDEs were hardly observed.

Adult female harbour porpoises had lower PCB, PBDE and HCB concentrations in their blubber compared with adult males. Female cetaceans can offload organic contaminants partly through metabolism (as do males) but particularly through transplacental transfer and lactation, resulting in a loss of contaminant body burden of the adult females of up to 60 to nearing 100% (Borrell et al. 1995; Fukushima & Kawai 1981, in: Yordy et al. 2010; Murphy et al. 2015; Wells et al. 2005).

The amount of offloaded contaminant as well as the exposure rates during lifetime are influenced by the age of the adult and the number of calves given birth to, and therefore varies per individual (Mongillo et al. 2016). The highest amount of offloading is thought to occur during the first successful pregnancy, predominantly through lactation (Borrell et al. 1995). This corresponds to our results, which showed that increasing total length (as a proxy for age) was related to a decrease in contaminants that were offloaded. After lactation, contaminants can again be taken up through prey ingestion, as this was observed in bottlenose dolphins (Yordy et al. 2010), although concentrations as in earlier life stages (prior to lactating) will not be reached again (Murphy et al. 2015).

Age, previous pregnancies, nutritional status and month may have influenced contaminant and lipid levels in milk. PCB concentrations in milk were lower in adult females with a greater total length and presumably older age. Besides, Lipid % in milk of emaciated females was lower than those that had a better nutritional status. Therefore, nutritional status of females presumably also strongly influenced how much contaminants were offloaded into the milk. Adult females with a poor nutritional condition had higher PCB concentrations in milk than those in a moderate or good nutritional condition, probably due to mobilisation of lipid stores and as such PCBs. Similar findings were reported for southern resident killer whales (*Orcinus orca*), where toxicant concentrations, which likely originated from endogenous lipid stores, were highest and had the greatest potential for toxicity in periods of low prey abundance (Lundin et al. 2016).

PCB and PBDE levels in milk were highest during early lactation (April-August) compared to late lactation, whereas lipid levels were highly variable and did not show a clear pattern between early and late lactation. Longitudinal studies on bottlenose dolphins showed that levels of organic compound releasement through milk were related to age, reproductive history and lipid content of the milk (Ridgway & Reddy 1995; Yordy et al. 2010). In grey seals (*Halichoerus grypus)* PCB levels increased from early to late lactation. This was explained by a higher mobilisation of PCBs through changes in the maternal blubber composition as a result of lipid loss by the mother (Debier et al. 2003). However, both the lactation period and intensity between grey seals and harbour porpoises differ greatly. Grey seals are capital breeders with a short lactation period (18 days on average) in which they mobilise maternal blubber stores to produce fat rich (50-60% lipid) milk (Hall and Russell 2018). Harbour porpoises have a lactation period of approximately 10 months and feed the entire time during lactation. Additional collection and analyses of milk samples of harbour porpoises will provide further insight into the variables influencing the contaminant levels in milk of harbour porpoises, despite the fact that it is challenging to acquire a sufficient number, volume and quality of milk samples in cetaceans.

Comparison to other studies

PCB concentrations in our study were in the same range to earlier reported PCB concentrations in harbour porpoises of the southern North Sea and UK waters (Jepson et al. 2016; Mahfouz et al. 2014; Pierce et al. 2008; Weijs et al. 2009; Williams et al. 2020b). However, variation between individuals is high and some maturity classes in earlier studies had higher average concentrations than animals in our study (Mahfouz et al. 2014; Weijs et al. 2009). Lower PCB concentrations can be found in harbour porpoises in the Black Sea (Weijs et al. 2010b) and southwest Greenland (Borrell et al. 2004), and higher concentrations in those from the Northwest Iberian Peninsula (Méndez-Fernandez et al. 2014).

PBDE concentrations in our study were similar to earlier reported PBDE concentrations in harbour porpoises inhabiting the southern North Sea, although concentrations in adults were slightly lower than what was reported before (Pierce et al. 2008; Weijs et al. 2009). Lower PBDE concentrations can be found in harbour porpoises of the Black Sea (Weijs et al. 2010b).

HCB concentrations in harbour porpoises of our study were lower than in earlier reported studies of the southern North Sea (Imazaki et al. 2015) and comparable to those reported from southwest Greenland (Borrell et al. 2004).

PCB concentrations in harbour porpoises were a factor 3-7 lower than in other larger toothed cetaceans of the Northeast Atlantic, such as the bottlenose dolphin, the striped dolphin and the killer whale (Andvik et al. 2021; Jepson et al. 2016). Population declines in bottlenose dolphins and killer whales in the Northeast Atlantic were thought to be predominantly driven by bioaccumulation of PCBs (Jepson et al. 2016). The higher PCB concentrations in these other odontocete species can be explained by their longer life expectancy, with a later age at sexual maturity, and the fact that they feed at higher trophic levels (Hall et al. 2006b; Desforges et al. 2018; Jepson et al. 2016).

PCB concentrations in harbour porpoises were in the same range as those in harbour seals (*Phoca vitulina)* in the North Sea, whereas PBDE concentrations were approximately a factor two higher in harbour porpoises than those in harbour seals (Weijs et al. 2009). Harbour porpoises contained a higher proportion of lower chlorinated PCBs than harbour seals. This was explained by the fact that harbour seals were thought to have a better capacity to metabolise PCBs than harbour porpoises (Boon et al. 1997a). This results in a higher bioaccumulation in harbour porpoises and could imply an increased risk for adverse health effects in this species (Weijs et al. 2009).

Potential health effects

Parasites

No significant correlation between the parasite loads and PCB concentrations in blubber was observed in our study. This is in contrast to a study conducted in the UK, where a significant, positive correlation between PCB levels and nematode burdens in stranded harbour porpoises was found, although the nature of the relationship was confounded by the porpoise's sex, age and cause of death (Bull et al. 2006). Also, individuals with the most severe nematode infestations did not have the highest PCB concentrations and therefore Bull et al. (2006) stated that while PCBs are important, they were clearly not the sole determinants of nematode burdens in harbour porpoises around the UK. Although scoring of parasite burden was done similarly to Bull et al. (2006), methodological differences could account for the different outcomes between the study by Bull et al. (2006) and ours. These authors used classification trees generated through recursive partitioning and fitted their models using binomial recursive partitioning, while we incorporated the parasite load data directly into the generalized linear modelling procedures. Additionally, more than twice as many cases were assessed in the UK compared to the current study. Bull et al. (2006) found the highest levels of Sum-25PCB to be associated with intermediate levels of both bronchiole and pulmonary nematodes and with high levels of nematodes found in the cardiac stomach but they could not provide a mechanistic explanation. Additional empirical studies on the relationship between contaminant levels and host immunocompetence would improve the understanding of the harbour porpoise-parasite relationship and the factors that may influence this.

Causes of death

Harbour porpoises with high PCB concentrations died more often from an infectious disease and/or debilitations (like severe emaciation) than from an acute cause of death, such as bycatch or predation. However, a significant relationship between causes of death and PCB concentrations could not be confirmed in our analyses. The number of animals with PCB concentrations above the thresholds, especially the threshold of 41 mg/kg lw (n=6), was low. However, with the odds ratio close to 0 (-0.004;0.055), a potential relationship between cause of death and PCB contaminants cannot completely be ruled out for this study. The interaction in the final model between sex and maturity class was explained by the difference in PCB concentrations between adult males and adult females due to offloading. Additionally, the interaction between cause of death and nutritional condition was expected, as animals dying from acute causes were often in better nutritional condition than those dying from prolonged health problems.

In harbour porpoises from UK waters and the southern North Sea the risk of death from infectious disease was previously associated with increasing PCB exposure (Hall et al. 2006a; Jepson et al. 2016; Mahfouz et al. 2014). Hall et al. (2006a) reported that for each 1 mg/kg increase in blubber PCBs, the average increase in risk of infectious disease mortality was 2% and a doubling of risk occurred at approximately 45 mg/kg lipid. In UK waters, all female porpoises with PCB burdens above 30 mg/kg lw died from either infectious disease or other non-traumatic causes of death (Murphy et al. 2015). This was also found in our study; the two adult females with very high PCB concentrations in their blubber (39.7 and 76.2 mg/kg lw) had died due to an infectious disease. Increasing the sample size of animals is recommended to further test this hypothesis.

Reproduction

In mammals at large, the reported effects of endocrine-disrupting chemicals such as PCBs and DDTs on reproduction include reproductive tract anomalies, polycystic ovarian syndrome, ovarian failure, uterine fibroids, endometriosis, neoplasm and ectopic gestation, all of which may lead to infertility, spontaneous abortion, changes in age at sexual maturity, and lactational and ovulational failure (reviewed in: Diamanti-Kandarakis et al. 2009). For marine mammals and specifically cetaceans, the number of cases where organic compound exposure could be associated with reproductive failure is low (Murphy et al. 2018) but cases of foetal and/or neonatal mortality in harbour porpoises (Murphy et al. 2015) and cases of increased firstborn-calf mortality in bottlenose dolphins (Schwacke et al. 2002; Wells et al. 2005) have been described. It has been proposed that if PCB levels exceed 11 mg/kg lw in resting, sexually mature female harbour porpoises, individuals should be regarded as nulliparous, as infertility or reproductive failure is expected (Murphy et al. 2015). Four out of 38 adult females in our study had PCB concentrations exceeding this level. Ovaries were assessed in 20 out of the 38 adult females. Within this small sample size, we found clear signs of reproduction failure in one case (1/20), where PCB concentrations of 39.7 mg/kg lw in blubber were found in combination with a high number of ovarian corpora scars (15) and low age (7 years). No significant further relation between the number of ovarian corpora scars in all other adult females and Sum-17PCB or Sum-17PBDE concentration in their blubber could be detected. This strongly suggests that reproduction failure in the form of infertility (e.g., failure of ovulation, conception and implementation) does not occur at large. This is supported by studies undertaken on both harbour porpoises and common dolphins (*Delphinus delphis*), where exposure to PCBs did not inhibit ovulation, conception or implantation but instead may have impacted foetal and new-born survival rates (Murphy et al. 2010, 2015, 2018).

In UK waters, resting harbour porpoise females were more likely to have higher PCB burdens than other maturity groups and, where data were available, these non-offloading females were shown to have previously been gravid, which also suggests foetal or new-born mortality (Murphy et al. 2015). As lower chlorinated and brominated compounds are more readily transferred via milk, this means that foetus and neonates are exposed to a more neurotoxic mixture of these contaminants than observed in adults (Williams et al. 2020b). Although the direct and indirect mechanisms are yet to be established, one explanation could be that even low levels of POP exposure or difference in the mixture of POPs can have adverse health effects when exposure occurs at critical periods of development. It is conceivable that the threshold levels of negative effects on e.g., foetuses, neonates and juveniles in puberty are therefore lower than the current threshold levels and therefore re-evaluation in light of age and development stages may be necessary, as previously suggested for bottlenose dolphins (Hall et al. 2006b).

Management implications

PBDE concentrations and more recently also PCB concentrations have declined in UK waters, after concentrations reached an initial plateau, as a consequence of legislation of the production and disposal (Law et al. 2012, 2014; Williams et al. 2020b). Over the past decade, mean blubber PCB concentrations fell below the proposed thresholds for toxic effects (Williams et al. 2020b). Trends for Dutch coastal waters cannot readily be determined based on the currently available data since sample size per year was too low for each maturity class. As trends vary over the region and measured PCB concentrations are still associated with increased rate of mortality due to infectious diseases, PCB mitigation is advised supplementary to earlier strict international regulations (Williams et al. 2020b). Open applications were reported to still release PCBs into the environment, and renewed mitigation measures may therefore provide further success to eliminate the release of these persistent contaminants into the environment (Stuart-Smith & Jepson 2017).

Monitoring of PCBs and other relevant POPs in stranded harbour porpoises is an efficient tool to assess trends and potential effects of these contaminants in the marine environment, if proceeded for the long-term. Three types of assessment are recommended. Firstly, to determine maximum levels and trends in population, it is advised to focus on concentrations in blubber of adult males, as this maturity group contains the highest PCB and PBDE concentrations, reducing the bias of offloading which is apparent in the mature females. Important confounding factors such as decomposition and nutritional status should be taken into account as these affect the observed contaminant levels. Secondly, since the correlation between PCB and PBDE levels in blubber of the harbour porpoise showed strong correlations, measured PCB concentrations can be used to predict PBDE levels in these individuals. It should however be noted that the observed range is rather broad. Therefore, such a prediction should only be used as an indicative value. Thirdly, to obtain a better insight into population reproduction parameters, it is advised to retain a focus on the functions of the female reproductive system. A further assessment of routes of generational transfer of pollutants from adult females to their offspring as well potential health effects, especially in the developing calf, is needed to better understand to what level pollutants as PCBs and PBDEs may impact the health of the harbour porpoise population.

Conclusions

In this study, we assessed the exposure level of harbour porpoises from the southern North Sea to PCBs, PBDEs, HCB and HCBD. Besides analysing these contaminants in the blubber of stranded neonate, juvenile and adult porpoises, we were able to analyse tissues of foetuses, as well as milk, placenta and umbilical cord samples. These samples are rarely analysed in cetacean research and this novel approach therefore sheds light on exposure levels and generational transfer through different pathways. We found that the contaminants were transferred from females to their offspring via both the placenta and via lactation, with the latter being the most dominant transfer route. Lactation resulted in a high concentration of chlorinated and brominated contaminants in calves, at the start of each new generation.

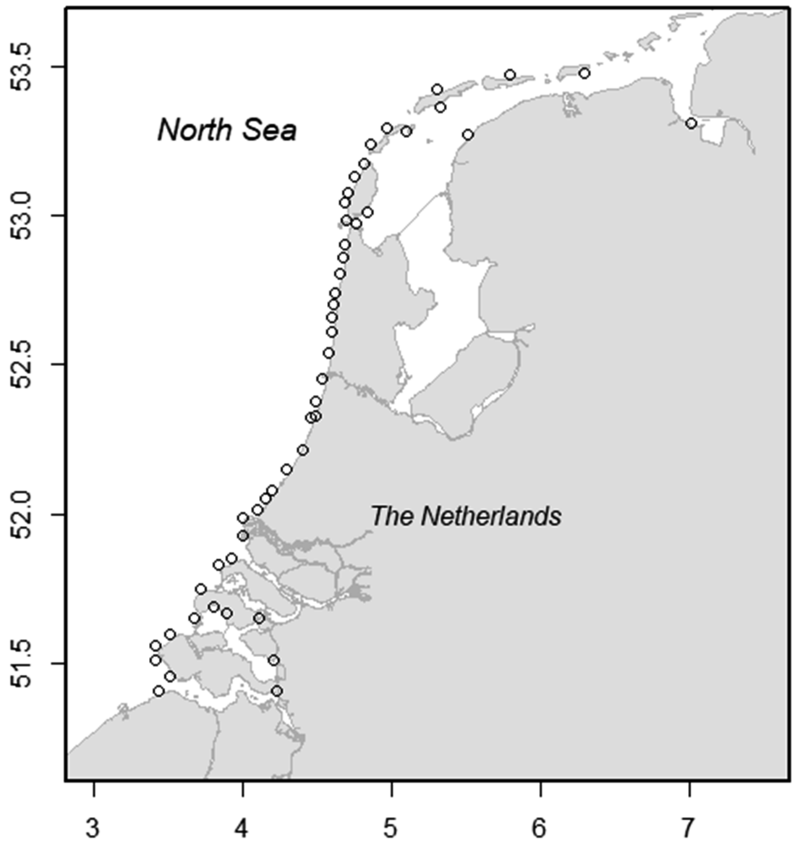
We assessed the relation between PCB levels with health status of porpoises and showed that specifically adult males, together with half of the neonates and juveniles of both sexes, had PCB concentrations that exceeded the threshold level of negative effects. Porpoises with PCB levels above the highest threshold level of 41 mg/kg died more often of infectious disease and/or debilitation. Finally, nutritional stress caused mobilisation of the endogenous lipid stores leading to higher offloading through milk, leading to a greater potential for toxicity in calves of nutritionally stressed females. These results provide further evidence of the potential health effects of PCBs on the reproduction system of harbour porpoises of the southern North Sea with consequences for population viability.

Acknowledgements

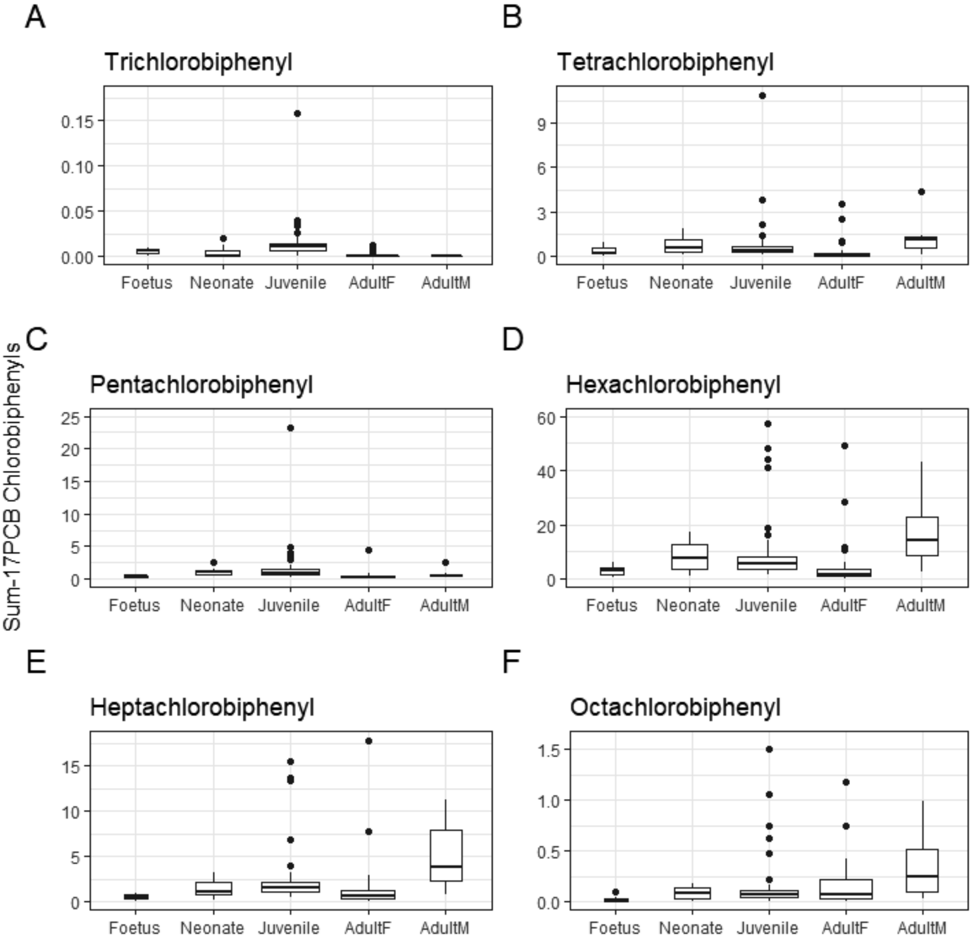
We are grateful for the help of numerous volunteers and organisations in reporting and collection of stranded harbour porpoises on the Dutch coastline. The contaminant analyses were conducted under the umbrella of the BO- and KRM-monitoring programmes on harbour porpoises commissioned by the Ministry of Agriculture, Nature and Food Quality and Rijkswaterstaat. The tissue samples were collected during necropsies as part of the Statutory Research Tasks Unit for Nature & the Environment funded by the Ministry of Agriculture, Nature and Food Quality. We acknowledge the help of staff of Utrecht University with necropsies and sample collection, in particular Lidewij Wiersma, Lineke Begeman, Sjoukje Hiemstra and Liliane Solé. We also thank Kayleigh Lambregts for her pilot study into the correlation between parasites and PCBs. Finally, we thank Mardik Leopold for reviewing a previous version of the manuscript.

Supporting information

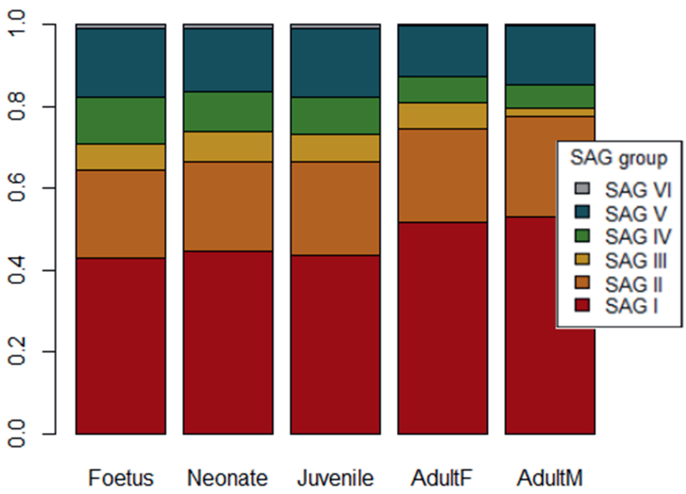
Supplementary data associated with this article can be found in the online version at doi: 10.1016/j.scitotenv.2021.148936.



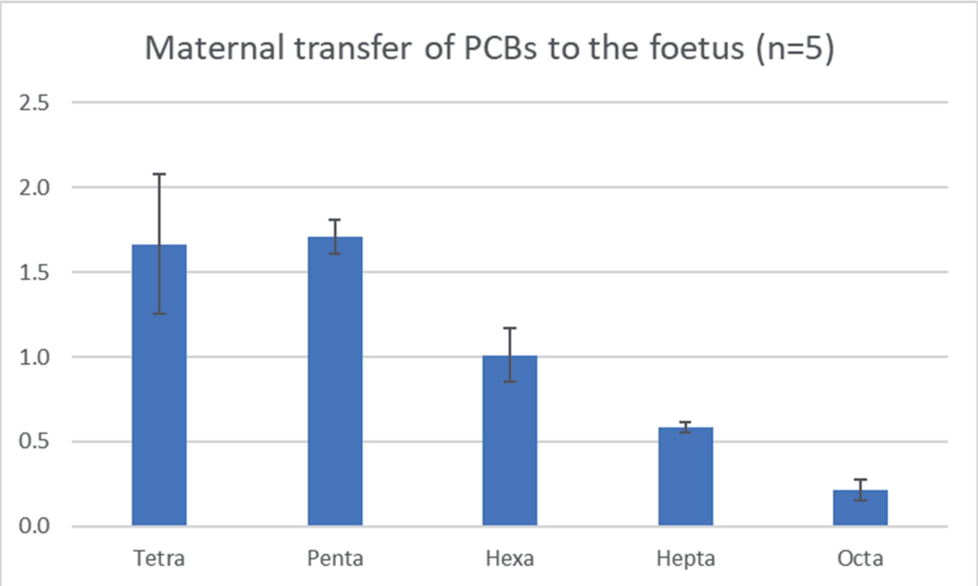
Supplementary Figure 1. *Stranding locations along the Dutch coast of porpoises that were included in this study (excluding the foetuses), in the period 2006-2019 (N=112).*



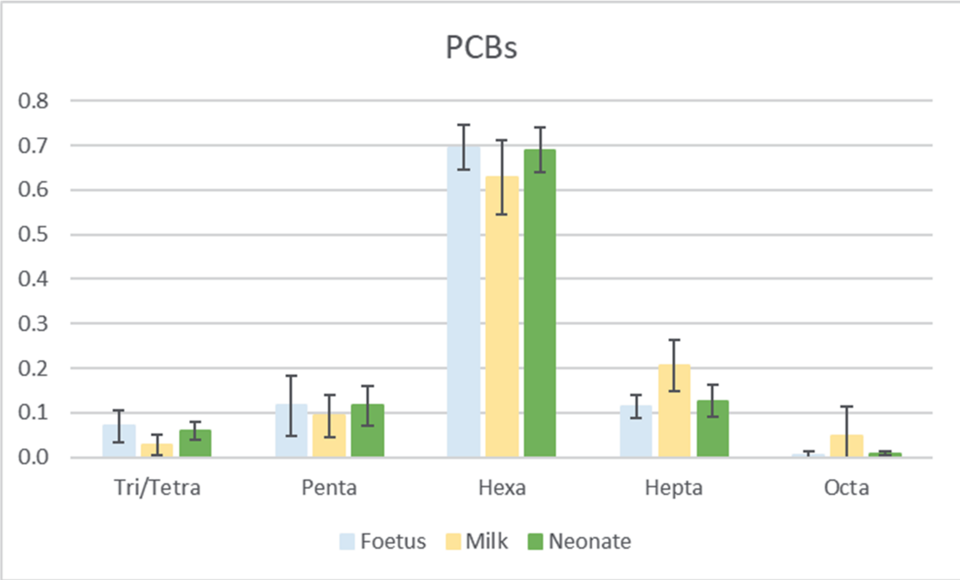
Supplementary Figure 2. *17PCB concentrations (mg/kg lw), divided per congener group, in blubber of all maturity classes of harbour porpoises stranded along the Dutch coast in 2006-2019 (n=120). The black dots represent the outliers in the dataset. The width of the bars represent the sample size, the horizontal lines the medians, the boxes the first to third quartile, and the tails the minimum and maximum.*



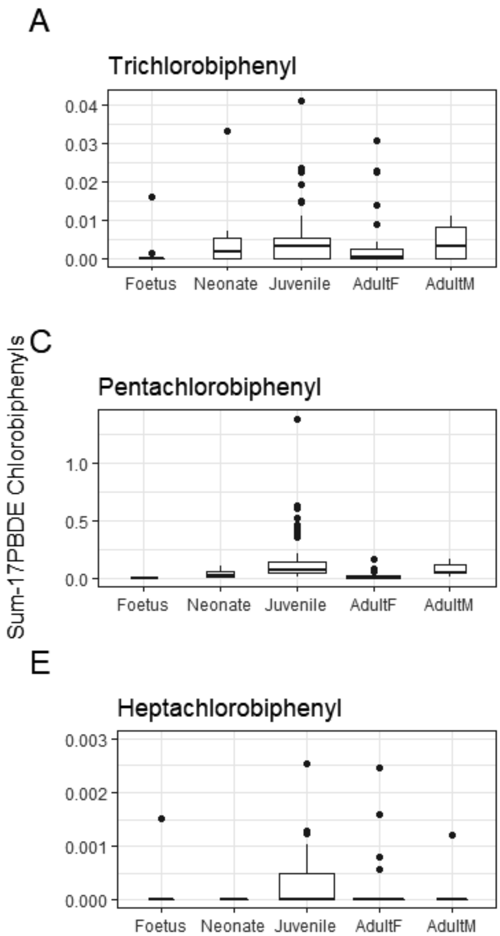
Supplementary Figure 3. *Relative PCB profiles grouped per Structure Activity Group (SAG) in blubber samples of all maturity classes of harbour porpoises stranded along the Dutch coast in 2006-2019.*



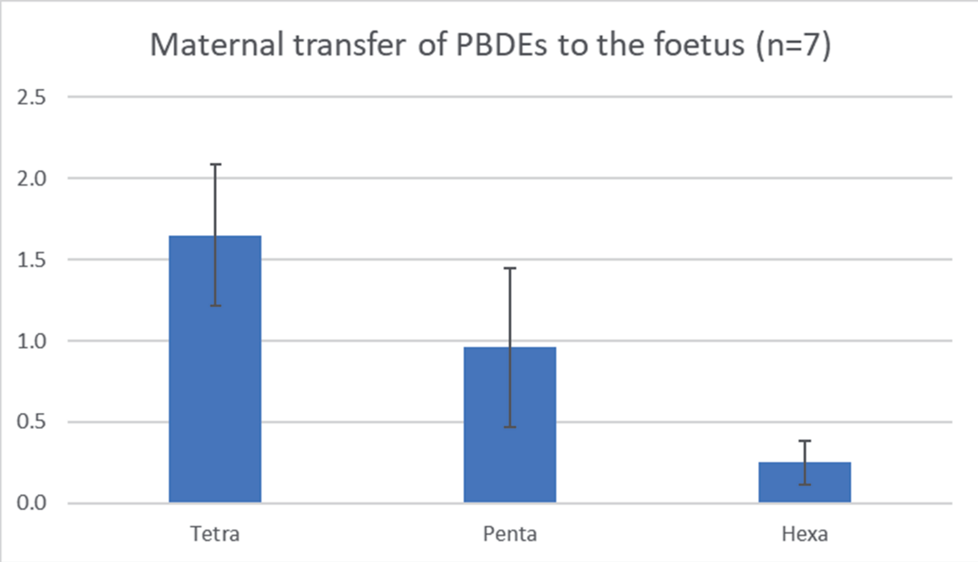
Supplementary Figure 4. *Relative PCB profiles in foetus blubber samples compared to blubber samples of their mother, in five mother-foetus pairs of harbour porpoises stranded along the Dutch coast in 2006-2019. PCB concentrations in foetuses were first corrected per congener group to obtain the same total-PCB concentrations as their mothers and then divided by the concentration of the mother to obtain the relative PCB profile.*



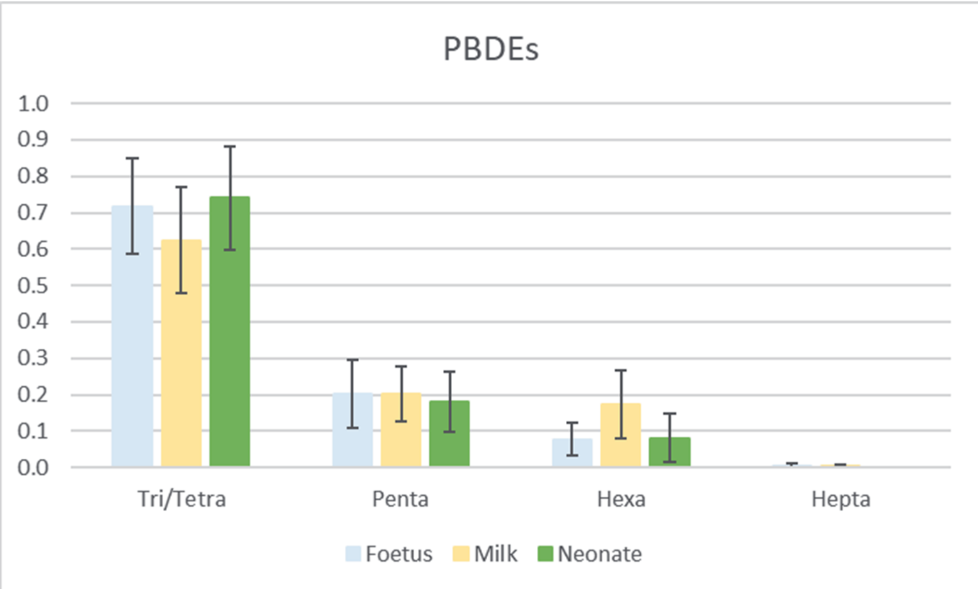
Supplementary Figure 5. *Relative PCB profiles in foetus blubber (n=7), neonate blubber (n=17) and milk of adult female (n=14) of individuals stranded along the Dutch coast between 2009-2019. Profiles were first summed per congener group and then divided by the Sum-17PCB concentration of the individual to obtain the proportion per congener group. Two foetus samples were removed due to low lipid levels in blubber (2.1-11%) in combination with strongly deviating profiles.*



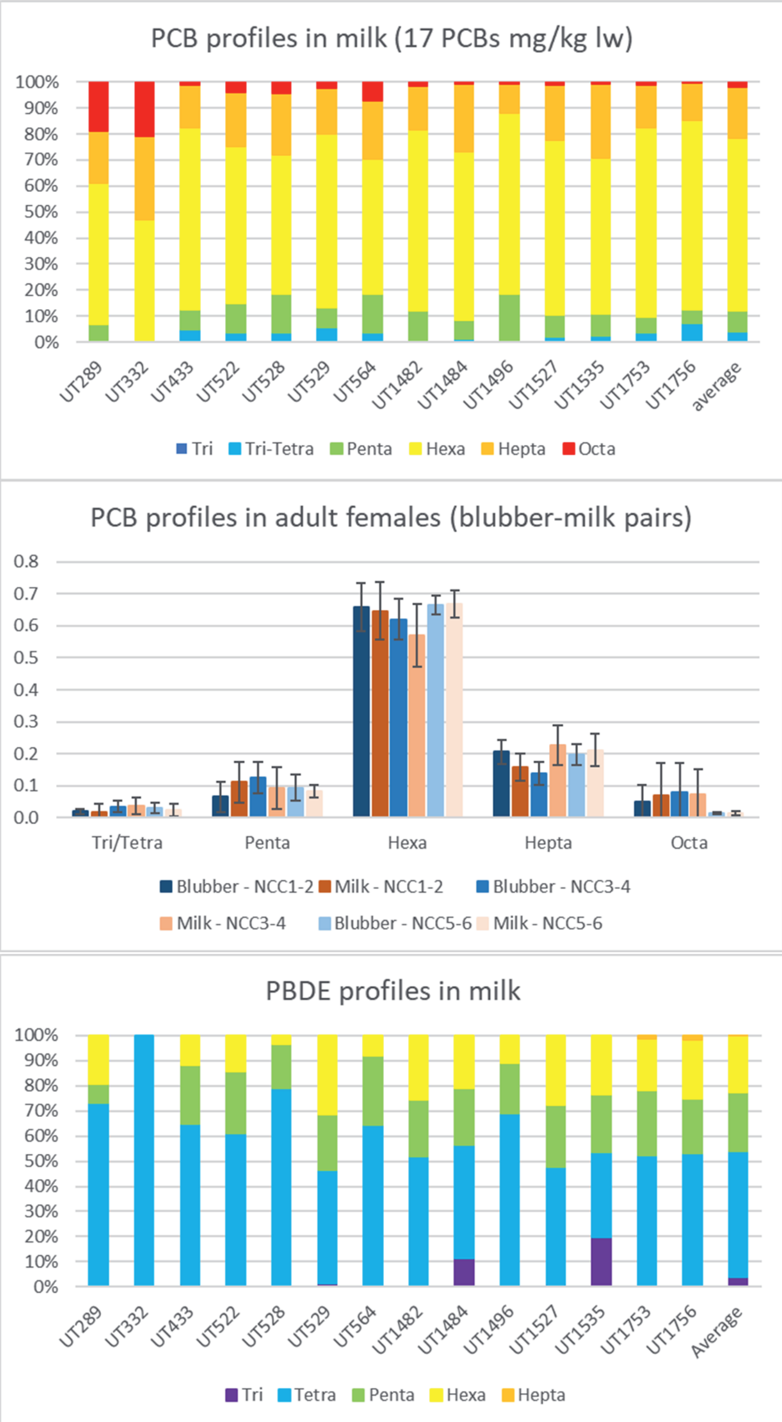
Supplementary Figure 6. *17PBDE concentrations (mg/kg lw), divided per congener group, in blubber of all maturity classes of harbour porpoises stranded along the Dutch coast in 2006-2019 (n=120). The black dots represent the outliers in the dataset. The width of the bars represent the sample size, the horizontal lines the medians, the boxes the first to third quartile, and the tails the minimum and maximum.*



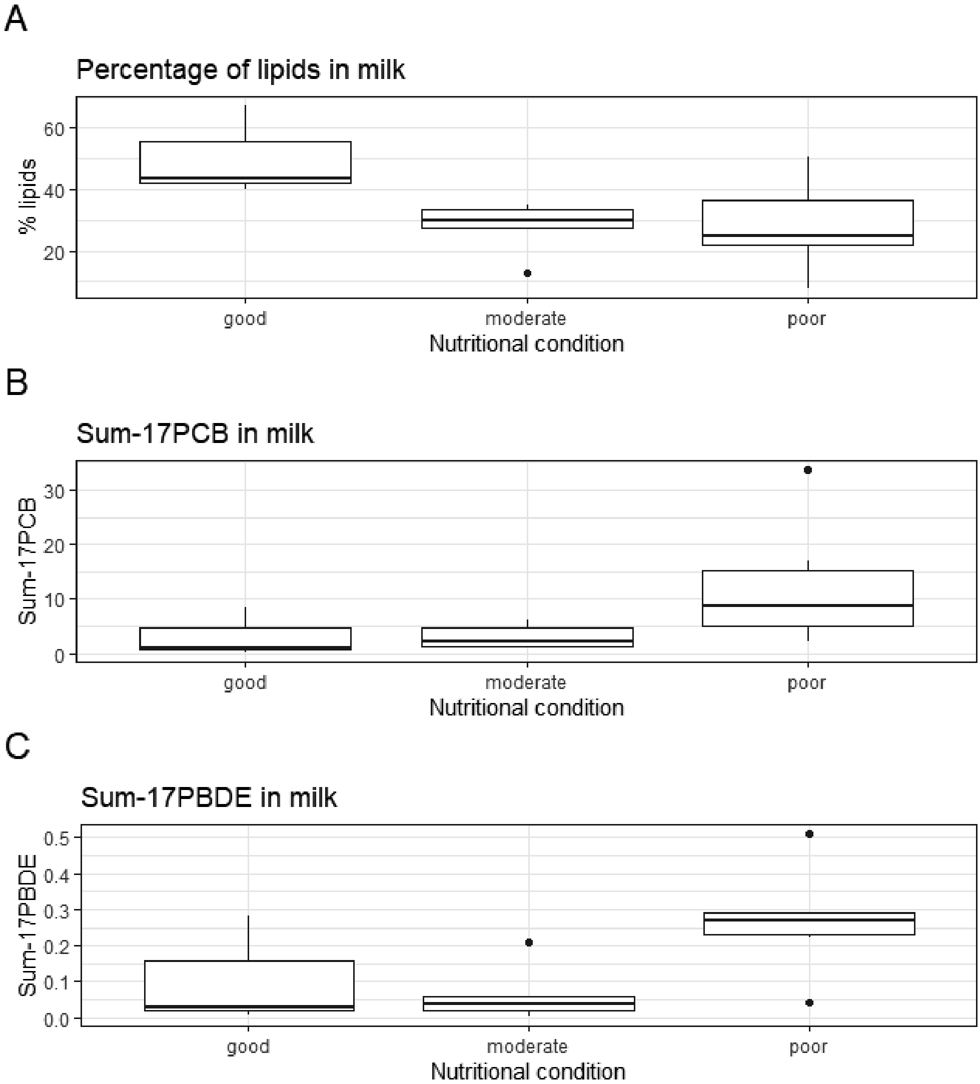
Supplementary Figure 7. *Relative PBDE profiles in foetus blubber samples compared to blubber samples of their mother, in five mother-foetus pairs of harbour porpoises stranded along the Dutch coast in 2006-2019. PBDE concentrations in foetuses were first corrected per congener group to obtain the same total- PBDE concentrations as their mothers and then divided by the concentration of the mother to obtain the relative PBDE profile.*



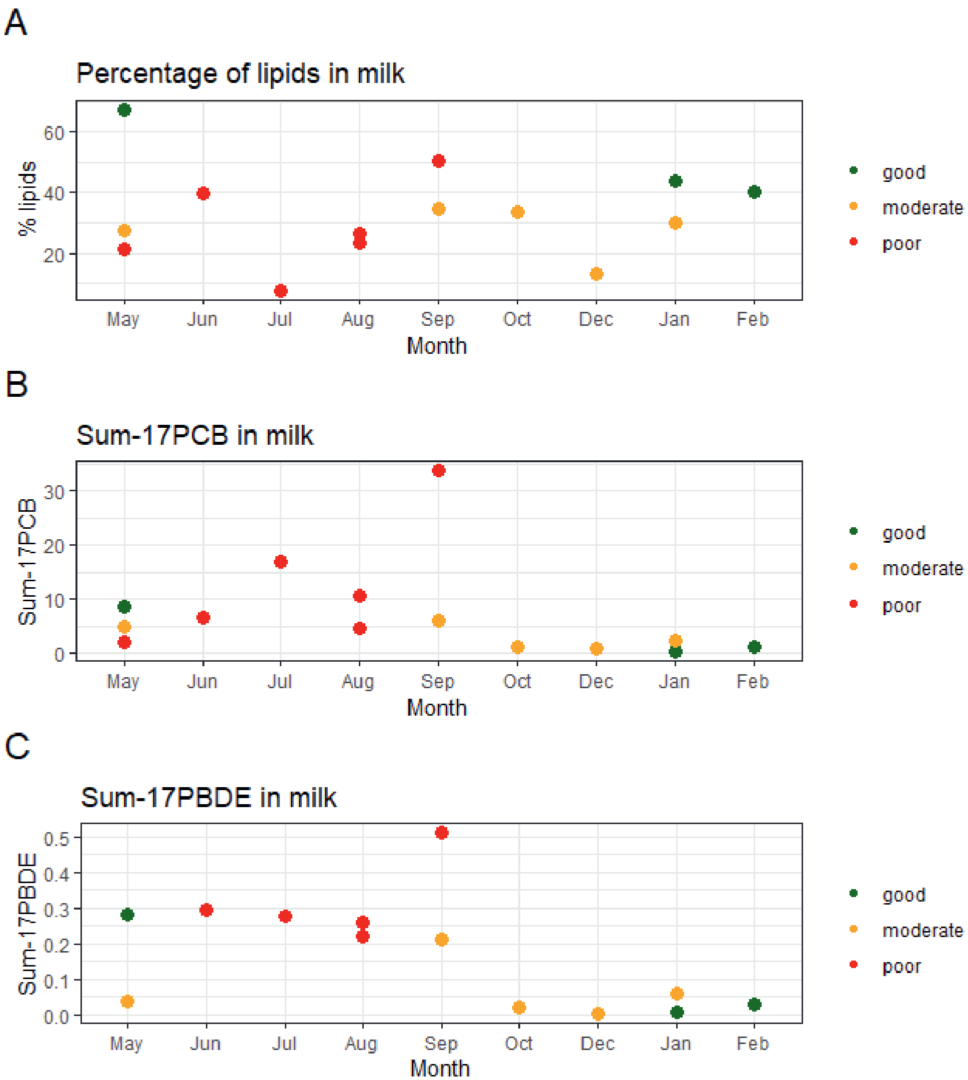
Supplementary Figure 8. *Relative PBDE profiles in foetus blubber (n=7), neonate blubber (n=17) and milk of adult female (n=14) of individuals stranded along the Dutch coast between 2009-2019. Profiles were first summed per congener group and then divided by the Sum-17PBDE concentration of the individual to obtain the proportion per congener group. Two foetus samples were removed due to low lipid levels in blubber (2.1-11%) in combination with PBDE concentrations being all below detection limit.*



Supplementary Figure 9. *Upper figure: PCB profiles in milk (n=14) of adult female harbour porpoises stranded along the Dutch coast in 2006-2019. Middle figure: relative PCB profiles in milk and blubber of adult female harbour porpoises (n=14) stranded along the Dutch coast in 2006-2019. Lower figure: PBDE profiles in milk (n=14) of adult female harbour porpoises stranded along the Dutch coast in 2006-2019*



Supplementary Figure 10. *A. Percentage of lipids measured in milk of lactating adult female harbour porpoises (N=14) in different nutritional conditions. B. Sum-17PCB levels (mg/kg lw) measured in milk of lactating adult female harbour porpoises (N=14) in different nutritional conditions. C. Sum-17PBDE levels (mg/kg lw) measured in milk of lactating adult female harbour porpoises (N=14) in different nutritional conditions.*



Supplementary Figure 11. *A. lipid %, B. Som-17PCB concentrations, and C. Sum-17PBDE in milk of lactating adult female harbour porpoises (N=13) (mg/kg lw) per month of stranding, showing differences in PCB concentrations in early lactation (months May – August) versus late lactation (months September - February). One outlier was excluded with a deviating high PCB concentration (76.2 mg/kg lw, month September).*

6

A version of this chapter has been published after minor revision in Scientific Reports (2021)

Nutritional status and prey energy density govern reproductive success in a small cetacean

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Abstract

A variety of mammals suppress reproduction when they experience poor physical condition or environmental harshness. In many marine mammal species, reproductive impairment has been correlated to polychlorinated biphenyls (PCBs), the most frequently measured chemical pollutant, while the relative importance of other factors remains understudied. We investigate whether reproductively active females abandon investment in their foetus when conditions are poor, exemplified using an extensively studied cetacean species; the harbour porpoise (*Phocoena phocoena*). Data on disease, fat and muscle mass and diet obtained from necropsies in The Netherlands were used as proxies of health and nutritional status and related to pregnancy and foetal growth. This was combined with published life history parameters for sixteen other areas to correlate to parameters reflecting environmental condition: mean energy density of prey constituting diets (MEDD), cumulative human impact and PCB contamination. Maternal nutritional status had significant effects on foetal size and females in poor health had lower probabilities of being pregnant and generally did not sustain pregnancy throughout gestation. Pregnancy rates across the Northern Hemisphere were best explained by MEDD. We demonstrate the importance of having undisturbed access to prey with high energy densities in determining reproductive success and ultimately population size for small cetaceans.

Introduction

The evolutionary causes and consequences of adaptions in life history strategies shape reproductive success and, consequently, long-term survival (Boness et al. 2002; Lummaa & Clutton-Brock 2002; Stockley & Bro-Jørgensen 2011). Most large mammals, ranging from whales and elephants to humans, are typical K-strategists that maximise lifetime reproductive output by having a low reproductive rate but high survivorship (Boness et al. 2002; Christiansen et al. 2014; Stockley & Bro-Jørgensen 2011;). Despite this, life history traits may vary spatiotemporally due to variance in social or cultural, ecological, and environmental factors (Boness et al. 2002), as demonstrated in a variety of mammals known to suppress reproduction in response to environmental harshness or poor physical condition impacting the reproductive system (Ellis et al. 2009; Kershaw et al. 2020; Smith 1947; Spitz et al. 2012, Stockley & Bro-Jørgensen 2011; Trites & Donnelly 2003; Wasser & Isenberg 1986; Williams et al. 2013). Ecosystems, and particularly the marine environment, are rapidly changing due to broad-scale natural and anthropogenic processes (Halpern et al. 2008, 2015, 2019). Consequently, marine mammals are increasingly exposed to a variety of stressors, including chemical and noise pollutants caused by e.g., ship traffic, seismic surveys and construction, as well as marine litter, disturbance, habitat loss and loss of prey through competition with fisheries (Fair & Becker 2000; Jepson et al. 2016; Lusseau et al. 2006; Murphy et al. 2009; Reijnders 1986; Sonne et al. 2020; Tyack 2008; Wisniewska et al. 2018).

Harbour porpoises (*Phocoena phocoena*) are K-strategists with their reproduction characterised by 10-11 months of gestation of a single offspring (Lockyer 2003). If conditions are good, porpoises may mature at 3-4 years of age and produce a calf every year thereafter (Lockyer 2007; Read & Hohn 1995). They inhabit cold temperate to sub-polar waters of the Northern Hemisphere and are one of the most abundant cetaceans (Hammond et al. 2008; Read 1999). Due to their small size, limited capacity for storing energy and their high metabolic rate, porpoises must eat large quantities of high energy prey to sustain themselves (Spitz et al. 2012; Wisniewska et al. 2018). They feed on a variety of prey and diets differ considerably across their range (Leopold 2015; Santos & Pierce 2003). It is therefore conceivable that regional differences in prey and habitat quality, as well as in anthropogenic activities, affect their health and subsequently their reproductive capacity, with cascading effects on population vital rates. Studies on reproductive impairment in harbour porpoises have predominantly been focussed on associations with PCB exposure (Jepson et al. 2016; Murphy et al. 2009, 2015; Pierce et al. 2008; Williams et al. 2020, 2021), while studies on the relative importance of other causes remain scarce.

Here, we present a comprehensive analysis into factors driving reproductive success of harbour porpoises. We investigate whether reproductive females abandon investment in their foetus when intrinsic or extrinsic conditions are poor, presumably to prioritise their own survival and thus long-term fitness. To study the role of intrinsic conditions, data on health, cause of death, fat and muscle mass, and diet of porpoises from Dutch waters were categorised and analysed. We expect a lower pregnancy rate (PR) in females in poor nutritional and health status compared to those in good nutritional and health status, and a positive relationship between foetal size and nutritional status, based on blubber thickness, visceral fat and muscle mass, of the mother. These results were used in the second part of this study, in which we examined the role of extrinsic conditions on life history parameters. We compiled records of PR and ages at sexual maturity (ASM) from 17 study areas across the distributional range of the harbour porpoise. For each area, we established proxies of environmental condition: mean energy density of prey that constituted porpoise diet (MEDD), cumulative human impact (CHI) based on a model containing data on climate change, fishing, land-based pressures, and other commercial activities (Halpern et al. 2008), and reported levels of polychlorinated biphenyl (PCB) contamination (Jepson et al. 2016; IWC 2019), with the aim of determining which of these environmental condition(s) best explain reported life history parameters.

Results

Dutch waters: Foetus growth

Between 2006-2019 199 mature female harbour porpoises were necropsied and a total of 52 foetuses were found: 50 singular and one twin. The smallest recorded foetus was approximately 10 mm, found August 18th, and the largest foetus was 76.5 cm, found on the 31st of May (SFig. 1). Foetus weight could not be assessed in all cases but where measured it was strongly correlated with foetus length, with length explaining 80% of the variance in the data (R2=0.8, n=34, of Model 2 in STab. 1). In order to identify which parameters influence foetus size, we assessed the effect of Julian date, to account for foetus growth which increases throughout gestation, the effect of total length of the mother, her health status (based on her cause of death), her nutritional status (firstly based on a for season corrected blubber thickness metric (corBT) and secondly based on a nutritional condition code (NCC), taken into account blubber thickness and assessment of visceral fat and muscle mass), as well as interactions between these parameters (Model 3 with corBT and Model 4 with NCC, see methods for full description). Foetus size was mostly influenced by Julian date (proxy for day in the gestation) and by the nutritional status of the mother but not by the length of the mother or her health status (AIC of final Model 3: 365.06, log odds ratio (OR) [95%CI] = -0.10[-0.15- -0.05] for Julian date and 1.47[0.29-2.65] for corBT. AIC of final Model 4: 366.96, log OR [95%CI] = -0.10[-0.15- -0.05] for Julian date and -17.99[-33.88- -2.06] for factor(NCC)poor). OR for Julian date was 9.04 (95%CI: 8.59-9.50), of nutritional status using corBT 4.36 (95%CI: 1.34-1.42) and of nutritional status using factor (NCC) poor 1.56 [95%CI: 1.94-1.27], indicative of strong positive relationships: increasing day in gestation increased foetus size, and additionally, an increasing nutritional status of the mother was positively correlated to an increased foetus size (Fig. 1, SFig. 2). No interactions between the predictor variables were retained in the final model. The lack of a significant interaction between Julian date and nutritional status indicates that the effect of nutritional status of the mother on foetus length remained the same throughout the study period.

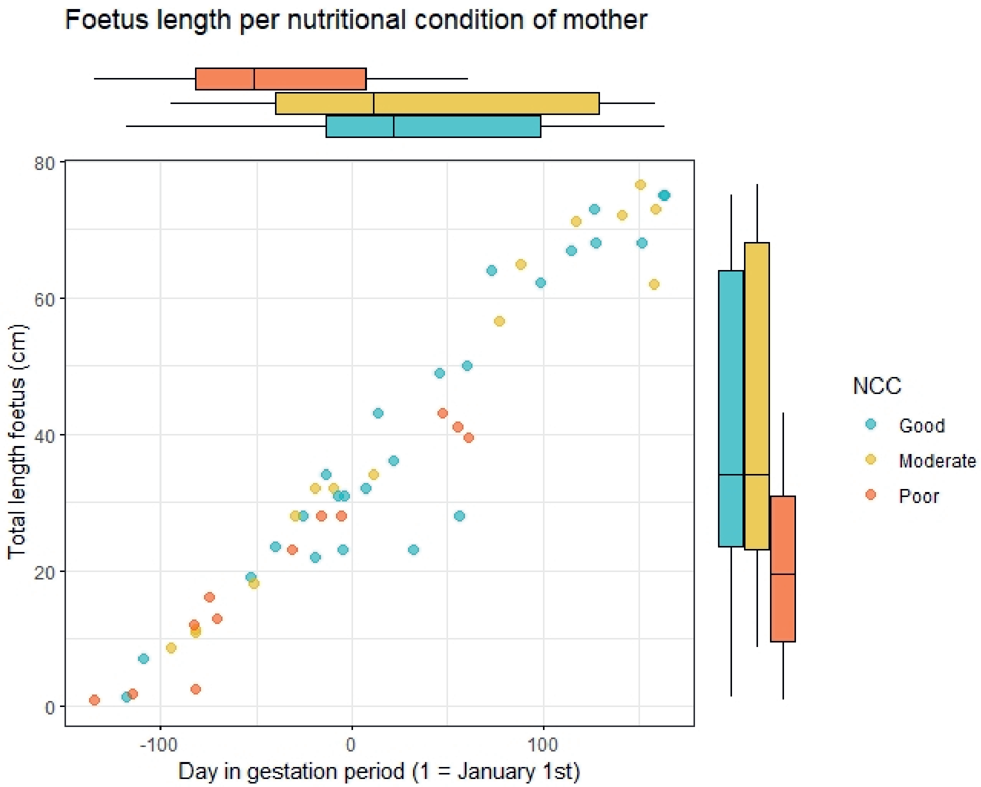

Figure 1. *Combined scatterplot and boxplots of foetus length (y-axis) throughout the gestation period (x-axis). Coloured by nutritional condition category (NCC, based on blubber thickness, visceral fat and muscle mass assessed during post-mortem examination) of the mother (n=50), with blue = good, yellow = moderate and orange = poor. One pregnant female carrying a twin was excluded.*

Dutch waters: Pregnancy rate

Excluding all mature females from the months May-August, to reduce the error of missing pregnancies as calving and conception occurs in these months, there were 119 mature females of which 41 were pregnant, giving a PR of 0.34 (95% CI: 0.26-0.43). Including the conception period (May-August) revealed a PR of 0.28 based on 51 pregnancies among 180 mature females (95% CI: 0.22-0.35). The PR for mature females in the health category which included mature females dying as a direct result of anthropogenic trauma or predation was higher: 0.58 (95% CI: 0.41-0.74, excluding conception period) based on 22 pregnancies among 38 mature females. The PR for mature females in the health category which included porpoises dying due to infectious disease or emaciation was lower: 0.24 (95% CI: 0.14-0.34, excluding conception period), based on 17 pregnancies among 71 mature females.

To test which variables influence pregnancy among the mature females, we assessed the effects of age, year, month, health status, nutritional status and several interactions among these variables (Model 5 with corBT and Model 6 with NCC, see methods for full description). Pregnancy was best explained by health status, and not by any of the other predictor variables or interactions between them (AIC of final model: 83.78, log OR [95%CI] = -1.79[-3.07- 0.66] for factor(Health)debilitated). OR was 0.17 (95%CI: 0.05-0.52), demonstrating that the odds or being pregnant reduce when in a debilitated health status.

Dutch waters: Prey energy density

Mean energy density of prey constituting porpoise diet (MEDD) was calculated for the entire dataset (all necropsied porpoises examined in The Netherlands with food remains in their stomachs (n=985)) and for various sub-sets of these data (Tab. 1). MEDD at large was relatively low (Tab. 1; <5.06 kJ/g,) and we found little or no difference between MEDD of adults and immatures, between adult females and adult males, or between pregnant and non-pregnant (adult) females. However, MEDD of mature females in the health category which included porpoises dying as a direct result of anthropogenic trauma or predation was on average 16% higher than the MEDD of mature females in the health category which included mature females dying due to infectious disease and/or due to emaciation of unknown origin. In addition, MEDD of mature females in good nutritional condition was also higher compared to mature females in moderate or poor nutritional condition (Tab. 1).

Table 1. Mean energy density of prey constituting porpoise diets (kJ/g) (MEDD) of the full dataset (all harbour porpoises from Dutch waters (new) (All HP-NL)) and for various sub-sets of animals. N-values give the numbers of animals with food remains in their stomachs. NCC= nutritional condition category, based on assessment of blubber thickness, visceral fat and muscle mass during post-mortem examination.

Category	N=	MEDD
All HP-NL	985	4.92
All adult	280	4.87
All immatures	702	4.97
Adult females	171	4.83
Adult males	110	4.88
Adult females-pregnant	45	4.76
Adult females-not pregnant	96	4.77
Adult females dying acutely, e.g., due to anthropogenic trauma or predation	55	5.06
Adult females dying as a result of infectious disease and/or emaciation	79	4.37
Adult females in good NCC	56	5.45
Adult females in moderate NCC	69	4.49
Adult females in poor NCC	58	4.47

Dutch waters: Age at sexual maturity

ASM of female porpoises found dead in The Netherlands was calculated from established ages of 154 individuals, comprising 32 immature and 122 mature females. The oldest immature female was 5.5 years, and the youngest mature female 3 years of age. ASM was calculated at 4.0 years (95% CI: 3.47-4.48 years, Model 7, SFig. 3). The maximum age at death was 24 years, however, the median age at death was 8 years, with a mean of 7.9 years and third quartile of 8.5 years, revealing that 75% of the mature females did not exceed 8.5 years.

Global: Life history parameters and environmental conditions

Nineteen studies were found that reported PR and sixteen that reported ASM for harbour porpoises, including the results of this study (STab. 2, the current study is referred to as 'Dutch waters - new'). PR ranged from a minimum of 0.28 in the Salish Sea to a maximum of 0.99 in Icelandic waters (STab. 3). ASM ranged from 3.1 in Eastern-Newfoundland to 5.5 years off the North-West Iberian Peninsula (STab. 4). MEDD and cumulative human impact (CHI) scores could be calculated for 17 of the study areas, PCBs (sum of all) for seventeen, of which thirteen remained when restricting the analysis to ≥∑17PCB-≤∑99PCB (Tab. 2, STab. 12). An overview of these data combined in provided Table 2, where the study areas are organised by the lowest to highest PR with associated data on ASM, MEDD, CHI, and contaminant scores.

Table 2. *Summary table of pregnancy rates (PR), age at sexual maturity (ASM), mean energy density of diet (MEDD), cumulative human impact (CHI) mean scores, and chemical pollution by PCBs (mg/kg lipid weight) of harbour porpoises in study areas across their distributional range. Note that PCB is given twice; PCB1 is without restrictions on the sum of congeners and mixtures reported, and PCB2 is restricted to studies reporting ≥∑17PCB-≤∑99PCB. The table is organised by study area arranged from lowest to highest PR with colour shadings corresponding to lowest (reddest) to highest (greenest) PR. All entries of the other columns are coloured as highest (reddest) to lowest (greenest). Time frames of studies and references are given in STab. 2. NA = not available.*

Study area	*Life history parameters*		*Environmental conditions*			
	PR	**ASM**	**MEDD**	**CHI**	**PCB1**	**PCB2**
Salish Sea	0.283	NA	4.15	1.323	13.66	9.13
English and Welsh waters	0.286	4.22	4.82	2.110	34.64	34.64
Dutch waters - new	0.345	4	4.92	1.718	24.00	24.00
Scottish waters	0.405	4.35	4.54	2.636	14.25	14.06
Baltic Sea	0.458	4.95	5.07	2.106	27.49	12.79
NW Iberian Peninsula	0.538	5.5	5.21	1.555	50.80	50.80
Kattegat and Skagerrak Seas	0.571	4.32	6.69	2.080	24.25	NA
German North Sea	0.571	4.95	5.11	1.916	2.20	NA
Dutch waters - old	0.590	NA	4.21	NA	52.11	52.11
Celtic and Irish Seas	0.600	4.26	6.79	2.106	43.30	43.30
Massachusetts	0.722	NA	6.24	1.212	NA	NA
Danish waters - newest	0.727	3.63	4.86	1.987	29.75	37.58
Bay of Fundy	0.743	3.44	6.82	1.208	82.79	NA
Danish waters - older	0.790	3.5	NA	NA	NA	NA
Danish Little Belt - oldest	0.840	NA	NA	NA	NA	NA
Norwegian Sea	0.850	4.3	5.15	1.942	21.31	27.14
Eastern-Newfoundland	0.882	3.1	7.22	1.833	11.14	9.78
Gulf of Maine	0.929	3.36	6.12	1.168	26.80	26.80
Icelandic waters	0.986	3.2	6.70	2.290	3.03	NA
West Greenland	NA	3.6	NA	1.697	1.59	1.59
Total number of studies for which this parameter was available:	*19*	*16*	*17*	*17*	*17*	*13*

The MEDDs ranged from 4.15 to 7.22 kJ·g-1 with a global average of 5.51 (SD: 0.96) kJ·g-1 (STabs. 5-9). Single diet studies comprised of various prey species but in each study, only a few prey species contributed substantially to the energy density of the diet. For study areas with a high MEDD there was a dominance of high-energy density prey (generally herring) while study areas with lower MEDD values showed a higher abundance of low-energy density prey (mostly *Gadidae* spp. like cod and whiting) (STab. 5-6). Although this geographical variation was evident, some prey species or prey groups were found to be included in the diet of porpoises at nearly all study sites: gadoids were found in all (17/17) studies, clupeids in 16/17, sandeels in 15/17, squid in 12/17 and gobies in 11/17.

According to previously published metrics of cumulative human impacts of world oceans (Halpern et al. 2008, 2015, 2019), North-west European waters had the highest maximum CHI scores, with maximum scores of 7.91-8.39 in the German, Dutch and English North Sea as well as the Norwegian Sea but the mean score was highest for Scottish waters (CHI mean score of 2.62). Waters around Canada and the US generally showed lower mean CHI scores compared to European waters (STab. 10).

PCB measures where available for a total of 484 individual adult male harbour porpoises, which are considered to be a better proxy to measure area specific environmental pollution than females or porpoises in other age classes (Berggrena et al. 1999; IWC 2019). Data was available from varying numbers per area of which the life history parameters were gained, ranging from only n=1 for the NW Iberian Peninsula (∑PCB of 50.8) to the maximum of n=127 for Scottish waters (mean ∑PCB of 14.47 with SD: 12.04). Lowest ∑PCBs were reported for West Greenland (∑PCB of 1.59), the German North Sea (∑PCB of 2.2) and Iceland (∑PCB of 3.02) and highest for the Bay of Fundy (∑PCB of 82.79). The number of congeners included by the different studies varied additionally, and therefore the analyses were conducted on two subsets of data: PCB1 refers to all studies, whilst for PCB2 the analyses were restricted to studies reporting ≥∑17PCB-≤∑99PCB (Table 2). Lowest ∑PCBs were found for West Greenland (∑PCB 1.59) the Salish Sea (∑PCB of 9.13), and Eastern-Newfoundland (∑PCB of 9.78), and highest for the water around the NW Iberian Peninsula (∑PCB of 50.8) and the older Dutch study (∑PCB of 52.11) when restricted the analysis to ≥∑17PCB-≤∑99PCB (STabs. 11-12).

The model which best explained the number of pregnant females (Npreg) in the total number of females (Ntotal) had both MEDD and PCB1 as the predictor variables, and not CHI (AIC=102.7). The confidence intervals for the estimated log-OR of this model revealed a strong, positive association with MEDD (log-OR[CI95%]: 0.98[0.46-1.51]), and a weaker, negative association with PCB1 (log-OR[CI 95%]: -0.02[-0.04-0]) (Model 8, Fig. 2, SFig. 4). When replacing PCB1 by PCB2 (thereby reducing the number of study areas), only MEDD was retained in the final model.

None of the environmental parameters assessed here explained the variance in ASM between the study areas (Model 9). However, the number of observations (the N total per study area), which was used to weigh the model, varied greatly between study areas (range n=32-354). The square root of the residual deviance divided by the degrees of freedom was large (6.29 for the model with PCB1 and 5.17 for the model with PCB2) and findings therefore likely dominated by the difference in sample size per study area.

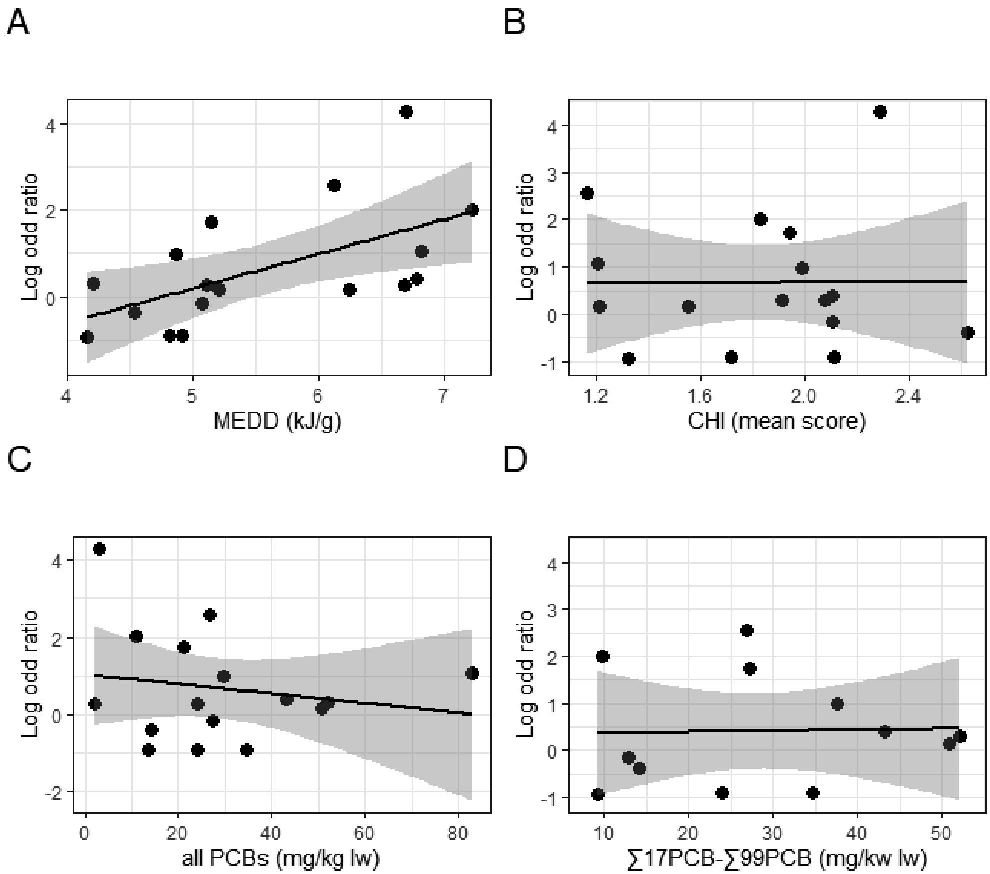

Figure 2. *Log odds ratios (y-axis) of being pregnant (versus being non-pregnant) in relation to the environmental conditions (x-axis): (A) the mean energy density of the diet (MEDD) (STab. 5), (B) cumulative human impact (CHI) mean scores (STab. 10), (C) chemical pollution by PCBs (PCB1) and (D) chemical pollution by PCBs restricted to studies reporting ≥∑17PCB-≤∑99PCB (PCB2) (STab. 11-12). A linear regression line is fitted to all graphs, with the grey shaded areas reflecting 95% CI.*

Discussion

Our study demonstrates that prey energy density, health status and nutritional status contribute importantly to reproductive success of harbour porpoises. Likewise, in mammals at large, maternal malnutrition has been shown to compromise conception, length of gestation, and foetal growth and development, with poor maternal health also affecting the onset of sexual maturity, and leading to pregnancy termination, preterm deliveries, small birth sizes and birth defects (Ashworth et al. 2009; Christiansen et al. 2014; Mellor & Murray 1982; Rondó et al. 2013; Smith 1947; Torheim et al. 2010; Trites & Donnelly 2003). We have shown, both at a local scale and globally, that comparable mechanisms likely occur in harbour porpoises. In our local study population (Dutch waters) we demonstrated that maternal nutritional and health status affect foetus size (Fig. 1) and the probability of being pregnant, respectively. Globally, pregnancy rates were best explained by the energy density of prey eaten and, to a lesser extent, by pollution with PCBs (Fig. 2, SFig. 4).

Reproductive impairment has previously predominantly been associated with PCBs, with numerous studies describing negative effects on marine mammal health, including implantation failure, abortion and sterility in pinnipeds (Helle 1976; Reijnders 1986; Reijnders et al. 2018), and increased first-born calf mortality, as well as infectious disease and the development of cancer in cetaceans (Jepson et al. 2016; Reijnders et al. 2018; Wells et al. 2005). Despite this, in our study, ambient PCB levels did not associate well with porpoise pregnancy rates and age at sexual maturity. This could be a result of the discrepancies between the samples used in previous studies compared to the PCB proxy used here; PCB levels measured in adult male porpoises as proxy of environmental contamination. This data was more abundantly available in literature, and it reduced the bias of offloading which occurs in mature females (IWC 2019; Evans 2003), thereby providing a more accurate representation of area specific maximum levels of contaminants. It is however possible that PCB levels measured in males, as well as the two life history parameters assessed here are (in combination) not suitable as biomarkers for the assessment of population effects of PCB pollution on cetaceans. We were additionally challenged by the many differences in PCB congeners examined, analytical protocols used between studies, and the level of detail at which the data was reported. Restricting to the more similar congener groups analysed ($\geq\sum$17PCB-$\leq\sum$99PCB) significantly reduced the sample size. Besides, data on other pollutants which may severely affect marine wildlife (Tian et al. 2020) was not available. This highlights the need for more consistent, long-term, global contamination data measured in the same tissues with similar calibrated techniques. Also, other approaches or biomarkers to assess pollution impact on small cetaceans should be established or further explored, e.g., through using individual-based model frameworks (Desforges et al. 2018; Hall et al. 2006; IWC 2019; Reijnders et al. 2018).

The increasing exposure of marine mammals to multiple stressors such as ambient noise and climate change is stated to be a major knowledge gap in marine ecology (Castellote et al. 2012; IJsseldijk et al. 2018a; Laidre et al. 2008; NAS 2017; Tyack 2008). The assessment of individual pressures as well as the cumulative effects of multiple stressors on marine mammal populations is highly challenging and a way of quantitatively assessing these effects is yet to be established (IJsseldijk et al. 2018a; NAS 2017). We explored the ecosystem-specific, multiscale spatial model by Halpern et al. (2008, 2015, 2019) and used this as a proxy of CHI on the study areas. Using this validated and published model had the advantage that CHI scores could be assessed equally for each study area, which was in contrast to the diet and PCB parameters for which it was unavoidable to use studies with different sample sizes, methodologies or spatiotemporal mismatches (STab. 2). The CHI model was however gained from a relatively recent study (Halpern 2015, 2019), whilst the life history studies were often conducted over several years and could also include data from previous decades (STab. 2). Additionally, the CHI model did not necessarily take into account the stressors which directly apply to or specifically affect small cetaceans, since it was built to quantify the effects of human activities on the world's oceans. The CHI scores were therefore a measure of stressors affecting the environment and although it is likely that negative effects on oceans directly or indirectly translate to the top predators living there (Bossart 2011), our analyses did not identify a direct negative association with the crucial life history parameters assessed. However, from a porpoise's perspective, the resolution for the CHI assessment used here are rather crude. This can be particularly true for areas where local, emerging and specific natural threats affect cetacean populations. Examples are the Baltic and North Seas, where there is large scale construction of offshore wind farms (Gilles et al. 2009; Teilmann & Carstensen 2012) as well as predatory pressure on porpoise populations (Leopold et al. 2015a). It is therefore recommended to add local threat layers, as well as to assess individual threat layers apart from a cumulative approach, before drawing smaller-scale or regional conclusions on the effects of anthropogenic stressors on cetaceans.

Our results present the current best overview of life history parameters of porpoise populations throughout their range, based on a long-term, large dataset containing information from stranded and bycaught individuals. Although there is uncertainty to which extent data from deceased cetaceans are representative of the at-sea population, it is unavoidable to use this, as longitudinal or alternative methods to determine reproductive parameters are currently lacking (IJsseldijk et al. 2018a, 2020; NAS 2017; Peltier et al. 2012; ten Doeschate et al. 2018). Pregnancy rates and MEDD differed between porpoises in the different health and nutritional categories, as demonstrated both in our sampling population (Dutch waters) (SFig. 2, Tab. 1) and in the global assessment. Globally, the highest pregnancy rates were reported in studies using specimens gained from fisheries (STab. 3). Results from studies on bycaught porpoises intrinsically differ from those based on stranded individuals. The latter constitute a mixture of animals dying of disease, bycatch, or e.g., interspecific interactions. Yet, regardless of an individual's eventual cause of death, a reliable assessment of health status should be conducted through extensive pathological assessment, since bycaught marine mammals may also display significant disease or poor nutritional conditions (IJsseldijk et al. 2021b; Siebert et al. 2020). Therefore, for future studies, both at global and local scales, it is vital to examine and report, in concert, pathology, ecology, toxicology and life history in order to estimate and correct for the effects of health, nutritional or confounding and interacting factors on life history parameters.

We assessed prey quality, expressed as mean energy density, as a determinant of reproductive success. Previous studies on a range of piscivorous top predators suggested that a shift towards low-quality prey, regardless of an availability of high-quality prey, may have detrimental effects on populations (Grémillet et al. 2008; Österblom et al. 2008; Trites

& Donnelly 2003; Wanless et al. 2005). Quantifying these effects on the predators' vital rates requires knowledge on population development, food-web structures, predator energetic requirements and prey energy densities; all of which may vary seasonally, geographically or temporally, both for the predators and for their prey (Fritz et al. 2005; Spitz et al. 2018; Trites & Spitz 2018). Studying the effect of nutritional stress in free-ranging animals is therefore complex and requires the understanding of many factors (Fritz et al. 2005). We did not assess prey availability, since this data is largely lacking for many of the non-commercial fish species which are part of porpoise diets but we were able to establish proxies of prey energy density for all but one of the study areas. It should however be kept in mind that we had to use mean prey energy densities, as well as mean CHI and PCB scores, since the input data each had their own limits and uncertainties. Modelling of mean values and thus data summaries per study area were conducted using a random area effect to at least partially account for unknown and unmeasured factors in which areas may differ but these approaches can result in hidden correlations or incalculable biases. Despite that there are still many uncertainties regarding the direct and indirect causes and consequences of nutritional stress on cetaceans, we here found a strong association between prey energy density and pregnancy, based on large numbers of animals and studies. The present study advances our understanding of risks that may affect harbour porpoise life history traits. We show the necessity of not focussing solely on chemical pollution when assessing reproductive impairment but to incorporate different and cumulative factors, and in particular we highlight the access to undisturbed prey with high energy density as a crucial factor for reproductive success.

Methods

Data collection and preparation – Dutch waters

Study specimens

Between 2006-2019, 1457 deceased harbour porpoises in The Netherlands were collected for post-mortem investigations and diet analysis, with the necropsies conducted following internationally standardised guidelines (IJsseldijk, Brownlow & Mazzariol 2019). For this study focussing on female life history, we selected all females >115 cm, as smaller animals may be maternally dependent and can be considered young of the year (Lockyer 2003). Cases in DCC5, which represent carcass remains, and of which reproductive organs were not assessable or present due to scavenging or incompleteness of the carcasses were excluded. The reproductive organs of the female porpoises >115 cm (n=328/1457) were macroscopically inspected to differentiate between immature and mature animals, with the presence of ovarian corporal scars used as indication of maturity (Learmonth et al. 2014). Exact age was determined by assessing tooth growth layer groups (GLG) for a subsample of cases (n=154), according to previously described methods (Hohn & Lockyer 1995). Data can be found in STab. 13.

Health and nutritional status

Porpoises collected for post-mortem investigation were necropsied with the primary aim to determine the animals' causes of death and their health status, with the quantity and quality of data and results strongly depending on carcass freshness and completeness as well as other logistical and financial factors (IJsseldijk, Brownlow & Mazzariol 2019). For this study, we established three proxies based on the findings and metrics taken and assessed at necropsy: a proxy for health status and two proxies for nutritional status. For the proxy for health status, we assessed the cause of death among the mature females (n=199/328) and divided all cases in two categories. In the first category, we placed all mature females which most likely died as a direct result of incidental bycatch (diagnosed following IJsseldijk et al. 2021b), as a direct result of a predatory attack (diagnosed following Leopold et al. 2015a) or as a direct result of another acute cause, such as sharp forced trauma or dystocia (obstructed labour, full-term foetus) which did not present signs of significant disease or debilitation. All other animals were placed in the second category, with these mature females displaying evidence of general and significant debilitation, including infectious disease (such as significant parasitism, bacterial, viral or mycotic infections) and/or emaciation. Cases that could not be grouped, mostly as a result of decomposition, were excluded from analyses that included this as a parameter.

The first proxy of nutritional status was based on the mean blubber thickness, measured during necropsies in a dorsoventral line on the left body flank just cranial to the dorsal fin, at three locations: dorsal, lateral and ventral. Blubber thickness in small cetaceans has previously been shown to decrease during periods of fasting (Kastelein et al. 2019; Koopman et al. 2002) and this metric has been used as proxy of nutrition by others (Beineke et al. 2005; Jepson et al. 2005b; Leopold 2015; Zeng et al. 2015) however, it should be noted that blubber thickness is not always a good reflection of individual health nor cause of death (e.g., animals dying of acute causes could also be debilitated (IJsseldijk et al. 2021b; Siebert et al. 2020)). There is uncertainty to what extent factors such as age, sex and season naturally influence blubber thickness, and this should be accounted for. Since we focus our analyses on mature females, no further correction for age and sex was done. However, to correct for season, we modelled the mean blubber thickness as a function of Julian date using a generalized additive model (GAM) to allow a smooth effect of the predictor variable (Julian date). This captures the sinus-shaped seasonal variation in blubber thickness (referred to as corBT) which naturally occurs as a result of changing water- and air temperature (Kastelein et al. 2019; Lockyer 2007) (SFig. 5). The residuals of that model were thereby indicative of an adult females' nutritional status independent of season; hence they were used as the proxy for nutrition (referred to in the main text as: nutritional status using corBT, Model 1 in STab. 1).

The second proxy of nutritional status used the categorical variable "nutritional condition" (NCC), which is assigned during necropsies as good, moderate or poor. Animals in good NCC generally presented a convex outline on a cranial perspective, no signs of muscle atrophy, and presented signs of visceral fat. Animals in moderate NCC generally did not have a fully round outline on a cranial perspective, showed possible signs of muscle atrophy and did not present visceral fat. Animals in poor NCC generally had a concave outline on cranial perspective, with visible aspects of vertebrae and/or scapula externally, a hollow appearance caudal to the skull and signs of muscle atrophy (based on IJsseldijk, Brownlow and Mazzariol 2019). Since this categorial differentiation is collinear with the first established proxy of nutritional status (SFig. 5), it was not used in the same modelling procedures. Therefore, models were run twice, first with corBT and secondly with NCC (for an overview see STab. 1).

Pregnancy rate and foetus size

The pregnancy rate (PR) was calculated as the proportion of pregnant females in the total sample of mature females (following e.g., Learmonth et al. 2014 and Norman et al. 2018). Pregnancy rates were also calculated separately for the animals in the two different health status categories (see above). To avoid missing the presence of very small, early embryos, samples from the period of conception (June-August (Lockyer 2003)) as well as samples from the period of calving (May-June (Lockyer 2003)) were excluded in the PR calculations. All foetuses were measured during necropsy (of the dam) and a proportion of these were also weighed.

Mean energetic density of diets

As a measure of the quality of prey species constituting the diet of harbour porpoises necropsied in The Netherlands, we calculated the mean energy density of their diet (MEDD). The energy density (ED) is defined as the energy per kilogram of wet weight of prey (Spitz et al. 2010, 2012). ED values were taken from the literature (STab. 7). If for a given prey species no value for ED could be found, the ED of a comparable species (mostly same genus), or the mean value of its family, was used. For species for which multiple ED values were available, values were averaged. ED values reported in kcal were multiplied by 4.184 to convert to kJ (following e.g., Perez (1994)). To calculate the mean ED of the diet for a group of porpoises (MEDD, kJ·g-1) we used:

$$MEDD = \frac{1}{\sum_{i=1}^{n} M_i} \sum_{i=1}^{n} (M_i * ED_i) \quad (1)$$

where i is the prey species and M the reconstructed preys mass in grams (following Spitz et al.[8]). The reconstructed preys mass per species is multiplied by the species-specific ED and the energy sum is divided by the total mass of all prey, resulting in the MEDD.

Data analyses and statistical models - Dutch waters

Data were explored prior to analyses following Zuur et al. (2007, 2009). Data exploration and analyses were performed using R version 3.6.3 (R Core Team 2017) with packages ggplot2, grid, gridExtra, rsq, glmTMB, mgcv and ggpubr. A number of statistical models were developed (for referencing in the text see overview in: STab. 1).

Influences on foetus size

In order to identify which variables influence foetus size, we firstly identified the best measure for foetus size. A Generalized Linear Model (GLM) for foetus length and weight was fitted (Model 2), with weight only available for a subset of all foetuses (n=34). This model indicated a close relationship between length and weight (R^2 of 0.8 for foetus length as a function of mass, SFig. 5), and foetus length was therefore used as representative for foetus size in the subsequent analysis, to increase sample size. GLMs with a Gaussian distribution were used (Model 3). The model selection tested for covariates and their influence on foetus length, with the predictor variables: Julian date to account for foetus length which increases throughout gestation, total length of the mother, health status of the mother, nutritional status of the mother. Interactions between length of the mother and her nutritional status were included following data exploration. Only cases with complete observation of all parameters were included (n=43). A backwards model selection approach was applied with the drop1 function from the R language used to assess which model terms could be excluded (R Core Team 2017). The best fitting model was selected using Akaike's Information Criterion (AIC), which provides a relative measure of the goodness of fit of statistical models. Model validation was done to identify potential violations of model assumptions by inspection of normalised residuals and assessment of residual probability plots. Likelihood profile confidence intervals (95%) and odds ratios of the most optimal model were calculated. Models were run twice, first using the first proxy of nutritional status based on a for season corrected blubber thickness metric (corBT) and secondly using the nutritional condition category (NCC), taken into account blubber thickness, visceral fat and muscle mass (for full descriptions, see above).

Influences on pregnancy

In order to identify which variables influence pregnancy, we firstly coded all mature, pregnant females as 1 and all mature, non-pregnant females as 0. Next, GLMs with a binomial error distribution and logit link were used (Model 4) to test the influence of included covariates on the likelihood of pregnancy. Only cases with complete observations of all parameters were included (n=65). The predictor variables included in the saturated model were age, year to assess temporal variance, month to assess seasonal variance, health status (proxy, categorical), and nutritional status. Interactions were added following data exploration: between health and nutritional status, between the health status and year and health status and month. Model selection, validation and interpretation was conducted following the protocol previously described above. Models were run twice, first using the first proxy of nutritional status based on a for season corrected blubber thickness metric (corBT, numerical) and secondly using the nutritional condition category, taken into account blubber thickness, visceral fat and muscle mass (NCC, categorical) (for full descriptions, see above).

Age at sexual maturity

The age at sexual maturity (ASM), or age at 50% maturity, was determined using binomial logistic regression models. Maturity, coded as 1 for mature females and 0 for immature females, was modelled as a function of age (in years) to assess ASM (n=154, Model 5). The model was fitted using a binomial error distribution and logit link, as is appropriate for binary data and the ASM was estimated by calculating the negative of the slope over the intercept.

Assessment of porpoise life history and environmental condition globally

The life history response variables assessed were PR and ASM. The environmental predictor variables used were quality of diet, expressed as mean energy density of diet (MEDD), cumulative human impact (CHI) with data on climate change, fishing, land-based pressures, and other commercial, and lastly chemical pollution expressed as polychlorinated biphenyls (PCBs). Details below.

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Life history

For PR the following were tabulated: (1) the number of pregnant females out of the total number of mature females in each study, (2) the determined conception period and whether this was accounted for in the calculation of the PR, (3) the method to assess pregnancy, which was either based on the presence of a foetus or presence of a corpora lutea (CL), and (4) the source of the specimens: either directly from fisheries, strandings including trauma cases, or a combination thereof (STab. 3). For ASM we provide: (1) how ASM was assessed in each study, and (2) the standard error (SE) or confidence interval (CI), if reported (STab. 4).

Energy density of prey

A literature search was performed for diet studies from stomach contents of porpoises from or near the study areas where PR and ASM were determined. When multiple diet studies were available the study was selected that best corresponded to the time frame at which PR and ASM were calculated. For the diet studies which reported the reconstructed preys mass in grams we used formula 1 (STab. 8). When the prey mass was reported as a percentage of relative abundance in terms of estimated biomass of prey (%M), we multiplied %M by the ED of the prey species and divided the total %M (STab. 9), using:

$$MEDD = \frac{1}{\sum_{i=1}^{n} \%M_i} \sum_{i=1}^{n} (\%M_i * ED_i) \qquad (2)$$

For the studies where the %M was presented in a bar chart, we measured the %M using digital callipers.

Cumulative human impact

An ecosystem-specific, multiscale spatial model containing high resolution data on the intensity of human stressors and their impact on marine ecosystems was developed by Halpern et al.[12,14] as part of their Ocean Health Index project. CHI values derived by this model are based on fourteen stressors related to human activities from four primary categories: (1) land-based drivers, including nutrient pollution runoff, organic chemical pollution runoff (pesticides), direct impact of humans (density of coastal human populations), and light; (2) five types of (commercial) fishing, including commercial demersal destructive, commercial demersal non-destructive high bycatch, commercial demersal non-destructive low bycatch, pelagic high bycatch, pelagic low bycatch, and artisanal; (3) climate change, including sea surface temperature, ocean acidification and sea level rise; and (4) shipping. Extensive descriptions of these drivers are published in the methods and supplementary material of Halpern et al. (2008, 2019), including information on the origin and validation of the data.

For this study we used the global CHI dataset that is publicly available via the Knowledge Network for Biocomplexity (Halpern et al. 2019). Data on CHI was based on the year 2008. We extracted the CHI scores for each of our study areas at ~1 km2 resolution and calculated the min, max, mean and median values. To do so, we defined our study areas using the standard georeferenced marine regions as published under the Flanders Marine Institute (2020). In most cases we combined 2 or more regions from the database to get full coverage of the study area. For the study areas where the marine regions did not provide full coverage, we used a manually created polygon. The list of regions is given in STab. 15. For areas with more than one life history study (Denmark and The Netherlands) we used the newest studies since these provided the better match to the time of the CHI score calculation (STab. 2).

Chemical pollution

Polychlorinated biphenyls were not included in the list of organic polluters by Halpern et al. (2019). However, PCBs have been specifically associated with reproductive impairment in many marine mammal species (Desforges et al. 2018; Jepson et al. 2016; Murphy et al. 2009, 2015; Reijnders et al. 1986), therefore the correlation with life history parameters for this industrial organic pollutant was assessed separately. Data was retrieved from the International Whaling Commission's (IWC) 'POP Contaminants Trend Explorer' tool, hosted on the portal of the Sea Mammal Research Unit (SMRU, University of St. Andrews, Scotland). This tool is established under the IWC Scientific Sub-Committee on Environmental Concerns (IWC SC/68A 2019) as part of the IWC Pollution 2020 Initiative and includes data from scientific publications from the 1970s-2000s (IWC 2019; Reijnders et al. 2018). The database was provided by the tool manager and included data restricted to adult males, to reduce the bias of biotransfer of chemicals, which occurs during gestation and lactation in females (Berggrena et al. 1999). The tool reports PCB concentrations in blubber, which is the most commonly assessed tissue in marine mammals for studying the burden of the highly lipophilic and stable PCB compounds (Evans 2003). PCB concentrations that were measured in porpoises in the same areas from which life history parameters were verified with the literature and included. In addition, the literature was searched for PCB analyses of harbour porpoises published in the 2010s, as well as own institutional databases, and data added to align time frame, where possible, with time frame of conducted life history studies (STab. 2).

The presentation of concentrations of pollutants was either on the basis of wet weight (ww) or lipid weight (lw). To allow comparison, the datapoints need to be converted to one common unit, with lw most frequently reported. Studies reporting only ww or dry weight were not included. Studies reporting ww and percentage of lipids (%lipids) were converted to lw, using:

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$$lw = \frac{ww}{\%lipids} * 100 \quad (3)$$

The datapoints were converted to mg/kg lw for all studies and the mean ∑TotalPCB is reported per area.

The variance of the sum of congeners reported ranged from ∑6PCBs up to ∑99PCBs, with several older studies reported Aroclor mixtures. Data per congener was however largely not available in literature. We therefore present two mean ∑PCB datapoints: firstly including all studies regardless of the sum of congeners or mixtures (referred to as PCB1), and secondly limited to studies reporting ∑17-99PCBs (referred to as PCB2).

Statistical models for global assessment

For the analyses we restricted to study areas with complete observations of the environmental conditions in order to compare models. A GLM fitted with a binomial distribution and logit link was used to determine the effect of environmental conditions on pregnancy rates (Model 6). The response variable was the number of pregnant females (Npreg) in the total number of females (Ntotal) (grouped binomial data, STab. 3). Since the differences between study areas can be large as a result of unknown effects, an individual normal random effect for area was added on the logit scale. Another GLM was conducted to determine the effect of the three environmental conditions on age at sexual maturity (Model 7) fitted with a Gaussian distribution and weighed by sample size (Ntotal) (STab. 4). This model was applied twice using two individual predictor functions: first, with the predictor variables MEDD, CHI and PCB1 and secondly with the predictor variables MEDD, CHI and PCB2. The latter restricted the analyses to a smaller number of study areas due to missing data but it reduced some of the bias as a result of very small (< ∑17PCBs) or very large (Aroclor mixture) reported ∑PCB datapoints.

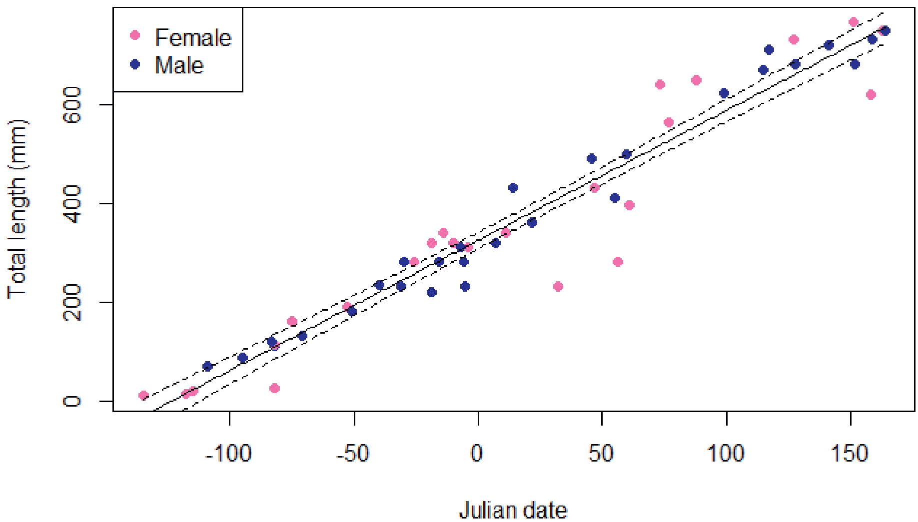
For all models, a backward stepwise model selection process using the drop1 function was conducted and the best models explaining the life history parameters pregnancy rate (Model 8) and age at sexual maturity (Model 9) were identified by assessment of the AIC. The logistic regression model yields log-odds ratios. This represents the odds (number of pregnant per non-pregnant) of being pregnant compared to non-pregnant in each study area, while accounting for differences in sample size. We also obtained the confidence intervals for the estimated log-odds ratios of the most optimal models.

Acknowledgement

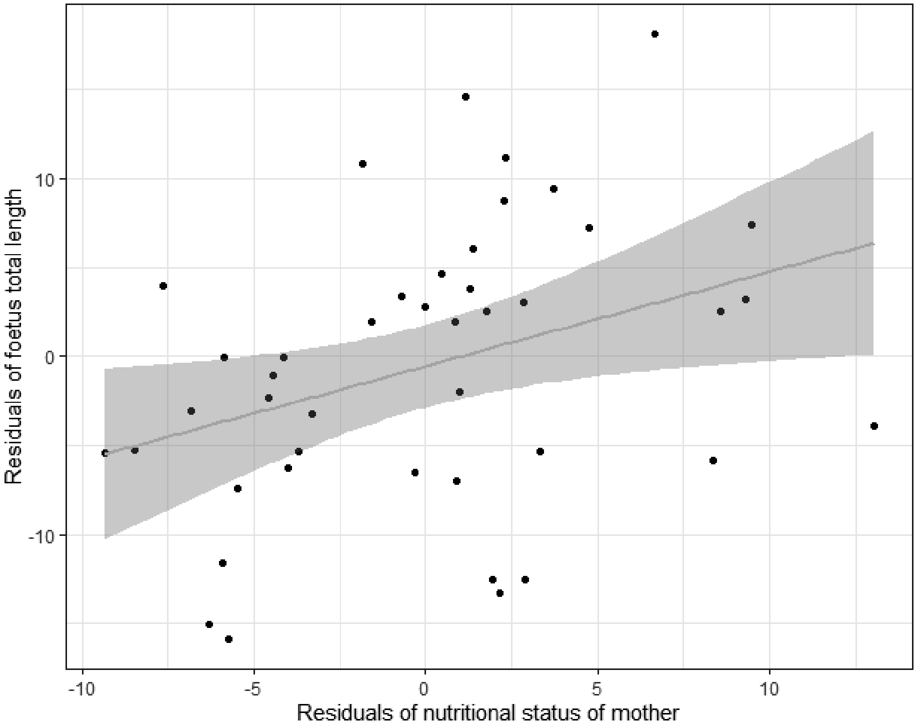
We sincerely thank the Dutch stranding network volunteers and organisations for their help with reporting and retrieving stranded specimens for post-mortem examination. In addition, we are thankful for the help of (former) colleagues, students and volunteers who conducted or assisted with the necropsies, and all students and volunteers who helped with the stomach content examinations. Benjamin S. Halpern and Ailsa Hall helped us and approved using the cumulative human impact data and the IWC/SMRU contaminant tool data respectively, and we thank Roos Voorvaart and Jonathan de Bruin for their help with data reproducibility.

Supporting information

Supplementary data associated with this article can be found in the online version at doi: 10.1038/s41598-021-98629-x.

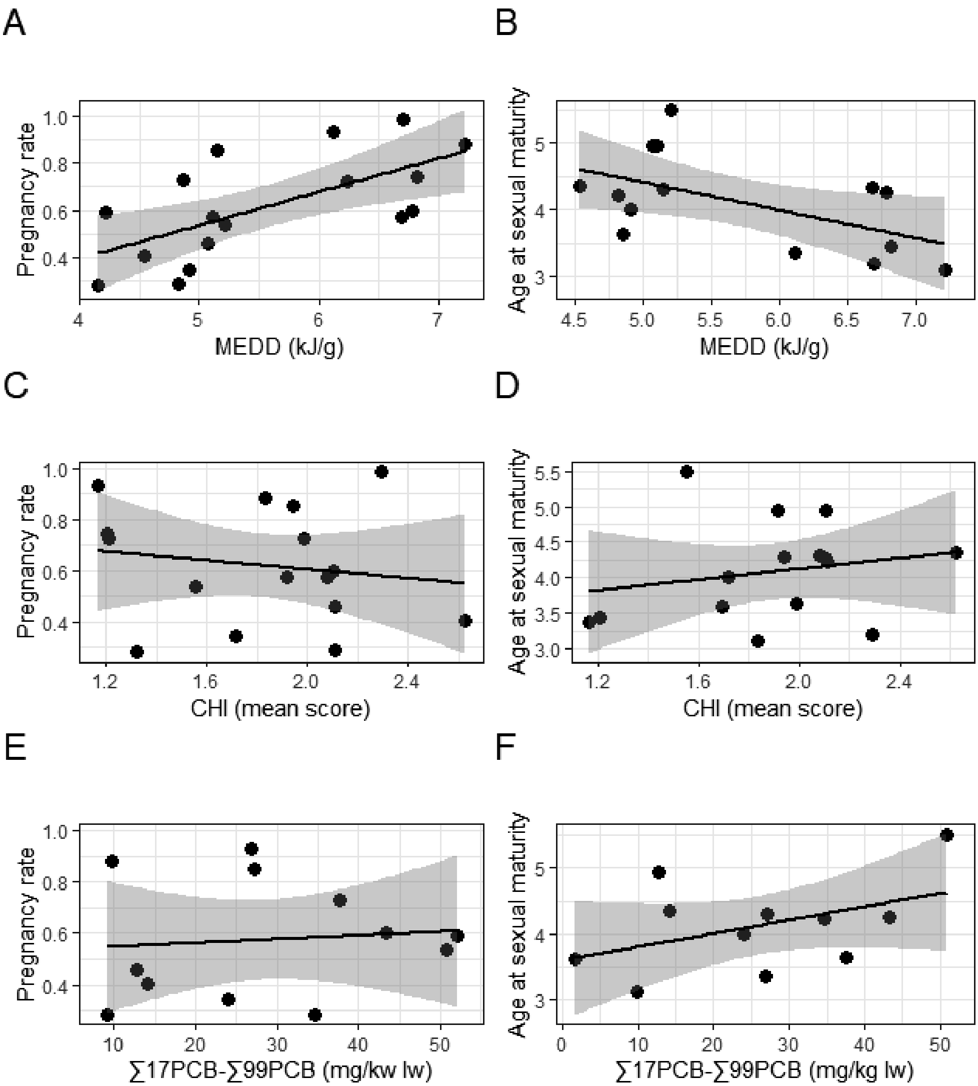


Supplementary Figure 1. *Foetal growth by day (coloured by sex with pink = females, blue = males) with linear regression line. Slope of the linear regression was 2.63, indicating growth of 2.63 mm per day. The R^2 was 0.94. Black dotted lines representing 95% CI.*

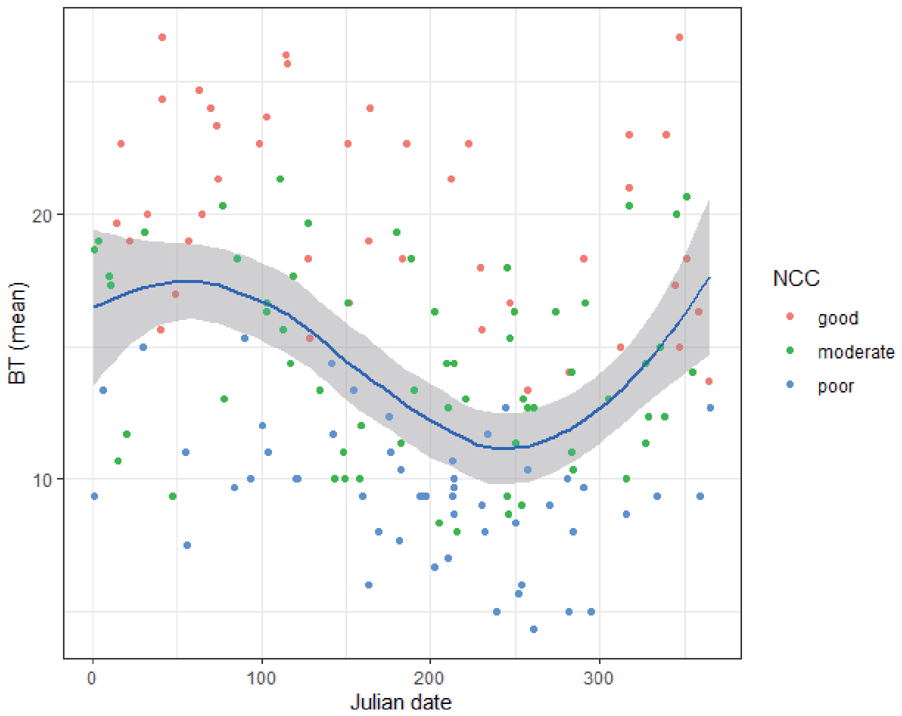


Supplementary Figure 2. *Residuals of linear model of foetus size (total length) as a function of nutritional status of the mother reveals a positive relation between parameters. Black dotted lines representing 95% CI.*

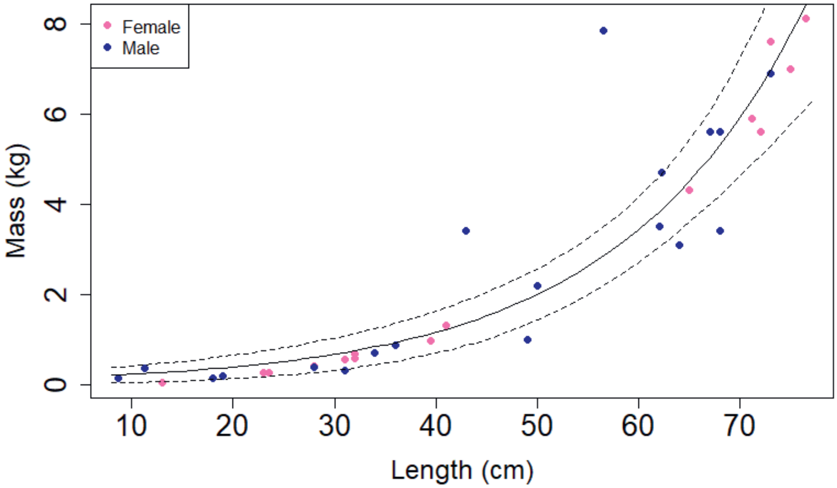
Supplementary Figure 3. *Boxplot of age (n=154) per maturity status (left) and probability of age at sexual maturity (right) with fitted regression lines (black dotted lines representing 95% CI) and red dotted line at 0.5 probability.*



Supplementary Figure 4. *Harbour porpoise pregnancy rate (STab. 4) and age at sexual maturity (STab. 5) in relation to environmental conditions: (A, B) the mean energy density of the diet (MEDD) (STab. 6), (C,D) Cumulative human impact (CHI) mean scores (STab. 11), (E,F) Chemical pollution by PCBs, restricted to studies reporting ≥∑17PCB-≤∑99PCB (STab. 13). A regression line is fitted to all graphs, with the grey shaded areas reflecting the 95% CI.*



Supplementary Figure 5. *Mean blubber thickness of mature females over the year (Julian date) shows the sinus-shaped, natural variance among all individuals. Datapoint are coloured by nutritional condition code (NCC), which are assessed during necropsies based on the animals shape, their fat reserves and musculature (see method section), with red = good, green = moderate, and blue = poor. A smooth regression line is fitted, with the grey shaded areas reflecting the 95% CI.*



Supplementary Figure 6. *Foetus mass versus total length (coloured by sex with pink = females, blue = males) with fitted regression line.* R^2 *for foetus length as a function of mass is 0.8. Black dotted lines representing 95% CI.*

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Forensic microbiology reveals that *Neisseria animaloris* infections in harbour porpoises follow traumatic injuries by grey seals

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Abstract

Neisseria animaloris is considered to be a commensal of the canine and feline oral cavities. It is able to cause systemic infections in animals as well as humans, usually after a biting trauma has occurred. We recovered *N. animaloris* from chronically inflamed bite wounds on pectoral fins and tailstocks, from lungs and other internal organs of eight harbour porpoises. Gross and histopathological evidence suggest that fatal disseminated *N. animaloris* infections had occurred due to traumatic injury from grey seals. We therefore conclude that these porpoises survived a grey seal predatory attack, with the bite lesions representing the subsequent portal of entry for bacteria to infect the animals causing abscesses in multiple tissues, and eventually death. We demonstrate that forensic microbiology provides a useful tool for linking a perpetrator to its victim. Moreover, *N. animaloris* should be added to the list of potential zoonotic bacteria following interactions with seals, as the finding of systemic transfer to the lungs and other tissues of the harbour porpoises may suggest a potential to do likewise in humans.

Introduction

The mucosal surfaces of the oropharynx are considered the principal habitats of *Neisseria* spp. recovered from humans and a number of domestic and experimental animals (Tøne 2005). Reports from wild animals are less common, however, *Neisseria zalophili* was described recently from the oral cavity of wild California sea lions (Volokhov et al. 2018), suggesting that pinnipeds may similarly harbour members of the *Neisseria* genus.

Neisseria animaloris was described for a group of organisms that had been placed originally in the Centers for Disease Control (CDC) group eugonic fermenter (EF)-4a (Vandamme et al 2006). The organism is considered to be a commensal of the oral cavity of cats and dogs and can cause systemic infections in both humans and animals (Ganière et al. 1995). Two biotypes, EF-4a and EF-4b, were described initially on the basis of arginine hydrolysis (Holmes & Ahmed 1981) and were further distinguished according to cellular fatty acid content (Dees et al. 1981) and whole-cell protein analysis (Holmes et al. 1990). The arginine positive strains (EF-4a) were subsequently described as *N. animaloris* (Vandamme et al. 2006).

Here, we report the recovery of *N. animaloris* from eight harbour porpoises (*Phocoena phocoena*) which stranded in four countries of Northern Europe: Scotland, England, The Netherlands and Belgium. Gross and histopathological evidence suggests these infections occurred following traumatic injury from a predator and the wounds fitted with those described to be inflicted upon porpoises by grey seals (*Halichoerus grypus*) (Leopold et al. 2015a).

Results

Gross and histopathology

The harbour porpoises were six adult females and two juveniles: one male and one female. All animals were in moderate to poor nutritional condition at the time of stranding. Six animals were found dead but all showed minimal decomposition, although one animal had severe scavenger damage. Two animals had live stranded and were subsequently euthanised on welfare grounds due to the severity of their condition (Table S1). Stranding locations varied temporally and spatially (Fig. 1).

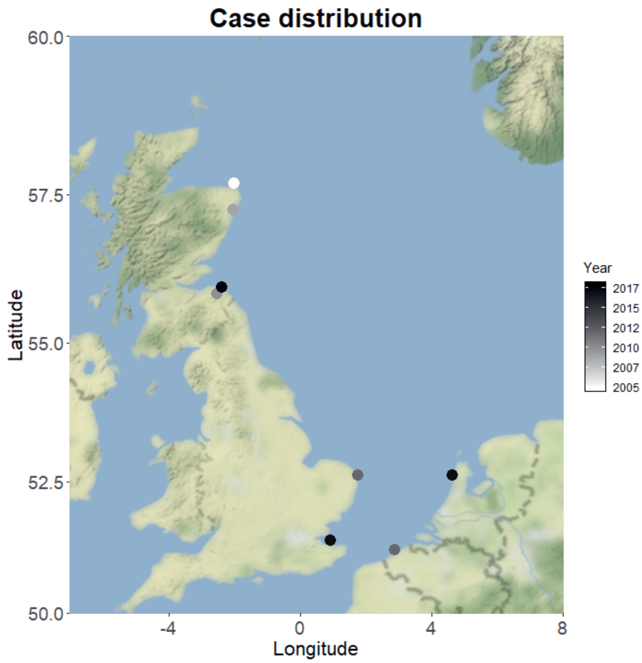

Fig. 1. *Case distribution throughout the area, with temporal colour scaling, where white is the oldest case (2005) and black is the most recent case (2018).*

The ultimate cause of death for all harbour porpoises was a disseminated bacterial infection causing abscesses in multiple tissues. All harbour porpoises where external assessment was possible (n=7) presented with abscesses and inflamed lacerations in the skin at specific sites: pectoral fins (n=3), tailstock (n=3) and in one case on a pectoral fin and the tailstock. In addition, abscesses were present in the lungs in all cases (n=8), in one or several lymph nodes in six of eight cases and in the spleen of one case. Abscesses were characterised as well circumscribed, large (up to 7 cm in diameter) masses, with fibrous walls containing caseous exudate on cut section (Fig. 2). Lung abscesses were randomly distributed throughout the lung parenchyma suggesting haematogenous spread. Lymph node abscesses occurred, varying per case, in pulmonary lymph nodes, prescapular lymph nodes and mesenteric lymph nodes. Histologically the abscesses were similar and characterised by well-demarcated masses with fibrous capsules and centrally large numbers of neutrophils and macrophages (pyogranuloma), few erythrocytes and intralesional aggregates of bacteria. Bacteria were non-filamentous, non-branching and surrounded by eosinophilic, acellular material that formed radiating clubs (Splendore-Hoeppli material) (Fig. 3).

Other pathological lesions which can be characteristic of bacterial sepsis and therefore might have been related to the *Neisseria* infection, were noted, including multifocal acute hepatic necrosis (n=2), haemorrhages in brain and adrenal glands (n=1); and mild acute cortical necrosis in the adrenal glands (n=1). Two livers exhibited fat accumulation (lipidosis), a change associated with an unknown period of anorexia prior to death.

Findings related to a helminth parasite burden, considered unrelated to the *Neisseria* infection, or the ultimate cause of death, were noted in lungs, liver and auditory sinuses (Table S2). Parasitic infections are common in free-ranging harbour porpoises and their clinical relevance is uncertain (ten Doeschate et al. 2017).

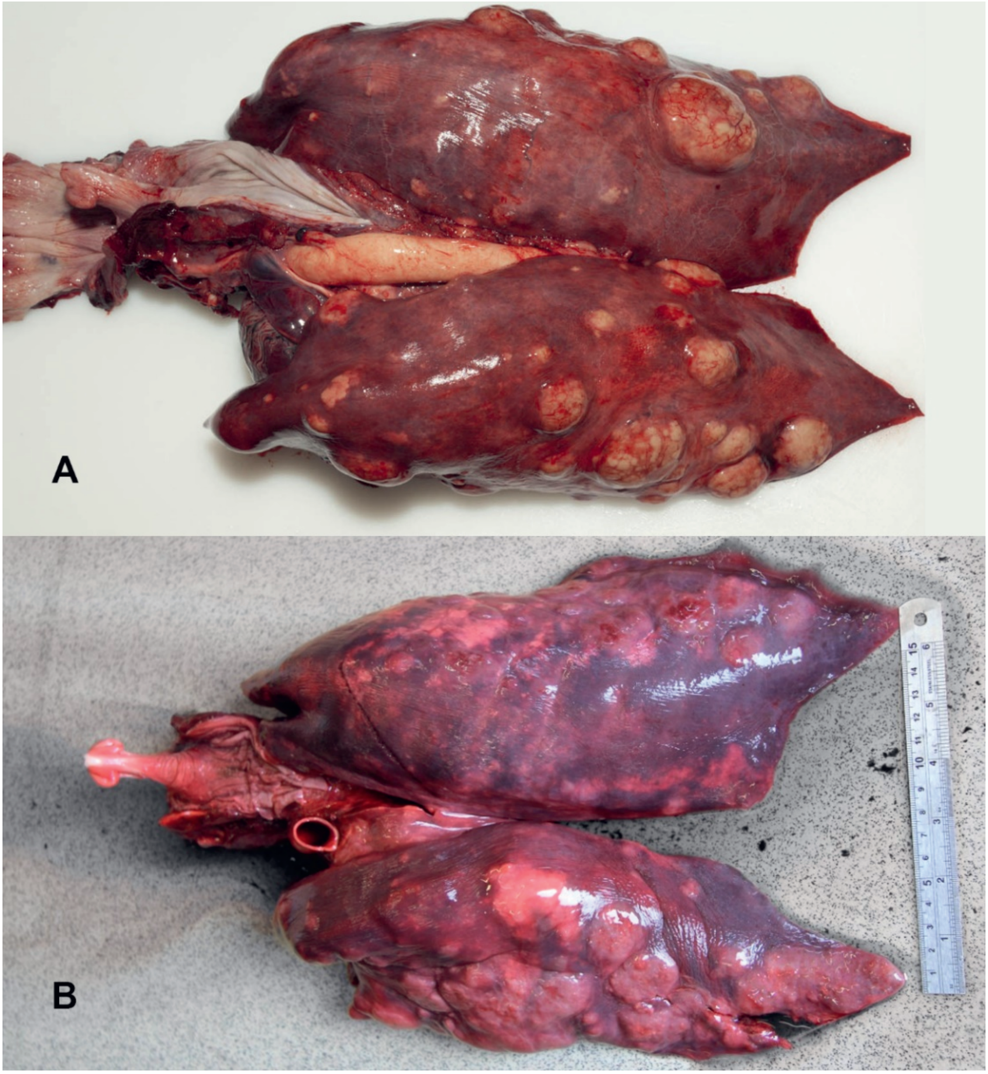

Fig. 2: *Respiratory tract of UT692 (A) and M175/18 (B) presenting with strong similarity in the macroscopic morphology of the lung lesions: multiple well demarcated, 5-60 mm yellow abscesses bulging from the surface. Photo's: Multimedia, UU (A) and SMASS (B).*

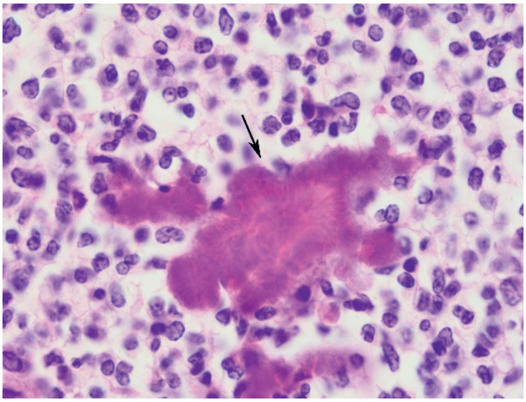

Fig. 3: *Lung of UT1576, HE stain, 100x magnification; typical example of a Neisseria abscess, with large numbers of degenerate neutrophils, admixed with typical aggregates of bacteria, which are surrounded by eosinophilic, acellular material that form radiating clubs (Splendore-Hoeppli material indicated by arrow).*

Macroscopic photos and/or detailed external descriptions were available from all cases for the evaluation of external evidence of lesions consistent with bite marks induced by grey seals, based on comparison with a previous study (Leopold et al. 2015a). Lesions were bilaterally or ventrodorsally, close to symmetrical, present on head, pectoral fins and/or tailstocks. Though these lesions were chronic, based on tissue response, they were otherwise consistent with lesions induced by grey seals based on location and morphology of the lesions (Fig. 4). For one case (M21/09) the carcass had been scavenged so severely that presence or absence of external lesions could not be assessed reliably. For this case it remains uncertain if the *Neisseria* infection could have been related to seal bites.

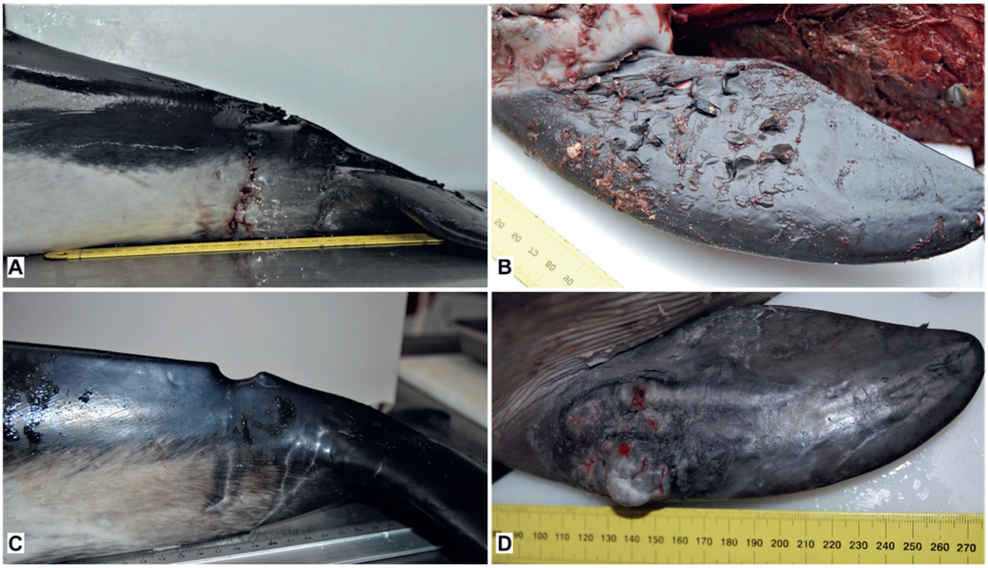

Fig. 4. *Acute grey seal bite wound on tailstock (A, case ref no. UT1004) and pectoral fin (B, case ref no. UT1007) from cases for which DNA analysis proved that lesions were inflicted by grey seals in a previous study (van Bleijswijk et al. 2014). Healed tailstock lesions (C, case no. UT1576) and an abscess on the pectoral fin (D, case ref no. UT692) show morphological similarities to the acute cases (A,B), although lesions presented in C and D are chronic.*

Microbiology

A Gram negative coccal-shaped organism which formed non-haemolytic, yellow, sticky colonies on CSBA was recovered from all abscesses cultured. A preference for additional CO_2 was demonstrated. The isolates were sensitive to 1 unit of penicillin but did not produce elongated cells at the edge of growth closest to the disc, indicating that they were Gram negative cocci. Growth was obtained anaerobically and at 25 °C but not at 42 °C. Growth on MacConkey agar without salt varied between isolates. Isolates from 6 animals (M29/05, M21/09, M78/10, SW12/463, UT1576 and M175/18) were tested in a panel of biochemical tests and results were consistent between each other and with those described for *N. animaloris*. Tests were positive for catalase and oxidase but negative for indole. Nitrates were reduced to nitrite with API 20E and 20NE. Acid production was produced from glucose with API 50CH but was negative with API20E and 20NE and produced varied results with glucose DIATABS. The only other positive carbohydrate detected was ribose in the API 50CH system. Arginine dihydrolase was positive with both API 20E and DIATABS, while all other tests in the API 20E, 20NE and DIATABS were negative. Using API ZYM, the only positive tests were weak reactions for esterase C3, esterase C8, leucine arylamidase and acid phosphatase. *Neisseria animaloris* is not included in the API databases, however, results, where available, were similar to those obtained by traditional methods in the species description (Vandamme et al. 2006). API ZYM reactions matched those for the type strain of *N. animaloris* available from the website of the Culture Collection of the University of Goteburg (CCUG 52597T), with the exception that the type strain had a weak reaction for alkaline phosphatase (http://www.ccug.se).

Whole-cell fatty acid analysis was performed on three isolates (M29/05, M21/09, M78/10) and results were consistent with those of Vandamme et al. (2006) (Table S3). Sequencing of the 16S rRNA gene was carried out for seven of the eight isolates with a closest match to *N. animaloris* on BLAST analysis. Over a 1390 bp contig available for all seven isolates either 8 (n=6), or 9 (n=1), nucleotide mismatches were apparent compared to the type strain of *N. animaloris* LMG23011^T, equivalent to 99.35% – 99.42% identity. Phylogenetic analysis, in comparison with type strains of all recognised *Neisseria* spp., where equivalent length 16S rRNA sequence was available, (equating to a 1348bp contig for the isolates described here), confirmed the identity of the isolates as most closely related to *N. animaloris* (Fig. 5). The porpoise isolates do appear to represent a clonal complex somewhat distinct from the species type strain, however there is a paucity of information on potential 16S rRNA gene diversity with this species. Further, while distinct from the *N. animaloris* type strain, even the most diverse sequence shares >99.3% nucleotide identity with it. While there are no strict criteria for species delineation based on 16S rRNA sequence this is consistent with the 98.7% - 99% threshold often quoted for confirmation of species identity (Drancourt et al. 2000; Stackebrandt & Goebel 1994). As further evidence of the relationship of these isolates to *N. animaloris* sequencing of a fragment of *rplF* was performed. This gene encodes the 50S ribosomal protein L6 and has been identified as a suitable target for differentiation within *Neisseria* spp. as phylogenies constructed from this fragment are consistent with phylogenies constructed from concatenated sequences of 53 whole-ribosomal protein genes (Bennett et al. 2014). Over a 414bp fragment, identical in all seven strains examined, sequences shared 18 nucleotide mismatches (95% identity) with the *N. animaloris* type strain sequence. Phylogenetic analysis with available equivalent sequences from type strains showed a similar relationship to that based on 16S rRNA with the porpoise isolates clearly most closely related to *N. animaloris* but again representing a clonal complex somewhat distinct from the type strain (Fig 6). Further, isolates of all cases were identified as *N. animaloris* with scores ≥2.0 using matrix-assisted laser desorption-ionisation time-of-flight mass spectrometry (MALDI-TOF MS), a result considered accurate to the species level according to the database supplied by the manufacturer.

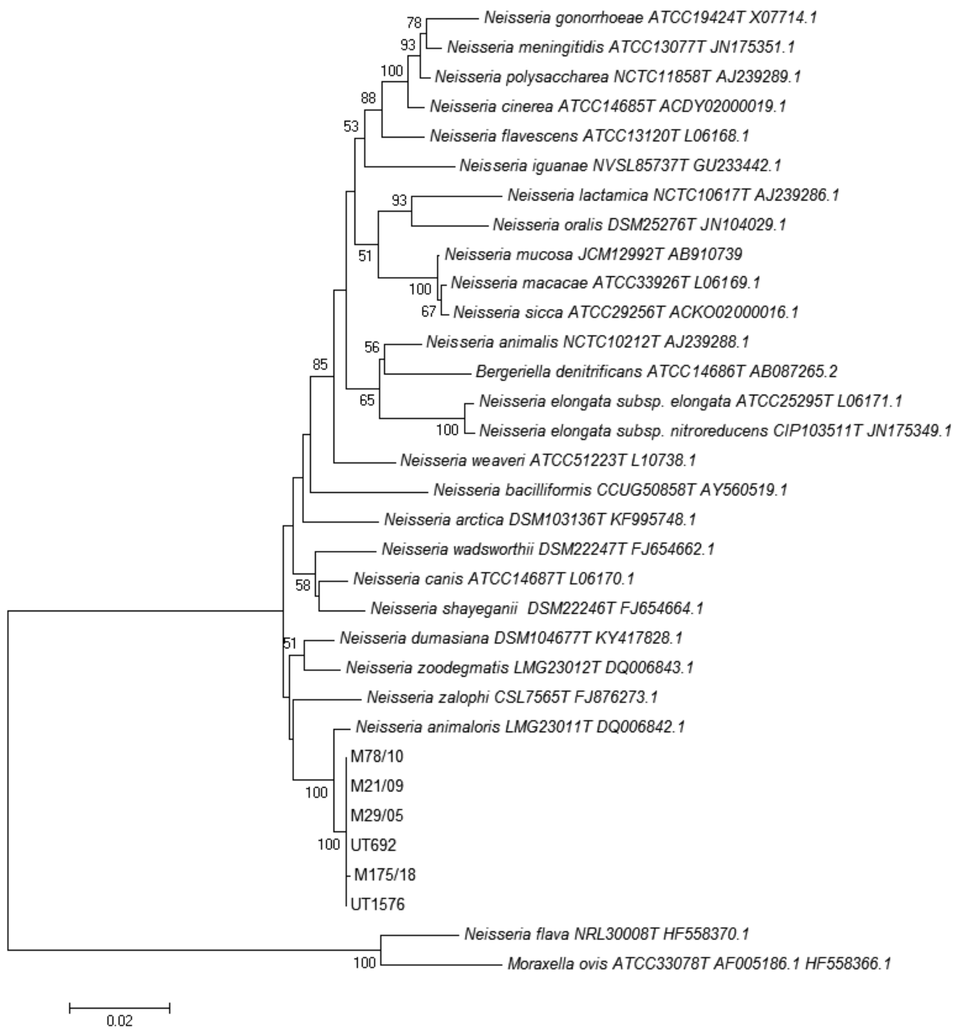

Fig. 5. *Phylogenetic analysis of the porpoise strains in comparison with type strains of members of the genus Neisseria inferred from 16S rRNA sequence. Labelling shows the strain name and the corresponding accession number for the sequence used in this comparison. Numbers at nodes correspond to proportions of 100 resamplings that support the topology shown with only values >50% indicated. Bar = 0.02 substitutions per nucleotide position.*

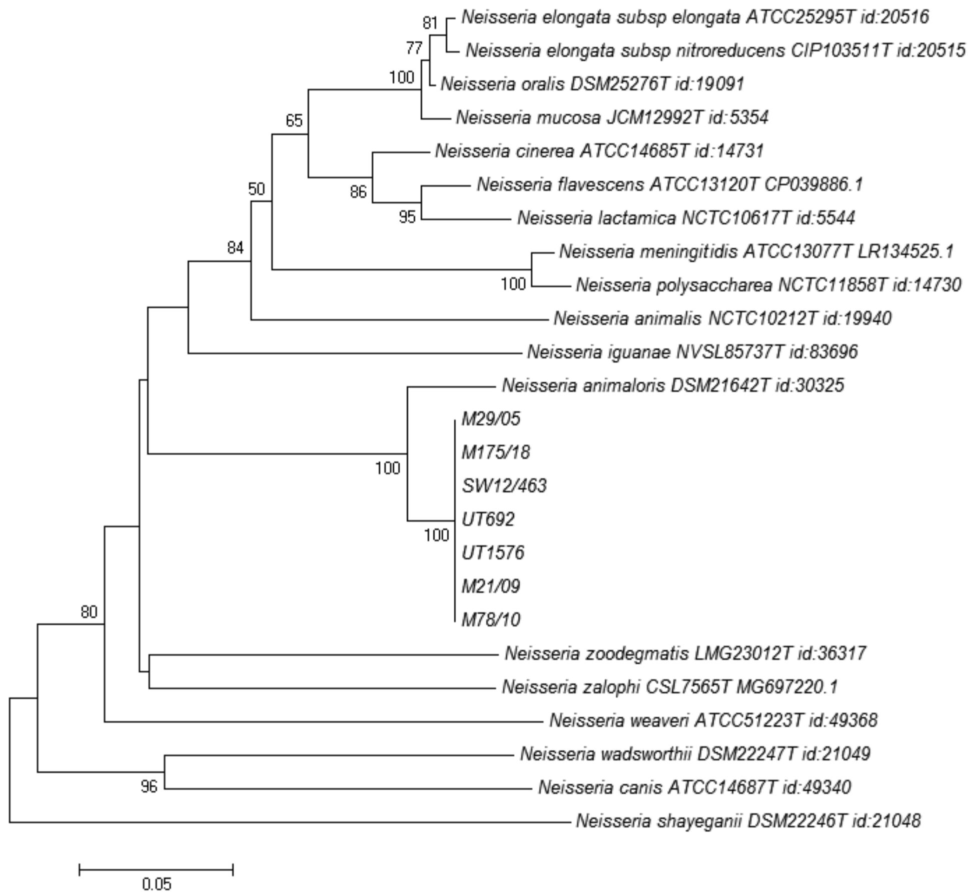

Fig. 6. *Phylogenetic analysis of the porpoise strains in comparison with type strains of members of the genus Neisseria inferred from rplF sequence. Labelling shows the strain name and the corresponding accession number for the sequence used in this comparison (see Methods). Numbers at nodes correspond to proportions of 100 resamplings that support the topology shown with only values >50% indicated. Bar = 0.05 substitutions per nucleotide position.*

Neisseria animaloris was the dominant isolate, often in pure growth, from lung abscesses of all cases (n=8). Additionally, *N. animaloris* was cultured from skin abscesses on pectoral fins (n=3), tailstock (n=1), inguinal area (n=1) and shoulder (n=1). Other recovery sites for *N. animaloris* and other isolates recovered are listed in Table S4. Additional isolates included *Brucella ceti* (M175/18) and a monophasic strain of *Salmonella enterica* with the antigenic formula, 4,12:a:-, which is host-adapted to porpoises (M29/05 and M21/09) (Table S4) but deemed unrelated to the ultimate cause of death of these cases.

Discussion

Here we report the identification of *N. animaloris* recovered from eight harbour porpoises. We could not find any previous reports of the isolation of *N. animaloris* from any marine mammal species. Furthermore, *N. animaloris* has not been recovered from any of the other harbour porpoises examined in the UK over the last thirty years, some of which had abscesses without evidence of grey seal bites. In all eight cases, the organism was recovered in sole or dominant growth from lung abscesses, with additional recovery from other tissues including pectoral fin and tailstock abscesses. The cases for which it was possible to assess (7/8), all demonstrated external lesions that were consistent with grey seal related injuries.

There are a number of other species known to interact with harbour porpoises but those can all be excluded as potential inflictors of the bite lesions as described on the eight cases. In the North Sea, aggressive behaviour towards harbour porpoises by white-beaked dolphins (*Lagenorhynchus albirostris*) (Haelters et al. 2011) and bottlenose dolphins (*Tursiops truncatus*) (Patterson et al. 1998; Ross & Wilson 1996) have been well-described. Porpoises that died following aggressive interactions with delphinids present clearly defined rake marks, which were not present on any of the cases from which *N. animaloris* was cultured. Killer whales (*Orcinus orca*) and large sharks do not inhabit the North Sea and only very rarely occur in adjacent waters. Finally, other animals known to interact with stranded porpoises are scavengers, like red foxes (*Vulpes vulpes*) or birds (Haelters et al. 2016) but scavenging does not explain the presence of *N. animaloris* in chronic lesions and internal organs of the eight cases presented here. The only other large marine animal in the North Sea is the harbour seal (*Phoca vitulina*) but seeing their significantly smaller body size and inter-teeth distance, it is less likely that harbour seals are responsible for the bite wounds on harbour porpoises and interactions between harbour seals and porpoises have to date not been described. This leaves the abundant grey seal as the most likely perpetrator of the infected wounds.

Neisseria animaloris is considered to be a constituent of the normal flora of the canine and feline oral cavity and has also been the cause of zoonotic infections following bites from these two domestic species (Tøne 2005). It is intriguing to consider that the route of infection in the harbour porpoises is similar, following seal bites. *Neisseria animaloris* has not been reported from grey seals previously and its culture from the mouth of these animals, where it may reside, possibly in low numbers in a highly populated and diverse microbial environment, may represent a significant challenge. Microbiome investigation of the oral cavity of nine grey seals, however, detected a *Neisseria* 16S sequence which showed 100% sequence ID with our isolates (based on a 437 bp sequence) in the oral cavity of four animals, indicating that at least some grey seals appear to carry *N. animaloris* (publication in progress). In contrast, this specific *Neisseria* 16S sequence was absent from the oral cavities of harbour seals (n=8) and harbour porpoises (n=6). The sequence showed 99% sequence ID with *N.*

animaloris obtained from terrestrial carnivores and humans, including the *N. animaloris* type strain.

Grey seal predation on harbour porpoises was first suggested by Haelters et al. (2012) who matched lesions present on mutilated porpoises to the inter-teeth-distance of grey seals. This theory was proven through the documentation of field observations of (fatal) interactions (Bouveroux et al. 2014; Stringell et al. 2015) and retrieval of grey seal DNA from bite marks on mutilated harbour porpoises (Jauniaux et al. 2014; van Bleijswijk et al. 2014). Characteristics of bite marks on harbour porpoises were retrospectively assessed evaluating post-mortem photos of harbour porpoises in The Netherlands and it was concluded that predation by grey seals is one of the main causes of death (Leopold et al. 2015a). It is known that not all grey seal attacks are (directly) fatal (Podt & IJsseldijk 2017). In the study of Leopold et al. (2015), 46 'possible escaped harbour porpoises' (6% of the studied sample) lacked large mutilations but had bite marks with associated inflammation. The observed and likely grey seal related injuries on the harbour porpoises in this study and the concurrent retrieval *of N. animaloris* from these cases, in addition to the absence of *N. animaloris* recovery from harbour porpoise lesions without evidence of grey seal assault, lead us to conclude that all these porpoises survived a grey seal attack. The bite lesions represent the subsequent portal of entry for bacteria to infect the animals, causing abscesses, as well as multiple organ infection, and eventually death.

The characterization and quantification of lesions on stranded marine mammals is vital, in order to differentiate between causes, with the aim of providing useful metrics to distinguish between lesions induced by direct human related causes of death, such as fisheries bycatch and from natural causes of death, such as inter-species interaction. There is an increasing need to assess any population level effects of interactions between sympatric species, which is of particular relevance for porpoise population assessments, given the observed exponential increase in grey seal numbers in many areas of the North Sea (SCOS 2017). The identification of a predator through forensic DNA has successfully been used in terrestrial settings (Blejwas et al. 2006; Williams et al. 2003) as well as in the identification and differentiation between grey seal predation and fox scavenging on marine mammals (Haelters et al. 2016; Heers et al. 2018; van Bleijswijk et al. 2014). In this study, we have demonstrated a further potentially powerful tool for the identification of a perpetrator: forensic microbiology through identification of *N. animaloris* in eight harbour porpoises and linkage to associated bite wounds from grey seals. Further support is provided by the finding of *N. animaloris* in grey seal microbiome studies whereby there was a 100% match with the porpoise isolates and a 99% match with the terrestrial strain NCTC 12227 based on 437 bp. Approaches to use bacteria in forensic science have been reported previously, with key examples in the identification of individuals through individual-specific skin bacterial communities (Fierer et al. 2010).

Finally, our findings add to concerns with respect to zoonotic infections of humans following seal bites and the handling of stranded seals and harbour porpoises. The association of *N. animaloris* to human infections following dog and cat bites suggests that seal isolates may have the potential to act similarly following infection from seals or porpoises. Therefore, *N. animaloris* should be added to the list of potential zoonotic organisms when handling marine mammals. *Neisseria animaloris* is absent from the databases of most commercial phenotypic identification kit suppliers, which potentially hinders its recognition from clinical samples. Diagnostic laboratories are increasingly turning away from commercial phenotypic biochemical kits, however, with MALDI-TOF MS becoming a preferred choice for first line identification of bacterial isolates. As *N. animaloris* is already included in the Bruker MALDI-TOF database and with 16S rRNA sequencing being a further option, the recognition of *N. animaloris*, whether from humans, dogs, cats, seals or other animals, should be more likely in the future.

Materials and Methods

All methods were carried out in accordance with relevant guidelines and regulations. No experiments were conducted on, or samples taken from, live animals as part of this study.

Specimens

The harbour porpoises reported here were all free-ranging animals that were found dead or died due to, or shortly after stranding. They were examined by different institutes depending on the geographic location of stranding: four harbour porpoises were examined in Scotland under the Scottish Marine Animals Strandings Scheme (SMASS); two animals from England under the Cetacean Stranding Investigation Program (CSIP) and two in The Netherlands at the faculty of Veterinary Medicine of Utrecht University (UU).

Gross- and histopathology

Post-mortem examinations and subsequent tissue collections were carried out following a recognised protocol for small cetacean necropsies (Kuiken & García Hartmann 1993). Tissues available for histologic review varied from animal to animal, but included: skin (with any lesions), muscle, prescapular lymph nodes, lung and associated lymph nodes, heart, liver, adrenals, kidney, oesophagus, stomachs, spleen, pancreas, intestine and associated lymph nodes, brain and spinal cord. Tissues were fixed in 10% neutral buffered formalin, processed routinely through graded alcohols, embedded in paraffin-wax, sectioned at 4-7 µm, stained with haematoxylin and eosin (HE) and examined by light microscopy. Additional stains were performed when appropriate, including the Periodic acid Schiff stain to detect fungi. For the cases in which photos of the external lesions were available the photos were evaluated for the presence of lesions characteristic of grey seal attacks based on methods previously described (Leopold et al. 2015a). If photos were not available, the macroscopic descriptions of the lesions were used to judge if wounds had been caused by seal bites.

Microbiology

Samples of lung and abscesses were collected and processed for microbiology from all harbour porpoises included in this study. Other tissues available for microbiology varied from animal to animal, but included liver, spleen, kidney, brain, mesenteric lymph node and intestine. Cultures were made on Columbia sheep blood agar (CSBA) (Oxoid, Basingstoke, UK), incubated at 37°C in air plus 5% CO_2 and MacConkey agar without salt (Oxoid), incubated aerobically at the same temperature. For cases processed at SMASS, selective cultures were set up for *Brucella* spp. as described previously (Foster er al. 2002) and anaerobic cultures were made on fastidious anaerobe agar with horse blood and on the same medium with nalidixic acid and vancomycin added (Oxoid). Further characterisation of *N. animaloris* isolates included API 20E, 20NE, 50CH and ZYM systems (BioMerieux, Basingstoke, UK), according to the manufacturer's instructions and with DIATABS (Rosco Diagnostica, Taastrup, Denmark) for the following tests: Voges-Proskauer, urease, aesculin hydrolysis, arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase, dulcitol, glucose, lactose, maltose, mannitol, mannose, sorbitol, sucrose, trehalose, xylose, α-fucosidase, α-galactosidase, β-galactosidase, α-glucosidase, β-glucosidase and β-xylosidase.

Whole-cell fatty acid analysis was carried out to compare three isolates (cases M29/05, M21/09, M78/10) with those described for *N. animaloris* previously[3]. For cellular fatty acid (CFA) analysis, cultures were grown on trypticase soy agar (TSA; Becton Dickinson Co., Oxford, UK) at 30°C aerobically for 24 hours. The CFAs were extracted and analysed by gas chromatography (MIDI Sherlock, Newark, DE) using the MIDI system's rapid TSBA database. Matrix-assisted laser desorption ionisation time-of-flight mass spectrometry (MALDI-TOF MS) was performed on the Bruker MALDI Biotyper. Sequencing of the 16S rRNA gene was performed, essentially as described previously (Hunt et al. 2013). Phylogenetic comparison of 16S rRNA sequences was carried out with MEGA5.2 using the neighbour-joining approach with the pairwise deletion option following CLUSTAL alignment of sequences trimmed to a 1348 bp consensus contig (= 1344bp of *N. animaloris* sequences). This analysis included all available near full length 16S rRNA sequences from *Neisseria* type strains based on consultation of Jean Euzeby's *List of Bacterial Names with Standing in Nomenclature* (http://www.bacterio.cict.fr/index.html) with the addition of the recently described *Neissera zalophi*[2]. Sequences from seven cases have been submitted to GenBank and assigned accession numbers MK441685 through MK441690. Amplification and sequencing of a fragment of *rplF* was performed as described previously[13]. Phylogenetic comparison of sequences was carried out with MEGA5.2 using the neighbour-joining approach with the pairwise deletion option following CLUSTAL alignment of sequences trimmed to a 414 bp consensus contig. Type strain alleles of *rplF* were obtained from either NCBI or the PubMLST database (denoted with id nr in Fig. 6) at https://pubmlst.org/neisseria/.

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Supporting information

Supplementary data associated with this article can be found in the online version at doi: 10.1038/s41598-019-50979-3.

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Manuscript in preparation

Anthropogenic and other causes of mortality of harbour porpoises from the southern North Sea

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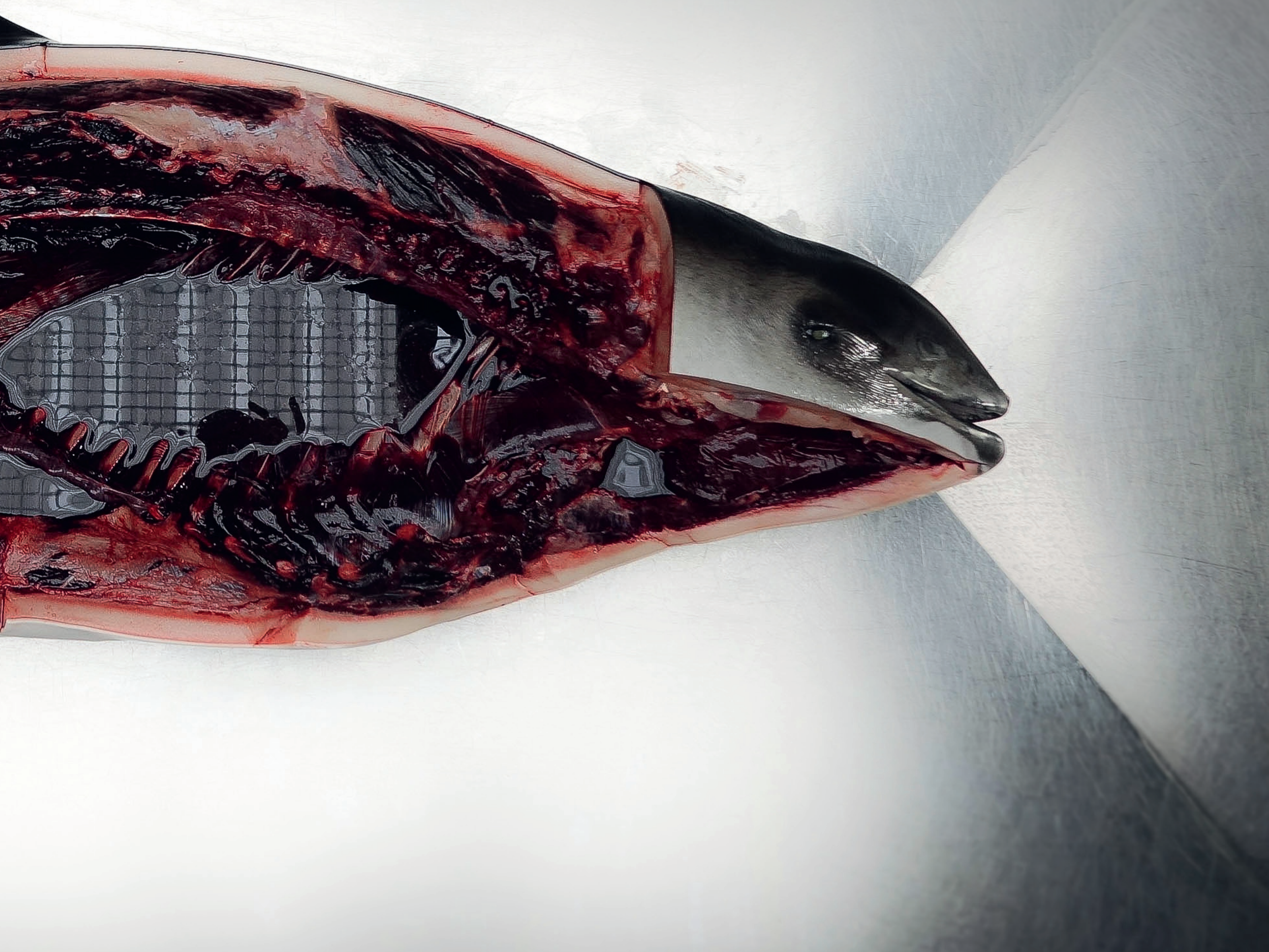

Introduction

Porpoises are the smallest of the toothed-whales, or odontocetes. Along with dolphins and the large whales they form the order of Cetacea, characterised mainly by their fully aquatic lifestyle, which sets them aside from all other mammals (Bjørge & Tolley 2018; Read 1999). Of the six species of true porpoises (*Phocoenidae*), the harbour porpoise (*Phocoena phocoena*) is by far the most abundant. Harbour porpoises are found in offshore and nearshore waters of the North Pacific and North Atlantic Oceans, as well as adjacent waters such as the Baltic and North Seas (Bjørge & Tolley 2018; Hammond et al. 2002, 2008; Nielsen et al. 2018). They are among the most frequently stranded marine mammals in Europe and North America, where they, unlike some other species of odontocetes, usually strand alone (Read 1999; IJsseldijk & ten Doeschate et al. 2020).

Knowledge of diseases and causes of death of stranded harbour porpoises in European waters comes from post-mortem investigations, including on individuals stranded in Belgium (Jauniaux et al. 2002), in the Baltic region, mostly in the western parts where porpoises are more abundant (Clausen & Andersen 1988; Siebert et al. 2001, 2020), and around the United Kingdom (Baker & Martin 1992; Kirkwood et al. 1997; Kuiken et al. 1994b). The most frequently reported causes of death in these studies were emaciation, parasitic and bacterial pneumonia and entanglement in fishing gear. In The Netherlands, there are historic investigations conducted by van Nie (1989), who reported pathological findings mainly related to parasitic infestation in 38 porpoises stranded from 1983-1986, and by Addink et al. (1995), who reported findings mainly due to bycatch (10-20%) and a high rate of broncho-pneumonia (50%) in 142 porpoises stranded between 1970 and 1994. In these years, porpoises were only rarely seen in the southern North Sea. Porpoise numbers gradually increased after 1990 and they are nowadays the most commonly found cetacean in Dutch waters (Camphuysen 2004; Camphuysen et al. 2008; IJsseldijk & ten Doeschate et al. 2020).

Due to the statutory requirement of several national, regional and international agreements and directives aiming at the protection of small cetaceans in their waters, such as the Agreement on the Conservation of Small Cetaceans of the Baltic, North East Atlantic, Irish and North Seas (ASCOBANS 1992), European Habitat Directive 92/43/EEC, the European Marine Strategy Framework Directive (MSFD) (2008/56/ EC), and Regional Sea Conventions, such as the Oslo-Paris Convention (OSPAR) (Hammond et al. 2002, 2008; IJsseldijk & ten Doeschate et al. 2020; LNV 2020), a post-mortem surveillance programme was established in 2006 in The Netherlands under the auspice of the Dutch government, aiming at monitoring the most common causes of death with a special focus on anthropogenic mortality. Although initiated by and at IMARES on the Dutch island of Texel, the programme was soon moved to the Division of Pathology of the Faculty of Veterinary Medicine at Utrecht University where it has been ongoing since 2008.

Metrics routinely collected at post-mortem investigation include pathological findings alluding to disease and causes of death, data on demographics, nutritional condition, diet and on marine debris ingestion. A special focus area since 2010 has been the investigation into hearing loss due to anthropogenic activities. In this chapter, we describe the causes of death of recently deceased animals and those in mild to moderate state of autolysis, stranded from 2008-2019, as well as the results from inner ear analyses on a subset of porpoises. Additionally, we investigate whether there are any age- and sex specific causes of mortality and if causes of death, such as bycatch in fisheries, have increased or decreased over time. The results of our study give an estimation on the occurrence and trends in anthropogenic and natural causes of mortality for harbour porpoises from different age classes from the southern North Sea. This knowledge is crucial for management of species or subgroups, which are particularly vulnerable to human interaction, like the harbour porpoise.

Materials and methods

Post-mortem examinations

Post-mortem examinations followed internationally standardised guidelines (IJsseldijk, Brownlow & Mazzariol 2019). In short: at the start of each post-mortem examination, external features were photographed. Data collection included date and location of stranding, sex, total body length (in cm), and mass (in kg). Based on length and gonadal appearance, animals were assigned in three age classes: neonate, juvenile and adult. Nutritional status was assessed based on visual examination of the dorsal musculature, visceral fat and blubber thickness, measured immediately anterior to the dorsal fin at three locations (dorsal, lateral and ventral, in mm). Nutritional condition was appointed on a six-point scale with 1 representing very fat and muscular animals and 6 emaciated animals. Additionally, all porpoises were given a decomposition condition code (DCC) at time of necropsy, from DCC1 representing very fresh carcasses to DCC5 representing carcasses remains. Parasite presence was recorded for four major organ systems: lungs, liver, cranial sinuses (peribullar and pterygoid) and tympanic cavity (referred to further as 'ears'), and stomachs, and if parasites were present, the infection was categorised as mild: approx. 1-20 nematodes or <10% affected with trematodes, moderate: approx. 21-50 nematodes or 10-30% affected with trematodes, or severe: approx. >50 nematodes or >30% affected with trematodes.

For this study, all cases for which a full post-mortem investigation was conducted, including at least gross- and histopathological assessment, and of which external photographs were available were selected (n=612). Of these 612 porpoises, a range of tissue samples were fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned at 4-7µm, stained with haematoxylin and eosin (HE) and microscopically examined. We refer to significant lesions when these were judged as multifocal mild, moderate or severe, and focal moderate or focal severe. In this manuscript, we present the main and most common findings and causes of death, largely following the subdivision of Arbelo et al. (2013). Despite the selection criteria of the 612 cases, which were included in this study, there could be missing data in some cases and therefore in the result section we give the number of cases out of the total number of cases assessed for each specific result between brackets.

Diet and marine debris investigation

Stomachs were firstly visually inspected and sampled during necropsy for pathology and stored at - 20˚C, with all the contents, until further analyses at Wageningen Marine Research in Den Helder. Here, contents were rinsed into a large beaker glass, placed under slowly running tap water so that light prey components such as tissue fragments and fluids were gently flushed out of the sample, whilst heavier prey remains could be collected from the bottom of the beaker glass (van Franeker et al. 2018). Since 2010, the beaker glass was placed on a metal 1 mm mesh sieve to make sure that no foreign items, especially small pieces, were overlooked. For full details on the methods as well as detailed results on plastic and other man-made litter in stomachs (2006-2013) see van Franeker et al. (2018). Prey remains were examined following earlier published methods (Leopold 2015). Briefly, first and foremost fish sagittal otoliths were used to identify fish species and to estimate fish length and weight. Prey mass was back-calculated following Leopold (2015) and Leopold et al. (2001). Detailed results of dietary analysis (2006-2015) are published in Leopold (2015). In this chapter, we include the data on presence/absence of marine debris in stomachs, and presence/absence of prey in the stomach, number of prey items, reconstructed preys mass (in grams), and the percentage of demersal prey mass out of the total reconstructed preys mass. If we assume that prey in the stomach represents the last meal, prey species composition (pelagic versus demersal) can be indicative of the location where the porpoise died within the water column and regardless of the quantity, may therefore be informative with regards to the cause of death (IJsseldijk et al. 2021b; Leopold 2015a,b).

Inner ear analysis

The inner ears of 50 stranded harbour porpoises from 2010 to 2019, sampled and fixed within 24 hours post-mortem, were collected following earlier published methods (Morell & André 2009). The ears were sent for analysis to the Technical University of Catalonia, Spain (UPC-Barcelona Tech) from 2008 to 2013, at the University of British Columbia (UBC), Canada from 2013 to 2017 and to the Institute for Neurosciences of Montpellier, France from 2017 to 2019 with appropriate CITES permits. Thirty-six inner ears were processed for scanning electron microscopy (SEM), three for transmission electron microscopy (TEM), and 11 for immunofluorescence. Protocols for processing the samples and results have been published previously (Morell et al. 2012, 2015, 2017a,b, 2020, 2021, in review). In short: the sensory cells of the organ of Corti were labelled with phalloidin (FluoProbes® X5 505, FP-AZ0130, 1:100), anti-prestin (Santa Cruz, USA SC-22692, 1:200 and from Dr. Zheng lab, 1:1000) and anti-myosin VI (Proteus Biosciences 25-6791, 1:500) antibodies. Type I afferent innervation was labelled with anti-neurofilament 200KD (Sigma-Aldrich N0142, 1:400) or anti-neurofilament H (Millipore AB5539, 1:5000). Nuclei were counterstained with DAPI (4', 6-diamidino-2'-phenylindole, dihydrochloride; Thermo Scientific 62247, 1:1000). The inner ears were observed using a Hitachi S-3500N (Institute of Marine Sciences, Spain), a Hitachi S-4000 (CRIC, Montpellier, France), and a Hitachi S-4700 (University of British Columbia, UBC, Bioimaging Facility) SEMs, a Hitachi H-7100 TEM (CRIC), Hitachi H7600 TEM (UBC Bioimaging Facility) and the FEI Tecnai G2 Spirit (Department of Cellular and Physiological Sciences, UBC), an Olympus FV1000 (UBC Bioimaging Facility) and a Zeiss LSM880 (Montpellier Resources Imagery) confocal microscopes.

Data exploration and analyses

Age- and sex specific differences in causes of death, as well as spatiotemporal changes were assessed. Data exploration was applied following Zuur et al. (2007, 2009) prior to analysis. Data exploration and analyses were performed using R version 3.6.3 (R 2017), with packages ggplot2 (Wickham 2016), grid, gridExtra and RColorBrewer of CRAN for visualization. We used a multinomial logistic regression model. This is an extension of binomial logistic regression with the response variable having more than two levels. Our response variable, the category 'cause of death', exists of eight groups (see result sections): accidental bycatch in fisheries, chronic or subacute lesions from previous bites, emaciation of unknown origin, infectious disease, perinatal death, acute predation from grey seals, acute starvation and sharp- and blunt force trauma. Two further categories, miscellaneous and unknown, were not included in the analyses. Multinomial regression is used to predict the nominal target variable (bycatch, see below), depending on a range of predictor variables. As predictor variables we assessed season (four categories: winter, spring, summer, autumn), year (2008-2019), location (five categories: Delta, Zuid-Holland, Noord-Holland, Noord Nederland (mainland of Friesland and Groningen) and Waddeneilanden), age class (three levels: neonate, juvenile, adult), nutritional condition (three levels: good, moderate, poor) and sex (two categories: male, female). We use the predicting 'cause of death' category and split the data in a training (bootstrap proportion of 0.7) and testing (remaining proportion of 0.3) set. Since we are, from a conservation point of view, most interested in assessing causes of death in light of the largest anthropogenic threat, the 'cause of death' category bycatch was set as the reference level, using the nnet package. Although multinomial regression models predict the probability of a particular observation to be part of the said level, here we use it to assess which predictor variables best explain the 'cause of death' categories. Model selection was applied by backward stepwise selection using the Akaike information criterion (AIC, Akaike 1974) to select the optimal model. From this final model, the coefficients are converted to odd ratios (OR) with upper bounds (coefficients +2*standard error) and lower bounds (coefficients -2*standard error) and assessed for their relevance. Finally, anthropogenic causes of death (indicated as 1 for bycatch and trauma and 0 for the other categories) were modelled as a function of body length (in cm, cases with unreliable length measures excluded) for all juvenile and adult porpoises using a General Linear Model (GLM) with a binomial distribution in order to determine and visualize the probability of an anthropogenic cause of death based on total length.

Results

Anthropogenic categories

Accidental bycatch in fisheries (103/612)

This category consists of harbour porpoises that died acutely and most likely as a result of underwater entrapment following accidental capture in fishing nets, referred to as bycatch. A total of 103 harbour porpoises (17%) were classified in this category, of which 41 were female and 62 males. These were mainly juveniles (73%, 75/103), followed by adults (21%, 22/103) and an additional six neonates most likely died due to bycatch (6%) as well. Nutritional conditions were judged as good for 55% porpoises (NCC1-2: 57/103), moderate for 35% others (NCC3-4: 36/103) and poor for the remaining 10% animals (NCC5-6: 10/103). Externally, 85% of the cases (88/103) had clear and distinct lesions consistent with net entanglement, including superficial cutaneous lesions on rostrum or on extremities, or encircling imprints (Figure 1), while for the other animals this was less apparent or could not be diagnosed with confidence due to significant external defects. The diagnosis of underwater entrapment due to bycatch was based on a combination of internal findings consisted with an acute and traumatic cause of death and the exclusion of another cause of death for these porpoises. Among all porpoises in the bycatch category, 51% of the cases (52/102) had signs of blunt force trauma, such as subcutaneous bruising, haemorrhage in (parts of) the central nervous system or acute skull or mandible fractures. Pulmonary oedema was present in 89% of the cases (92/103) and multiple organ congestion in 29% of the cases (28/96). Excluding neonates (6/103) and one case without stomach assessment, prey remains were present in 86% of all porpoises for which the most likely cause of death was bycatch (83/96). Number of reconstructed preys varied widely, with a mean of 261 (range: 1 to 4453, median: 50), and a mean reconstructed summed preys mass of 1349.66 gram (range: 0.35 gram to 14673.14 gram, median: 569.24 gram). The percentage of total demersal prey species constituting diets was on average 71%. This was higher for juvenile porpoises (on average 76%) than for adults (on average 54%).

Figure 1. *Prominent linear imprint encircling the rostrum (left) and several nicks in the edges of the fluke (right).*

Bycaught porpoises deemed healthy

No significant macroscopic or microscopic lesions or signs of poor nutritional status were detected in 29% of the individuals (24/83) and therefore these animals were considered 'healthy' at the time of bycatch. Mild dermatitis was however diagnosed among those porpoises, as two of these animals presented chronic lesions from previous bites, of which one, as well as three other porpoises, had a mild amount of round-to-oval or longitudinal skin lesions with a likely viral origin. Nematodes were present in mild numbers in the gastrointestinal tract of one porpoise, in mild numbers in the respiratory tract of six and in moderate numbers in the respiratory tract of another animal. There were higher prevalence's of nematodes affecting the middle ear and sinuses: four animals with mild infestation, three with moderate and one with severe numbers. Trematodes were present in the liver of one of these porpoises in moderate numbers.

Bycaught porpoises deemed unhealthy

Additional and significant lesions were seen in a majority of the porpoises (n=79/103), which indicates that despite that the most likely cause of death of these animals was considered to be bycatch, the health status of these individuals was probably compromised prior to the entanglement. Most frequently diagnosed was moderate to severe respiratory pathology (70%, 53/76). Moderate to severe dermatitis was seen in five animals, and three animals presented external, chronic lesions from previous bites, indicative of earlier, non-fatal interspecific interactions. Ulceration of the alimentary tract was noted for 18% of the cases (14/76). Pathological changes were apparent in the central nervous system in four porpoises: one case of focal gliosis in the brainstem, and three cases with focal perivascular cuffing in the cerebrum and meninges. Additional lesions included moderate to severe hepatitis and/or cholangitis in 38% of the cases (28/74), three porpoises presented changes in the heart (8%, 3/67), among which one case with macrophage infiltrates in the myocardium, one case with mild fibrosis of the left ventricle and one animal with focal acute necrosis of the cardiomyocytes. There were two porpoises (3%, 2/71) with moderate to severe nephritis and eight animals had inflammatory lesions in multiple organs (three or more). Parasites were detected in the gastrointestinal tract in 23% of the cases (18/78): in mild numbers in 13, moderate in three and severe in two. Pulmonary nematodes were present 81% of the cases

(64/79): in mild numbers for 25, moderate for 26 and severe in 13 of these porpoises. Trematodes were present in the hepatic ducts in 25% of the cases (23/79), of which mild in 14 and moderate in eight. Finally, nematode infestation in the middle ear and sinuses was apparent for 78% of the cases (58/74): mild in 28 cases, moderate in 24 and severe in six porpoises.

Pathology due to foreign bodies (2/612)
This category represents animals with lesions associated with a physical aetiology of anthropogenic origin, including the ingestion of foreign bodies with associated pathological changes and animals chronically entangled. There were two harbour porpoises which most likely died as a result of foreign bodies (0.3%): one adult male and one juvenile male. Both animals were in good nutritional condition. The adult male had an encircling, chronic lesion just cranial to the pectoral fins (Figure 2), which was indicative of a (previous) prolonged entanglement. However, no monofilament or other man-made material was detected in the lesion. The other porpoise, the juvenile male had an orange nylon fishing line wired around the epiglottis (Figure 2), with a fishing hook attached on one end of the line. The fishing hook was not ingested but instead protruding outside the oral cavity, and here it was penetrating the blubber cranial to the pectoral fins. The hook and fishing line caused a moderate necropurulent and ulcerative chronic dermatitis and a necrotic laryngitis, and the animal suffered additionally from a multifocal bronchopneumonia.

Figure 2. *Harbour porpoises with foreign body pathology. Left: adult male with sign of encircling, chronic lesions indicative of chronic entanglement. Right: fishing line wired around the larynx of a juvenile male porpoise, causing a necrotic laryngitis. Pictures taken by Kees Camphuysen (left) and Lineke Begeman (right).*

Marine debris was detected in the stomachs of 7% of all harbour porpoises (39/599), of which 25 were juveniles and 14 were adults. There was one notable case with a fishing hook in the forestomach. At first, a 5 cm in diameter ball of algae, mostly *Sargassum muticum*, was found with a straight J-shaped (37x15.4 mm) fishing hook with a 6x3.3x1 mm bait stopper and a single barb inside the algal mass. The hook was rusty but the tip was sharp and attached was 45 mm, 0.55 mm cross-section, length of white nylon fishing line. This juvenile male was in a poor nutritional condition and the surface of the stomach was affected by multifocal 1 mm in diameter black ulcerations. There weas a focal moderate chronic granulomatous gastritis with intralesional trematodes, morphologically consistent with *Pholeter* *gastrophilus*. Other pathological findings included multiple small longitudinal ulcerations in the larynx, oesophagus and a small amount of black content in the third and fourth stomach and intestines (presumably digested blood). Since upon necropsy, the fishing hook was centrally placed in the ball of algae and there was no sign of perforation of the stomach wall, a causal relationship between the fishing hook and the gastric lesions could not be established. This animal is listed under the subheading 'infectious disease'. At large, marine debris ingestion could not be associated with poor nutritional condition, alimentary pathology or pathology of the central nervous system. Harbour porpoises with ingestion of marine debris died due to a variety of causes and none died as a direct result of the ingestion of debris. In most cases, ingested materials were small pieces of rope or netting, or small fragments or sheets of plastic, all smaller than the average whiting otoliths found in the stomachs, and these were not considered directly detrimental to porpoise health (van Franeker et al. 2018; Bravo Rebolledo 2020).

Trauma consistent with ship collision (11/612)

This category refers to those cases that suffered a severe trauma resulting in rapid loss of life most likely due to collision with a vessel. Severe and extensive sharp trauma was apparent in eleven stranded harbour porpoises (2%). These were nine juveniles and two adults, of which six males and five females. Nutritional conditions were mostly good (NCC1-2: 67%) or moderate (NCC3-4: 33%), with eight animals with prey in their stomachs, one empty stomach and two missing stomachs. Number of reconstructed preys varied, with a mean of 377 (range: 99 to 781, median: 318) reconstructed preys, and a mean reconstructed summed preys mass of 1761.22 gram (range: 564.71 gram to 6229.24 gram, median: 1074.02 gram). The percentage of total demersal prey species constituting diets was on average 77%. This was higher for juvenile porpoises (on average 88%) than for the one adult (3%). All eleven animals presented penetrating lacerations with underlying fractures in ten, affecting the vertebral column (n=7) (Figure 3), ribs (n=2) and head (n=1). Two had amputated tail flukes and one an amputated rostrum. Although the lesions fit with anthropogenic trauma, lacerations with some similarity to grey seal-induced mutilations were suspected in 2/11 cases. Additionally, the presence of net-marks could not be ruled out in 9/11, which was mostly due to extensive external defects. This highlights a difficulty in differential diagnosis among these two anthropogenic causes of acute mortality.

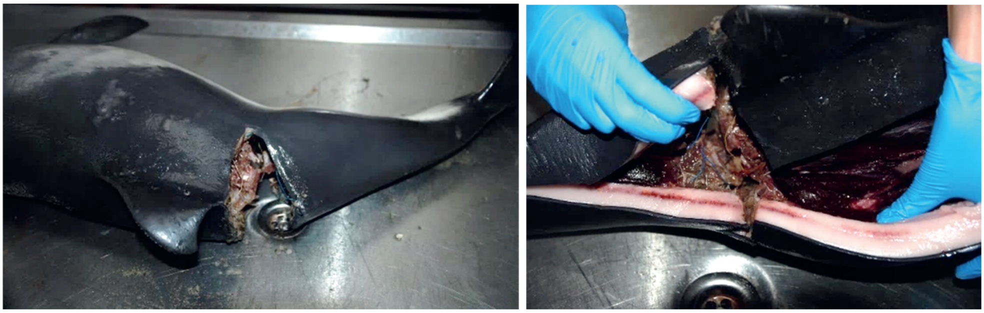

Figure 3. *Harbour porpoise with severe sharp trauma to the vertebral column, just caudal to the dorsal fin (left) and associated haemorrhage in the blubber layer around the trauma (right). Pictures taken by Mariel ten Doeschate.*

Other types of trauma (14/612)

Severe blunt force trauma was the main diagnosis in thirteen stranded harbour porpoises. These were ten juveniles and three adults, among which eight were males and five females. The nutritional status of these animals varied, with 33% of these porpoises in good nutritional status (NCC1-2: 5/12), two animals in moderate nutritional status (NCC3: 17%) while the other six were in poor to emaciated state (NCC5-6: 50%). Nine of these porpoises had prey in their stomachs, while the other four stomachs were empty. Number of reconstructed preys was generally low, with a mean of 37 (range: 1 to 128, median: 30), and a mean summed reconstructed preys mass of 295.48 gram (range: 4.36 gram to 1152.07 gram, median: 189.98 gram). The percentage of total demersal prey species constituting diets was on average 48%. In contrast to the bycatch and sharp trauma cases, this was lower for juvenile porpoises (on average 38%) than for the two adults (81%). None of these porpoises presented external evidence of sharp trauma. Fractures of the mandible were most commonly seen (46%, 6/13). One porpoise with a mandible fracture also had a complete skull and cochlea fracture. One other porpoise had a skull fracture and additional fractures to the vertebral column. There was haemorrhage around fractures in all cases, demonstrating pre-mortem injury. Porpoises without fractures presented extensive, subcutaneous haemorrhage and haemorrhage surrounding the spinal cord (n=1) or haemorrhage in the brain (n=3). Two porpoises live stranded, one of them had a mandible fracture as well as haemorrhage in the spinal cord and cerebrum and an additional pneumonia. The other presented extensive subcutaneous haemorrhage and severe ulcerations of the upper alimentary tract. For all thirteen porpoises listed here, it is unclear what the cause of blunt force trauma was. No obvious external lesions consistent with bycatch were detected. Collisions with the hull of ships are possibilities, especially for the cases with fractures. However, contact with other objects at sea or in coastal waters, estuaries or harbours, or during stranding may have also been at play.

A final case, an emaciated juvenile male, had small but multifocal, deep lacerations on the left lateral flank and caudal to the right pectoral fin. The presumable knife-induced injuries penetrated the abdominal cavity and ruptured the diaphragm and liver after which the animal most likely bled-out.

Noise-induced hearing loss
From all the ears analysed in the period 2010-2019, we found six cases with focal haemorrhages in the cochlea but there was no conclusive evidence of hearing loss in the majority of these cases, either because there were no lesions detected, or because the organ of Corti was not in good enough condition for diagnosis in some parts of the cochlea. However, there were four cases with missing sensory cells in the apical region of the cochlea. While the pattern of sensory cell loss in two of them suggested to be cases of individual apical variability (Morell et al. 2021), the other two showed evidence of sensory cell loss that are compatible with noise exposure. More details on these cases are published elsewhere (Morell et al. 2015, Morell et al. in review, respectively). The inner ears from the best-preserved porpoises were used to establish an anatomical baseline to further distinguish between normal and pathological changes but also to describe ultrastructural adaptations of the sensorineural epithelium of the cochlea for very high frequency hearing (Morell et al. 2015, 2020, 2021).

Categories of non-anthropogenic pathology

Peri-neonatal pathology (63/612)
A total of 84 neonates were necropsied, of which twenty died due to interspecific interaction, bycatch or infectious disease and are therefore listed under these subheadings. The cause of death of one neonate could not be established due to decomposition. Results for the remaining neonates are presented here. This category refers to neonatal harbour porpoises whose morphological and etiological characteristics were most likely related to problems in gestation, birth or nursing. For 22 neonatal porpoises the most significant finding were general, internal, diffuse congestive-haemorrhagic lesions. Six neonates live stranded, the other 16 were found dead. One porpoise had complete foetal atelectasis, this was most likely a stillborn animal. Three neonates had ulcers in the alimentary tract. Parasitic infestation was not diagnosed, with the exception of one nematode detected in the auditory system of one neonate, morphologically consistent with *Stenurus minor*. The cause of death of these 22 porpoises is referred to further as perinatal death. Their mean length was 76.2 cm, consistent with the reported length at birth for porpoises in the North Sea (Lockyer 2003). For an additional 41 calves, the most significant finding was hepatic lipidosis and their cause of death was most likely starvation. Seven of these live stranded. The mean length of these 41 porpoises was 83.4 cm. They likely lived for a short period of time but had insufficient resources or additional problems related to nursing. Nutritional conditions varied, with 20% in good condition (NCC1-2: 8/41), 46% in moderate condition (NCC3-4: 19/41) and the other 34% calves (NCC5-6: 14/41) being in poor to emaciated condition.

Infectious disease (198/612)

This category is defined as pathological changes associated with the presence of subacute and/or chronic pathological findings that were sufficiently severe to be considered ultimately responsible, alone or in combination, for the death and/or stranding of the animal. These lesions can be caused by various aetiological agents but the ancillary assessment of biological agents varied widely between cases. Since the category of infectious disease is large, 198 cases comprising 32% of all porpoises included in this study, we present summary findings per organ system. We separate pathologies associated with good nutritional conditions (NCC1-2), as indicative of acute disease, from those in moderate-poor nutritional conditions (NCC3-6), as indicative of prolonged debilitation. Except for the prevalence of parasitic infestations in the respiratory tract, gastrointestinal tract, liver and auditory system, no further information on aetiologies is provided.

Pathology associated with good nutritional condition (50/198)

This category refers to harbour porpoises dying of significant infectious disease, which presented a good nutritional condition (NCC1-2). Fifty porpoises were placed in this category. These were mostly adult porpoises (50%) and juveniles (44%), with additionally three neonates (6%). Of the fifty cases, 21 live stranded (42%). Most porpoises presented pulmonary oedema (90%, 45/50). Despite the good nutritional conditions of these porpoises, significant pathological changes were present. Most commonly diagnosed was moderate to severe respiratory pathology (88%, 43/49) and moderate to severe liver pathology (53%, 25/47), both often associated with parasitic infestations. Seventeen porpoises had lesions or inflammatory infiltrates in their central nervous system (36%, 17/47), among which three cases of extensive meningoencephalitis with fungal elements with an associated otitis in one (see also Kapetanou & IJsseldijk et al. 2020), six porpoises with meningitis and six with encephalitis. Six porpoises had lesions or inflammatory infiltrates in their cardiovascular system, including a case of mycotic myocarditis and two cases for which a bacteraemia was suspected. Significant urogenital pathology was detected in eleven porpoises, among which three cases of nephritis and two adult porpoises with endometritis. Dermatitis of likely viral origin was determined in 27 porpoises (54%), although mostly only few skin lesions were detected. Alimentary pathology was apparent in 14% of the porpoises (7/50). Strikingly, 70% of all cases (32/46) had inflammation in multiple organs (three or more), which demonstrates the extensiveness of lesions for most porpoises within this category. Parasites were detected at large: in the gastrointestinal tract in 32% (16/50) in mild numbers in nine cases, moderate in four and severe in three. Pulmonary nematodes were present in 86% of the porpoises (43/50): in mild numbers for 13, moderate for 14 and severe in 16 of these porpoises. Trematodes were present in the hepatic ducts in 50% of the cases (25/50), of which mild in 15, moderate in eight and severe in two. Finally, nematode infestation in the middle ear and sinuses was apparent for 86% of the cases (37/43): mild in 16 cases, moderate in 16 and severe in five. Despite the significant pathological findings, most of these porpoises had prey in their stomachs. Excluding neonates (3/50) and one case without stomach assessment, prey remains were present in 67% of the cases (31/46). Number of reconstructed preys varied but was generally lower compared to the anthropogenic causes of mortality, with a mean of 47 (range: 1 to 395, median: 17), and a mean summed reconstructed preys mass of 986.73 gram (range: 0.15 gram to 5874.14 gram, median: 139.89 gram). The percentage of total demersal prey species constituting diets was on average 67%. This was lower for juvenile porpoises (on average 58%) than for adults (on average 78%).

Pathology associated with moderate-poor nutritional condition (144/198)

This category refers to harbour porpoises dying of significant infectious disease and had (some) signs of deterioration based on their moderate to (very) poor nutritional conditions (NCC3-6). Hundred forty-four porpoises were placed in this category. These were mostly adult porpoises (58%, 83/144), followed by juveniles (40%, 58/144), and an additional three neonates (2%). Of all cases, 48 were live stranded (33%) and 76% of the cases (109/144) had pulmonary oedema. Most animals demonstrated moderate to severe respiratory pathologies (86%, 122/142), followed by moderate to severe liver pathology (51%, 69/135). Ulcers or other alimentary lesions were found in 40% of the cases (57/143). Lesions and inflammatory infiltrates were detected in the central nervous system of 31% of the cases (39/126). Less frequently, lesions in the heart (12%, 15/129) and urogenital tract (16%, 22/137) were observed. A total of 77 cases had multiple organ inflammation (53%, in 3 or more organs), demonstrating the extensiveness of lesions in these porpoises. Parasites were detected at large: in the gastrointestinal tract in 55% of all porpoises (78/142): in mild numbers in 35 cases, moderate in 23 and severe in 20. Pulmonary nematodes were present 96% of the cases (138/144): in mild numbers for 40, moderate for 52 and severe in 46 of these porpoises. Trematodes were present in the hepatic ducts in 53% of the cases (76/144), of which mild in 41, moderate in 31 and severe in four. Finally, nematode infestation of the middle ear and sinuses was apparent for 89% of the animals (112/126): mild in 43 cases, moderate in 36 and severe in 33. Despite the moderate to poor nutritional conditions, most of these porpoises had prey in their stomachs. Excluding neonates (2/144) and four cases without stomach assessment, prey remains were present in 76% of the cases (105/138). Number of reconstructed preys varied but was generally lower compared to the anthropogenic causes of mortality, with a mean of 88 (range: 1 to 1280, median: 17), and a mean summed reconstructed preys mass of 941.52 gram (range: 0.62 gram to 7606.08 gram, median: 177.7 gram). The percentage of total demersal prey species constituting diets was on average 72%. This was higher for juvenile porpoises (on average 77%) than for adults (on average 69%).

Insufficient food or feeding (non-neonates) (53/612)

This category refers to non-neonatal harbour porpoises for which the most significant pathological finding pointed towards food shortage as a direct cause of mortality. For eight young juveniles (length ranging from 93-113 cm) hepatic lipidosis was the most significant finding. An additional 45 porpoises, both juveniles (87%, 39/45) and adults (13%, 6/45), were in poor to very poor nutritional conditions but no cause for the emaciation could be established. Alimentary pathology was diagnosed in over half of these cases (52%, 22/42). Moderate to severe respiratory pathology was seen in 38% of these porpoises (15/40), and moderate to severe liver pathology in 35% of them (13/37). There was one adult female with fibroleiomyoma in the reproductive tract (3%, 1/37), one porpoise with a minimal diffuse meningitis (3%, 1/36) and one case of degeneration and necrosis of the cardiomyocytes (3%, 1/36); which were deemed unrelated or not severe enough to explain the severe emaciation of these individuals. Similar to the other categories, parasites were detected in many of these harbour porpoises: in the gastrointestinal tract in 20% of the cases (9/45): in mild numbers in eight cases and moderate in the other. Pulmonary nematodes were present in 64% of the porpoises (29/45): in mild numbers for 14, moderate for seven and severe in eight. Trematodes were present in the hepatic ducts in 20% of the cases (9/45), all in mild numbers. Finally, nematode infestation in the middle ear and sinuses was apparent for 71% of the porpoises (32/40): mild in 15 cases, moderate in nine and severe in eight. Excluding one animal without stomach assessment, prey remains were present in 67% (35/52) despite frequently observed poor nutritional conditions and/or signs of lipidosis. Number of reconstructed preys varied, with a mean of 223 (range: 1 to 5255, median: 8), and a mean summed reconstructed preys mass of 439.85 gram (range: 0.62 gram to 5180.18 gram, median: 106.71). The percentage of total demersal prey species constituting diets was on average 75%. This was slightly lower for juvenile porpoises (on average 74%) than for the three adults (on average 79%). There was one notable case, a juvenile porpoise found far inland, after the animal passed the harbour locks and swam up a canal. When it was found dead, there were no prey remains in its stomach and it was severely emaciated and had an extensive dermatitis most likely as a result of the prolonged period (approximately 11 days) of residing in fresh water. There were additional signs of substrate (mud) inhalation and associated acute pneumonia. For the other porpoises in this category, no signs of significant infectious disease or acute mortality from anthropogenic or interspecific causes could be detected and therefore the underlying causes of their poor nutritional state remained unclear.

Interspecific traumatic interactions (144/612)

This category refers to harbour porpoises with characteristic lesions consistent with grey seal attacks. A total of 144 harbour porpoises (24%) where classified within this category. We divide this category into two subcategories: pathology associated with acute interaction and with the presence of large, sharp-edged mutilations and pathology associated with interactions that did not immediately result in death. For more information on both categories, also see: Leopold et al. 2015a and Foster et al. 2019.

Pathology associated with acute interactions (78/144)

This category refers to harbour porpoises with ante-mortem mutilation that took place immediately before death and were the most likely cause of death, with the presence of associated claw- and bite marks most likely induced by grey seals in a predatory attack. Seventy-eight porpoises had these typical lesions. These were mostly juvenile porpoises (76%, 59/78), although adults and neonates were on occasion also targeted (17 and two respectively). The majority of these predated porpoises were in good nutritional condition (63%, 49/78), 33% in moderate condition (26/78) and the final three porpoises were in poor nutritional condition (4%, 3/78). Excluding neonates (3/78) and one case without stomach assessment, all but one had prey in their stomachs (99%, 74/75). Number of reconstructed preys varied but was generally large, with a mean of 375 (range: 1 to 6557, median: 96), and a mean summed reconstructed preys mass of 1426.41 gram (range: 1.58 gram to 9104.12 gram, median: 746.59 gram). The percentage of total demersal prey species constituting diets was on average 62%. This was higher for juvenile porpoises (on average 70%) than for the adults (on average 37%). None of these porpoises live stranded. Despite the good nutritional conditions of the majority of these porpoises, additional pathological changes were detected. Pulmonary oedema was a common finding (79%, 61/77). Over half of these porpoises suffered from moderate to severe respiratory pathologies (57%, 37/65) and a third presented moderate to severe liver pathology (32%, 19/59), mostly associated with parasitic infestations. In addition, ulcers and other alimentary lesions were detected in 4% of the cases (3/75), while two animals had mild meningitis and another presented gliosis. Lesions in the heart were detected in 5% of the predated porpoises (3/59), one case of necrosis and degeneration of myocytes, another lymphocytic and neutrophilic myo- and epicarditis, and the third subendocardial fibrosis. Finally, there was one porpoise with glomerulonephritis.

Six harbour porpoises presented clear signs of chronic lesions from previous bites in addition to the acute ones, and for another six animals there was some suspicious skin lesions which could also have been from previous bites (8-15%, 6-12/78). In total, there were eight cases of multiple organ inflammation (three or more organs) with dermatitis due to previous grey seal bites as a likely contributing cause in half of these. Harbour porpoises with chronic lesions from previous bites but without large, sharp-edged mutilation are discussed in the next paragraph. Parasitic infestations were detected in the gastrointestinal tract in 28% of the predation cases (22/78): in mild numbers in 14 cases, moderate in four and severe in one. Pulmonary nematodes were present 84% of these porpoises (65/77): in mild numbers for 25, moderate for 26 and severe in 13. Trematodes were present in the hepatic ducts in 24% of the porpoises (18/74), of which mild in seven, moderate in eight and severe in two. Finally, nematode infestation in the middle ear and sinuses was apparent for 78% of the porpoises (53/68): mild in 24 cases, moderate in 22 and severe in seven.

Pathology associated with interactions that did not immediately result in death (66/144)

This category refers to harbour porpoises with subacute to chronic lesions from previous bites, most likely induced by grey seals in a predatory attempt. These animals lacked large, sharp-edged mutilation typically seen in acute grey seal victims (previous paragraphs). All bite lesions of porpoises in this category had inflamed appearances. As for the acute predatory cases, most animals with bites were juveniles (73%, 48/66), followed by adults (23%, 15/66) and neonates (4%, 3/66). Unlike the acute cases, good nutritional conditions were seen in fewer animals with chronic lesions from previous bites (26%, 18/66 in NCC1-2; 35%, 24/66 in NCC3-4; 35%, 24/66 in NCC5-6). Excluding neonates (3/66) and one case without stomach assessment, 76% had prey in their stomachs (47/62). Number of reconstructed preys varied but were generally less compared to the acute predation cases, with a mean of 96 (range: 1 to 885, median: 40), and a mean summed reconstructed preys mass of 769.71 gram (range: 0.47 gram to 10661.66 gram, median: 210.53 gram). The percentage of total demersal prey species constituting diets was on average 76%, near equal for juveniles and adults (76% versus 77%, respectively). Eleven of these porpoises live stranded (16%). The percentage of cases with multiple organ inflammation (3 or more organs) was high in this cause of death category: 45% (29/64). Alimentary pathology was apparent in 32% of the cases (21/66), moderate to severe respiratory pathology in 68% of the cases (44/65) and moderate to severe liver pathology in 38% of the cases (24/64). In addition, there were nine porpoises with lesions in the central nervous system (14%, 9/64). Lesions in the heart were detected in 10% of the cases (6/62) and in the urogenital tract in 3% of the cases (2/64). Parasites were detected in the gastrointestinal tract in 33% of the cases (22/66): in mild numbers in 11 cases, moderate in five and severe in six. Pulmonary nematodes were present 76% of the cases (50/66): in mild numbers for 21, moderate for 17 and severe in 12 of these porpoises. Trematodes were present in the hepatic ducts in 22% of the cases (15/68), of which mild in nine, moderate in two and no cases with severe parasite infestation. Finally, nematode infestation in the middle ear and sinuses was apparent for 68% of the cases (38/56 cases assessed): mild in 12 cases, moderate in 13 and severe in 13.

Miscellaneous (5/612)

Five adult females had acute causes of natural deaths, namely dystocia (n=3) and intestinal volvulus (n=2). The three cases of dystocia were found stranded in May, corresponding to the calving season of porpoises in the North Sea (Lockyer 2003). One animal had a partly protruding foetus from the genital opening, which apparently got stuck at the height of the pectoral fins (Figure 4). This adult was in good nutritional condition, had prey in the stomach and no significant other pathological findings. The second porpoise, a fresh adult in poor nutritional condition without any prey in its stomach, had a moderately decomposed foetus located in the left uterus horn. There was extensive necrosis in the endometrium as well as in the uterus wall with additional peritonitis. The third porpoise presented extensive, ante-mortem external and internal injuries as a result of ‘breech’ position of the foetus, which was located with its dorsal fin in the genital opening. This had caused an ante-mortem uterus- and abdominal wall rupture (Figure 4).

Figure 4*. Two cases of dystocia. Top: protruding foetus from the genital opening, which was stuck at the height of the pectoral fins. Bottom: abdominal wall rupture as a result of foetus in 'breech' position, with the dorsal fin protruding from the genital opening (arrow).*

One live-stranded porpoise had an intestinal volvulus, with findings previously published (Begeman et al. 2013). No possible predisposing factors were noted for this particular case. The animal was in good nutritional condition although there was no prey in the stomach. There were moderate numbers of trematodes in its liver and mild nematode infestations in the middle ear and sinuses. The other animal with an intestinal volvulus stranded in May and was pregnant. This was a relatively small female (to be gravid), measuring only 139 cm. With a foetus just under half its size (63 cm total length). The pregnancy could have been a predisposing factor of the intestinal twist. The animal was in good nutritional condition and had prey in the stomach. It had an additional parasitic pneumonia and there were moderate amounts of trematodes in liver and a mild nematode infestation of the middle ears and sinuses. Additionally, there was one other adult resting (non-pregnant and lactating) female with an intestinal volvulus but that porpoise is listed under the infectious disease category since it suffered from a severe multiple organ inflammation, including an encephalitis, pneumonia and peritonitis.

Unestablished cause of death or stranding

For nineteen harbour porpoises, there was no clear cause of death or stranding established. These were five adults and fourteen juveniles. Five porpoises live stranded, two of which were euthanised. No signs of significant infectious disease or clear signs of anthropogenic pathological changes could be detected in any of the nineteen porpoises. Most of these harbour porpoises were in good nutritional condition (NCC1-2: 63%, 12/19) and the others in moderate nutritional condition (NCC3-4: 37%, 7/19), which points towards acute causes of mortality or stranding for these animals.

Age-, sex, spatial and temporal differences in causes of death

Eight cause of death categories were established: accidental bycatch in fisheries, chronic or subacute lesions from previous grey seal bites, emaciation of unknown origin, infectious disease, peri- and neonatal mortality, acute predation from grey seals, acute starvation, and sharp- and blunt force trauma (combined). The category miscellaneous, containing five animals dying of dystocia and intestinal volvulus, and nineteen cases with unestablished cause of death or stranding are not further included here. Data exploration indicated a clear age-related difference in causes of death, which was confirmed in the multinomial regression model. Neonates or calves were, logically, predominantly listed under the peri-neonatal and starvation categories. Juveniles were largely present in the grey seal related causes of death (both chronic lesions from previous bites and acute predation) as well as the trauma, bycatch and emaciation categories. Adults dominated the infectious disease category (Figure 5, Supplementary Figure 1). When we group the anthropogenic causes of mortality (bycatch and trauma) and compare these to other categories among the juveniles and adults, the probability of dying due to anthropogenic causes decreases with increasing total length (Figure 6). No sex difference was apparent, although males were overrepresented in all but one (emaciation of unknown origin) cause of death categories (Supplementary Figure 2). Bycatch was the predominant cause of mortality among the juvenile porpoises until up to around 2017, when cumulative numbers of infectious disease overtook (Supplementary Figure 1). One notable spike in the category emaciation of unknown origin was detected in the year 2011, especially when we assess the absolute numbers (Figure 6) and mainly juveniles were found (Supplementary Figure 1). Bycatch predominantly occurred in March and September-October. Acute predation cases, and to a lesser extent porpoises with chronic lesions from previous bites, were mainly seen throughout late winter and early spring (January-March). Emaciation seemed to be a late-summer phenomenon (August-September). Peri- and neonatal porpoises were mainly seen from May into June, corresponding to the calving season, and cases of starvation following that period, peaking in July. These findings were consistent, both in terms of absolute numbers (Figure 8) and relative numbers (Supplementary Figure 4).

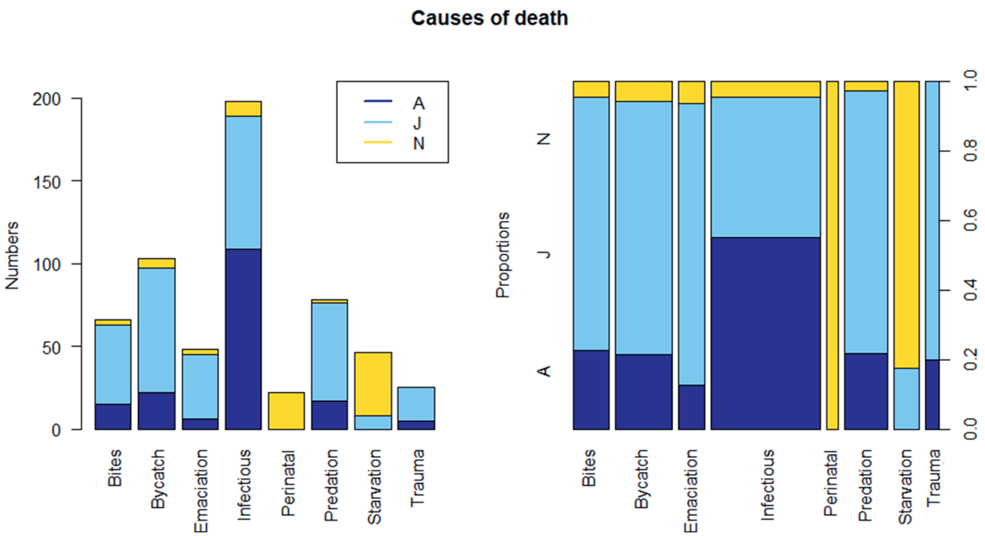

Figure 5. *Age class per cause of death category. Left: stacked absolute numbers. Right: proportions with the width of the bars representative of sample size. Dark blue is adult (A), light blue is juvenile (J), yellow is neonate (N).*

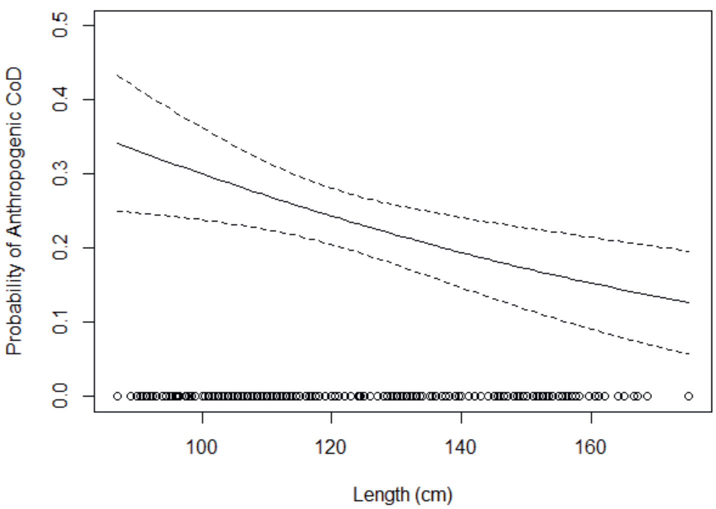

Figure 6. *Probability of an anthropogenic cause of death based on total length (in cm).*

The final multinomial regression model contained the predictor variables season, age class and nutritional condition, but not location, sex and year. This shows that at large, a sex and spatiotemporal difference could not be detected, but seasonal, nutritional and age-related variance was apparent (Supplementary Table 1). Despite that, the relative numbers in the category bycatch seemed to have decreased since 2013 (Figure 7), also in the numbers corrected for sample size (Supplementary Figure 3). Additionally, the acute predation cases seemed to be more frequently diagnosed in the earlier study years, with the number of cases with chronic lesions from bites becoming more apparent in recent years (Figure 7 and Supplementary Figure 3). Odd ratios (OR) of the final model are provided in Supplementary Table 2, but most apparent findings included that for age class, the odds of dying of emaciation of unknown origin versus bycatch was 2.95 (95% CI: 0.99-8.80) times higher for juveniles compared to adults, while the odds of dying as a result of bites, predation and trauma versus bycatch were similar for the juvenile and adult age classes (OR of ~1, see Supplementary Table 2 for 95% CI). The odds of dying due to infectious disease instead of bycatch was lower for the juveniles compared to the adults (OR=0.24, 95%CI: 0.13-0.45). As expected, the OR for the neonates was high in the cause of death categories perinatal and starvation (see Supplementary Table 2). Porpoises in poor nutritional condition (NCC5-6) had greater odds of dying due to infectious disease (OR=10.38, 95%CI: 4.51-23.91), animals with chronic lesions from bites (OR=11.59, 95%CI: 4.33-31.03), trauma (OR=3.40, 95%CI: 1-11.51) and emaciation of unknown origin (OR=5.42E+09, 95%CI: 2.77E+09 – 1.06E+10) versus bycatch, compared to those in good nutritional condition. Porpoises found in winter had higher odds of dying due to infectious disease (OR=4.36, 95%CI: 1.88 -10.08), chronic lesions from bites (OR=11, 95%CI: 3.23-37.43), and predation (OR=4.67, 95%CI: 1.67-13.06) versus bycatch, compared to those found in autumn, while spring was dominated by the perinatal cause of death (OR=1.28E+07, 95%CI: 3.86E+6 – 5.52E+06) (Figure 8).

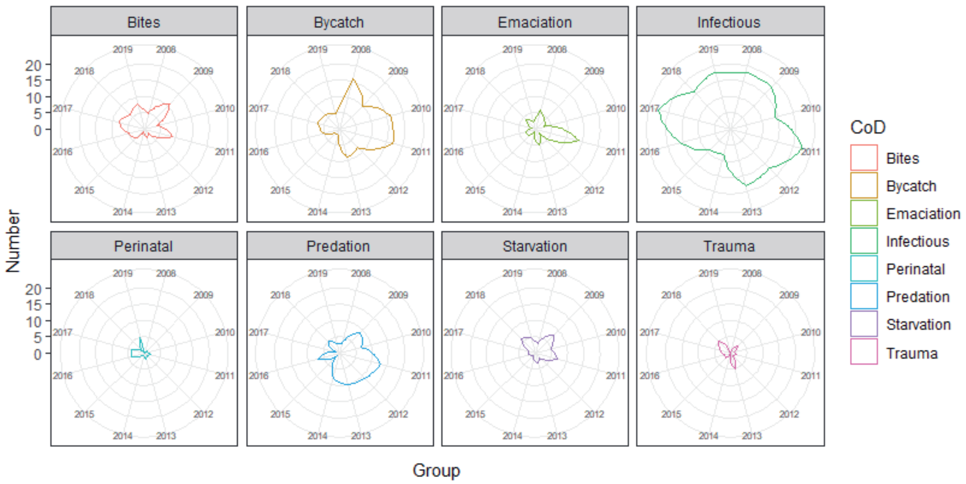

***Figure 7**. Radar plots of causes of death categories per year (2009-2019).*

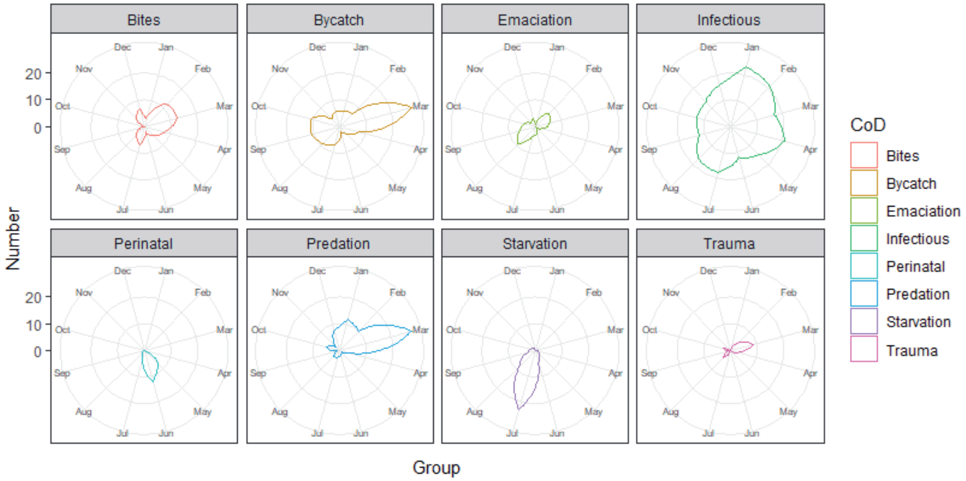

Figure 8. *Radar plots of causes of death categories per month.*

8

Discussion

Here, we provide an estimation on the occurrence and trends in anthropogenic and natural causes of mortality of stranded harbour porpoises in The Netherlands over a twelve-year period (2008-2019). The largest anthropogenic cause of mortality was bycatch (17%), with mainly juveniles affected and peak periods in March and September-October. There were signs of a decreasing trend over time, with bycatch being relatively less frequently diagnosed in more recent years. Other anthropogenic causes of mortality were infrequently diagnosed: trauma (2%), possibly due to ship collision, and marine debris ingestion and entanglement (0.3%). Lesions compatible with noise induced hearing loss could only be investigated for a small subset of cases but was apparent in two harbour porpoises. Infectious disease was by far the largest cause of death category (32.4%), mainly for adults, and rather constant over time. Lesions consistent with grey seal attacks were detected in 23.5%, with more acute predation cases in the earlier years, while porpoises with pathology associated with interactions that did not immediately result in death (chronic lesions from previous bites) were diagnosed more recently. Two third of all stranded neonates had morphological and etiological characteristics most likely related to problems in gestation, birth or nursing, while others died due to infectious disease, interspecific interaction or bycatch. Acute starvation or emaciation with unknown origin (non-neonates) were the most significant findings for 8.6%. There was one notable spike in the category emaciation of unknown origin in 2011, especially for juveniles. The risk of dying due to anthropogenic causes was highest among the juveniles, although season and nutritional status were also relevant variables in the categorization of cause of death.

Anthropogenic causes of death

Accidental bycatch in fisheries

The impact of fishery activity on different species of cetaceans is considered one of the main threats for small cetaceans (Dolman et al. 2016; Jefferson & Curry 1994; Moore 2014). The overall proportion of harbour porpoises that most likely died as bycatch was 17% in our study. In other geographic areas in the North-western EU waters, reported bycatch proportions varied widely. For the United Kingdom, several studies overlapping in time and space report bycatch percentages ranging from 11.1% (England, 8/72 between 1990-1993 (Kuiken et al. 1994b)) and 15.3% (Wales, 16/105 porpoises between 1989-1993 (Baker 1994)), to as much as 37.5% (England and Wales, 66/176 between 1990-1995 (Kirkwood et al. 1997)). For the southernmost parts of the North Sea, specifically Belgium and Northern France, bycatch proportions were below those we report for The Netherlands (8/55 between 1990-2000, 14.5% (Jauniaux et al. 2002)), although mass bycatch mortality has also been reported from Belgium (Haelters & Camphuysen 2009) before recreational set-netting was banned there. Harbour porpoise mortality related to bycatch in the German North and Baltic Seas were not provided, instead, authors gave the number of stranded porpoises with net-marks. This was 42.4% and 20.2% respectively for the German North and Baltic Seas (98/231, 42.4% and 17/84, 20.2% between 1990-2015 (Siebert et al. 2020)) and probably runs in the thousands of animals per year in the North Sea (Vinther & Larsen 2004). Stranded porpoises suspected to be bycaught were often deemed too decomposed for extensive necropsy which hampered the establishment of a bycatch prevalence (Siebert et al. 2020). This is likely also true for other areas, including The Netherlands. Here, only approximately 10-15% of all stranded porpoises are necropsied annually due to financial restrictions, with subsequently a focus on very fresh to moderately fresh carcasses for post-mortem examination. It is conceivable that among the putrefied stranded porpoises, prevalence's of bycatch related mortality are higher since discarded porpoises might not make immediate landfall or they do not strand at all, depending on environmental factors such as tides and wind (IJsseldijk et al. 2021b; Moore et al. 2020; Peltier et al. 2013). This results in advanced time between death and retrieval from the beach, which subsequent negative effects on carcass freshness. Bycatch-related mortality among harbour porpoises is therefore likely higher (Siebert et al. 2020). Additionally, diagnosing bycatch among stranded cetaceans comes with its challenges (IJsseldijk et al. 2021b; Kuiken 1996; McEwen & Gerdin 2018). Previously used criteria that were thought to aid in the diagnosis of underwater entrapment due to bycatch were a favourable health status, the absence of disease, and good nutritional conditions (Kuiken 1996). However, these did not apply to the majority of bycaught porpoises gained directly from fisheries in The Netherlands (IJsseldijk et al. 2021b). In the current study we have also shown that a large number of stranded harbour porpoises that most likely died as bycatch had signs of underlying disease or debilitation. This is indicative of an overall reduced fitness of porpoises inhabiting the southern North Sea, or it could indicate that debilitated porpoises have higher chances of becoming bycaught (IJsseldijk et al. 2021b). Collaborations with fisheries for the direct retrieval of bycaught specimens could improve bycatch and health monitoring for harbour porpoises in the southern North Sea.

Marine debris ingestion and entanglement

The wide distribution and abundance of man-made litter, in particular plastics, affects a broad range of marine organisms through entanglement and ingestion (van Franeker et al. 2018; Kuhn 2020; Kuhn et al. 2015; Unger et al. 2016). A previous study on harbour porpoises was conducted with individuals overlapping our study (van Franeker et al. 2018). The incidence of marine debris in stomachs was between 7-15%, depending on methodology used. We have shown here that ingestion of marine debris occurred more frequently than entanglement in debris and that fishery related debris is of particular concern (van Franeker et al. 2018; Bravo Rebolledo 2020; this study). We presented one case of fatal debris ingestion, probably of a fish hooked on lost angling gear (wired epiglottis), and another porpoise with signs of severe, external and prolonged entanglement. Although this marks the number of porpoises dying as a direct result of marine debris as very low (0.3%, 2/612), the welfare state of both individuals was severely compromised as a result of the prolonged entanglement. This demonstrates the harmful effects that marine debris, specifically macro items, can have on marine animals. The detection of small particles ingested by cetaceans is hampered by protocols and methodologies, and the prevalence of microplastics (<1 mm) in porpoises is likely higher (van Franeker et al. 2018; Philipp et al. 2021). The prevalence of ingestion, and the rate and effect of specifically micro- and nanoparticle intake therefore remain an area where future work needs to be conducted.

Sharp and blunt force trauma

Fourteen porpoises had sharp and/or blunt force trauma as main and most significant pathological finding (14/612, 2.2%). Blunt forced injuries may result from collision with non-rotating features, such as the bow or hull of a ship, or slowly rotating tidal energy turbines, with common pathological findings being well-defined focal areas of subcutaneous haemorrhage and oedema, physically disrupted muscle, fractured bones and disruption of organ systems (IJsseldijk et al. 2014; Martinez & Stockin 2013; McLellan et al. 2013) and as seen in porpoises of this study. Collisions with ship propellers are often associated with major injuries such as incised wounds and lacerations, and amputations, as seen in porpoises of this study, inflicted by heavy metal blades rotating at high speed (Byard et al. 2012; Costidis et al. 2013; Lightsey et al. 2006). However, necropsy findings in the blunt force trauma category were sometimes also consistent with bycatch criteria, such as the presence of bruising and fractures (Cox et al. 1998; Jepson et al. 2013; Moore et al. 2013a). Although the most reliable indicator of bycatch is the presence of net-marks or encircling imprints (IJsseldijk et al. 2021b; Jepson et al. 2013; Kuiken 1996), post-mortem and stranding-related degradation of the skin may have hampered the detection of such external lesions. This highlights several diagnostic difficulties among these categories of acute death. Bycaught porpoises can directly be retrieved from fisheries or fishermen, which can lead to targeted investigations into common findings in these animals which aid in the diagnosis of this cause of mortality among stranded individuals (IJsseldijk et al. 2021b). Collisions on the other hand often go unnoticed, remain unreported or carcass collection following collisions is hampered, e.g., animals may sink or drift away (Laist et al. 2001). Besides, the severity of injuries is associated with the size and speed of the propeller or vessel, the nature or behaviour of the victim and the angle of impact (Byard et al. 2012; McLellan et al. 2013) and may present large individual variance in the inflicted damage. This influences our ability to pinpoint exact causes of induced trauma diagnosed during post-mortem investigations. Natural causes of mutilations on porpoises are commonly seen following interspecific interactions. Grey seal attacks are characterised by sharp-edged mutilation but the distinct bite lesions, demonstrated as repetitive and often bilateral punctures, aids in further subdivision of these cases (Leopold et al. 2015a). Violent behaviour of other cetaceans, like the bottlenose dolphins (*Tursiops truncatus*), may result in large hematomas and fractures on porpoises attacked (Barnett et al. 2009; Patterson et al. 1998). However, delphinids are infrequently observed in the southern North Sea (Hoekendijk et al. in review; Wells & Scott 2018). This makes it less likely that the trauma cases investigated in The Netherlands were attributable to inter-specific interactions, thus increasing the likelihood of anthropogenic sources causing these injuries.

Noise-induced hearing loss

The quantification of the number of harbour porpoises dying as a direct result of underwater noise over-exposure through the assessment of the organ of Corti can only be conducted up to 24-30 hours after death (Morell et al. 2017b). Decomposition alters these structures quickly, hampering this investigation in the vast majority of all stranded cetaceans. Despite these significant challenges, we were able to investigate the well-preserved inner ears of 37 individually stranded harbour porpoises between 2010-2019. Two harbour porpoises had scattered loss of sensory cells in the apical turn of the inner ear which was compatible with noise-induced hearing loss (Morell et al. 2015, in review). Currently, there is no other analysis that can directly relate acoustic trauma to death in cetaceans, while the detection of noise-induced hearing loss is key to understand the physiological impact that anthropogenic noise may have on these species (Morell et al. 2017b). The large number of very fresh stranded porpoises that are found annually in The Netherlands, as well as the well-established stranding network, aids in the evolvement of this focus research area.

Categories of non-anthropogenic pathology

Infectious disease

Bronchopneumonia was the most commonly detected pathology, not only in the category infectious disease but also among the porpoises dying of acute causes (both anthropogenic and natural). Often this was associated with a high prevalence of nematodes. The relative pathogenicity of the different parasite species is, however, uncertain and the role of parasites as a cause of stranding and death has widely been discussed in scientific literature (Arbelo et al. 2013; Jepson et al. 2000; Siebert et al. 2020). Parasite occurrence increases with total body length as a proxy of age (ten Doeschate et al. 2017). Besides the endoparasite, ectoparasites may be present on stranded porpoises. A prevalence of ectoparasitic crustaceans of 7.6% was found on fresh stranded porpoises in The Netherlands, which was higher than the prevalence calculated for in Germany stranded porpoises (1.6%) (Lehnert et al. 2021). This was deemed indicative of a difference in severity of skin lesions in which these ectoparasites occur (Lehnert et al. 2021). Chronic skin lesions were indeed present in high numbers on our stranded harbour porpoises. Some of these lesions were attributed to grey seal bites, others deemed more likely of viral origin (e.g., herpesvirus, see van Beurden et al. 2015). Structural microbiological screening was beyond the scope of our study. The investigation into infectious agents is hampered by finances, logistics and tissue quality, and an overview and estimated prevalence's of pathogens are lacking. Several infectious agents have however been targeted and published separately. Between 2008-2011, the presence of *Brucella* spp. in stranded harbour porpoises was assessed, and an overall prevalence of 6.3% was established, although associated pathology could not be investigated (Maio et al. 2014). Mycoses, specifically *Aspergillus fumigatus*, had a prevalence of 2.4% among the stranded porpoises in The Netherlands (2009-2019), with most commonly affected organs being the respiratory tract, central nervous system and auditory system (Kapetanou & IJsseldijk et al. 2020). *Toxoplasma gondii* antibodies were detected in two porpoises stranded on the Dutch coast but without associated lesions (van de Velde et al. 2016) and a third porpoise died due to encephalitis with intralesional *T. gondii* (Morell et al. in review). Morbillivirus has not been detected in harbour porpoises found stranded on the Dutch coast to date (unpublished data).

Grey seal interaction

The detection of grey seal DNA in attack wounds on harbour porpoises revealed the (for porpoises) fatal interaction between these two marine mammals (Jauniaux et al. 2014; van Bleijswijk et al. 2014). A retrospective analyses of post-mortem findings demonstrated the large scale of grey seal predation on harbour porpoises in the southern North Sea (Leopold et al. 2015a) and predations have subsequently been reported for a much wider geographical range (Bouveroux et al. 2014; Foster et al. 2019; Jauniaux et al. 2014; van Neer et al. 2020). Immediately it became apparent that the acute predation cases were just the tip of the iceberg, with potentially many more porpoises suffering from bite injuries with occasionally deadly outcomes (Foster et al. 2019; Leopold et al. 2015a). Several of our mutilated porpoises had clear signs of chronic lesions from previous bites in addition to the acute mutilations, suggesting previous failed predatory attempts. This demonstrates frequent interactions between seals and porpoises in the southern North Sea. Here, grey seal numbers have been on the rise after a successful rehabilitation programme aiming for their conservation when numbers were at their lowest (Brasseur 2017). With increasing numbers of seals as well as the increase in harbour porpoises in the southern North Sea, interactions have undoubtedly become more common in the last decades. The direct and indirect effects of these interactions is currently unclear but it is conceivable that the large number of seals may negatively affect harbour porpoise health, behaviour and fitness. This may particularly be true in areas such as the Eastern Scheldt, with a small resident population of porpoises, from which seal victims are frequently observed (Podt & IJsseldijk 2018).

Peri- and neonatal mortality
Perinatal mortality was diagnosed for 26.2% of necropsied neonates in this study, mostly the smallest new-borns found stranded with lengths corresponding to reported length at births (Lockyer 2003)[40]. Causes of death may be related to asphyxiation due to aspiration of meconium or amniotic fluid and subcutaneous haemorrhages which indicate foetal suffering (Arbelo et al. 2013). Larger neonates more often died with signs of acute starvation (which can be fat animals) and/or emaciation (skinny animals) (48.8% of all neonates necropsied). Hepatic lipidosis was a common finding. Nursing calves have high intake of carbohydrates which results in the accumulation of excessive triglycerides in the hepatocytes, resulting in pale and waxy livers (St. Leger et al. 2018). In neonates, this is considered physiological (Jaber et al. 2004; St. Leger et al. 2018) with the significance of the neonatal hepatic lipidosis deemed unclear (Hiemstra et al. 2015; Siebert et al. 2001). In harbour porpoises stranded in Germany, diffuse hepatocellular lipidosis was found in 13 of 29 (44%) calves (Siebert et al. 2001) and neonatal mortality attributed to starvation was also reported to be high amongst calves stranded in the United Kingdom (Kirkwood et al. 1997). Neonatal death may be a result of maternal separation or nursing difficulties but in the majority of neonates it is impossible to further pinpoint exact causes. It is possible that maternal inexperience or failure played a role in our cases, although other explanation could be disturbance from natural or anthropogenic threats.

Management implications
There is an increasing need to understand marine mammal health and how it changes over time to determine the effects of persisting, new, emerging and cumulative threats affecting wild populations (Bossart et al. 2011; IJsseldijk et al. 2018a; NAS 2017; Siebert et al. 2020). Knowledge on temporal differences in health as well as mortality is crucial for management of species or subgroups, which are particularly vulnerable to human interaction, like the harbour porpoise. Accidental bycatch in fisheries has historically been a focus topic within cetacean conservation and management programmes (ASCOBANS 1992; Camphuysen & Siemensma 2011; Dolman et al. 2016). The high incidence of bycatch mortality in our study (17%) suggests that bycatch is a persisting issue and the number of diseased and debilitated animals within the bycatch category is, to say at least, worrying. A more emerging, anthropogenic threat, both in terms of scale and focus, is over-exposure to anthropogenic noise. High intensity sonars, seismic surveys, pile driving for offshore windfarm construction, and shipping among other activities, can result in masking, behavioural and physiological changes, or even hearing impairment in exposed cetaceans (Morell et al. 2017b; Wisniewska et al. 2018). There is clearly a need for a better understanding of the many consequences of exposure to anthropogenic sound and the effects of noise pollution on marine mammals.

Continuation of a comprehensive research program on fresh, stranded harbour porpoises with targeted studies on the hearing organ are a means to increase our knowledge on the anatomy of and pathologies in the auditory system, which could ultimately help to distinguish between lesions attributable to noise pollution and other causes of hearing loss such as aging or infectious disease.

The grey seal was exposed as a major predator of harbour porpoises relatively recently. This meant a shift in cause of death from most likely anthropogenic origin to a natural cause for numerous harbour porpoises, after the diagnostic difficulties regarding differentiation of these two categories were overcome (Leopold et al. 2015a). However, the increase in grey seal abundance in the southern North Sea was a result of multiple, long-term, successful rehabilitation programs. Although dedicated studies on the indirect effects of grey seal interactions on porpoise health and population resilience are lacking, one could state that conserving one species might have diminishing effects on others.

Reported infectious disease outbreaks in marine mammals have been on the rise, with factors causing these still being mostly unclear (Gulland & Hall 2007; Sanderson & Alexander 2020). There are however signs that especially chemical pollutants may be involved, since the persisting organic pollutants in the marine environment have been associated with disrupted endocrine and immune function in cetaceans (Jepson et al. 1999, 2005, 2016). Climate change, another emerging conservation focus, may also result in indirect and thus more nebulous health effects, likely in terms of habitat degradation, changes in resource quantity and quality and increasing infectious disease outbreaks (Bossart 2011; Learmonth et al. 2006; Sanderson & Alexander 2020; Simmonds & Isaac 2007). This has already been demonstrated for seabirds, where breeding success in the northern Hemisphere was compromised as a result of ocean warming and human impacts (Sydeman et al. 2021). It may therefore become more pressing to expand the monitoring efforts to infectious agent screening, and an important factor to investigate the effects of those human-related threats that do not leave distinct lesions on animals but rather have indirect effects on their immune system, behaviour or reproduction.

Acknowledgements

We sincerely thank the Dutch stranding network volunteers and organisations for their help with reporting and retrieving stranded specimens for post-mortem examination. The necropsies and diet analyses were conducted with the help of many colleagues, research assistants, students and volunteers. Although we are thankful to every one of them, in particular we would like to acknowledge Johan van Amerongen, Louis van den Boom, Kees Camphuysen, Mariel ten Doeschate, Natashja Ennen-Buijs, Sjoukje Hiemstra, Guido Keijl, Thijs Kuiken, Manon Lock, Liliane Solé and Ruby Wagensveld. Necropsies and diet analyses of harbour porpoises in The Netherlands were commissioned by the Ministry of Agriculture, Nature and Food Quality, and since 2016 embedded under the Legal Research Tasks Nature & Environment unit of Wageningen UR (project reference numbers 140000353, WOT-04-009-045). The inner ear collection, preservation and analysis were funded by Rijkswaterstaat (WoZEP 2016), the Natural Sciences and Engineering Research Council (NSERC) of Canada Discovery and Accelerator grants RGPAS 446012-13 and RGPAN 312039-13, the Spanish Ministry of the Environment (grant number: 083/SDGTB/2007) and Marie Skłodowska-Curie Individual Post-doctoral Fellowship (M. Morell: grant number: 751284-H2020-MSCA-IF-2016).

Supporting information

Supplementary data associated with this chapter will eventually be part of the final publication and in the meantime can be requested by e-mail.

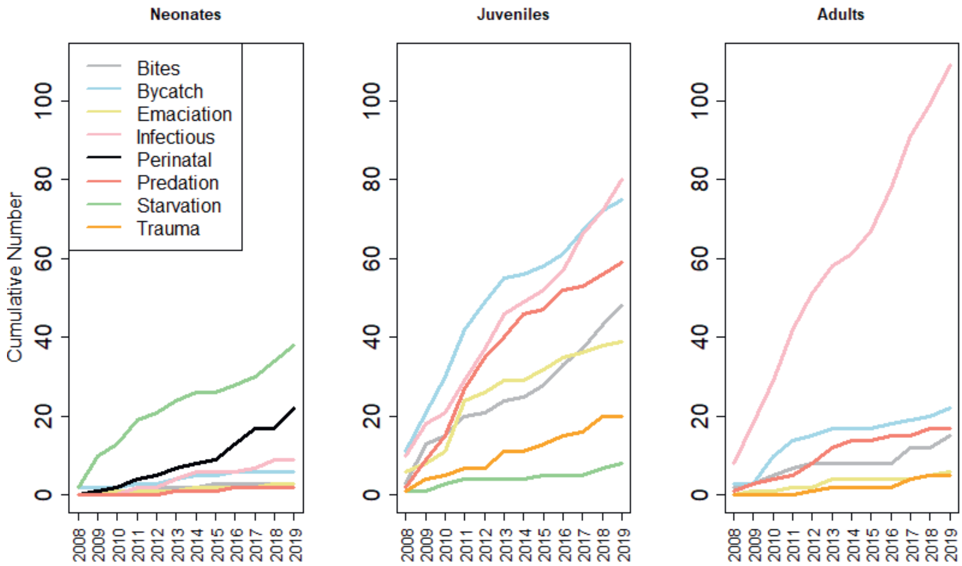

Figure S1. *Cumulative number of causes of death per year, framed per age class*

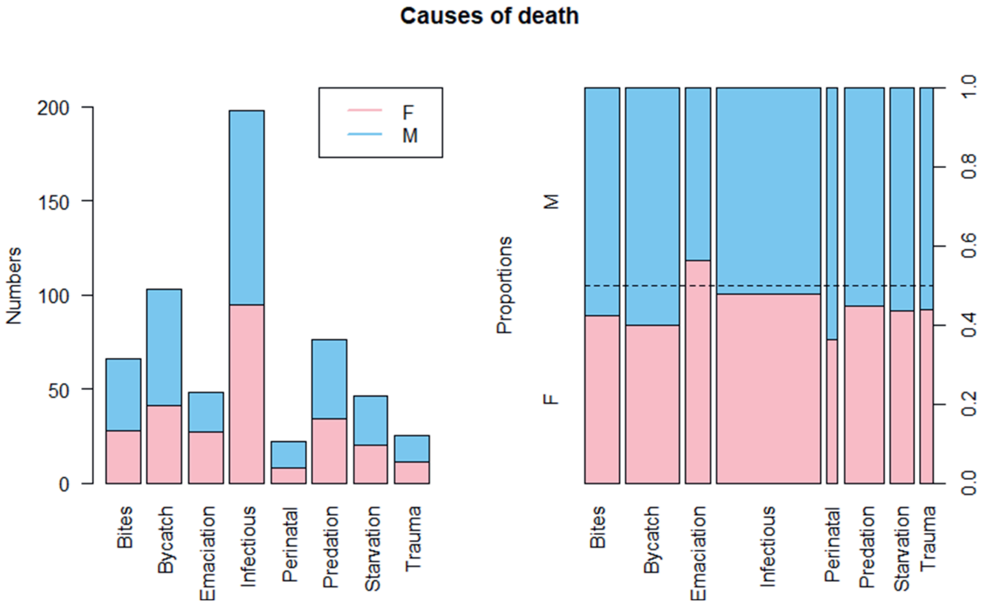

Figure S2. *Sex per cause of death category. Left: stacked absolute numbers. Right: proportions with the width of the bars representative of sample size and dotted horizontal line at 0.5. Blue = males. Pink = females.*

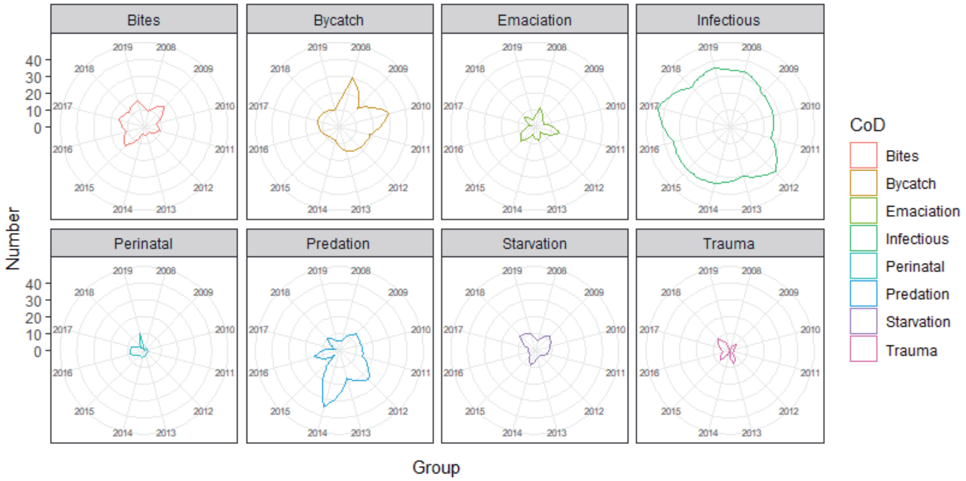

Figure S3. *Radar plots of causes of death categories per year (2009-2019) corrected for n/year*

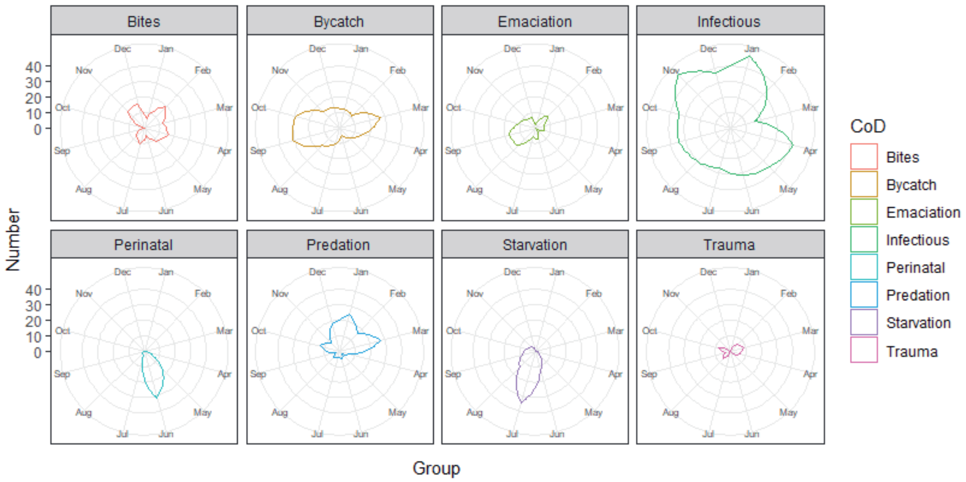

Figure S4. *Radar plots of causes of death categories per month corrected for n/month*

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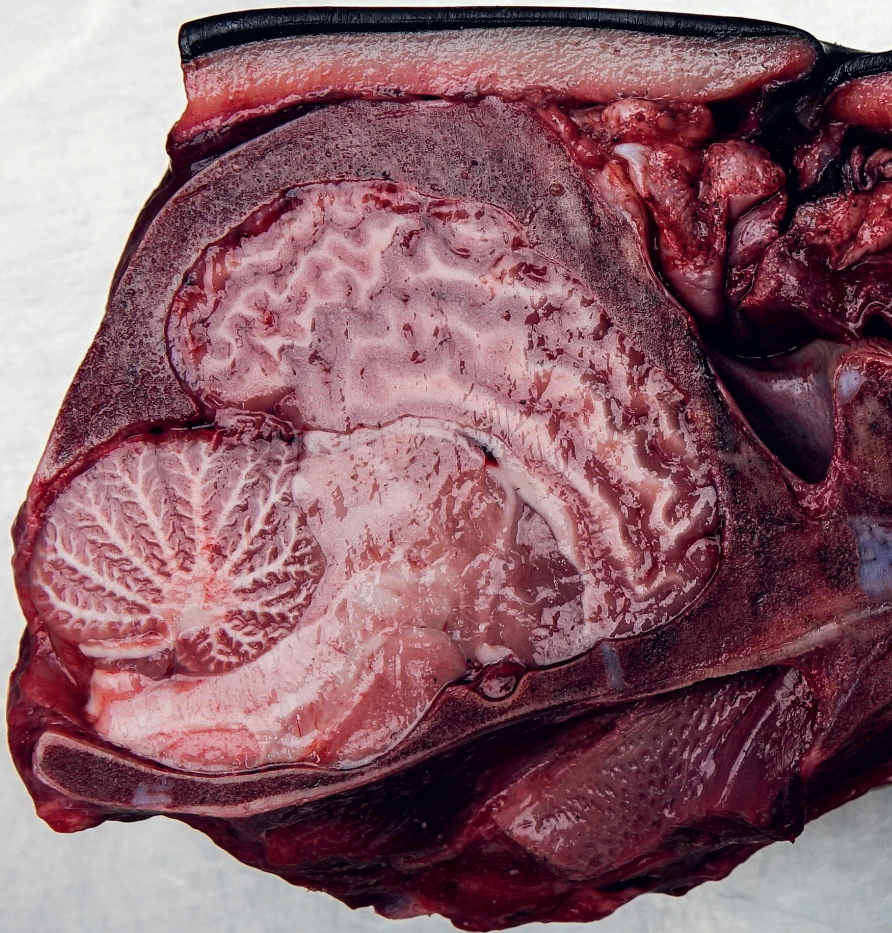

Manuscript in preparation

Exploring biological, ecological and pathological data of harbour porpoises using supervised and unsupervised classification methods

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Introduction

Animal autopsies, so-called necropsies, are often a key element in wildlife surveillance programs (Ryser-Degiorgis 2013). Necropsies can be performed for varying reasons, including (but not limited to) characterising normal and abnormal gross and morphologic anatomic features, to establish baseline health parameters and to identify cause(s) of morbidity and mortality in individuals, groups or populations (McAloose et al. 2018; Ryser-Degiorgis 2013). In terrestrial wildlife research, the goals of health surveillance programs are mainly disease-driven: the recognition of exotic or emerging disease and its potential for transmission to domestic animals or humans (Artois et al. 2009; Ryser-Degiorgis 2013). Many marine mammal monitoring programs, however, originated with a primary conservation and management focus and the main aim of determining the rate and extent of human-related causes of morbidity and mortality. Ideally, studying cetacean pathology — the study of diseases, their cause or aetiology, pathogenesis and the structural and functional changes they give rise to (McAloose et al. 2018) — should be extended with ecological and other biological disciplines and quantitative analysis, to go beyond the emphasis on specific individuals and to be able to make population inferences.

Studies reporting pathological findings in commonly stranded small cetaceans in the North-East Atlantic and adjacent waters often focus on descriptions of the most frequently diagnosed lesions and causes of death (Jauniaux et al. 2002; Kirkwood et al. 1997; Schick et al. 2020; Siebert et al. 2001, 2006, 2020). Specifically, bycatch has been a primary research focus (Clausen & Andersen 1988; IJsseldijk et al. 2021b; Kirkwood et al. 1997; Kuiken 1996), and so have reports on the health effects of anthropogenic noise, chemical pollution and marine debris for a number of marine mammal species (Desforges et al. 2018; Fernández et al. 2005; Jepson et al. 2005a; Kuiken et al. 1994b; Sonne et al. 2020; Unger et al. 2016). Although these studies are highly important in order to extensively describe typical or atypical causes of mortality and new or emerging infectious and non-infectious diseases, they are often limited from an analytical point of view. Analysing data and results collected as part of post-mortem examinations is challenging because of several factors, including a large individual variance, varying methods and procedures between cases, insufficient numbers of individuals or a lack of long-term studies; all of which may hamper comprehensive meta-analyses. There is, however, an urgent need for assessments of patterns in biological, ecological and pathological profiles across individuals, in order to detect changes over space and time for trend analysis and timely detection and follow-up of unusual health events affecting populations. This is particularly relevant given the rapid changes of marine ecosystems as a result of human influences and the reported rise of disease in marine organisms (Bossart 2011; Gulland & Hall 2007; Halpern et al. 2008, 2019).

Here, we use supervised and unsupervised machine learning techniques to explore and assess data of a marine mammal in order to expand the methods of surveillance as a basis of targeted biological, ecological and pathological investigations and comparisons across individuals. Unsupervised learning is the process of grouping data into clusters using automated methods or algorithms on unclassified data. This means that there is no *a priori* response variable and no prior labelling or classification of the data, and one seeks to understand the relationships between the measured variables using cluster analysis. With supervised learning there is a response variable, and these models have the ability to generalize knowledge from labelled or categorised data to predict new or unlabelled cases (Berry et al. 2019).

We use the harbour porpoise (*Phocoena phocoena*), an abundant small whale species occurring in the North Sea, as a case study. A detailed collection of data was available from a large number of individuals examined post-mortem in The Netherlands (chapter 8). We use classification and correspondence analyses to investigate the links between data collected upon stranding and necropsy, and the subsequently conducted dietary and marine debris investigations. This is aimed at determining the most important variables, finding associations between variables, and classification of variables. Next, using the powerful machine learning technique 'random forest' we quantify relationships between predictor variables and causes of death to estimate the relative importance of each variable collected during post-mortem examinations for determining the cause of death. With the large number of individuals included in these analyses, robust trends in post-mortem findings in terms of anthropogenic and natural threats can be detected, resilient to small or short-term variations and thus relevant at the population level. It therefore serves a detailed example demonstrating the usefulness of extensive, long-term mortality data of a protected species, applicable for wildlife across different environments and taxa.

Methods

Post-mortem investigations

Necropsies

Post-mortem examinations on stranded harbour porpoises have been conducted at the Faculty of Veterinary Medicine, Utrecht University since 2008 (n=1768), with the necropsies conducted following internationally standardised guidelines (IJsseldijk, Brownlow & Mazzariol 2019). The primary aim of the post-mortem examinations is to determine the animals' most likely causes of death and their health status prior to death. At the start of each post-mortem examination, external features are photographed for documentation purposes. Data collection of each case additionally includes date and location of stranding, sex, total body length (in cm), and mass (in kg). Based on total length, animals were assigned an age class, with animals ≤90 cm classified as neonates, >90 cm to 130 cm considered juvenile, and >130 cm classified as adults. For animals in the range of 120-140 cm, assessments of reproductive organs were conducted in order to make the final differentiation between juveniles and adults. All porpoises were given a decomposition condition code (DCC) at time of necropsy, with DCC1 representing very fresh carcasses and DCC5 representing carcasses remains. Organs of all cases were assessed grossly, and a range of tissues samples were collected from cases in DCC1-3 for histopathological assessment (see data preparation and chapter 8). Finally, each case was assigned to one of ten cause-of-death categories reflecting the most likely cause of death or stranding: accidental bycatch in fisheries, acute grey seal predation, chronic lesions from a previous grey seal attack, infectious disease, emaciation, starvation, perinatal death, blunt forced- and sharp trauma, other and unknown (chapter 8). Descriptions on anthropogenic and other causes of death for harbour porpoises in the southern North Sea, spatiotemporal and age- and sex related differences in mortality patterns are presented in chapter 8.

Diet and marine debris

For diet and marine debris investigation, stomachs were visually inspected and sampled during necropsy and stored at - 20˚C until further analyses at Wageningen Marine Research. Here, contents were collected following methods described in van Franeker et al. (2018) and in chapter 8, and prey remains were examined following earlier published methods (Leopold et al. 2015b). Here, we include presence/absence data of marine debris and prey in stomachs. Any non-dietary, non-man-made items, such as plants, stones and wood, were additionally recorded. Detailed results of dietary analysis are published in Leopold (2015).

Data preparation

For this study, the necropsy reports and results from the diet- and marine debris analysis in the period 2008-2019 were retrospectively assessed in a uniform way, and results were categorised and scored for analytical purposes. The quantity and quality of data and results strongly depend on carcass freshness and completeness as well as other factors (IJsseldijk, Brownlow & Mazzariol 2019). Only cases for which a full post-mortem investigation was initially conducted, this includes availability of at least gross- and histopathological assessment reports and external photographs, were included in the retrospective scoring. These criteria exclude cases in advanced decomposition stages (DCC4-5). In addition, cases with unknown age class and/or sex were excluded as well as all necropsied foetuses. The number of individual harbour porpoise cases ultimately included in this study was 612. A total of 49 variables were assessed for these 612 cases. An overview of the variables, a description, assessment and scoring range can be found in Table 1.

***Table 1**. Categories and scores of necropsy reports and results from diet- and marine debris investigations for analytical purposes. Examination: Stranding = collected during stranding, Photo = retrospectively assessed using the photo database, Macro = based on the macroscopic examination, Histo = based on the histologic examination, Diet = based on the stomach content analysis.*

Variable	Description	Examination	Range or categories
Month	Month of stranding	Stranding	January-December
Year	Year of stranding	Stranding	2008-2019
Season	Grouped months	Stranding	1 = winter (Dec, Jan, Feb) 2 = spring (Mar, Apr, May) 3 = summer (Jun, July, Aug) 4 = autumn (Sep, Oct, Nov)
Location	Location of stranding categorised by geographical area	Stranding	1 = Delta 2 = Zuid-Holland 3 = Noord-Holland 4 = Noord NL 5 = Waddeneilanden
Sex	Sex	Macro	1 = female 2 = male
Age class	Based on total body length (TL), assessment of reproductive organs	Macro	1 = neonate 2 = juvenile 3 = adult
DCC	Decomposition condition code of carcass at necropsy	Macro	1 = very fresh 2 = fresh 3 = putrefied
NCC	Nutritional condition code visually assessed based on the dorsal musculature, presence of visceral fat and blubber thickness	Macro	1 = very good 2 = good 3 = moderately good 4 = suboptimal 5 = poor 6 = very poor
Live stranding	Observed or based on ante-mortem scavenging or other ante-mortem stranding related artefact	Stranding, macro	0 = no 1 = yes
External defects	Measure of % of body externally damaged incl. all predation, scavenging, stranding related and post-mortem defects	Photo	0 = none 1 = mild, <10% defects 2 = moderate, 10-50% defects 3 = severe, >50% defects
Imprints	Acute, encircling lesions, incision wound(s) or other imprints that may represent line, net or rope entanglement. Characteristics described in Kuiken (1994) and IJsseldijk et al. (2021)	Photo	0 = absent: no sign of line or net imprints 1 = possible: suspicious marks but difficulties interpretation, e.g., due to scavenging, stranding or post-mortem defects 2 = present: clear imprints, multiple nick and notches
Mutilation	Ante-mortem, acute blubber defect > 10x5cm^2, sharp edged, additional	Photo	0 = absent 1 = present

	bilateral bite marks, which could be located on the tailstock, pectoral fins, as well as on the head or rostrum and/or fluke. Characteristics described in Leopold et al. (2015)		
Chronic bite lesions	Parallel running punctures interpretated as bite lesions, chronic, active, e.g., clear sign of inflammation, which could be located on the tailstock, pectoral fins, as well as on the head or rostrum and/or fluke. Characteristics described in Leopold et al. (2015) and Foster et al. (2019)	Photo, macro, histo	0 = absent: no sign of bite lesions 1 = possible: suspicious lesions but difficulties in interpretation, e.g., due to scavenging, stranding defects or post-mortem damage 2 = present: clear parallel running, chronic bite lesions
Skin lesions	Chronic lesions or scars affecting predominately the (epi)dermis. Mostly round to oval or longitudinal, characterised by e.g., necrosis and/or haemorrhage, with a suspect viral cause or morphology, inflammatory infiltrates presence (e.g., INI's) (herpesvirus (van Beurden et al. 2015), poxvirus (Barnett et al. 2015))	Photo, macro, histo	0 = none 1 = mild: affecting approx. 1-9% of body surface 2 = moderate: affecting approx. 10-20% of body surface 3 = severe: affecting >20% of body surface
Blunt trauma	Subcutaneous or intra-musculature bruising or contusion, (acute) fractures, haemorrhage in central nervous system with no (or very mild/acute) amount of (associated) inflammatory cells. Animals with signs of (acute) sharp-edged mutilation or trauma are given a score 0	Photo, macro, histo	0 = absent 1 = present
Pathology alimentary tract	Chronic and sub-acute lesions in oral cavity, e.g., gingivitis, oral ulcers, oesophagus ulcers, gastritis and gastric ulcerations	Macro, histo	0 = no or mild abnormalities 1 = moderate to severe abnormalities
Ingestion of prey	Hard prey items, varying from partly (un)digested prey to otoliths	Diet	0 = absent 1 = present
Ingestion of plants	Plant material in the stomach	Diet	0 = absent 1 = present
Ingestion of stones	Natural, non-plant and non-prey items in the stomach, like stones or wood	Diet	0 = absent 1 = present
Ingestion of debris	Plastics and other man-made materials in the stomach	Diet	0 = absent 1 = present
Parasites gastrointestinal tract	Nematodes, cestodes, trematodes in stomach lumen, stomach wall and/or intestine	Macro	0 = none 1 = mild: approx. 1-20 nematodes in stomach lumen, and/or mild extensive infestation of wall, no cestode in intestine 2 = moderate: approx. 21-50 nematodes in stomach lumen,

			and/or focal severe infestation of wall, cestode in <50% of intestine 3 = severe: approx. >50 in stomach lumen, and/or severe extensive infestation of wall, cestode in >50% of intestine
Oedema respiratory tract	Foam (blood tinged or white) in trachea and/or pulmonary oedema in the upper respiratory tract and/or pulmonary airways. Pulmonary congestion with intra-alveolar oedema	Macro, histo	0 = no oedema detected 1 = oedema detected
Pathology respiratory tract	Description contains: granulomatous, suppurative/purulent and/or necrotising (broncho)pneumonia and/or pleuritis. There could be signs of fungal elements (PAS-stain)	Histo	0 = no or mild abnormalities 1 = moderate to severe abnormalities
Parasites respiratory tract	Nematodes in bronchi and/or pulmonary vessels	Macro	0 = none 1 = mild: approx. 1-20 nematodes 2 = moderate: approx. 21-50 nematodes 3 = severe: approx. >50 nematodes
Pathology central nervous system	Description contains: granulomatous, suppurative/purulent and/or necrotising meningitis, encephalitis or meningoencephalitis, incl. PAS-stain positive, cuffing, INIs, sign of mild to severe amount of inflammation or inflammatory cells	Histo	0 = no abnormalities detected 1 = abnormalities detected
Parasites liver	Trematodes in hepatic ducts	Macro	0 = none 1 = mild: <10% affected 2 = moderate: 10-30% affected 3 = severe: >30% affected
Pathology liver	Description contains: hepatitis and/or cholangitis, granulomatous, suppurative/purulent and/or necrotising	Histo	0 = no or mild abnormalities 1 = moderate to severe abnormalities
Iron accumulation Kupffer cells	Golden-brown pigment: presence/absence based on HE, with confirmation of severity in positive cases using Prussian blue stain	Histo	0 = none 1 = mild 2 = moderate 3 = severe
Iron accumulation hepatocytes	Golden-brown pigment: presence/absence based on HE stain, with in positive cases confirmation of severity using Prussian blue stain	Histo	0 = none 1 = mild 2 = moderate 3 = severe
Parasites middle ear and sinuses	Nematodes in the peri-bullae sinuses and tympanic cavity	Macro	0 = none 1 = mild: approx. 1-20 nematodes 2 = moderate: approx. 21-50 nematodes

			3 = severe: approx. >50 nematodes
Pathology cardiovascular system	Description contains: myo- or pericarditis, granulomatous, suppurative/purulent and/or necrotising, degeneration	Histo	0 = no abnormalities detected 1 = abnormalities detected
Pathology urogenital system	Description contains: nephritis, mastitis, endometritis, orchitis, severe obstructions e.g., calcification causing blockage, dystocia; severe and extensive macroscopically visible inflammation reproductive organs	Macro, histo	0 = no or mild abnormalities 1 = moderate to severe abnormalities
Multiple organ congestion	Hyperaemia/congestion in ≥ 2 organs: incl. adrenal, brain, heart, kidneys, liver, lung, spleen	Histo	0 = absent 1 = present
Multiple organ inflammation	Moderate to severe inflammation ≥ 3 organs: brain, heart, lung, liver, spleen, kidney, GIT, peritoneum/pleura, blubber, skeletal muscle	Macro, histo	0 = absent 1 = present
Miscellaneous	Other significant abnormalities which significantly contributed to or explained death or stranding of the animal, e.g., obstruction, neoplasia, sharp-edge trauma/ amputations, chronic entanglement	Stranding, photo, macro, histo	0 = absent 1 = present

Data analyses

Data exploration and analyses were performed using R version 3.6.3 (R 2017), with packages ggplot2 (Wickham 2016), grid, gridExtra and RColorBrewer of CRAN for visualization. Specific packages for supervised and unsupervised classification of data are listed at the appropriate subsections below.

<u>Missing data</u>

Despite the criteria applied in the case selection, variables could still be missing on a case-to-case basis. Of the 612 cases, 405 (66%) had complete observations of all 49 variables listed in Table 1. We will focus the unsupervised and supervised classification methods on these complete cases but it is important to have a good understanding of the data limitations before conducting more in-depth analyses or drawing conclusions. Missing data can be generated by predictive analytical procedures but understanding why data is missing is needed to avoid inaccurate prediction in future analyses. We therefore constructed a generalized linear model (GLM) with a binomial error distribution to test for the included covariates on the likelihood of having missing data, with complete cases coded as 0 and incomplete cases (one or more missing variables) coded as 1. Predictor variables included were: 'DCC', 'age class', 'month', 'year', 'external defects' and 'cause of death', which were all treated as factor variables. A backwards model selection approach was applied with the drop1 function from the R language to assess which model terms could be excluded (R 2017). The best fitting model was selected using Akaike's Information Criterion (AIC) as relative measure of the quality of the statistical model (Akaike 1974). Models with a smaller AIC were interpretated as different and more optimal if the difference with the prior model was larger than 2. Odds ratios (OR) of the most optimal model with their likelihood profile confidence intervals (95%) were calculated to assess which predictor variables best explained the missing of data.

Unsupervised learning: Correlation matrix using Cramer's V

We used the scored variables from photographs, macroscopic- and histologic reports, and diet- and marine debris analysis of the 405 cases with complete observations, as well as the stranding variables 'season' and 'location' (Table 1). Because we applied unsupervised learning techniques, the concluding category 'cause of death' was not included. Firstly, the correlation between all variable factors was assessed using a correlation matrix with R package corrplot (version 0.84). Cramer's V correlation coefficient was calculated for every pair of variables, using the vcd and DescTools libraries. Cramer's , is defined as the square root of a normalised chi-square value and measures the strength of associations between categorical variables and ranges between 0 and 1. Similar to Pearson's r, a value close to 0 means no association (Akoglu 2018). For further interpretation of Cramer's V we followed Akoglu[35]: > 0.05 indicates a weak association, > 0.10 a moderate association, > 0.15 a strong association and > 0.25 a very strong association. Because we measure association between categorical variables, the direction of associations (positive or negative) is not reflected in the Cramer's V. However, some of the categories represent Likert scales (such a parasite loads) or interval data (such as age class or NCC) and hence we have an interest in the direction of the relation between these variables. For those associations with Cramer's V > 0.25, we further assessed the relationships using stacked bar charts and mosaic plots.

Unsupervised learning: Multiple Correspondence Analysis and Hierarchical Classification

Cluster analysis attempts to group points, representing individuals, and their multivariate sample in a multidimensional Euclidean space, into disjoint sets (Gower 1967). Different methods of clustering can be applied, depending on whether the variables are quantitative, qualitative or a combination. Here, a Multiple Correspondence Analysis (MCA) using a Burt table was conducted using the FactoMineR package for analysis and the factoextra package for visualization (Berry et al. 2020; Husson et al. 2016). MCA is an extension of correspondence analysis for summarising and visualising a data table consisting of more than two categorical variables. As such, it is considered a generalization of Principal Component Analysis (PCA) with categorical instead of quantitative variables (Abdi & Williams 2010). MCA shows associations between variable categories and groups individuals with similar profiles in their scores of the variables. We focussed mainly on the categorical variables and not on the individuals. We extracted the eigenvalues, reflecting the proportion of variance retained by the different dimensions (axes) and explored the percentage of inertia explained by the dimensions using scree plots. For interpretation we assessed the (square of the) correlation ratio between each variable and each dimension (eta2), the Test Values (v.test) to judge association between categories and a factor following a Gaussian distribution, and the R2 correlation ratio of the variables in the different dimensions. Positive Test Values represent positive associations, negative values represent negative associations. For visualization, we focused on the quality of the representation of variables on the factor map (squared cosine, cos2) and the contribution of variables to the definition of dimensions (contrib). Finally, the coordinates of the individuals (coord) on the principal components of the MCA were used for hierarchical classification. The main output, the dendrogram generated by the hierarchical clustering, shows the hierarchical relationship between the clusters and distance between clusters (Murtagh & Contreras 2012).

Supervised learning: Random Forest Classification
As a method of supervised learning, we conduct random forest classification on the complete cases (n=405), including the category 'cause of death', using the R-package randomForest. Individuals with cause-of-death categories 'other' and 'unknown' were excluded. The random forest algorithm is based on classification (if response is a factor) and regression (if response is continuous) tree analysis, where nodes of a tree are split using the best subset of randomly chosen predictors. This is in contrast to standard trees, which splits nodes into binary groups by identifying regions with the most homogenous set of a response to predictor variables (Breiman 2001; Liaw & Wiener 2002). Random forests have two parameters: the number of variables in the random subset at each node and the number of trees in the forest. The technique is robust against overfitting and yields accurate predictions (Breiman 2001), for a detailed explanation see: Liaw & Wiener (2002).

We split our dataset randomly in a training set (bootstrap samples, set at a proportion of 0.7 of the cases) and a test set (the remaining 0.3 cases). The predictive ability was tested on out-of-bag (oob) observations. Two settings needed to be chosen: the numbers of trees in the forest (Ntrees) and the number of variables randomly drawn at each split (m) (James et al. 2017). Each random forest tree can provide an estimate of the importance of the variables for the predictions and of the structure of the data (proximity of data points to one another) based on the oob-data. The variable importance was assessed by estimating the mean decrease accuracy, which indicates how much accuracy the model loses by excluding each variable, and by the mean decrease in Gini coefficient, which measures how each variable contributes to the homogeneity of the nodes and leaves in the resulting random forest (Liaw & Wiener 2002). For both measures, the higher the mean value, the greater the accuracy of the variable to the successful classification or importance of the model.

Results

Missing data

Before restricting the analyses to complete cases, we assess why cases have missing data. This is a vital step prior to predictive analytics in the future. The GLM to test whether specific variables correlated with cases showing missing data revealed that ‘age class’ and ‘month’ had no correlation, but that ‘DCC’, ‘external defects’, ‘cause of death’ and ‘year’ did. Confidence intervals of the final model and odds ratios indicated that the effect of DCC was most significant, with likelihood of having missing data increasing with an increasing decomposition status (95% CI for factor(DCC)2: 0.51-1.75 and for factor(DCC)3: 2.07-3.56) and with the odds of having missing data 3.03 times higher in DCC2 (95% CI: 1.67-5.77), and 16.3 times higher in DCC3 (95% CI: 7.91-35.12).

For the predictor variable ‘external defects’, there was no significant difference between the first two categories (‘mild’ and ‘moderate’), but the last category (‘severe’) was positively correlated with missing data (95% CI 0.28-3.33), with the odds of having missing data 5.35 times higher when external defects were severe (95% CI 1.32-28.06). Two cause-of-death categories were positively correlated with missing data: perinatal (95% CI: 0.57-2.92 and OR of 5.6 [95% CI 1.77-18.62]) and unknown (95% CI: 0.26-2.75 and OR of 4 .42 [95% CI 1.3-15.58]). Finally, the year 2009 was negatively correlated with missing data (95% CI: -2.71- -0.6 and OR of 0.2 [95% CI: 0.07-0.55]). We can conclude that ‘DCC’ is most influential on the likelihood of having missing data and followed by ‘external defects’ (category 3: severe external defects); both of which can be expected. In addition, we can conclude that porpoises dying perinatally, as well as animals with an unknown cause of death, cannot always be completely sampled. Finally, there was a minor year difference in the data, with more complete cases in 2009.

Correlation matrix using Cramer’s V

We assessed the correlation between all variables in Table 1 and calculated the Cramer’s V correlation coefficient (Figure 1, Supplementary Table 1). The variables ‘age class’, ‘pathology of the respiratory tract’ and ‘multiple organ inflammation’ were the most relevant and were associated with multiple other variables. Values with none or little relevance within the dataset appeared to be ‘ingestion of debris’, ‘other significant pathology (miscellaneous)’ and ‘ingestion of plants’. Figure 1 shows all associations between the variables, with the most relevant on the left and with the graph following the order of the first principal component. There were 46 associations with Cramer’s V > 0.25 (Supplementary Table 1), of which six indicative plots are provided (Figure 2A-E) to illustrate the directions of the associations.

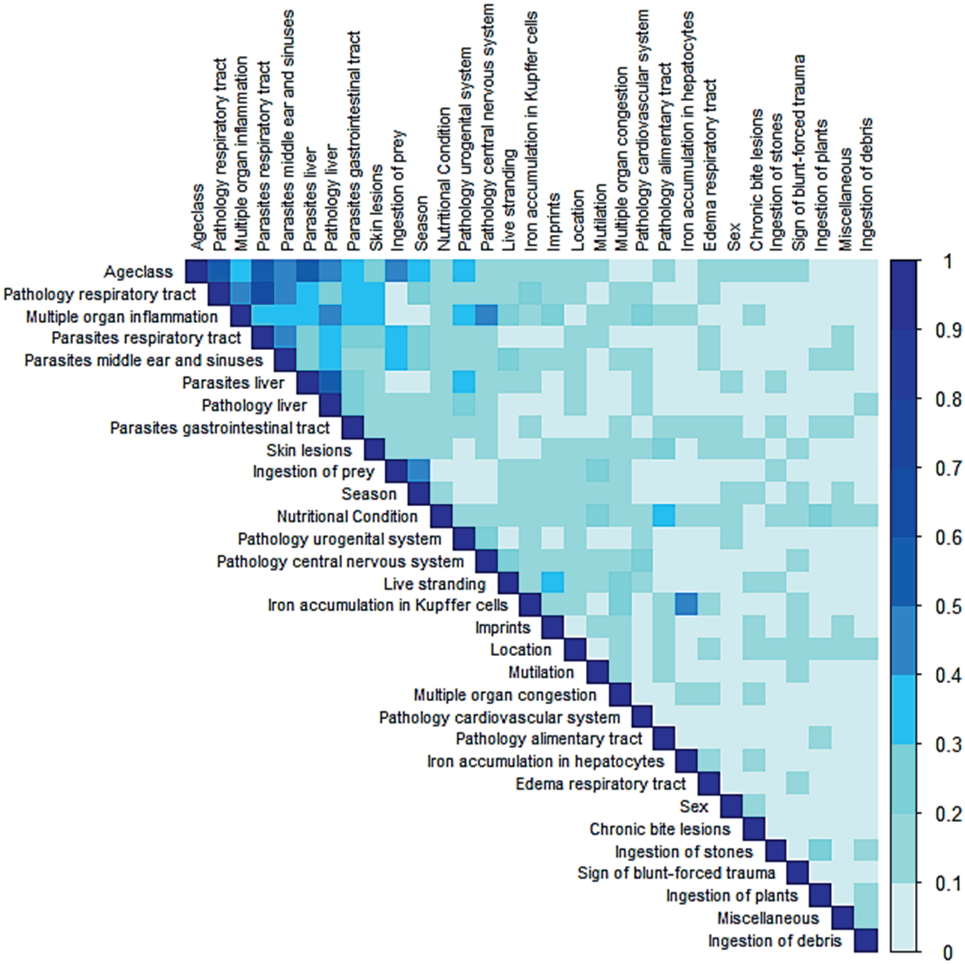

Figure 1. *Correlation matrix with Cramer's V correlation coefficient. Cramer's V close to 0 means no association, > 0.05 indicates a weak association, > 0.10 a moderate associated, > 0.15 a strong association and > 25 a very strong association (Akoglu, 2018).*

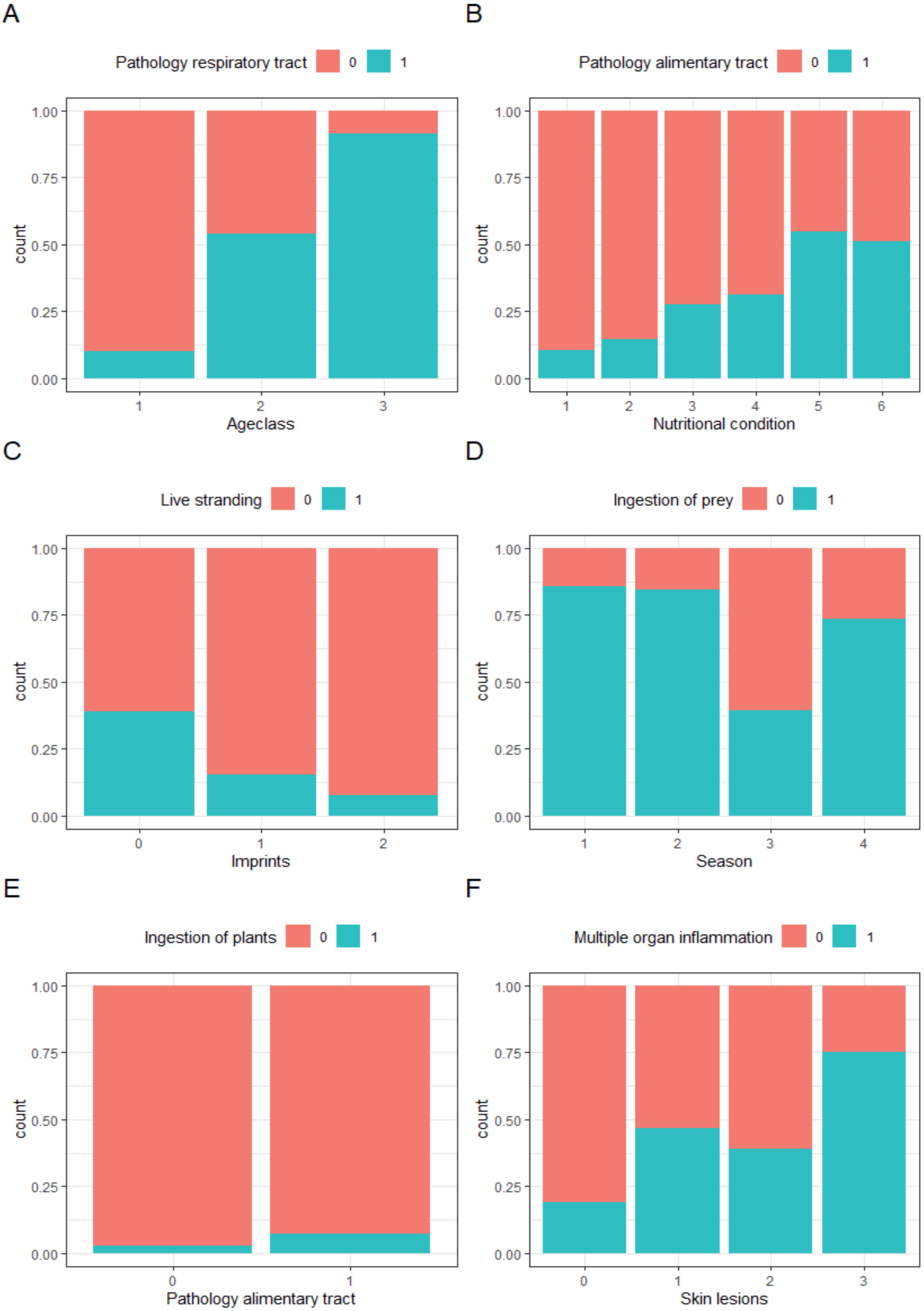

Figure 2. *Barplots of six associations with Cramer's V > 0.25 to explore direction. Where possible, the variable with most categories is plotted on the x-axis.*

Multiple Correspondence Analysis

The percentage of inertia (variance) explained by the first three dimensions was 42.5% (Figure 3) of the (in total) 56-dimensional space (eigenvalues, percentage of variance and cumulative variance are given in Supplementary Table 2 and the variable output of the MCA are given in Supplementary Tables 3-7).

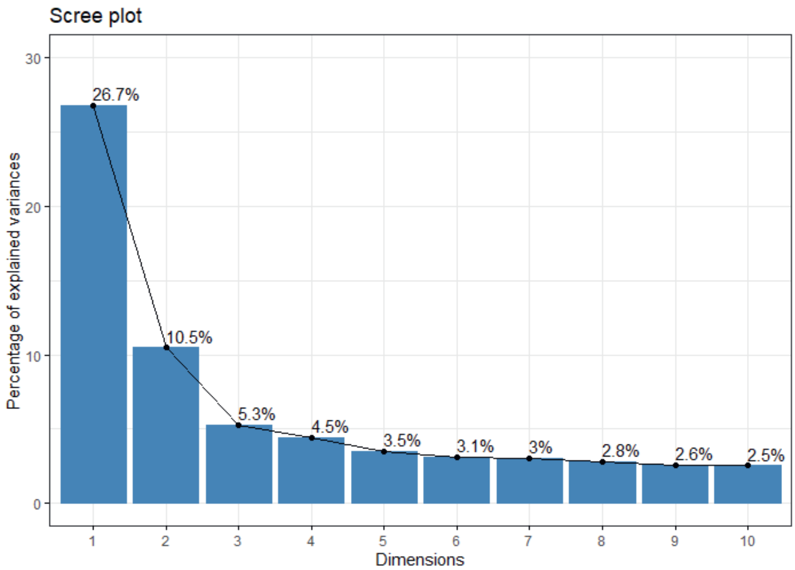

Figure 3. *Scree plot showing the percentage of explained variance in the first ten dimensions. When using the 'elbow rule', the most optimal number of dimensions is three.*

The first two dimensions are used in the plotting of the MCA-maps. The variables with the highest contribution to the first dimensions were 'age class' (R2 = 0.63), 'parasites of the respiratory tract' (R2 = 0.59), 'pathology of the respiratory tract' (R2 = 0.51) and 'parasites of the middle ear and sinuses' (R2 = 0.48). Top categories represented in this dimension were the absence of respiratory and ear parasites (scores 0), age class adult (score 3), absence of pathology in respiratory tract (score 0), and multiple organ inflammation (score 1). Variables with the highest contribution to the second dimension were 'season' (R2 = 0.43), 'age class' (R2 = 0.42) and 'ingestion of prey' (R2 = 0.42). Top categories represented in this dimension were the absence of prey ingested (score 0), season summer (score 3), age class neonate (score 1) and live stranding (score 1) (Figures 4-6, Supplementary Figures 1-7, Supplementary Table 8).

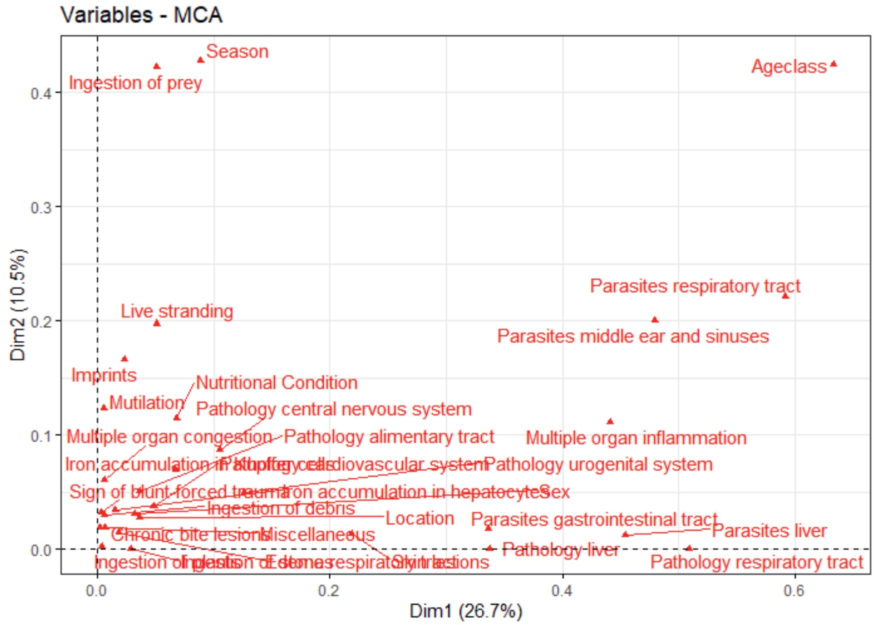

Figure 4. *Two-dimensional plot of the variables on the first two dimensions of the Multiple Correspondence Analysis. Variables close to 0 can be considered uninformative in explaining the dimensions.*

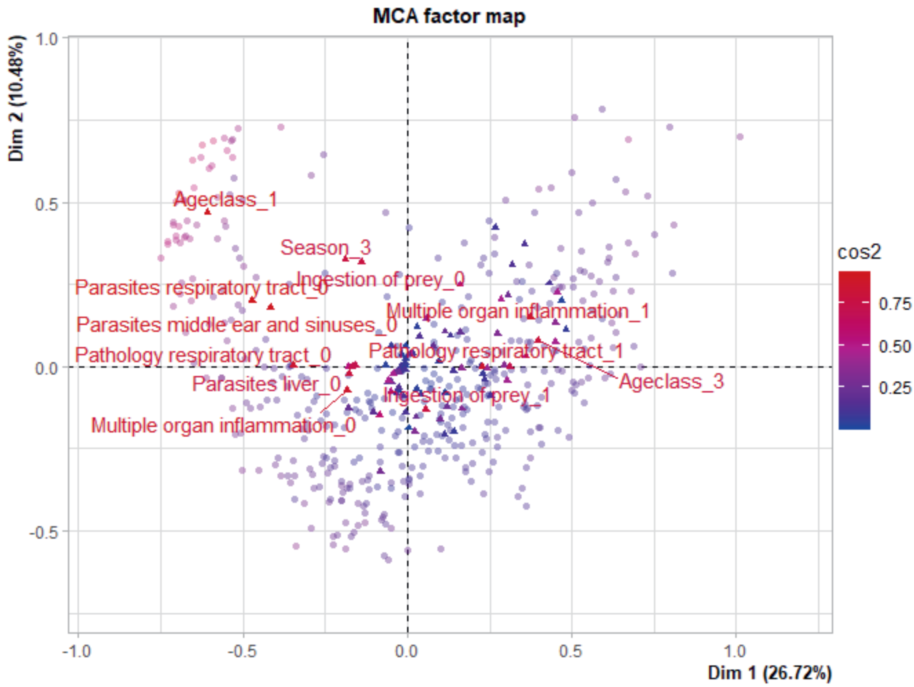

Figure 5. *Plotted variable categories (triangles) and individuals (circles) on the Multiple Correspondence Analysis factor map. Categories most influential (cos2 > 0.75) are indicated in red and the categories are given.*

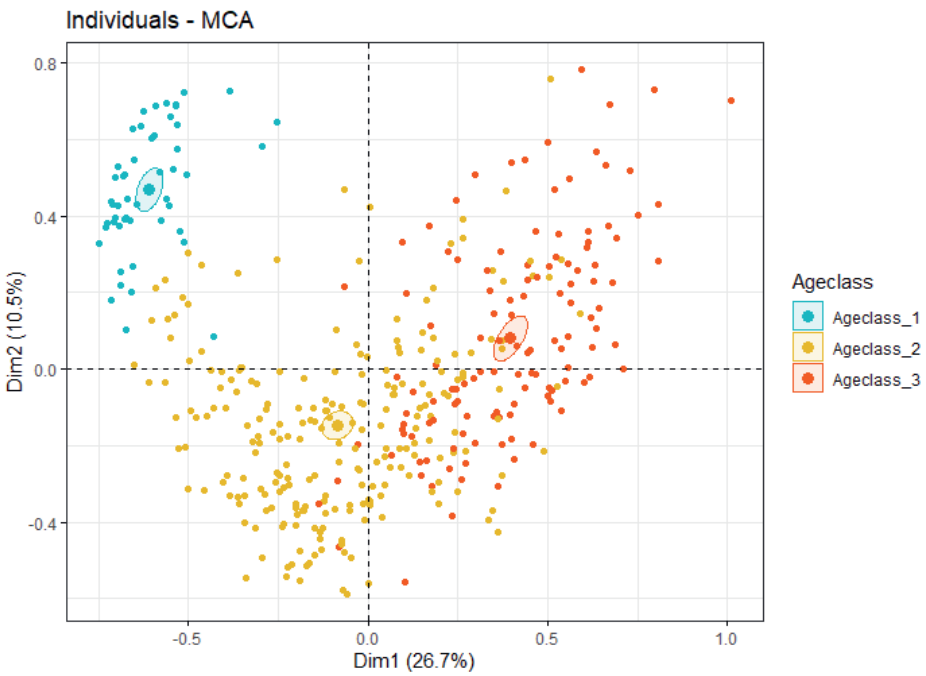

Figure 6. *Plotted individuals on the Multiple Correspondence Analysis factor map, colored by ageclass. Ageclass 1 are the neonates (blue), 2 the juveniles (yellow) and 3 the adults (orange). The ellipse of the different ageclasses do not overlap, suggesting that the three ageclass categories are part of distinct clusters.*

Hierarchical Classification

The coordinates of the individuals on the principal components of the MCA are used for hierarchical classification (Figures 7-8). When we assess which of the causes of death are most abundant in each of the three clusters, it becomes clear that cluster 1 predominantly consists of animals dying perinatally or due to starvation. This is the smallest cluster of the three. Cluster 2 contains mostly animals dying due to acute causes, including bycatch, predation and trauma, although animals with grey seal bite lesions as well as animals dying of emaciation with unknown cause are also represented. In the third cluster are the porpoises dying predominantly of infectious disease (Table 2).

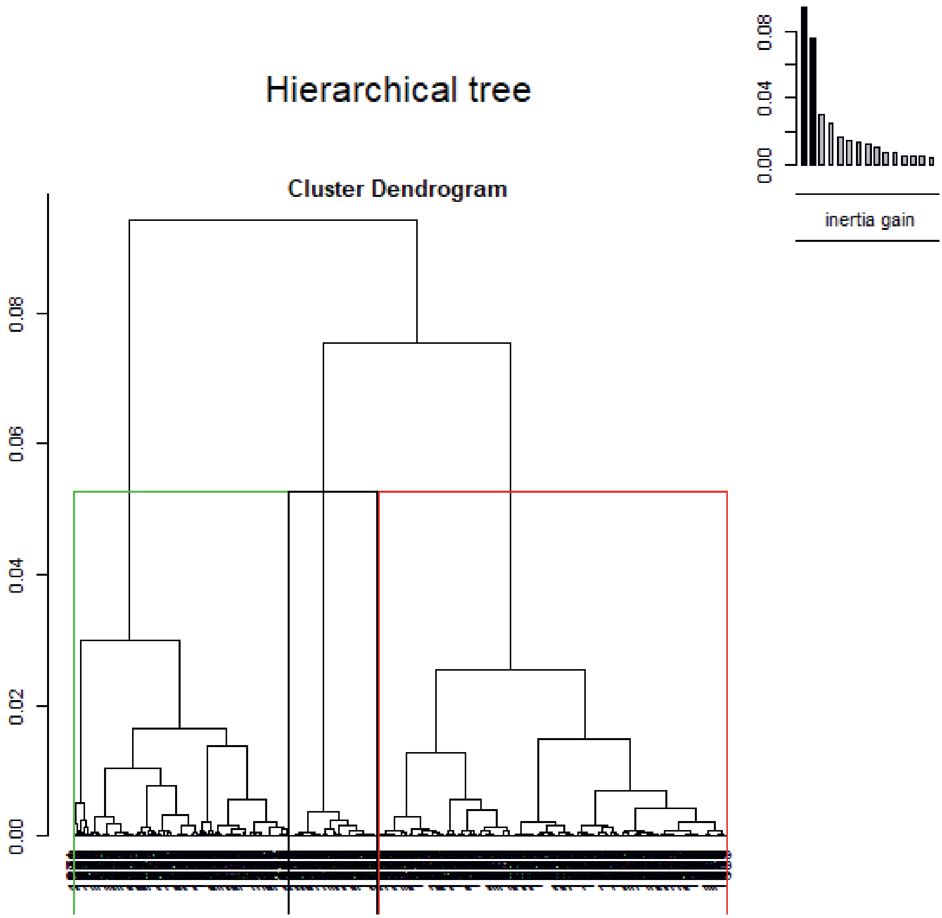

Figure 7. *Hierarchical tree or cluster dendrogram indicating three distinct clusters (see Figure 8)*

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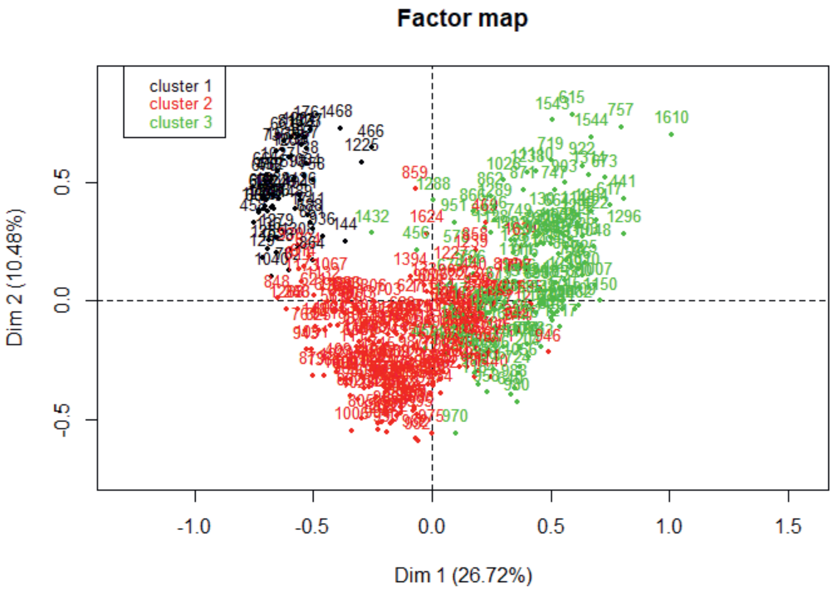

Figure 8. *Plotted individuals on the factor map, with the three identified clusters colored (See Figure 7).*

Table 2. *Representation of causes of death categories in the three clusters assigned through the multiple correspondence analysis.*

	Cluster 1	Cluster 2	Cluster 3
Bites	4	39	9
Bycatch	7	53	17
Emaciation	3	22	4
Infectious	5	51	84
Other	0	1	4
Perinatal	9	0	0
Predation	2	29	9
Starvation	23	4	1
Trauma	0	13	4
Unknown	2	4	2

Random Forest Classification

The model constructed with 700 trees and m=5 classified 74.82% of the causes of death categories correctly. Different runs with varying m (3-7) resulted in marginally different classification accuracies (varying from 73.72% at m=3 to 75.91% at m=7). A decrease in Ntrees to 100 (with m=5) also did not change the accuracy of the model with still 72.63% of the data accurately predicted.

The most important variables for accurately predicting cause-of-death categories (MeanDecreaseGini) were: ‘imprints’, ‘chronic bite lesions’, ‘mutilation’, ‘nutritional condition’ and ‘location’. Unimportant variables in the model were ‘iron accumulation in the hepatocytes’, ‘pathology of the cardiovascular system’, ‘ingestion of plants’, ‘ingestion of marine debris’ and ‘pathology of the urogenital system’ (Figure 9).

The most important variables in the classification (MeanDecreaseAccuracy) were slightly different: ‘mutilation’, ‘imprints’ and ‘chronic bite lesions’ followed by ‘multiple organ inflammation’ and ‘age class’. Unimportant variables were ‘ingestion of marine debris’, ‘sex’, ‘pathology of the cardiovascular system’, ‘iron accumulation in the hepatocytes’, ‘ingestion of stones’ and ‘multiple organ congestion’ (Figure 10).

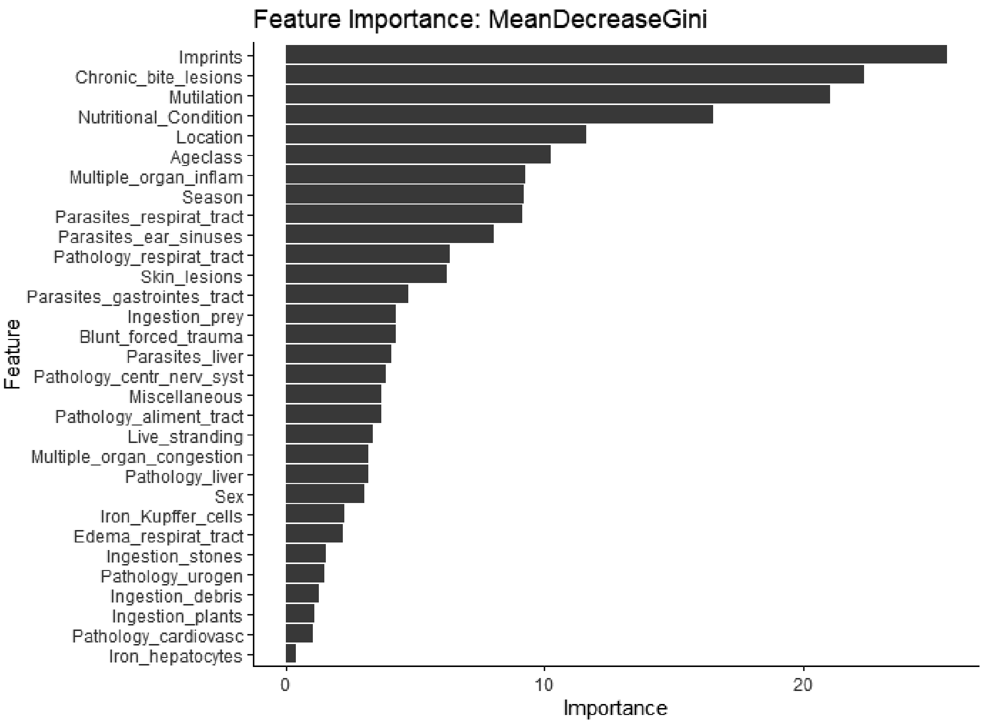

Figure 9. *The most important variables for accurately predicting cause of death categories (MeanDecreaseGini) in decreasing order.*

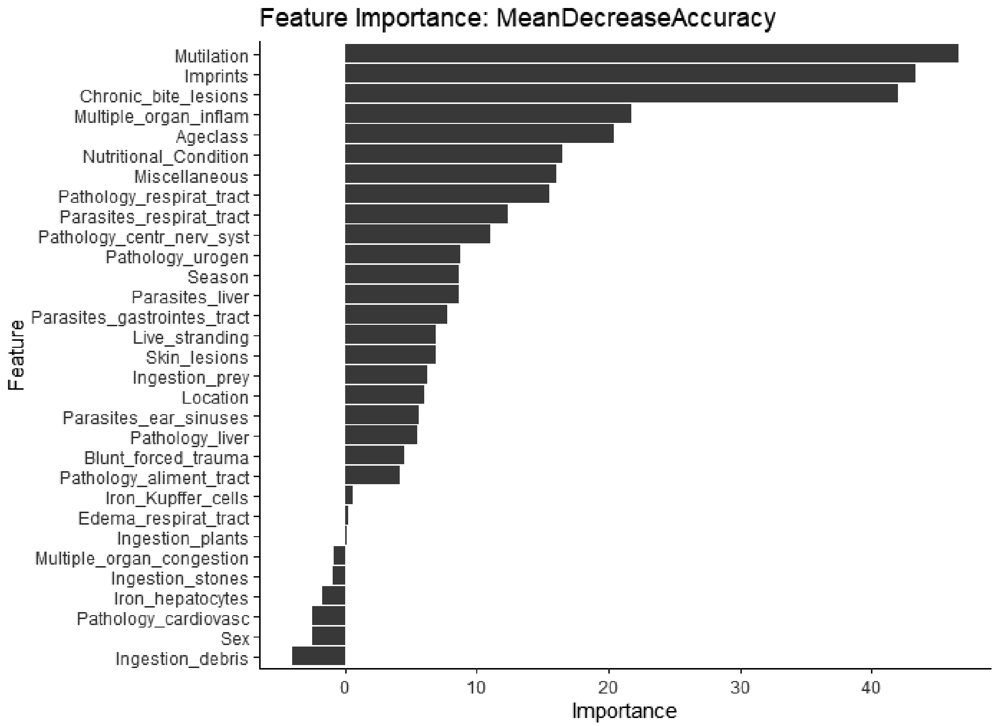

Figure 10. *The most importance variables in the classification (MeanDecreaseAccuracy) in decreasing order.*

Discussion

Here, we demonstrated the usefulness of supervised and unsupervised classification methods for the retrospective exploration and analysis of biological, ecological and pathological data of harbour porpoises found stranded in The Netherlands between 2008-2019. Through these analyses we determined the most relevant post-mortem findings in terms of causes of death, reflecting both anthropogenic and natural threats to cetacean populations. We additionally assessed which variables were deemed most informative in order to predict causes of death. The three clusters defined through the unsupervised classification methods were mainly a result of age class, pathology of the respiratory tract and multiple organ pathologies. Through the supervised classification methods, we defined imprints, grey seal bite lesions, mutilation and nutritional condition as the most important variables for predicting cause of death categories, with a model prediction accuracy of 74.82%. In both supervised and unsupervised classification methods, ingestion of plants and debris, iron accumulation and sex were deemed uninformative to explain or predict cause of death.

The results of the multiple correspondence analyses and subsequent hierarchical clustering identified three distinct clusters, collectively explaining 42.5% of the variance in the data. The first cluster existed predominantly of neonates. The individuals in this cluster were associated with the absence of disease, parasites and solid stomach content. The second cluster consisted of mainly of the juveniles. The variables indicative of acute causes of death were predominantly grouped here, revealing similarity in pathological profiles among porpoises dying of bycatch, grey seal predation and trauma. However, also the category emaciation was prominent in this cluster, whilst poor nutritional conditions suggest prolonged debilitation rather than point to underlying acute processes. These animals had no signs of significant disease or other abnormalities that were explanative for their poor nutritional status and pathological profiles of these porpoises therefore showed commonality with the acute and anthropogenic causes of death. This suggests that nutritional condition alone can be considered a non-explanative variable for subdividing causes of death. The third cluster consisted of mainly adults. This cluster was dominated by the infectious disease category, although numerous bycaught individuals were also part of cluster 3. This indicates similar profiles among bycaught porpoises and those dying of infectious causes. This could suggest overall reduced health of harbour porpoises inhabiting the southern North Sea, as previously described for certain bycaught harbour porpoises in Dutch waters (IJsseldijk et al. 2021b). Subsequently, diagnosing bycatch based on the absence of disease will therefore inherently result in an underestimation of bycatch numbers (IJsseldijk et al. 2021b; Siebert et al. 2020).

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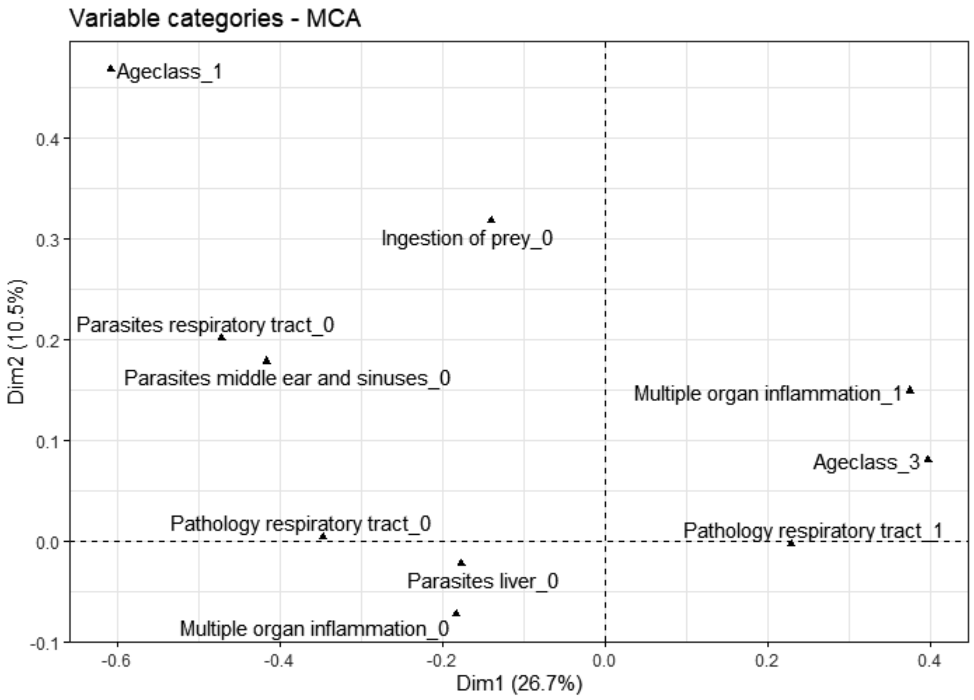
Through the unsupervised classification procedure, the ability to identify pathologically relevant characteristics and association between the variables within this large dataset was demonstrated. The analyses are based on a large number of individuals and hence allow the detection of robust trends not influenced by minor individual variance. They may therefore inform upon threats relevant to the population. However, although the pathological relevant characteristics are deemed indicative in the subdivision of the clusters, they do not address the full range of lesions found in an individual. Especially the cases with rare findings can be difficult to qualify (Warns-Petit et al. 2010). This explains why categories with many zeroes, such as 'ingestion of plants' or 'marine debris' but also the category 'miscellaneous pathology', and urogenital and cardiovascular tract lesions were deemed irrelevant in order to subdivide or predict causes of death. In addition, the grouping of cases into categories as either presence/absence or on Likert scale can be considered rather crudely. The significance of lesions for an individual and its vulnerability might differ between cases as a result of age, season, severity or undetermined mechanisms. The methods applied here are therefore not able to study pathogenesis, aetiological agents, or cause-effect relationships and should thus be considered complementary and not as an alternative to in depth or targeted biological, ecological and pathological investigations.

Through the supervised classification random forest method, we were able to accurately predict cause of death categories based on the scored variables. This allows us to identify common but rather unspecific lesions, such as congestion in multiple organs or pulmonary oedema, compared to findings which are directly informative upon causes of death, such as the presence of net-marks of mutilations. Logically, predictions are highly influenced by the variables included in the model, as well as their mutual relationship and variance. We were able to include large datasets containing post-mortem findings, with information on pathology, nutritional status, marine debris and stomach content, but information on a range of other factors reflecting potential threats for small cetaceans was not generally available. This includes the assessment of chemical pollution, a persistent threat to cetaceans which influences reproductive capability but could also disrupt immune- and endocrine functions (Jepson et al. 2005b, 2016; Murphy et al. 2015; van den Heuvel-Greve et al. 2021). In future, expanding the dataset with measured persistent organic pollutants, or other chemicals, in tissues of these individuals, as well as any other relevant biomarker would allow to investigate their relevance and potential effects in terms of health and causes of death. Additionally, we have shown that mainly decomposition and scavenging hampers data collection, as these two variables were deemed most relevant in explaining missing data. If in future missing data is generated through predictive analytical procedures, when interpretating the results it should be taken into consideration that the freshest cases are often porpoises which die on or very close to shore. This suggests that animals dying offshore, such as bycaught and subsequently discarded individuals, which take longer to make landfall, may therefore be underrepresented in the sample. Also, environmental factors such as tides, wind, and temperature affect carcass freshness (Moore et al. 2020), and thus limit the sample size to very fresh-moderately fresh carcasses, which may result in unknown or confounding effects on conclusions.

Using multiple correspondence analysis for disease monitoring, or to determine risk factors associated with disease outbreaks and mortality events, is a more frequently used approach in terrestrial wildlife health studies (Orozco et al. 2020; Sichewo et al. 2019, 2020; Warns-Petit et al. 2010). These studies aim to define distinct pathological entities which can be used in early outbreak detection or to determine most frequent disease and their distinctive characteristics (Warns-Petit et al. 2010). We show the importance of incorporating data on biological and ecological factors as well, such as interspecific or predator interaction, age and diet, and anthropogenic threats like bycatch and marine debris ingestion, to generate a complete picture of the health status of individuals and their association to causes of mortality. The classification of cases based on their pathological profile has practical value, because when precise diagnosis is not needed, this is a rapid, reliable and rather inexpensive means of analysing wildlife health data (Warns-Petit et al. 2010). Infectious disease outbreaks in marine mammals have been on the rise, however, with factors causing these events being mostly unclear (Gulland & Hall 2007; Sanderson & Alexander 2020). Extrinsic ecological factors may play a critical and increasing role under the current climate change predictions (Harvell et al. 2002; Sanderson & Alexander 2020). Increased and improved marine mammal and infectious disease surveillance programmes are therefore vital for long-term population health monitoring and to provide essential data for developing early warning frameworks, forecasting models, and species- and location-specific adaptive management plans (Burek et al. 2008; Burge et al. 2008; Sanderson & Alexander 2020). Our study serves as a detailed example demonstrating the usefulness of different analytical approaches to pathology and health data of a long-studied small cetacean species, translatable to different taxa, disciplines and geographical areas.

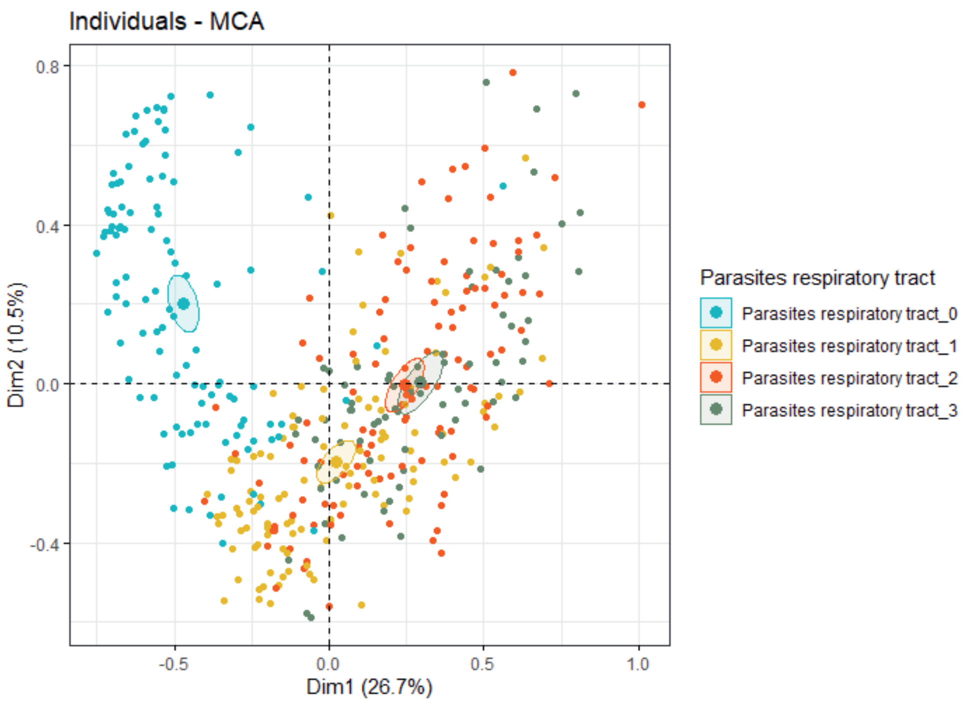
Supporting information

Supplementary data associated with this chapter will eventually be part of the final publication and in the meantime can be requested by e-mail.

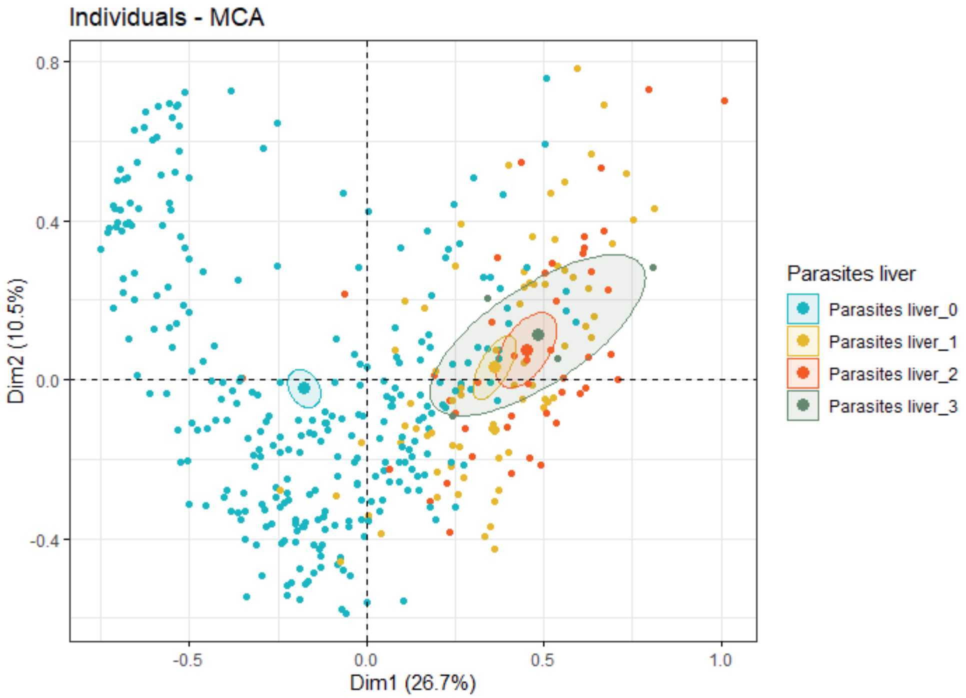


Supplementary Figure 1. *Top ten variable categories of the Multiple Correspondence Analysis which contribute mostly to the first two dimensions.*

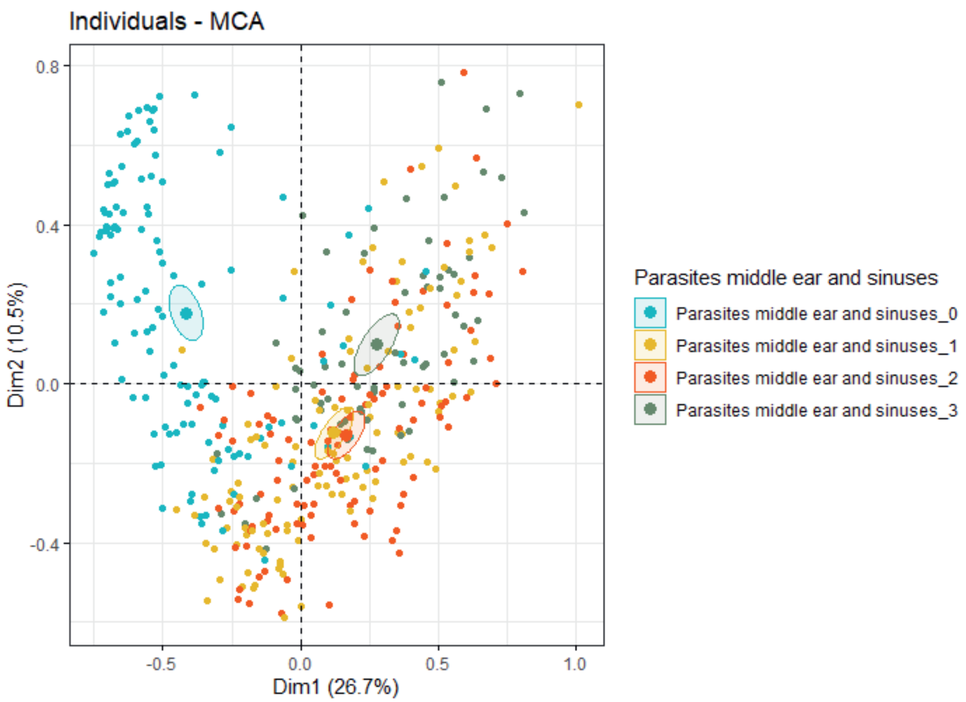
Supplementary Figure 2*.* *Plotted individuals on the Multiple Correspondence Analysis factor map, colored by pathology of the respiratory tract. Category 0 are animals with no or mild pathological changes (blue) and category 1 are animals with moderate or severe pathological changes (yellow). The ellipses of both categories do not overlap, suggesting separate clusters.*



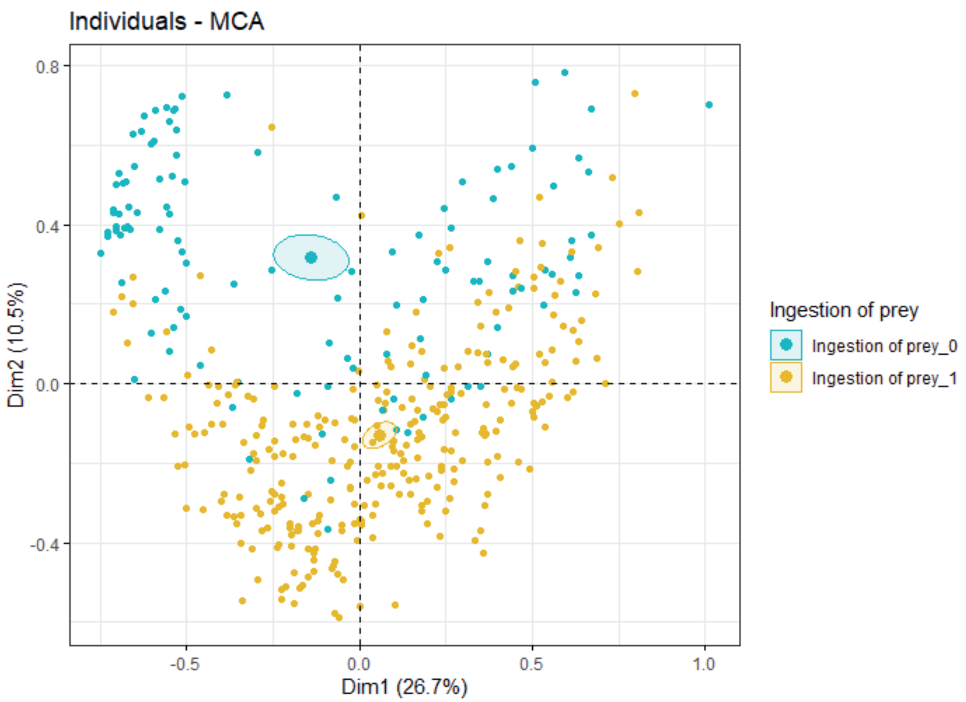
Supplementary Figure 3. *Plotted individuals on the Multiple Correspondence Analysis factor map, colored by parasite presence in the respiratory tract. Category 0 are animals with no parasites detected (blue), category 1 are animals with mild parasitic infestation (blue), 2 moderate parasitic infestation (yellow) and 3 severe parasitic infestation (darkgreen). The ellipses of categories 0 and 1 do not overlap suggesting separate clusters but 2 and 3 cluster together.*



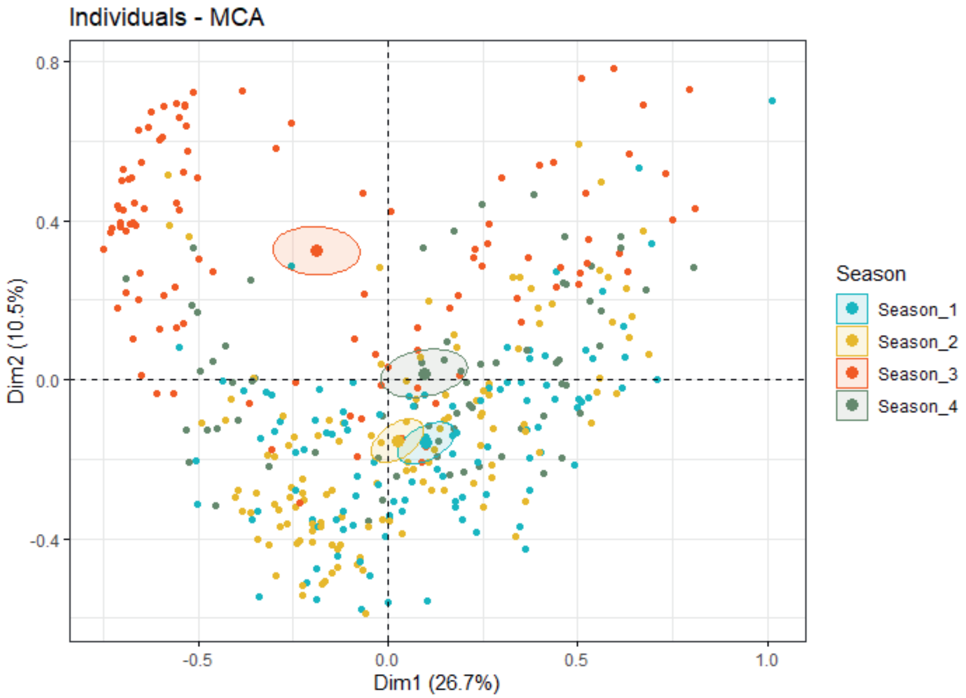
Supplementary Figure 4. *Plotted individuals on the Multiple Correspondence Analysis factor map, colored by parasite presence in the liver. Category 0 are animals with no parasites detected (blue), category 1 are animals with mild parasitic infestation (yellow), 2 moderate parasitic infestation (orange) and 3 severe parasitic infestation (darkgreen). The ellipses of category 0 does not overlap with the other three clustered categories.*



Supplementary Figure 5. *Plotted individuals on the Multiple Correspondence Analysis factor map, colored by parasite presence in the middle ear and sinuses. Category 0 are animals with no parasites detected (blue), category 1 are animals with mild parasitic infestation (yellow), 2 moderate parasitic infestation (orange) and 3 severe parasitic infestation (darkgreen). The ellipses of categories 0 and 3 do not overlap suggesting separate clusters but 1 and 2 cluster together.*



Supplementary Figure 6. *Plotted individuals on the Multiple Correspondence Analysis factor map, colored by ingestion of prey. Category 0 are animals with no prey in the stomach (blue) and category 1 are animals with prey in the stomach (yellow). The ellipses of both categories do not overlap, suggesting separate clusters.*



Supplementary Figure 7. *Plotted individuals on the Multiple Correspondence Analysis factor map, colored by season. Category 1 in winter (blue), category 1 is spring (yellow), 2 is summer (orange) and 3 is autumn (darkgreen). The ellipse of categories 3 and 4 do not overlap with the others and therefore likely represent separate clusters, while 1 and 2 cluster together.*

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Synthesis


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The primary **research aims** of this thesis were to establish methods and metrics of health which aid in the assessment of the most relevant natural and anthropogenic threats to harbour porpoises in the North Sea overtime. Throughout North-western Europe, studies on harbour porpoises have been ongoing for decades, because this species is listed under several national, regional and international agreements and directives (ASCOBANS 1992; Camphuysen & Siemensma 2011; Dolman et al. 2021; Evans 2019). These studies aim to achieve and maintain a favourable conservation status and to protect marine mammal species in European waters. In The Netherlands, a range of investigations have been initiated, under the auspices of the Dutch government, on abundance and distribution, strandings and pathology, and contaminants and diet (Camphuysen & Siemensma 2011; Jansen 2013; Leopold 2015; LNV 2020). These monitoring projects were the imperative drivers for, and are the foundation of, this thesis, with each chapter representing in-depth investigation into several topics or issues related to porpoise health. During the time I have been working on this thesis, I have often wondered what "a favourable conservation status" actually means for a harbour porpoise, or any small cetacean. How can we measure "good environmental conditions" or "healthy populations" for a species that lives largely out of human sight and is by nature elusive, shy and small? Stranded and bycaught harbour porpoises were the primary source from which information and samples were collected, and subsequently utilised, in the majority of the chapters of this thesis. Sampling the living population is near impossible and considered unethical in many countries including The Netherlands, thus stranded and bycaught animals provide a unique sample of the living population and offer a relatively low cost method of surveillance (ten Doeschate et al. 2018). Data and tissue archives from dead animals contain valuable time-series of information and ecological insights unobtainable through other means of surveillance. But when handling so many deceased porpoises, I have also often asked myself "are there healthy porpoises"? And subsequently, how successful will we really be at "conserving a species" in our oceans under the present, rapid and increasing, direct and indirect human use and influence?

Harbour porpoise health

The perception of wildlife health has gained significant interest, since both humans and animals are confronted with increasingly complex issues of global change and the subsequent rising concerns for their health (Gibbs 2014; Zinsstag et al. 2011). Health has been defined by the World Health Organization (WHO) in 1948 as a state of complete physical, mental and social wellbeing and not merely the absence of disease (Gibbs 2014). For wildlife however, health is a difficult concept to define and assess, because the term is not static and can be framed in different ways depending on the discipline in which it is used (Hanisch et al. 2012; Patyk et al. 2015). The definition of harbour porpoise health as posed in this thesis (**chapter 2**) was based on an established definition for the polar bear (*Ursus maritimus*) (Patyk et al. 2015). The multidisciplinary concept and multiple factors involved were judged as important by a selected panel of experts, but resilience to factors in their environment and long-term sustainability was deemed vital in characterising healthy individuals and (sub)populations. As such, there are several ways to assess multiple components reflecting cetacean health. Studying free-ranging cetaceans at sea, the currently used metrics are body condition and growth, external lesions or markings, and hormones or microbiomes gained from biopsies, faeces, or blow samples (Apprill et al. 2017; Burgess et al. 2018; Christiansen et al. 2020; Hart et al. 2012; Rolland et al. 2012; Stewart et al. 2013; Van Bressem et al. 2009). Additionally, photo-identification or satellite tagging aids in studying changes in health over time, and can be conducted for a range of whale species (Balmer et al. 2014; Cates et al. 2019; Mizroch et al. 2011; Schwacke et al. 2014). Harbour porpoises, however, are one of the more difficult species to study at sea. Longitudinal sampling is almost impossible due to the inability to approach these animals and the scarcity of distinct natural or inflicted markings to recognise and follow individuals. Instead, causes of death, presence of disease, and nutritional status gained from stranded or bycaught animals are (paradoxically) used as indirect indicators of health (Bennett et al. 2001; Jepson et al. 1999; Murphy et al. 2015). Often, specimens dying from bycatch are seen as a healthy representative of the population, hence used as a control group that can be compared to porpoises dying of e.g., infectious disease. I have, however, shown in this thesis that 'being healthy' is not the same as 'dying acutely'. This became most apparent in the study on porpoises retrieved directly from fisheries as bycatch (**chapter 4**). Two thirds of these animals were found to have lesions, and half of them presented severe or extensive morphological changes that likely compromised the health of these animals. Lesions were also seen in 77% of the stranded porpoises that most likely had died from bycatch, indicative of a compromised health status prior to the entanglement (**chapter 8**). The reliability of the frequently used 'nutritional status', or more specifically blubber thickness, as a proxy of nutritional condition in the context of assessing individual health, also caused debate. As blubber thickness is naturally influenced by age, sex and season (**chapter 6**), and did not correlate well with cause of death at large (**chapter 8 & 9**), it is not a recommended metric to use without further consideration for other factors involved.

Living on a knife-edge

Harbour porpoises are amongst the smallest of cetacean species, with limited capacity to store energy (Bjørge & Tolley 2018). The cold environment in which they live, in combination with their large body surface to body volume ratio results in a high metabolic demand (Kastelein et al. 1997, 2019; Lockyer 2007). Harbour porpoises must eat continuously and need large quantities of high-energy prey to sustain themselves (Spitz et al. 2012). They feed on a variety of prey species with diets differing per life stage (Leopold 2015). In the chapter on reproductive success, it was shown that when mature female porpoises feed on high-calorie prey, their chances of pregnancy and staying pregnant throughout gestation were highest (**chapter 6**). This suggests a vulnerability to shifts in prey quality during the breeding season and throughout gestation. After a successful pregnancy, mature females face the challenge of ensuring sufficient intake of prey while losing energy through lactation. Although porpoises are capable of conceiving again right after birth, and can thus experience concurrent pregnancy and lactation (Lockyer 2003), it comes as no surprise that this was not commonly found (**chapter 6**). The high rate of loss of fat and thus energy through lactation, however, does not only come at a cost for the adult. Lactation was shown to be the most effective way of releasing lipophilic chemical pollutants, with adult females offloading their pollutants to their offspring (**chapter 5**). This means that calves, especially those first-born can receive excessively high concentrations of detrimental chemicals through suckling (**chapter 5**). This was particularly true for calves of nutritionally stressed females, since nutritional stress caused mobilisation of the endogenous lipid stores leading to higher offloading through milk and subsequently to a greater potential for toxicity (**chapter 5**). With polychlorinated biphenyls (PCBs) not seemingly inducing reproduction failure in the form of infertility (e.g., failure of ovulation, conception and implantation) (**chapter 5 & 6)**, pollutants are more likely to impact new-born survival rates (Murphy et al. 2009, 2015).

Data from dead porpoises represents only a snapshot of their lifetime, thus metrics like pregnancy rates should be interpreted with caution and might not be directly translatable to reproductive success of the population. For the southern North Sea, a calculation using basic life history parameters from **chapter 6**, however, gives a rather worrying prospect. Given that the majority of mature females do not reach an age above 8.5 years and considering an age at sexual maturity of 4 years and pregnancy rate of 0.58 at maximum (this was the pregnancy rate established for mature females dying as a direct result of anthropogenic trauma or predation), an average adult female porpoise would produce 2-3 calves in her lifetime. The chance that all offspring survive to reproductive age is very slim considering the many natural and anthropogenic stressors they come across, already early in life (**chapter 5 & 8**). If population numbers are really stable, as has been suggested based on aerial surveys

(Geelhoed & Scheidat 2018; Hammond et al. 2002, 2013, 2017), the population in the southern North Sea should be fuelled with (im)migrants from more flourishing areas. These might not be healthy (im)migrants, as suggested in **chapter 3**: the southern North Sea may act as a population sink, or paradoxically reflect a more supportive environment for weak animals to survive longer in poorer body condition. Further evidence for a difference in porpoise distribution between the southern and other North Sea areas can be found in a study on 71 free-ranging porpoises tagged in the Danish part of the North Sea (Nielsen et al. 2018). These were all wild-captured animals and may therefore represent a subset of the porpoise population in good condition. None of the porpoises tagged in Danish North Sea waters entered the southern North Sea, instead they used western and northern parts (Nielsen et al. 2018). It is recommended to conduct assessment of genetic variation of harbour porpoises from across the North Sea to further investigate the sink hypothesis or the potential presence of subpopulations, as well as isotopic analysis to inform on (changes in) long-term feeding habits (Fontaine et al. 2010; Jansen 2013).

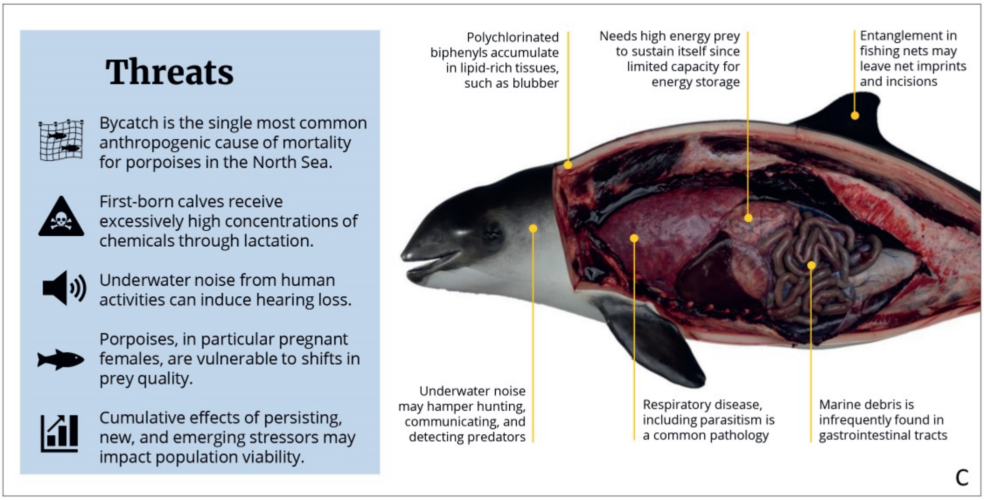
The costs of living dictate what a porpoise eats (Spitz et al. 2012), but porpoises face additional nutritional challenges due to the presence of predators. Predator presence could result in hunger-dependent risk-taking: a phenomenon of weight loss in organisms at risk of predation, which has been reported to occur in many species, also of other taxa (Houston et al. 1993; Lima 1986, 1998). It has been suggested that porpoises coexisting with bottlenose dolphins (*Tursiops truncatus*) adapt by losing weight to allow efficient manoeuvrability in response to violent dolphin behaviour (MacLeod et al. 2007). Hunger-dependent risk-taking makes harbour porpoises more prone to emaciation (Leopold et al. 2015a). Growing numbers of grey seals and harbour porpoises in the southern North Sea have undoubtedly led to increasing interactions between both species (**chapter 8**) (Leopold et al. 2015b; Podt & IJsseldijk 2018). Survival after a grey seal attack does occur, although how often this happens is currently unknown. Some of the porpoises that do survive the initial attack succumb later as a result of the bite injuries, which can act as entry point for bacterial infections, such as with the zoonotic pathogen *Neisseria animaloris* (**chapter 7**). Other porpoises may survive the attack, which allows them to learn and adapt to avoid their predator in the future, for example through inducing changes in behaviour, abundance, habitat use, fitness, and hunger-dependent risk associated with energy intake (Lima 1998a,b). The southern North Sea and especially coastal areas with high seal abundance, such as the Wadden Sea and Dutch Delta area, may therefore have become 'landscapes of fear' for harbour porpoises. This concept describes the spatial variation in predation risk perceived by prey and the subsequent behavioural and physiological outcomes (Gaynor et al. 2019). Becoming leaner in response to potential predation risk makes porpoises more prone to emaciation, infectious disease or reproductive failure and an overall increase in stress levels. Harbour porpoises clearly live on a knife-edge, with a constant need to balance themselves between individual health and reproductive success, while responding to environmental pressures, all with the aim of increasing the chances of individual and ultimately population survival.

Anthropogenic and natural threats

In order to assess individual and population health, knowledge on the full range of threats and pressures affecting harbour porpoises is important, as well as understanding how porpoises respond to intrinsic and extrinsic factors. Through the expert elicitation study (**chapter 2**), bycatch was judged as the main concern for porpoises over the next twenty years, followed by chemical and noise pollution. Cetacean bycatch has been a major conservation and welfare concern for decades, but management acts have, to date, failed to assess, mitigate and reduce bycatch numbers (Dolman et al. 2016, 2021; Rogan et al. 2021). Without a comprehensive plan to conserve cetaceans in European waters (Rogan et al. 2021), it is not surprising that bycatch will persistently be the dominant concern for harbour porpoises and other cetaceans.

The exposure to chemical contaminants was additionally identified as a major concern for the health of harbour porpoises (**chapter 5**). The production of PCBs has been banned since the 1980s, leading to a slow but consistent reduction of concentrations measured in blubber from porpoises in the North Sea (Jepson et al. 2016; Law et al. 2012, 2014; Williams et al. 2020). Yet, PCB levels measured in the environment are still high today, and there are still new sources released into the sea, so further mitigation is advised (Stuart-Smith & Jepson 2017). Besides this, toxicity of organic pollutants differs between age classes (Williams et al. 2020), as suggested in **chapter 5**, and re-evaluation of previously reported threshold levels in the light of age and development stages seems necessary. Other pollutants which may severely affect marine wildlife, such as Perfluoroalkyl substances, have not yet been determined in porpoises at large from the North Sea (Galatius et al. 2013; Gebbink et al. 2016; Spaan et al. 2020; Tian et al. 2020) and studies on the effects of these other pollutants are subsequently lacking.

It is obvious that the threats perceived as most important for harbour porpoises in the next twenty years in **chapter 2** (bycatch, chemical and noise pollution, see also infographic C) reflect the current dominant research focus. When it comes to studying deceased animals and determining causes of death, diagnosis and the interpretations of lesions are limited by the knowledge and experiences of those conducting these investigations. One key example of this is the identification of the grey seal as a major predator of harbour porpoises (Leopold et al. 2015a). Mutilated porpoises were initially thought to have died from bycatch, as the large mutilations were judged to be ‘man-made cuts’ and the bite lesions as ‘rope-marks’ (Camphuysen & Oosterbaan 2009). Once the grey seal was suggested as a predator of porpoises (Haelters et al. 2012), laboratory DNA investigations were conducted (Jauniaux et al. 2014; van Bleijswijk et al. 2014), as well as field studies on grey seal and porpoise interactions (Bouveroux et al. 2014). A retrospective analysis of the post-mortem database in The Netherlands eventually revealed that the deaths of numerous porpoises firstly believed to be a result of bycatch, should actually be attributed to grey seal attacks (Leopold et al. 2015a). The extensive photo- and tissue archives allowed retrospective adjustment of research findings due to the renewed insights in characterisation of lesions. Inaccurate conclusions may also occur for other causes of mortality, simply because of a current lack of knowledge, experience, data, or methods to properly assess and diagnose these. This may be particularly true for the more cryptic or complex threats, such as climate change, which are not associated with distinct markings or direct observations. This makes these more nebulous threats particularly difficult to quantify at current time. This emphasises the importance of systematic and long-term collection of data and samples to allow retrospective investigation when our knowledge evolves.



Multidisciplinary and cross-border approaches

The “One health” initiative advocates the combination of human- and veterinary medicine in response to zoonoses, and recognises the connection between the health of people, animals and the environment. It constitutes a global strategy for holistic, transdisciplinary and multisector approach (Destoumieux-Garzón et al. 2018). Despite this need for multidisciplinary and cross-border approaches when studying wildlife, many investigations on harbour porpoises and other cetaceans have been conducted in a relatively monodisciplinary way and on a small scale that mainly follows the boundaries of national borders and management units. For free-ranging porpoises, who do not comply to national borders and can travel long distances (Nielsen et al. 2018), this approach makes little ecological sense, in particular when one is aiming to detect trends relevant at the population level.

In the North Sea, two international studies on harbour porpoises have been conducted: the large scale multinational survey series of cetaceans in European Atlantic waters, SCANS (three surveys to date (Hammond 2002, 2013, 2017)) aimed at determining trends in distribution and abundance, and the study presented in **chapter 3** of this thesis, based on strandings. Both initiatives have markedly different designs and associated biases and challenges. The most important difference is probably the temporal scale at which these surveys are conducted: the three dedicated SCANS surveys were run during summer months at decadal intervals, while strandings data were opportunistically but continuously recorded and hence offered a higher temporal resolution. Directly comparing or combining the survey results is therefore challenging. There are, however, several smaller-scale initiatives that report combined sighting and stranding data in order to detect fine-scale trends in abundance, distribution and mortality (Jung et al. 2009; MacLeod et al. 2005; Siebert et al. 2006a). In The Netherlands, long-term datasets on nearshore sightings and strandings were recently combined and analysed to assess if stranding frequency could be explained by local population density (IJsseldijk et al. 2021a). Harbour porpoise stranding numbers were corrected for local abundance based on sightings to flag higher or lower stranding rates than expected, and to determine unusual mortality events (IJsseldijk et al. 2021a). Expanding these analyses across the border, and with information on diet, life history, contaminant and mortality data, mostly presented in this thesis (**chapters 5, 6, 8**), and combining this with time-area information of anthropogenic activities would be a recommended follow-up in order to investigate the impacts of human activities on porpoise populations.

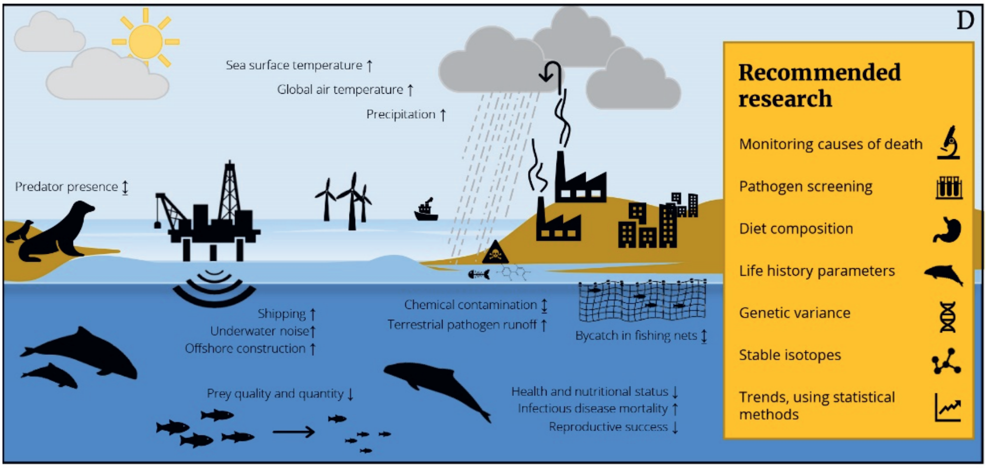
The detection of the effects of large-scale environmental changes on cetaceans also requires multidisciplinary and transboundary approaches. The impact of climate change on Arctic species is already evident (Moore & Huntington 2008), but there are also indications for range shifts of delphinids with temperate distributions, including the North Sea, likely in direct or indirect response to a changing climate (MacLeod et al. 2005; IJsseldijk et al. 2018b; Williamson et al. 2021). Studying the consequences of large-scale environmental change on cetaceans will likely be most evident through indirect health effects, like those related to habitat degradation, changes in prey quality and quantity, or decreased immune function (Bossart 2011; Learmonth et al. 2006; Sanderson & Alexander 2020; Simmonds & Isaac 2007). Infectious disease outbreaks causing mass mortalities in marine mammals are likely to intensify due to increases in sea surface temperatures, with increasing precipitation resulting in higher terrestrial pathogen pollution and runoff (Sanderson & Alexander 2020). Increased and improved infectious disease surveillance, including pathogen screening as part of cetacean monitoring programmes, will thus become critical for long-term population health monitoring, and comprehensive investigations incorporating all life science disciplines are required (see infographic D).

Implications for conservation management

The number of threats affecting the harbour porpoise is only rising, and measures to assess the cumulative effects of the range of stressors are highly needed. Where small changes to the environment could under optimal circumstances have little effect on individuals, this might not be true when conditions have worsened for a longer period of time. What could be a small or short-term disturbance or injury on an otherwise healthy animal, can have devastating effects on debilitated individuals with subsequent negative outcomes for their fitness and survival. Tipping points, marked by abrupt shifts in conditions, could eventually result in population collapse (Dakos et al. 2019). The identification of metrics that can be observed and act as early warning signs for population changes are therefore needed (NAS 2017). Long-term data and archived samples, with well documented conditional variables gained from pathological analyses, provide unique opportunities to detect such changes. This allows the detection of suitable life-history, or fitness-related, traits to be used as indicators of imminent biological transitions.

Endangered species are often the focus of conservation management plans. Harbour porpoises are currently stated to be of 'least concern' by the International Union for Conservation of Nature, except for the Baltic Sea and Black Sea subpopulations, despite the fact that their global population trend is currently unknown (Braulik et al. 2020). In The Netherlands, the conservation status of harbour porpoises has recently been upgraded to 'favourable', based on the assessment of range, population and habitat, while their future prospect was assessed as 'unknown' (LNV 2021). However, being abundant is not synonymous with being healthy, nor does it automatically reflect a favourable state. This may particularly be true for porpoises inhabiting the southern North Sea. In this area, as demonstrated in this thesis, mostly juveniles and animals in poor health conditions are detected. Healthy harbour porpoises did not represent a large proportion of the sample but were occasionally found among the stranded individuals and those retrieved as bycatch (**chapter 4 & 8**). The most healthy porpoises were found among the acutely killed grey seal victims (**chapter 8** and Leopold et al. (2015a)). In order to establish control samples for future studies, it is advised to use a subset of porpoises who died acutely due to natural or anthropogenic causes, regardless of their source of mortality. These animals need to be completely screened for lesions and pathogens in order to rule out any potential contributing factors or underlying health problems, before they can act as a reliable control.

The biggest advantage of working with abundant species is that this allows investigations on large numbers of individuals, to detect trends or findings resilient to small or short-term variations, or simply chance. In The Netherlands, data and samples of just over a decade have now been collected and stored, reflecting at least one generation of porpoises. It is vital to include abundant and wide-spread species in conservation and management plans, besides only focussing on endangered and thus rarer species, in order to be able to make population inferences, so mitigation measure can be taken in a timely manner. For harbour porpoises in the North Sea, there are several fundamental population aspects yet to be investigated, such as age-, sex-, seasonal- and health condition-specific distributional patterns, including the potential subpopulations that may occur in the region. The ability and potential of analysing large datasets containing biological, ecological and pathological information was demonstrated in **chapter 9**. This dataset can be expanded with data from following years and from other geographical areas. Additionally, metrics that could not be included because they were not routinely analysed for all the necropsied porpoises, such as contaminant concentrations or the presence of micro-organisms, should be added in the future. The current data can be seen as a baseline or used in comparison to future cases sampled under changing environments or health conditions. Despite the challenging task, a more comprehensive investigation of post-mortem investigated harbour porpoises, in the past and in the future, can significantly improve our understanding of harbour porpoise biology, demographics and ecology. Furthermore, this would allow the detection and quantification of new, emerging and cumulative stressors affecting individuals, populations and ecosystem health, aiming to promote the long-term successful conservation of harbour porpoises in our waters.



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12

Addenda

Summary / samenvatting

English summary

Harbour porpoises are charismatic and protected animals. This has resulted in a wide social, scientific and political interest into their health and well-being. Porpoises are an integral part of the marine food web, and along with their general distribution, they are the focus species in many environmental monitoring and research programs. These programs generally focus on increasing knowledge about human-related marine activities that may affect harbour porpoise populations, and serve the ultimate goal of protecting and preserving harbour porpoises and their habitat.

The North Sea is one of the busiest marine areas in the world, where many human activities, often simultaneously, take place. Porpoises are the most common whale species in the North Sea, with an estimated number of about 350,000 individuals. A high number of porpoises combined with a lot of human activities means that many animals are exposed to various threats. These include fishing activities, chemical pollution and underwater noise from different sources, such as shipping, seismic surveys and unexploded ordnance detonations. In addition, there is an increase in the number of offshore activities for the construction of wind farms in recent years. Also, the large-scale effects of climate change, such as changes in prey quantity and quality, are becoming more and more apparent. Individual and cumulative stressors threaten the immediate survival of individual animals, but may also cause non-lethal effects that affect population viability and environmental health.

One fifth of the North Sea porpoise population lives on the Dutch Continental Shelf. A high number of porpoise sightings in The Netherlands corresponds to a high number of strandings. Stranding recording is done by volunteers of the stranding network. A proportion of the stranded animals is examined post-mortem. This is to determine their cause of death and health status. Samples are also taken for additional research, including on reproduction, diet and contaminants. These investigations should provide insight into the health of the population and, in a broader sense, into the health of the environment. However, the available knowledge about threats and the effects on harbour porpoises is still incomplete. This is mainly because most research and management efforts have been conducted at the national level. Cross-border and multidisciplinary approaches would provide a more accurate assessment of population and ecosystem health. After all, harbour porpoises do not stick to national borders. Combining multiple disciplines, including biology, ecology, toxicology, epidemiology and pathology, provides opportunities to develop methods and measurement tools that aid in the determination and assessment of the most relevant natural and human-related threats to harbour porpoises in the North Sea. The ambition of this thesis is to contribute to this development and its application.

In **chapter 1**, the harbour porpoise is introduced as one of the smallest whales (cetaceans), and a warm-blooded marine mammal fully adapted to live in the ocean. That does not come without its challenges, particularly with regard to energetics. Porpoises are opportunistic

predators and require continuous food to meet their energetic requirements. They need up to 10% of their own body weight in prey per day. From a metabolic point of view, this gives them little 'leeway': they live on a knife-edge.

Chapter 2 describes the current knowledge gaps, future pressures or threats, and essential indicators for continuing research on harbour porpoises in the North Sea. This overview was gained by collecting the most recent expert knowledge on threats to harbour porpoises, using the so-called Delphi approach. The expert panel consisted of people working in various disciplines of harbour porpoise research and management and from all countries around the North Sea. The three main knowledge gaps listed by the experts were bycatch, population dynamics, and cumulative effects of multiple stressors. Bycatch was rated as the top concern for harbour porpoises over the next 20 years, followed by chemical and noise pollution, respectively. The most essential indicators, which can serve as means to monitor these threats and health status of harbour porpoises in the future, were: research into cause of death, distribution, abundance, habitat use and diet composition. These results guided the themes for the following research chapters of this thesis.

A large-scale and international research effort followed (**chapter 3**), investigating spatio-temporal patterns in population dynamics of harbour porpoises in the North Sea area. A dataset containing 16,181 stranding records over 28 years, obtained from national stranding networks of five countries adjacent to the North Sea, was analysed. There was a high density of new-born porpoises on the North Sea coasts of Denmark and Germany. This indicates that this area is important during and after the calving season. There were large numbers of young males found along the southern North Sea area, including on the Dutch coast. This may be a sign that weaker animals in the population are mainly found in the southern parts of the North Sea. It could also indicate a wider distribution of the young males in particular. Since 1990, the number of strandings increased throughout the region. This was most apparent since 2005 in the southern parts of the North Sea and emphasises the need to focus the research specifically on this area.

Bycatch was listed as the main concern for harbour porpoises in the North Sea area (**chapter 2**) and thus became the focus of **chapter 4**. Bycatch numbers can be determined by onboard observers, remote electronic monitoring, and when fishermen voluntarily report this. All is, however, not systematically done. Necropsies on stranded animals can additionally provide insight into bycatch numbers. However, there are uncertainties when it comes to assessing bycatch in stranded cetaceans, mainly due to the lack of diagnostic tools specific for underwater suffocation. Through a literature study, 25 criteria were listed that are used in the assessment of bycatch in small cetacean species. The presence or absence of these parameters were scored on harbour porpoises obtained from gillnets in The Netherlands (n=12). Presence of 'superficial incisions', 'circling marks' and 'recent food' were seen in the vast majority of the bycatch cases. Other criteria, such as 'pulmonary oedema', 'emphysema' and 'organ congestion' were also commonly diagnosed. These are, however, nonspecific features of underwater suffocation and commonly seen in cetaceans dying from other causes. It was striking that the previous parameters 'a favourable state of health', 'the absence of disease' and 'a good nutritional status' did not apply to the majority of the porpoises in this study. Cases with such notable pathological findings are often excluded as bycatch when assessing stranded animals. This can lead to an underestimation of the proportion of stranded porpoises which die as a result of fishery activities.

The next study (**chapter 5**) focused on chemical pollution, in particular the transfer of contaminants in harbour porpoises. Persistent organic pollutants, such as polychlorinated biphenyls (PCBs), bioaccumulate in marine ecosystems. Top predators, such as the harbour porpoise, therefore contain high levels of PCBs in their tissues. Analyses were done on different tissues, including blubber, of porpoises from all age groups. PCBs passed to foetuses via the umbilical cord, however, concentrations increase significantly after birth through lactation. Milk mainly contains lower halogenated substances, which are more toxic. New-born porpoises are thus exposed to high levels of pollutants already early in life. Of all animals, 38.5% had PCB concentrations that exceeded a threshold value for negative health effects. This was especially true for adult males (92.3%). Adult females had relatively low PCB levels (10.5%) because they were able to get rid of them through lactation. In addition, it became clear that nutritional stress leads to a higher release of PCBs through the milk, which resulted in a greater potential for toxicity in calves of nutritionally stressed females.

In a large body of literature it is advocated that reproductive disorders in small cetaceans are mainly due to PCB contamination. However, in **chapter 5** reproductive disorders were observed in a minority of adult females. Therefore, the following study (**chapter 6**) focused on the life history parameters: age at sexual maturity, pregnancy rates and foetal growth of female harbour porpoises. It was investigated whether adult females abandon investment in their foetus when they themselves are in poor physical condition or experience deteriorating environmental conditions, presumably to prioritise their own survival. Data on disease, diet, fat reserves and reproductive status were collected from post-mortem examined porpoises. This was combined with life history parameters from sixteen other harbour porpoise habitats gained from literature. These data were then correlated with variables reflecting environmental condition: mean energy density of prey from local diets, cumulative human impact and PCB load. Maternal nutritional status was found to have significant effects on foetal size. Females in poor health had a lower chance of being pregnant and generally did not sustain the pregnancy throughout gestation. Pregnancy rates were best explained by the energy density of local prey: the group of mature females of populations which fed on fish of higher energy levels had a higher percentage of pregnancies compared to the group of mature females who fed on low caloric prey. The quality of the prey eaten therefore seems to determine the reproductive success of this species.

In addition to anthropogenic threats, natural stressors may influence the health and survival of harbour porpoises. Grey seals (*Halichoerus grypus*) are abundant in the North Sea. Previously, this marine mammal was revealed as a large-scale predator of harbour porpoises. But not all seals are successful in their predation attack. Using forensic microbiological approaches, in **chapter 7** is described that the bite wounds inflicted by grey seals, from which harbour porpoises do not immediately die, can result in chronic and ultimately fatal infections, namely with the bacterium *Neisseria animaloris*.

Anthropogenic and other causes of death identified in stranded harbour porpoises from 2008-2019 were further described and analysed. **Chapter 8** focussed mainly on the directly human-related threats such as bycatch and hearing loss. Bycatch was the largest human-related cause of death (17%), mainly affecting young animals. Peak periods were March and September, although there was a sign of a downward trend over the years. Other human-related causes of death were rarely diagnosed: trauma presumably from collisions with ships (2%), and ingestion and entanglement in marine litter (0.3%). Inner ear abnormalities could only be examined in fifty porpoises, but were found in two of these. Infectious diseases was by far the largest cause of death category (32.5%), mainly for adult porpoises. Grey seal attacks were the suspected cause of death for 23.5%. Previously, more acute predation cases were noted, while recently more harbour porpoises with pathology associated with interactions that did not immediately lead to death were diagnosed. Two-thirds of the neonates died following problems during pregnancy, birth or lactation. Acute starvation or severe emaciation of unknown origin (non-neonates) was the most important finding for a further 8.6% of all stranded harbour porpoises examined.

Assessing patterns in biological, ecological and pathological profiles of individuals is very important to detect changes over time and space. However, **chapter 8** lacked an analytically robust approach. Therefore, and as a final study, an exploratory analysis was performed of combined biological, ecological and pathological data collected during post-mortem investigations (**chapter 9**). Using different statistical methods, the most relevant post-mortem findings to categorise and predict the cause of death were determined. Three clusters were defined, with age class, respiratory pathology and multiple-organ inflammation being the most important in the subdivision of those clusters. External incisions (from fishing nets), grey seal wounds, and nutritional status were the main variables for predicting causes of death. Other data, such as plants and litter in the stomach, iron accumulation in the liver and sex, may be considered uninformative to explain or predict cause of death. These data analyses were based on a large number of individuals and thus make it possible to detect robust trends that are unaffected by small, individual variation. The database should be completed by adding information on chemical pollution, among other factors. The results can then provide information about threats that are relevant at the population level.

Extensive research of stranded, dead harbour porpoises provides us with a general understanding of their biology, demographics and ecology, and also gives us insight into the health of these animals and threats to individuals and populations. Currently, monitoring of stranded animals is one of the very few ways to effectively investigate and quantify the effects of new, emerging and cumulative stressors on harbour porpoises. Continuing this monitoring work will be crucial to gain and increase knowledge about the state of the animals and the North Sea. This knowledge is vital in order to take targeted measures that aid in the protection and conservation of harbour porpoises in our waters.

Nederlandse samenvatting

Bruinvissen zijn charismatische en beschermde dieren. Dit heeft als gevolg dat er een brede maatschappelijke, wetenschappelijke en politieke interesse is in hun gezondheid en welzijn. Bruinvissen zijn een integraal onderdeel van het mariene voedselweb en hebben een algemene verspreiding, wat erin resulteert dat ze een focussoort zijn in veel onderzoeksprogramma's die de toestand van het milieu in de zee monitoren. Deze programma's zijn over het algemeen gericht op het vergroten van de kennis over de invloed van mensgerelateerde activiteiten op zee en op de bruinvispopulaties, met als doel het beschermen en behouden van deze dieren en hun leefgebied.

De Noordzee is wereldwijd gezien een enorm druk gebied waar veel menselijke activiteiten, veelal tegelijkertijd, plaatsvinden. Bruinvissen zijn in de Noordzee de meest voorkomende walvissoort, met een geschat aantal van zo'n 350.000 individuen. Een hoog aantal bruinvissen gecombineerd met veel menselijke activiteiten betekent dat veel dieren worden blootgesteld aan verschillende bedreigingen. Dit zijn onder andere visserij, chemische vervuiling en onderwatergeluid van verschillende bronnen, zoals de scheepvaart, seismisch onderzoek en ontploffingen van oude munitie. Aanvullend daarop is er de laatste jaren een stijging van het aantal offshore-activiteiten voor de bouw van onder andere windparken. Ook worden de grootschalige effecten van klimaatverandering, zoals veranderingen in prooi kwantiteit en kwaliteit, steeds duidelijker zichtbaar. Individuele en cumulatieve stressoren bedreigen de directe overleving van individuele bruinvissen, maar kunnen ook niet-dodelijke effecten veroorzaken die van invloed zijn op de levensvatbaarheid van de populatie en de gezondheid van het milieu.

Een vijfde van de gehele bruinvispopulatie leeft in het Nederlandse gedeelte van de Noordzee. Een hoog aantal kustwaarnemingen in Nederland correspondeert met een hoog aantallen strandingen. Strandingsregistratie wordt door het vrijwillige strandingsnetwerk gedaan. Daarnaast wordt een deel van de gestrande bruinvissen postmortaal onderzocht om hun doodsoorzaak en gezondheidsstatus te monitoren. Tijdens de secties worden ook monsters genomen voor aanvullend onderzoek, onder andere naar reproductie, dieet en chemische vervuiling. Deze onderzoeken moeten inzage geven in de gezondheid van de populatie of, in bredere zin, in de gezondheid van het milieu. De beschikbare kennis over bedreigingen en de effecten op bruinvissen is echter onvolledig. Dat komt vooral omdat de meeste onderzoeks- en beheersinspanningen worden uitgevoerd op nationaal niveau, terwijl een grensoverschrijdende en multidisciplinaire benadering een nauwkeurigere beoordeling van de gezondheidstoestand zou opleveren. Bruinvissen kennen immers geen landgrenzen. Het combineren van meerdere disciplines, waaronder biologie, ecologie, toxicologie, epidemiologie en pathologie, biedt de mogelijkheid om methoden en meetinstrumenten te ontwikkelen die helpen bij het bepalen en beoordelen van de meest relevante natuurlijke en antropogene bedreigingen voor bruinvissen in de Noordzee. De ambitie van dit proefschrift is om bij te dragen aan deze ontwikkeling en de toepassing daarvan.

In **hoofdstuk 1** wordt de bruinvis geïntroduceerd als een van de kleinste walvisachtigen en een warmbloedig zeezoogdier, die volledig is aangepast aan het leven in zee. Dat brengt verschillende uitdagingen met zich mee, met in het bijzonder het behouden van een gunstige voedingstoestand. Bruinvissen zijn opportunistische roofdieren en hebben continu voedsel nodig om aan hun energiebehoefte te voldoen, aangezien zij tot 10% van hun eigen lichaamsgewicht aan prooi per dag nodig hebben. Dit geeft ze metabolisch gezien weinig 'speelruimte', ze leven op het scherpst van de snede.

In **hoofdstuk 2** volgt een overzicht van ontbrekende kennisonderwerpen, met welke bedreigingen bruinvissen te maken hebben en een lijst van onderzoeksindicatoren voor toekomstige monitoring. Dit overzicht is tot stand gekomen door het verzamelen van de meest recente kennis van experts over bedreigingen voor bruinvissen, aan de hand van de zogenaamde Delphi-benadering. Het panel van experts bestond uit mensen die werkzaam zijn binnen bruinvisonderzoek en -beheer, in alle landen rond de Noordzee. De drie belangrijkste onderwerpen waarvan kennis nodig is, zijn volgens het expertpanel: bijvangst, populatiedynamiek en de cumulatieve effecten van meerdere stressoren. Bijvangst werd beoordeeld als de grootste bedreiging voor bruinvissen in de komende 20 jaar, gevolgd door respectievelijk chemische vervuiling en geluidsoverlast. Als meest essentiële indicatoren, die in de toekomst kunnen dienen om de gezondheidstoestand van bruinvissen te monitoren, werden genoemd: onderzoek naar doodsoorzaak, verspreiding, populatiegrootte, habitatgebruik en dieetsamenstelling. Deze resultaten vormden de leidraad voor de thema's van de opvolgende hoofdstukken van dit proefschrift.

Een grootschalige en internationale samenwerking volgde (**hoofdstuk 3**), waarbij tijdruimtelijke patronen in het aantal bruinvisstrandingen in het Noordzeegebied werden onderzocht. Een dataset met gegevens van 16.181 strandingen over 28 jaar, verkregen van nationale strandingsnetwerken van vijf landen grenzend aan de Noordzee, werd geanalyseerd. Er werd een hoge dichtheid van pasgeboren bruinvissen op de kust van Denemarken en Duitsland gevonden. Dit kan betekenen dat deze regio belangrijk is voor bruinvissen tijdens of vlak na de geboorte. Daarnaast werden grote aantallen jonge mannetjes langs het zuidelijke deel van de Noordzee gevonden, waaronder op de Nederlandse kust. Dit kan een teken zijn dat zwakkere dieren in de populatie vooral in het zuidelijke deel van de Noordzee voorkomen. Ook kan het duiden op een ruimere verspreiding van specifiek jonge mannetjes. Sinds 1990 nam het aantal strandingen in de hele regio toe, met specifiek in het zuidelijke deel van de Noordzee een opvallende toename vanaf 2005. Dit benadrukt de noodzaak om het onderzoek specifiek op dit gebied te richten.

Bijvangst in de visserij werd door experts naar voren gebracht als grootste zorg voor bruinvissen in de Noordzee (**hoofdstuk 2**) en om die reden werd het de focus van **hoofdstuk 4**. Bijvangstaantallen kunnen worden vastgesteld door waarnemers aan boord van schepen, elektronische monitoring en het vrijwillig rapporteren door vissers. Dit alles wordt echter niet systematisch gedaan. Secties op gestrande bruinvissen kunnen aanvullend inzicht verschaffen in bijvangstaantallen. Er zijn alleen onzekerheden als het gaat om het vaststellen van bijvangst bij gestrande dieren, voornamelijk door het ontbreken van diagnostische hulpmiddelen die specifiek kunnen bepalen of een dier is overleden als gevolg van verstikking onderwater. Uit de literatuur werden 25 criteria verzameld, die gebruikt worden bij de beoordeling van bijvangst bij kleine walvisachtigen. De aan- of afwezigheid van deze kenmerken zijn vervolgens in kaart gebracht bij bruinvissen die in Nederland uit kieuwnetten verkregen waren (n=12). De aanwezigheid van 'oppervlakkige incisies', 'omcirkelende afdrukken' en 'recent voedsel' werd bij het overgrote deel van de bijvangstgevallen geconstateerd. Andere criteria, zoals 'longoedeem', 'longemfyseem' en 'congestie van organen' werden ook vaak gediagnosticeerd, maar zijn niet-specifieke kenmerken voor verstikking onderwater, omdat dit ook veel wordt gezien bij walvisachtigen met een andere doodsoorzaak. Opvallend was dat met name de criteria 'een gunstige gezondheidstoestand', 'de afwezigheid van ziekte' of 'een goede voedingstoestand' niet van toepassing waren op de meerderheid van de bruinvissen. Bij het beoordelen van de bijvangst onder gestrande bruinvissen worden gevallen met dergelijke pathologische bevindingen vaak uitgesloten. Dit kan leiden tot een onderschatting van het aandeel strandingen dat verband houdt met de visserij.

De volgende studie (**hoofdstuk 5**) had als focus chemische vervuiling. Met name de overdracht van chemische stoffen bij bruinvissen. Persistente organische verontreinigende stoffen, zoals polychloorbifenylen (PCBs), stapelen op in mariene ecosystemen. Toppredatoren, zoals de bruinvis, hebben daardoor hoge PCB-niveaus in hun weefsel. Er werden metingen gedaan in verschillende weefsels, waaronder blubber, van bruinvissen uit alle leeftijdsgroepen. PCBs worden al aan foetussen doorgegeven via de navelstreng. Het PCB-gehalte neemt vervolgens aanzienlijk toe na de geboorte en zodra het kalf gaat zogen bij de moeder. Moedermelk bevat vooral lager gehalogeneerde stoffen, welke meer toxisch zijn. Pasgeboren bruinvissen worden dus al vroeg in hun leven blootgesteld aan hoge niveaus van verontreinigde stoffen. Van alle onderzochte dieren had 38,5% PCB-concentraties die de drempelwaarde voor negatieve gezondheidseffecten overschreden. Dit waren met name volwassen mannetjes (92,3%). Bij de volwassen vrouwtjes werden veel lagere PCB-niveaus vastgesteld (10,5%), omdat zij die via het geven van melk konden kwijtraken. Daarnaast werd duidelijk dat nutritionele stress leidt tot een hogere afgifte van PCBs via de melk, wat een groter potentieel voor toxiciteit veroorzaakt bij kalveren van vrouwtjes die vermagerd zijn.

In veel literatuur wordt beschreven dat reproductiestoornissen bij kleine walvisachtigen voornamelijk komt door PCB-vervuiling. In **hoofdstuk 5** werd echter bij de minderheid van de volwassen vrouwtjes een reproductiestoornis waargenomen. Daarom richtte de volgende studie (**hoofdstuk 6**) zich op de voortplanting van vrouwelijke bruinvissen, met daarbij in het bijzonder de focus op leeftijd bij geslachtsrijpheid, zwangerschapspercentages en foetale groei. Er werd onderzocht of vrouwtjes de investering in hun foetus opgeven wanneer ze zelf in een slechte fysieke conditie zijn of in verslechterende omgevingsomstandigheden verkeren, vermoedelijk om de eigen overleving voorrang te geven. Gegevens over ziekte, dieet, vetreserves en reproductieve status, verkregen uit postmortaal onderzoek, werden gebruikt. Dit werd aangevuld met informatie over zwangerschappen en geslachtsrijpheid uit de literatuur, vanuit zestien andere leefgebieden van de bruinvis. Deze gegevens werden vervolgens gecorreleerd aan variabelen die de omgevingsconditie weerspiegelen: gemiddelde energiedichtheid van prooi uit de lokale diëten, menselijke activiteiten en PCB-belasting. De voedingsstatus van de moeder bleek significante effecten op de grootte van de foetus te hebben. Vrouwtjes in slechte gezondheid hadden een lagere kans om zwanger te zijn of konden de zwangerschap niet voldragen. Zwangerschap werd het best verklaard door de energiedichtheid van lokale prooi: de groep volwassen vrouwtjes uit populaties die vis aten met hogere energetische waardes had een hoger zwangerschapspercentage dan de groep die vis van lagere energetische waarde had gegeten. De kwaliteit van de gegeten prooi lijkt dus bepalend voor het reproductiesucces van deze diersoort.

Naast antropogene bedreigingen kunnen ook natuurlijk stressoren de gezondheid en het voortbestaan van bruinvissen beïnvloeden. In de Noordzee leeft de veelvoorkomende grijze zeehond (*Halichoerus grypus*). In een eerdere studie werd dit zeezoogdier al als grootschalige predator van bruinvissen ontmaskerd, maar niet alle zeehonden zijn succesvol in hun predatieaanval. In **hoofdstuk 7** wordt een forensische, microbiologische benadering beschreven waarmee werd ontdekt dat de bijtwonden die grijze zeehonden toebrengen, maar waaraan bruinvissen niet direct overlijden, tot chronische en uiteindelijk dodelijke infecties kunnen leiden, namelijk met de bacterie *Neisseria animaloris*.

Verschillende doodsoorzaken die zijn vastgesteld bij gestrande bruinvissen tussen 2008 en 2019, in het bijzonder door direct-menselijk toedoen, werden beschreven en geanalyseerd in **hoofdstuk 8.** Hier bleek bijvangst de meest voorkomende doodsoorzaak die wordt veroorzaakt door mensen (17%) en waarbij vooral jonge dieren worden getroffen. Piekperiodes zijn in maart en september, al werd er wel een dalende trend over de jaren gezien. Sommige mens-gerelateerde doodsoorzaken werden zelden gediagnosticeerd, zoals trauma als gevolg van een aanvaring met een schip (2%) en het inslikken van en verstrikking in zwerfvuil (0,3%). Specifieke afwijkingen in het binnenoor konden worden onderzocht bij vijftig bruinvissen en er werden afwijkingen geconstateerd bij twee van deze dieren. Infectieziekten komen naar voren als de grootste doodsoorzaak (32,5%) voornamelijk onder volwassen bruinvissen. Aanvallen van de grijze zeehond waren in 23,5% van de onderzochte gevallen de vermoedelijke doodsoorzaak. In het verleden werden meer acute predatiegevallen geconstateerd, terwijl recentelijk meer bruinvissen met ontstoken bijtwonden werden gevonden. Twee-derde van de pasgeborenen stierf na problemen tijdens de zwangerschap, de geboorte of het zogen. Acute verhongering of ernstige vermagering van onbekende oorsprong was de belangrijkste bevinding voor nog eens 8,6% van de dode bruinvissen.

De beoordeling van patronen in biologische, ecologische en pathologische profielen van individuen is erg belangrijk om veranderingen in de tijd en ruimte te ontdekken. Omdat **hoofdstuk 8** een analytisch robuuste benadering miste, is ten slotte een verkennende analyse uitgevoerd van gecombineerde biologische, ecologische en pathologische gegevens die zijn verzameld tijdens het postmortaal onderzoek (**hoofdstuk 9**). Gebruikmakend van verschillende statistische methoden werden de meest relevante postmortale bevindingen bepaald om doodsoorzaken te categoriseren en voorspellen. Er werden drie clusters in de data gevonden, waarbij leeftijdsklasse, pathologie van de luchtwegen en ontstekingen in meerdere organen het belangrijkste waren in de onderverdeling van die clusters. Externe afdrukken (vermoedelijk van visnetten), wonden door grijze zeehonden en voedingstoestand waren de belangrijkste variabelen voor het voorspellen van doodsoorzaken. Andere kenmerken, zoals planten en zwerfvuil in de magen, geslacht en ijzerophoping in de lever bleken niet informatief om de doodsoorzaak te verklaren of te voorspellen. Deze data-analyses zijn gebaseerd op een groot aantal individuen en maken het dus mogelijk om robuuste trends te ontdekken die niet worden beïnvloed door kleine individuele variatie. De database moet nog worden aangevuld met onder andere data over chemische vervuiling. Daarna kunnen dergelijke analyses informatie verschaffen over bedreigingen die relevant zijn voor de gehele populatie.

Uitgebreid onderzoek van gestrande, dode bruinvissen geeft, naast een algemeen begrip van hun biologie, demografie en ecologie, ons ook inzage in de gezondheid van deze dieren en bedreigingen op individuen en populaties. Momenteel is het monitoringsonderzoek van gestrande dieren één van de zeer weinige efficiënte en effectieve manieren om de invloed van huidige, nieuwe, opkomende en cumulatieve stressoren op bruinvissen te achterhalen. Het voortzetten van dergelijke monitoringswerkzaamheden is cruciaal om kennis te blijven vergaren over de toestand van de dieren en de Noordzee. Deze kennis is nodig om doelgerichte maatregelen te treffen die bruinvissen beschermen en behouden in onze wateren.

About the author

Lonneke Liza IJsseldijk was born in Alkmaar, The Netherlands on November 12, 1991. She started volunteering with post-mortem investigations on harbour porpoises at Utrecht University in the final year of her senior general secondary school (HAVO). After completing the HAVO in Bergen, Noord-Holland, IJsseldijk spent her summer break in Plettenbergbay, South-Africa, to study whales and dolphins at the Centre for Dolphin Studies. This involved informing tourists on whale-watching boats and here she gained her first experiences with marine mammal observations. Then, she moved to Leeuwarden to study Integrated Coastal Zone Management at Van Hall Larenstein (VHL). After successfully completing her propaedeutics, the next summer break was spent in Piran, Slovenia at Morigenos Dolphin Research, studying bottlenose dolphin behaviour both at sea and computer-based, conducting the capture-recapture method photo-identification.

During the additional three years at VHL, IJsseldijk continued to volunteer at Utrecht University, interspersed with several internships abroad. First, a five-month Erasmus placement at the Irish Whale and Dolphin Group in 2011. The goal of the internship was to explore the work field, and IJsseldijk alternated stranding investigations with at-sea cetacean observations, both in nearshore as in offshore waters. Additionally, she processed and matched photographs of bottlenose dolphins collected during dedicated surveys or submitted by the public, which she continued back in The Netherlands. In 2012/2013, she conducted a five-month research internship at Murdoch University, which was split between working in Hawaii, USA, and Perth, Australia. The research focussed on the effects of tourism on spinner dolphins of The Big Island. Additional to the already known photo-identification techniques, she got familiar with more (analytical) approaches for long-term studying of wild dolphins. In the final year at VHL, IJsseldijk moved to Texel. At the Royal Netherlands Institute of Sea Research and IMARES (now: Wageningen Marine Research), she studied harbour porpoises in the Marsdiep area. After several weeks of conducting line-transect surveys from the TESO ferry, she investigated the tidal influence on the occurrence of harbour porpoises. This research, which was later also published, sealed her Bachelor degree.

During the time on Texel, a research assistant position became available at Utrecht University in the stranding research programme. IJsseldijk moved from Texel to Utrecht and started, what was supposed to be, a one-year contract at the Division of Pathology in 2013. The one-year became a two-year position, which was again extended and IJsseldijk was able to move up as junior researcher. Highly motivated to continue in academia she enrolled into the Master Environmental Biology at the Faculty of Life Sciences of Utrecht University. A challenging two years followed, balancing fulltime work and study. A four-month break from work was unavoidable, but allowed to do one more focussed and final internship abroad, at the Scottish Marine Animal Stranding Scheme. Here she further developed her necropsy skills gaining experience with a wide range of species and undertook a Delphi study focussing on harbour porpoise conservation in the North Sea. She also furthered the "best practise in cetacean post-mortem investigations", as a joint ACCOBAMS-ASCOBANS document, sealing her Master degree.

After completing the MSc, IJsseldijk continued her work at the Division of Pathology as researcher and manager of the stranding research programme. With professors Gröne and Heesterbeek, the 'PhD-by-paper'-plan was discussed in 2017, the final result of which is lying in front of you. Meanwhile, IJsseldijk continued cetacean post-mortem investigations, supervised students and interns, and became an advisor for Dutch governments and NGO's. She is a member of several working groups and expert panels, including the panel on strandings of the International Whaling Commission, and the Cetacean Specialist Group of the International Union for Conservation of Nature. In the years ahead, IJsseldijk hopes to continue her work furthering the field of harbour porpoises and other cetacean research and conservation, both nationally as international.

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De bruinvissen lagen opgestapeld in een andere koelcel. Een van de twee snijzalen was geheel in gebruik door de internationale snijploeg die speciaal voor de bruinvissectie was samengesteld. Na wat rondkijken bij het magenonderzoek van Mardik Leopold en op de snijtafel van Thierry Jauniaux, heb ik mij uiteindelijk gevoegd bij het team van Kees Camphuysen, die de externe beoordelingen deed. Tot op heden mijn favoriete onderdeel, daar waar de bruinvis nog gewoon een bruinvis is. Daar leerde ik onder het kritisch oog van Kees, in gezelschap van Coby en Dirk Kuiken, Marijke de Boer en Polona Kotnjek, wat kenmerken van bijvangst, huid laesies en een goede voedingstoestand waren. Ik heb de rest van de week in dat gezelschap doorgebracht. Terugkijkend heb ik in die periode veel mensen leren kennen, waar ik later in mijn carrière nog veel mee in aanraking ben geweest.

> Kees, je woorden zijn me altijd bijgebleven: "ga een master doen" en "wie schrijft die blijft". Dank je, dat je de jonge ik welkom heette en me (ook later nog regelmatig) de "goede" richting in hebt geduwd.

Na dat jaar en die snijweek verplaatste het bruinvisonderzoek officieel naar Utrecht. Onder leiding van prof. Andrea Gröne en destijds met Lidewij Wiersma *in charge* werden de secties niet meer opgespaard, maar regelmatig uitgevoerd. Met Lidewij ontstond een band. Ik vond het leuk om te helpen bij de secties en Lidewij kon de extra paar schone handjes vaak wel gebruiken. Met regelmaat spijbelde ik in Leeuwarden, waar ik toen studeerde, om vervolgens in Utrecht te assisteren. Na enige tijd schrijven en samplen tijdens de secties, werd mij niet heel veel later ook het mes in eigen hand toevertrouwd.

Lidewij, je openheid, vertrouwen en welwillendheid om mij nieuwe dingen te leren ben ik je tot op de dag van vandaag dankbaar. Dit proefschrift ligt er mede dankzij jou, jouw enthousiasme en hulp aan de start van mijn loopbaan was cruciaal! Ik kijk met veel plezier terug op mijn eerste snijweek en de dolfijnensecties!

De eindstage van mijn bachelor heb ik op Texel volbracht, bij IMARES en NIOZ, onder leiding van Kees Camphuysen en Geert Aarts. Dit werd mijn eerste, tot nu toe ook laatste, onderzoek met levende bruinvissen. Voor een periode van pakweg zes weken in hartje winter, ging ik bijna dagelijks mee op de TESO-veerboot. Ik voer dan, verpakt in een fel oranje survival suit, heen-en-weer op de zijvleugel van de brug. Vanaf daar was er perfect zicht over het Marsdiep en heb ik dagelijks bruinvissen gezien.

Geert Aarts, bedankt voor de fijne begeleiding, de introductie van data analyse en R. Ook dank voor je hulp na de stage, toen we het rapport hebben verwerkt tot een wetenschappelijke publicatie.

Ook dank aan alle andere (oud-) WMR'ers, van wie vele nu nog altijd als 'echte' collega's voelen: Steve, Meike, Hans, Elisa, Martine, Christiaan, Michiel, maar ook Marin, Suse, Jan Andries, Simon, André, Piet-Wim, Jessica & Sophie. Ik kijk uit naar nog vele samenwerkingen, fijne gesprekken en nuttige discussies!

Toen Lidewij in Utrecht vertrok, was daar Lineke Begeman om het mes over te nemen. Gelukkig mocht ik blijven komen en was de band met Lineke minstens net zo goed als met Lidewij. Lineke kreeg hulp van Sjoukje Hiemstra. Met z'n drieën hebben we nog heel wat bruinvissen weggesneden. Eenmaal aanbeland in het laatste jaar van mijn HBO-opleiding besloot Sjoukje voor de liefde naar de Caribbean te verhuizen en kon ik als onderzoeksassistent en directe collega van Lineke in Utrecht komen werken. Na een jaar of twee intense samenwerking met (en in de leer bij) Lineke, vertrok zij voor een PhD naar Erasmus. Het bruinvisproject kreeg verlenging en ik ook. Mijn onderzoeksfunctie werd gecombineerd met een master Environmental Biology en zo kon ik mijn wetenschappelijke carrière bij de Universiteit Utrecht voortzetten.

Lineke, jij hebt mij de fijne kneepjes van het vak geleerd. Naast collega's werden we bevriend en ondanks het weinige contact, heb ik altijd het gevoel gehouden dat ik je kan bellen op de momenten waarop ik je hulp of advies nodig had. Bedankt voor je vertrouwen en bovenal de gezellige, leerzame tijd. Ik kijk met veel plezier terug op de secties (klein en groot) die wij samen hebben uitgevoerd!

Na het vertrek van Lineke naar Rotterdam, nam Marja Kik het pathologie-stokje over. Dat was rond de tijd dat ik startte met de fulltime master naast mijn werk. Dit was alleen maar mogelijk door een enorme steun vanuit de afdeling. In het bijzonder de collega's die mij als stage begeleiders vooruithielpen. Hélène Verheije & Steven van Beurden, maar ook Geert de Vrieze en Alinda Berends hebben mij met erg veel geduld ingewerkt op het lab om PCRs te doen. Liliane Solé heeft het bruinvis-team een jaartje versterkt, zodat ik onder andere een laatste stage in het buitenland kon volbrengen. Daarna nam Natashja Ennen-Buijs haar herintreden in het bruinvisteam en oud-stagiaire Linde van Schalkwijk, die als combi SIO/onderzoeker werkt, maakt het huidige team compleet.

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Marja, bedankt voor al je hulp en geduldige uitleg over alles wat met microscopie te maken heeft. Je constante drive om nieuwe ziektes te ontdekken is op z'n zachtst gezegd bewonderingswaardig.

Hélène & Steven, het gepriegel op het lab ging me niet altijd even goed af. Wel heb ik kennis en ervaring opgedaan die nog vaak van pas komt. Dank voor jullie geduld, heldere uitleg en bovenal de leuke gesprekken!

Liliane, ondanks de korte tijd als collega's kijk ik met plezier terug op onze samenwerking! Bedankt voor al je hulp, niet alleen in de sectiezaal, maar ook als vrijwilliger op het strand.

Natashja, de échte miss-bruinvis. Ik ken niemand die zo secuur werkt als jij. Je bent een ontzettend fijne, gezellige en betrouwbare collega waar ik nog jaren mee hoop samen te werken!

In diezelfde, eerste snijweek van 2008 leerde ik de rest van het Utrechtse sectiezaalteam en mijn huidige (fantastische!) collega's kennen: Louis van den Boom, Ruby Wagensveld en oud collega Johan van Amerongen. Later kwamen daar Manon Lock en Darryl Leydekkers bij. De afgelopen jaren, in de sectiezaal en tijdens de onderzoeken op de gestrande bruinvissen werd ik regelmatig vergezeld door stagiaires, oproepkrachten en vrijwilligers: Amy, Annemarie, André, David, Elisa, Els, Eveline, Jasper, Javier, Jeroen, Jesse, Jolanda, Laura, Linda, Linde, Mariel, Marijke, Martijn, Mia, Marianthi, Mariska, Nasia, Nowell, Pieter, Sterre & Thom.

Het is misschien maar goed dat dit proefschrift gaat over de bruinvissen, want anders had dit dankwoord vol vieze anekdotes gestaan over mensen in door-smurrie-doordrenkte kledingstukken, uitglijders op of vliegende stukken blubber en wrong kidneys (en zou Louis' naam veel vaker voorkomen…). De meeste avonturen beleven we veelal rondom de walvisstrandingen. Daar waar we in weer en wind ons werk aan zee mogen doen, zijn er altijd de thuisblijvers die alle ballen hoog houden: teamwork. Dat begrip en de interesse komt niet alleen uit de sectiezaal, maar uit alle delen van het departement: huidige en oud collega's van het histolab, de administratie, DWHC, pathologen, onderzoekers, SIO's, studenten en het management.

Lieve UU-collega's, ik ben jullie enorm dankbaar voor alle hulp die jullie bieden (al moet ik soms eerst een hoop gemopper aanhoren, vooral wanneer de al wat belegen, gedroogde of goed gefermenteerde walvisachtigen zich aandoen). Jullie steun, toewijding en enthousiasme is de afgelopen jaren alleen maar groter geworden. Dit maakt de dagen die soms zo stressvol zijn, ook heel erg leuk! Ik kijk uit naar alle avonturen die ons nog te wachten staan!

Ook wil ik mijn (oud-) collega's van de afdeling Marketing, Communicatie en Multimedia noemen, met wie ik ontzettend veel mooie media output heb gegenereerd en de prachtige landingspagina www.uu.nl/strandingsonderzoek heb gemaakt. En ons laatste pareltje "De Bruinvis", ons publieksmagazine, met in het bijzonder dank aan Josien, Bas en Lisanne.

De opdrachtgevers van het bruinvisonderzoek zijn en waren ook altijd erg bevlogen. Dit maakt het ontzettend prettig om samen te werken aan het gemeenschappelijke doel: bruinvis behoud en bescherming. Een beetje voor mijn tijd, maar toch meegekregen, was daar Folchert van Dijken, die opgevolgd werd door Jeroen Vis er daarna Anne-Marie Svoboda, bij het ministerie van Landbouw, Natuur en Voedselkwaliteit. Ook vanuit Rijkswaterstaat, met name Inger van den Bosch en Aylin Erkman, en een beetje tussen alle instanties en partijen door, Marije Siemensma.

Naast collega's en opdrachtgevers, is het project niks zonder de enorme lading aan strandingsnetwerkvrijwilligers, die er vooral buitengewone acties op na hielden om ons onderzoek en dus dit proefschrift mogelijk te maken. Ik heb met zoveel mensen gesproken in de afgelopen jaren. Mensen die geholpen hebben bij het registreren, veiligstellen en transporteren van gestrande bruinvissen naar de faculteit (in alle vormen, maten en staten). Specifiek herinner ik de vele acties van Arnold, Jaap, Kees en Rinus. Nooit te beroerd om voor dag en dauw de auto in te stappen, strandingen te coördineren en st(r)a(n)d en land af te rijden om achter meldingen aan te gaan. Ook Annemarie van den Berg, ik kijk uit naar wat de toekomst ons gaat brengen! Hetzelfde geldt eigenlijk voor het gros van de mensen die betrokken zijn bij het vrijwillige strandingsnetwerk: medewerkers van Eerste Hulp bij

Zeezoogdieren, Ecomare, ReddingsTeam Zeedieren, Dierenambulances, Pieterburen, Zeehondencentrum Terschelling, Eemsdelta, Aseal en Naturalis. Dank allen, jullie weten wie jullie zijn: toppers!

Tussen de sectiezaalwerkzaamheden door waren daar ook de vele meetings, congressen en workshops. Daar leerde ik mijn niet-UU collega's kennen: my dear UK-friends Andrew Brownlow, James Barnett, Paul Jepson, Nick Davison, Mark Dagleish, Matt Perkins and Rob Deaville (and of course Mariel ten Doeschate, who also belongs to this list). Also, Jan Haelters, Thierry Jauniaux, Sandro Mazzariol, Maria Morell, Sinéad Murphy, Misty Niemeyer, Brian and Sarah Sharp, Ursula Siebert and Karen Stockin.

Dear colleagues from abroad, you are my workfamily and I am thankful for all drinks we have had! And for the conversations, of course, about cool cases, pathology, ecology, toxicology and so many other things. I feel blessed to have you on speed-dial in case of horrible mass stranding events, ranging from porpoise heads to full-size sperm whales; you love them all as much as I do. Thank you for all the interesting discussions and conversations, I hope many more will follow!


Voordat ik bij mijn 'promotieteam' aankom, wil ik vier mensen in het bijzonder bedanken voor hun bijdragen aan mijn PhD hoofdstukken. Te beginnen met Martine van den Heuvel-Greve, met wie ik het toxicologie hoofdstuk geschreven heb. Hoewel we zo nu en dan de zweep van Steve Geelhoed nodig hadden, is het maar mooi gelukt om dat project tot een zeer succesvol einde te brengen. Jan van den Broek, wie ik altijd mocht storen met mijn statistiek vragen. En last but not least, Guido Keijl, altijd welwillend om mee te denken, zowel over gestrande dieren als het onderzoek. Dank!

Andrea Gröne, mijn promotor, ontmoette ik in 2008, maar leerde ik pas echt kennen toen ik in 2013 op de afdeling Pathologie kwam werken. Er zijn zoveel dingen te vertellen, maar een verhaal is kenmerkend. Die ene dag, avond eigenlijk, toen ik besloot een sectie te doen op een levend gestrande bruinvis die dezelfde avond bij Bloemendaal overleden was. Met mijn vader reed ik vanuit de kop van Noord-Holland, via Bloemendaal naar Utrecht, de bruinvis achterin. Hoewel de sectie voorspoedig verliep, was daar dat ene moment dat mij (en Andrea waarschijnlijk ook) altijd is bijgebleven: ik zou de kop zagen met de zaagmachine, maar drukte op de verkeerde knop, waarmee ik niet de zaag, maar het noodalarm aanzette. In no-time stond de security in de sectiezaal. Het was inmiddels ruim na middernacht. Geen van ons wist de uitknop te vinden en er zat niets anders op dan Andrea wakker te bellen, om ons met de simpele woorden: "ingedrukte knop weer uittrekken" van de herrie te verlossen.

Andrea, mijn promotor, tot op de dag van vandaag gebruik ik de zaagmachine niet, maar weet ik wel dat ik jou op elk moment om hulp kan vragen. Bedankt voor je vertrouwen in mij!

Hans Heesterbeek kwam via Andrea als tweede promotor in beeld. En dat bleek een goede match. Vanaf het eerste moment hadden we interessante gesprekken en daagde Hans mij vaak uit om groter te denken. De ervaring van Hans met het schrijven van wetenschappelijke artikelen en het overbrengen van een boodschap hebben mij verder geholpen tijdens mijn onderzoek en het schrijven van dit proefschrift.

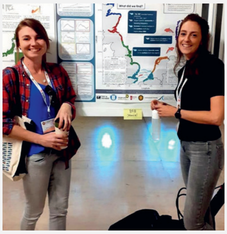
Hans, mijn promotor, ik heb je ervaren als een zeer betrokken en enthousiaste begeleider en ik heb veel van je geleerd. Ik hoop dat we dat de komende jaren zullen doorzetten!

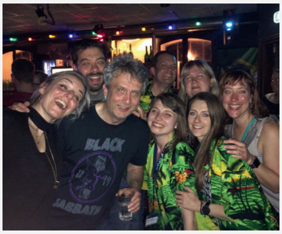
Mijn promotieteam werd versterkt door Mardik Leopold, die vanwege het dieetwerk al erg betrokken was bij het bruinvisonderzoek. Door ons regelmatig samenkomen, ontstonden vaak discussies en bovenal nieuwe ideeën. Iedereen die Mardik kent weet dat hij een ster is in het bedenken (en starten) van publicaties. Gecombineerd met het regelmatig herinneren en doorpakken van mijn kant (lees: stalken) heeft dat tot prachtige resultaten geleid.

Mardik, mijn copromotor, in het dankwoord van jouw proefschrift schreef je: '*als ik zie hoeveel we samen nog op de stapel hebben staan, gaan we nog een mooie toekomst tegemoet*'. Laat dit proefschrift daar één van de uitkomsten van zijn, al kan ik niet anders dan, zes jaar na dato, hetzelfde zeggen: ik kijk ernaar uit om de komende paar jaren nog vele vraagstukken samen aan te pakken en uit te zoeken! Dank voor je mentorschap en zoveel meer dan dat.

En toen, de laatste loodjes. Het was een gemakkelijke keus wie ik op dé dag het liefst naast me wilde hebben staan. En gelukkig wilden zij dat ook…

Mariel, mijn paranimf, where to start? Er schieten zoveel momenten door m'n gedachte, de ene nog vreselijker dan de ander, de meeste beter niet te beschrijven noch te herhalen. Conferences would be so much less fun without you! Ik hoop dat we nog ontelbaar veel meetings, trips, workshops, happy hours, conferences, gins, necropsies zullen delen! Bedankt voor al je input in dit proefschrift. You are an amazing friend and colleague, iemand waar ik altijd op kan vertrouwen, maar het bovenal zó leuk mee heb gehad. Bij jouw PhD zal ik er voor je zijn zoals jij er voor mij bent geweest! (muhaha) **searching nice pictures of us at work……. Kansloze missie…**



Linde, mijn paranimf en daarnaast mijn collega met wie ik het afgelopen anderhalf jaar lief en leed gedeeld heb. Wat is het fijn om weer een lotgenoot te hebben! Ik was zo blij dat je na een paar keer aandringen toch besloot om in het bruinvisproject te komen werken. Hoewel we op sommige punten (te) veel op elkaar lijken, vullen we elkaar op andere punten feilloos aan. Je bent een enorme harde werker en een super betrouwbaar en gezellig mens!! Bedankt voor je steun bij de laatste, zware loodjes van m'n proefschrift, te midden van massa's bruinvissen, dolfijnen en vinvissen hebben we ons maar mooi staande gehouden!

Pap en mam, jullie bijdrage is van onbeschrijfelijke waarde geweest. Ik koos een studie en loopbaan met slecht baanperspectief, maar zonder enige twijfel en met volledige steun van jullie. Jullie boekten mijn reis naar Zuid-Afrika toen ik nog maar 17 jaar was, hebben mij zo'n 15 keer verhuisd tot ik uiteindelijk in 't Veld (*of all places*…) neerstreek. Jullie hebben mij altijd geleerd om hard te werken voor wat ik echt wilde. Dit proefschrift had zonder jullie onvoorwaardelijke steun, liefde en hulp niet tot stand kunnen gekomen.

Mijn lieve vriendinnen uit Noord-Holland, Annemijn en Fleur, m'n zusje Céline en schoonfam. Jullie volgen het onderzoek op de voet (of worden er door bekenden op geattendeerd). Een aantal van jullie is zelfs mee geweest bij secties! Het is fijn om mensen om mij heen te hebben die snappen dat je last minute afzegt, omdat er ergens iets gestrand is. Jullie zijn de beste! En ik beloof na m'n verdediging ook weer heel sociaal te zijn…

(Mijn) Geert, a picture says more than a thousand words.

Wat is het fijn om thuis te komen bij iemand die met zoveel plezier over mijn werk praat. Je bent altijd geïnteresseerd en helpt me ook nog eens bij alles waarbij je dat kunt, van communicatievragen tot assistentie bij weekendsecties. Zonder jouw steun was dit boekje er nu niet geweest. Je biedt een luisterend oor als dat nodig is en zorgt dat ik altijd met beide beentjes stevig op de grond blijf staan. Ik heb geen enkele seconde spijt gehad van het terugverhuizen naar Noord-Holland, om met jou in 't Veld te kunnen wonen. Wat de toekomst ons ook maar brengen zal, als we het samen kunnen doen, dan komt het altijd goed. Love you.

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COLOFON

Credits

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