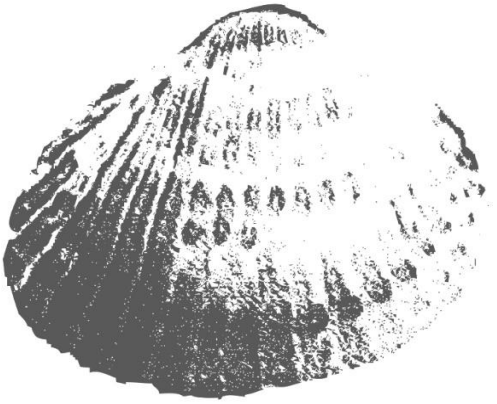
Microalgae diets for land-based aquaculture of the cockle *Cerastoderma edule*:



impacts of dietary fatty acids on growth

Isabel Reis Batista

Microalgae diets for land-based aquaculture of the cockle *Cerastoderma edule*: impacts of dietary fatty acids on growth

Isabel C. dos Reis Batista

Thesis committee

Promotor
Prof. Dr A. C. Smaal
Professor of Sustainable Shellfish Culture
Wageningen University

Co-promotors
Dr P. Kamermans
Researcher, IMARES, Wageningen UR

Dr M.C.J. Verdegem
Senior lecturer, Aquaculture and Fisheries Group
Wageningen University

Other members
Dr N. Nevejan, Ghent University, Belgium
Dr R. Robert, Ifremer, Plouzané, France
Dr L. Sijtsma, Wageningen University
Prof. Dr R.H. Wijffels, Wageningen University

This research was conducted under the auspices of the Graduate School of Wageningen Institute of Animal Sciences

Microalgae diets for land-based aquaculture of the cockle *Cerastoderma edule*:

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Thesis

submitted in fulfilment of the requirements for the degree of doctor
at Wageningen University
by the authority of the Rector Magnificus
Prof. Dr A.P.J. Mol
in the presence of the
Thesis Committee appointed by the Academic Board
to be defended in public
on Wednesday 11th of November 2015
at 1.30 in the Aula.

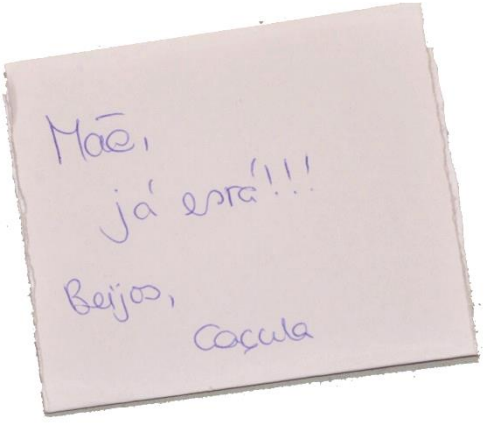
Isabel C. dos Reis Batista

Microalgae diets for land-based aquaculture of the cockle *Cerastoderma edule*: impacts of dietary fatty acids on growth,
132 pages.

PhD thesis, Wageningen University, Wageningen, NL (2015)

With references, with summary in English

ISBN 978-94-6257-587-5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Contents

Chapter 1: General Introduction

During the last decades, the interest in land-based marine aquaculture in Europe has been growing. Traditionally, fish and shellfish have been cultured (semi-) extensively, either separated or combined, in land-based systems in Southern Europe (mainly Portugal, Spain, France, Italy and Greece). However, an integrated land-based culture system combining fish, shellfish and algae has yet to be successfully implemented in Europe. In recent years, a pilot project in the Netherlands explored the feasibility of a system growing Dover sole (*Solea solea*), ragworms (*Neanthes viridus*), phytoplankton (various species) and bivalves (common cockle *Cerastoderma edule,* Linnaeus, 1758, mussels *Mytilus edulis*, Manila clams *Ruditapes philippinarum*) in an integrated system.

As part of this pilot project, this thesis focuses on the effect of dietary fatty acids on growth, survival and fatty acid composition of juvenile common cockles. New knowledge of the impact of dietary fatty acids on juvenile common cockle performance will generate insights allowing the development and optimization of its land-based culture. The assessment of the dietary modulation of the fatty acid content of the cockles will also be evaluated, as this is a relevant issue considering their role in human nutrition. The value of specific n-3 and n-6 unsaturated fatty acids (commonly known as omega-3 and omega-6) for human health has received a lot of attention in the last decades. The intake ratio of n-6/n-3 fatty acids is high in western society, due to diets rich in n-6 fatty acids and deficient in n-3 fatty acids. The n-6 fatty acids are typically provided by meat products, while the importance of eating fish products, a good source of n-3 fatty acids, is reduced in western society. Diets with high n-6/n-3 ratio and excess of n-6 fatty acids have been correlated to increased risks of cancer and cardiovascular, inflammatory and autoimmune diseases (Simopoulos, 2008). This reinforces the importance of increasing the consumption of n-3 fatty acids. Dietary n-3 fatty acids such as eicosapentaenoic acid (EPA, 20:5 n-3) and docosahexaenoic acid (DHA, 22:6 n-3) have been recognized as important for human health given their importance on brain development and function (e.g. Innis, 2007) and positive effects on prevention of cardiovascular disease (e.g. Kris-Etherton *et al.*, 2002, Breslow, 2006), cancer (e.g. Leitzmann *et al.*, 2004) and inflammatory and autoimmune diseases (e.g. Simopoulos, 2002). n-3 fatty acids, specifically the polyunsaturated fatty acids (PUFAs) such as EPA and DHA, are mainly made by aquatic primary producers such as microalgae and are subsequently accumulated throughout the aquatic food web (Gladyshev *et al.*, 2009), making seafood a major source of PUFAs in the human diet.

1. Coastal land-based aquaculture

Coastal land-based aquaculture refers to marine or brackish water inland aquaculture operations in tanks or ponds. Although most land-based aquaculture systems focus on monocultures, this research is associated with an integrated multi-trophic aquaculture (IMTA) system. In a land-based IMTA, organisms that occupy different trophic levels, such as fish, molluscs and algae are co-cultured (e.g. Neori *et al.*, 2004, Troell *et al.*, 2009) for environmental and economic benefits (e.g. Neori *et al.*, 2000, Troell *et al.*, 2003). When compared with open sea IMTA operations, the main disadvantages of a coastal inland IMTA include land use, facility and food production costs. A land-based IMTA recycles water, reduces overall water use and minimizes nutrient discharge, while maximizing profits by optimizing nutrient cycling. One possible concept of a land-based IMTA is to rely on nutrient-rich waste water from fish cultivation to grow algae (microalgae for suspension feeders or macroalgae for grazers) as feed for shellfish. This concept can, for instance, be fine-tuned to grow microalgae to feed filter-feeding shellfish. Three research projects have addressed intensive and semi-extensive land-based integrated aquaculture in Europe: the international projects GENESIS (Sustainable integrated marine multi-trophic aquaculture in Europe) and SEACASE (Sustainable Extensive and Semi-Intensive Costal Aquaculture in Southern Europe), and the Dutch project Zeeuwse Tong. While GENESIS and SEACASE aimed to improve existing culture systems throughout Europe, Zeeuwse Tong aimed at the development of a locally adapted land-based integrated multi-trophic aquaculture system. This PhD research contributed to the Zeeuwse Tong project.

Zeeuwse Tong (2007-2014) determined the technical and economic feasibility of a land-based IMTA for the co-culture of fish, ragworms, shellfish and microalgae (Stichting Zeeuwse Tong, 2012). The shellfish culture was based on the grow-out of juveniles into commercial size. An IMTA pilot farm was established in 2009 in Colijnsplaat in the Province of Zeeland, the Netherlands, where juvenile shellfish were co-cultured with ragworms in earth ponds supplied with seawater (Ketelaars and Ruizeveld de Winter, 2014). Shellfish was fed by natural phytoplankton community of the seawater that developed in the ponds due to the nutrient load of ragworm faeces as well as uneaten ragworm feed. This type of co-culturing has the advantage of reducing nutrient load of the output (due to recycling into microalgae) while reducing the costs of shellfish feed production. However, just as in any other land-based shellfish culture, there are other costs that need to be

considered when evaluating the economic feasibility of such a culture system. These are seed purchase, high seawater use and pumping, labour of seeding and harvesting. Furthermore, there are disadvantages of co-culturing shellfish with ragworms. The differences between the growth cycle of the ragworms (harvest 1 year after seeding) and Manila clam (typically 2 years) preclude a joint pond harvest, and the use of the natural phytoplankton community does not ensure the highest nutritional quality and quantity of the algae being consumed by the shellfish. The viability of land-based duo-aquaculture of shellfish and microalgae was also tested at two pilot locations in Wilhelminapolder and at Zeeland Aquacultuur (Smaal *et al*, 2014). At Wilhelminapolder mussels were grown in small tanks fed nutrient-rich ground water with natural phytoplankton communities. In this case, the mussel production was also affected by the nutritional quality as well as insufficient microalgae production to sustain shellfish growth. Zeeland Aquacultuur worked on the pond production of specific microalgae species usually used in shellfish hatcheries (mainly *Skeletonema* sp., but also *Chaetoceros* sp. and *Tetraselmis* sp.), that were then supplied to shellfish ponds (Roem van Yerseke & Prins en Dingemanse, 2014).The economic aspects of such culture system indicated that microalgae production is the major bottleneck for land-based shellfish culture, as the costs of algae production needed to produce shellfish was €2 per Kg of fresh weight, for Manila clam sold at €4,50 per kg fresh weight. The remaining costs such as purchase of seed and equipment, labour, electricity and use of water, for example, are not covered by the differences between feeding costs and market value. Optimisation of the algal production can lead to cost reduction.

2. Cockles

The common cockle (*Cerastoderma edule*) is a marine burrowing bivalve species (Figure 1), found in sandy intertidal and subtidal areas from the Barents Sea and the Baltic Sea in the North to Mauritania and West Africa in the South. Isolated populations are also found in the south-western Mediterranean (FAO, 2014). They are suspension feeders, i.e., they capture food with their gills (Barnes, 1980). Water containing oxygen and particles is brought into the gills by the inhalant siphon (Ruppert *et al.*, 2004). Cockles then filter organic (mainly phytoplankton) and inorganic particles larger than 3 µm (Dare *et al.*, 2004) into the frontal cilia, where they are grouped into mucus that is then directed towards the mouth and stomach.

This mucus is then consolidated, by its own protein matrix and by the enzymes that are produced by the surrounding sac, the style sac. The cilia of the style sac cause the crystalline style to rotate and release enzymes. The rotation of the crystalline style also allow the movement of the food particles into the stomach and the mixing of the enzymes with the stomach contents, starting the extracellular digestion. The heavy particles are then directed to the intestine and eventually expelled, whereas the fine particles are directed towards the digestive glands (Banes, 1980). Intracellular digestion of the fine particles occurs in the digestive gland, after which the material is directed into the intestine, where they are absorbed. Cockles can control their food intake either by changes in the filtration rate (Iglesias *et al.*, 1996) or by the production of pseudofaeces, which contain mainly sediment particles or excess organic matter and mucus that are expelled through the inhalant siphon (Ruppert *et al.*, 2004).

Figure 1: *Cerastoderma edule* and its natural habitat.

Cockles live up to 6 - 10 years and adults can reach 5 cm shell length (Reise, 1985). Sexual maturation and spawning occurs at different times between the northern and southern populations. Cockle populations in the North spawn between May and July (Newell & Bayne, 1980), producing pelagic larvae that settle in early summer, whereas gametogenesis of the cockle populations in the South starts in autumn and spawning occurs in March-April (Reise, 1985).

Major mortality risks for cockle populations are predation by starfish, flatfish, gulls and oystercatchers (Reise, 1985). Population survival is mainly affected by food availability, low temperatures during severe winters or summer heat and parasites (Reise, 1985). Nevertheless, Reise (1985) considers the common cockle as the most virile bivalve.

Common cockle fisheries have always been important in Europe, with a record landing of 106 494 tons, in 1987 (FAO, 2012). Although since then landings have declined steadily, reaching only 7 181 tons (live weight) in 2012, cockles are still an important economic resource (Figure 2). Cockle fisheries are located mainly in the United Kingdom, the Netherlands, Spain, Portugal and France. In the Netherlands, wild stocks of common cockle are fished in the Wadden Sea, the Voordelta, the Oosterschelde and the Westerschelde (Kamermans & Smaal, 2002). Due to environmental concerns, the mechanical dredging practice was banned in the Dutch Wadden Sea in 2004, and only hand dredging is allowed since**.** The highest cockle fisheries landings recorded in the Netherlands occurred in 1989 (76 349 tons), after which fisheries have drastically declined (Asch *et al.*, 2014). Cockle abundance follows a pattern of a high peak after massive spat fall and a subsequent decline of the stock until a new successful reproduction occurs (Beukema & Dekker, 2006). In 2013 the observed high peak of cockle abundance resulted in fisheries landings, which are restricted to a maximum of 5% of the total stock, of 8 080 tons of live weight (Asch *et al.*, 2014). The consumer interest and restrictions for fisheries on wild stocks make the common cockle an interesting candidate for land-based aquaculture. In 2012 aquaculture production of the common cockle amounted to 1826 tons in live weight, originating from France (1010 tons), Portugal (449.2 tons), Spain (43.5 tons) and United Kingdom (5 tons) (FAO, 2012). Currently, cockle aquaculture production consists of wild settled seed that is collected and transplanted to protected areas with adequate food supply for grow-out (e.g. Robert *et al.*, 2013).

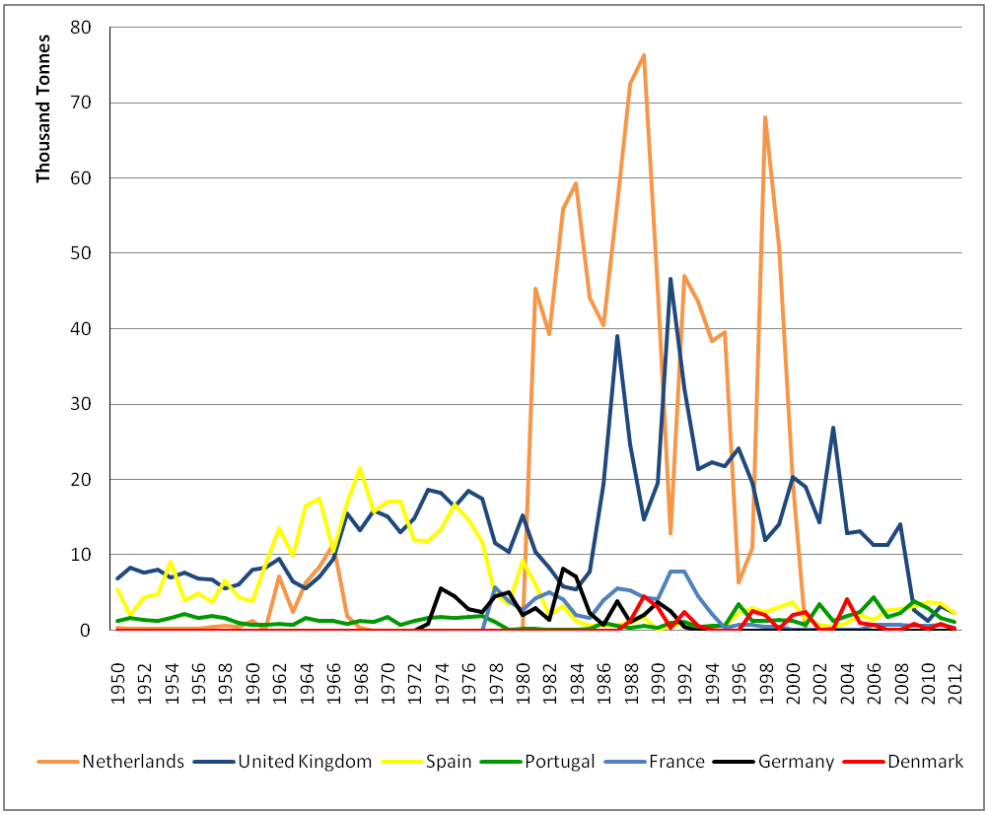


Figure 2: *Cerastoderma edule* landings (thousand Tonnes of live weight) per production area from 1950-2012. Data retrieved from FAO Fishstat Global Capture Production.

In these cases, the animals are grown under natural conditions and are therefore exposed to natural food supply and potentially extreme environmental conditions such as exposure to high or low temperatures at low tide in the summer and winter respectively, as well as parasite and pathogens (Longshaw & Malham, 2013), predators (Malham *et al.*, 2012), storm damage and food shortage. When animals are grown-out in land-based ponds it is possible to guarantee a stable and diverse (different species) food supply as well as favourable environmental conditions, by means of controlling water depth (thus controlling temperature) and carrying out water treatment, thus lowering the risk of mortality.

With the development of aquaculture techniques, nutrition (e.g. Knauer & Southgate, 1999, Marshall *et al.*, 2010) of oysters (e.g. Ronquillo *et al.*, 2012), mussels (e.g. Sánchez-Lazo & Martínez-Pita, 2014) and clams (e.g. Albentosa & Moyano, 2008)

has become a very important topic. Less is known about the nutrition of *C. edule*, although a few studies focused on natural food (e.g. silt, mixtures of silt and microalgae, mono-cultures of microalgae and detritus) acquisition (Iglesias *et al.*, 1996, Navarro *et al.*, 1992) and digestion and absorption (Ibarrola *et al.*, 1996, 1998, 2000, 2008). It is important to point out that dietary requirements of bivalves are species specific (Albentosa *et al.*, 1996a) with each development phase having its own specific requirements (for example, lipids for larvae (Marshall *et al.*, 2010), proteins for juvenile *Mytilus trossulus* (Kreeger & Langdon, 1993) and carbohydrates and essential fatty acids for juvenile *Placopecten magellanicus* (Parrish *et al.*, 1998). In the literature, different developmental phases are not clearly defined, neither in age nor sizes. In this thesis, the development stages of shellfish were defined as spat (<1 mm), seed (1-5 mm), juveniles (>5mm) and adults (>15mm). Juveniles are used for the grow-out of shellfish in the land-based aquaculture, and the growth, survival and composition of juvenile cockles was therefore the focus of the thesis. Juveniles also have growth potential, allowing for short-term experiments in which significant growth can be observed. Most literature on nutrition for culturing shellfish focusses on animals that are kept under controlled conditions in in a hatchery or nursery (broodstock, larvae and spat) and much less in known about grow-out of juveniles as this phase usually takes place in the field.

3. Fatty acids in bivalves

Dietary lipids and fatty acids are considered important factors in bivalve nutrition. Studies on the impact of dietary fatty acids on growth and development have mainly focused on larvae (e.g. Fernández-Reiriz *et al.*, 2011), spat (e.g. Albentosa *et al.*, 1996a) and seed (Fernández-Reiriz *et al.*, 2006), while studies on dietary requirements of juvenile common cockles (shell size 5mm and above) are lacking. In the studies determining which factors contribute to food quality for bivalves, special attention has been given to the n-3 PUFAs such as eicosapentaenoic acid (20:5n-3,EPA) and docosahexaenoic acid (22:6n-3, DHA) (Langdon & Waldock, 1981, Chu & Webb, 1984, Enright *et al.*, 1986), and n-6 PUFAs such as arachidonic acid (20:4n-6, ARA) (Milke *et al.*, 2008, Pettersen *et al.*, 2010). Fatty acid are designated by their number of carbons, followed by the number of carbon-carbon (or double bonds) in each molecule. For example, palmitic acid is a saturated fatty acid (no carbon-carbon bonds) with 16 carbons, 16:0, whereas EPA has 20 carbons and

5 carbon-carbon bonds, 20:5n-3. The terminology “n-3” and “n-6” (also known as ω3 and ω6) is used to indicate the position of the first carbon-carbon bond, either in the third or sixth carbon-carbon bond, respectively, from the methyl end of the carbon chain (Figure 3). The “Δ” numbering indicates the number of carbon-carbon bond from the carboxyl-terminus (COOH-terminus).

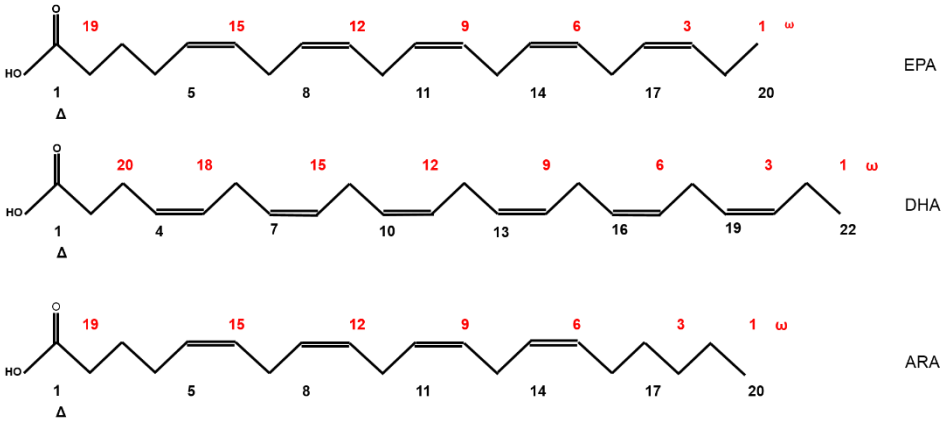


Figure 3: Chemical structure of eicosapentaenoic acid (20:5n-3, EPA), docosahexaenoic acid (22:6n-3, DHA) and arachidonic acid (20:4n-6, ARA). Numbers in red indicate the first carbon-carbon number, from the methyl end and black numbers indicate the number of carbon, counted from the carboxyl-terminus.

EPA and DHA are important in the regulation of the membrane fluidity and functionality (Knauer & Southgate, 1999), whereas EPA and ARA are important precursors for the production of eicosanoids. The latter play an important part in the immune response of bivalves (Delaporte *et al.*, 2006, Hurtado *et al.*, 2009). Previous studies on the clams *Ruditapes decussatus* (Albentosa *et al.*, 1996b) and *R. philippinarum* (Caers *et al.*, 1998) have suggested that EPA and/or DHA are important for growth. In general, EPA and DHA are considered essential dietary ingredients in diets for growth and development of bivalves (Langdon & Waldock, 1981, Chu & Webb, 1984, Enright *et al.*, 1986) since most bivalve species are considered to have no or limited capacity to synthesize *de novo* EPA and DHA from their precursors 18:3n-3 (De Moreno *et al.*, 1976; Langdon & Waldock, 1981).It has been suggested that presence of one of these fatty acids in the diet is sufficient to fulfil the n-3 PUFA requirements (Langdon & Waldock, 1981). The major pathways of fatty acid desaturation and elongation in animal tissues are shown in Figure 4.

Black arrows indicate major pathways in animal tissues and blue arrows indicate the pathways to the most encountered non-methylene-interrupted (NMI) fatty acids in lipids from marine invertebrates.

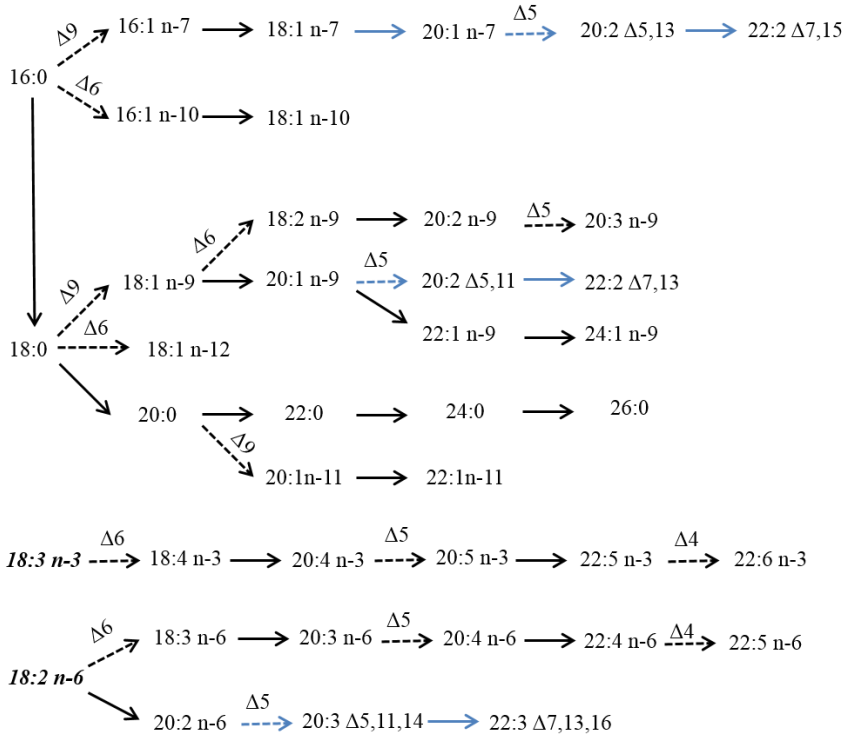


Figure 4: Fatty acid synthesis in animal tissue (black arrows) and in marine invertebrates (blue arrows). Plain arrows indicate chain elongation and dashed arrows desaturation. Adapted from Cook, (1991), Garrido & Medina (2002) and Zhukova, (1986). Eicosapentaenoic acid (20:5n-3, EPA); Docosahexaenoic acid (22:6n-3, DHA); Arachidonic acid (20:4n-6, ARA).

These NMI fatty acids are a result of elongation of dietary fatty acids and are commonly found in bivalves (Zhukova, 1986; Garrido & Medina, 2002). NMI fatty acid synthesis might be correlated with periods of starvation (Klingensmith, 1982) and their synthesis strongly depends on dietary fatty acid composition (Delaporte *et al.*, 2005). It has been suggested that 20 and 22-NMI could have similar functions for the stability and fluidity of the membranes as 20:5n-3 and 22:6n-3, and can be used as a replacement for these fatty acids when they are not provided by the diet (Klingensmith, 1982, da Costa *et al.*, 2011). However, the reasons for synthesis and accumulation of these NMI fatty acids is still not completely understood or explained

(Delaporte *et al.*, 2005; Barnathan, 2009). This is particularly true for the common cockles, for which this topic has not been studied so far.

4. Microalgae

Microalgal production is an important component of aquaculture, although production data are not always reported since the algae are not the end product (Neori, 2012). Microalgae are the preferred food source for filter-feeding bivalves, and the source of nutrients for bivalves in land-based aquaculture systems. The microalgae composition varies greatly between different classes and culture conditions, but generally microalgae are built by proteins (12–35% of dry weight), lipids (7.2–23% of dry weight) and carbohydrates (4.6–23% of dry weight) (Becker, 2004).

Alternatives to live microalgae have been successfully tested as partial replacements in small-scale experiments with the clam *R. decussatus* (e.g. 25% algae+75 % industrial cheese whey, Enes & Borges (2003)), the oyster *C. gigas* (50 % algae + 50% balanced micro-particulate diet, Badillo-Salas *et al.*, 2009) and the mussel *M. galloprovincialis* (algae+2,8% live weight formulated feed, Nevejan *et al.* (2007)). However, 100% replacement of the algae in shellfish diets has never been achieved.

Microalgae used in shellfish aquaculture are chosen based on their non-toxicity to the animals and final consumers, their size (adequate to each shellfish development stage) and nutritional composition (e.g. de Pauw *et al.*, 1984).The most commonly used algal species fed to shellfish belong to the classes Bacillariophyceae (diatoms), Chlorophyceae, Prasinophyceae and Prymnesiophyceae (Muller-Feuga, 2005), due to their size and specific nutritional compositions. Flagellated species are usually combined with diatoms. Mostly a mix of algal species is necessary to fulfil the nutritional requirements of shellfish for protein, carbohydrates, lipids, vitamins and minerals, which vary between shellfish species and development stages. Of all the microalgal components, lipids have received a lot of attention due to their role for energy and as nutrient supply in marine ecosystems (Guschina & Hardwood, 2009) .Microalgae are also the source of molecules that are considered essential when the animals have little or no capability of *de novo* synthesize them, such as sterols and fatty acids has been identified. Sterols are important constituents of the cellular membranes, and are provided by the microalgae, as marine bivalves have little or

no capacity of *de novo* synthesise these molecules (Soudant *et al.*, 1998). Fatty acids, specifically polyunsaturated fatty acids, have been correlated with survival (e.g. Fernández-Reiriz *et al.,* 2011) growth (e.g. Ronquillo *et al.,* 2012) and reproduction (e.g. Hendriks *et al.,* 2003). Using a mixture of different live microalgae is considered the best option to provide a diet with required fatty acid profile (e.g. with EPA and DHA or with only EPA). Each class of microalgae comes with a characteristic fatty acid profile, offering a limited range of essential fatty acids. Diatoms such as *Skeletonema costatum, Thalassiosira pseudonana*, *Chaetoceros muelleri* and *Phaeodactylum tricornutum* are known to have high EPA contents (e.g. Volkman *et al.*, 1989). Chlorophyceae such as *Brachiomonas submarina* and *Dunaliella tertiolecta* contain no DHA, whereas Prasinophyceae such as *Pyramimonas parkeae* have a high DHA content (Dunstan *et al.*, 1992). *Tetraselmis suecica* is an exception, as this Prasinophyceae has high levels of EPA and no DHA (Volkman *et al.*, 1989).

Microalgae are susceptible to changes due to culture conditions, such as nutrient concentrations (e.g. Harrison *et al.*, 1976), temperature (e.g. Eppley, 1972), light (e.g. Goldman, 1979) and salinity (e.g. Brand, 1984). Microalgae can be cultured indoor under controlled light and temperature, or outdoor under seasonal and diurnal fluctuations of light and temperature (Lee, 2001). Microalgae can be produced in batch culture, semi-continuous culture or continuous culture. Batch culture is the most labour intensive, since new cultures have to be started every 10-14 days, as nutrients are quickly depleted (Hoff & Snell, 1987). For the development of land-based shellfish aquaculture, large-scale microalgae production is of great importance. Large-scale microalgae cultures can be produced in either open-systems such as raceways and open ponds, or closed-systems such as photobioreactors or fermenters. In both systems, cultures can be grown indoors or outdoors. The different systems give different productivities. For example, a high-tech closed tubular reactor can produce 90 ton ha^{-1} $year^{-1}$ (Tredici & Zittelli, 1998), while yields for low-tech open ponds are 27 ton ha^{-1} $year^{-1}$ (Jimenez et al., 2003). As the value of shellfish is lower than some other algae products (e.g. astaxanthin) a low cost system such as an open pond culture is needed. For large-scale microalgae cultures, the water supply and the culture media can be an important cost fact, for example in locations where there is no easy/inexpensive access to a suitable water source such as clean nutrient-rich groundwater. Farmer have then added costs for water treatment and addition of lacking macro or micro nutrients. Apart from the

culture medium price, which can be prohibitive high in large operations, easy and fast medium preparation and application helps to reduce costs. Most recipes for microalgae culture media contain all necessary macro and micro nutrients, as well as vitamins (Grobbelaar, 2004), which may sometimes be supplied in excess as default in order to assure maximum productivity. The availability of an effective simplified culture medium (with less components, different nutrient source and easier preparation) would greatly improve the cost-efficiency of microalgae production for land-based aquaculture.

5. Aims, research questions and outline of the thesis

Grow-out of juvenile cockles in a land-based aquaculture system requires the supply of live algae diets as source of nutrients and energy. However, the dietary requirements of these animals are still not known.

Lipids have been one of the most studied nutritional aspect of microalgae. Within the lipids, the importance of fatty acids has been established for (i) larvae and seed of oysters (e.g. Rivero-Rodriguez *et al.*, 2007) and clams (e.g. Fernández-Reiriz *et al.*, 2006), and (ii) bivalve broodstock conditioning (e.g. González-Araya *et al.*, 2011), but not for juvenile cockles. Although there are other nutritional aspects that may play a role in the growth of juvenile cockles, one of the aims of this thesis was to further understand the role of dietary fatty acids in survival, growth and composition of the animals (shell size above 5-mm). For this, endemic microalgae species were chosen due to their adequate size for shellfish growth and use in aquaculture. Several different mono-algal and mixed algal diets, with different fatty acids profiles were designed and fed to juvenile cockles in short-term experiments.

Apart from identifying the importance of dietary fatty acids for juvenile cockles, it is of extreme importance to solve one of the bottleneck of the development of land-based shellfish culture (Roem van Yerseke & Prins en Dingemanse, 2014) – the costs associated with the large-scale production of the microalgae diets. The mass production of an affordable high quality, diet for juveniles remains a priority and the second aim of this thesis was therefore to overcome this bottleneck. The use of an affordable, practical, simplified culture media using less nutrients while maintaining quality of nutritionally interesting microalgae was investigated. In order to achieve the research goals, the following research questions were formulated:

1. How do live microalgal diets with similar total lipids and total PUFA contents and different composition in terms of fatty acid profiles (amounts EPA, DHA and ARA) influence growth and survival of *C. edule* juveniles?

Hypothesis: The supply of fatty acids (namely EPA, DHA and ARA) through microalgae diets are a requirement for the growth and survival of juvenile cockles.

In order to test this hypothesis, mono-cultures of indigenous microalgal species were monitored for their nutritional quality and used to formulate live mixed microalgal diets differing only in ARA, EPA, and DHA content, while maintaining similar proximate composition. Linear programming was used to formulate the live diets (Chapter 2). The formulated diets were fed to *C. edule* juveniles, monitoring growth and survival, and analysing the fatty acid composition. The importance of dietary EPA, DHA or ARA for growing *C. edule* juveniles was analysed (Chapter 3).

2. a) Is EPA or DHA or the combination of both essential for juvenile cockles? b) What is the impact of dietary fatty acids on fatty acid profiles of the cockles?

Hypothesis: EPA or DHA can be sufficient for short term growth of cockles, given the structural role of these fatty acids. The dietary fatty acids will have an impact on the fatty acid profiles of the cockles, with increases of EPA and DHA in the cockles fed diets rich in these fatty acids.

To describe and understand the use of dietary fatty acids by *C. edule* as well as the importance of EPA and DHA for growth and survival of this species, microalgal diets with clear deficiencies in specific fatty acids were fed to juvenile cockles. To investigate the impact of the dietary fatty acids on cockle composition, the polar and neutral lipid profiles in juvenile cockles was described (Chapter4).

3. Are mixed live microalgae diets combining different taxonomic classes better than single class diets?

Hypothesis: Mixed diets of two different microalgae classes are beneficial for short term growth of cockles.

To determine the effect of mixed taxonomical classes on growth of juvenile cockles mixed diets consisting of either two Chlorophyceae species or of one Bacillariophyceae (diatom) and one Chlorophyceae species were fed to juvenile cockles and their growth and survival was recorded (Chapter 4).

4. (a) Can cockles produce EPA and DHA from the precursor 18:3n-3? (b) Can cockles, like other marine bivalves, *de novo* produce NMI fatty acids? (c) Is the NMI synthesis a direct result of the dietary fatty acid composition or a replacement for lacking EPA or DHA in the diet?

Hypothesis: Cockles, like other marine invertebrates, cannot synthesize EPA or DHA from their precursors at a rate high enough to overcome dietary shortage. However, cockles can produce *de novo* NMI fatty acids that could be used to overcome EPA and/or DHA dietary shortage.

To understand the use of dietary fatty acids for *de novo* synthesis (EPA, DHA or NMI fatty acids) by juvenile *C. edule* the animals were fed live microalgal diets with clear deficiencies in certain fatty acid contents (no DHA, no long chain C20 fatty acids) (Chapter 4).

5. Is the production and the duration of a batch culture of *C. muelleri* different between the standard Walne's medium and a simplified medium?

Hypothesis: Diatom cultures grown with simplified culture medium (with less nutrients but with adequate nutrient ratios) can have similar lipid content and productivity as Walne's medium.

To evaluate *C. muelleri* microalgae response to a simplified, less expensive medium suitable for large-scale microalgal production for the grow-out of cockles, three simplified media were formulated and compared to the control Walne's medium. All simplified media contained fixed concentrations of P, Si, Fe, Mn and vitamins, but used either nitrate or ammonium as N-source, with different N: P ratio. The evaluated parameters were growth, productivity (organic matter), culture duration and lipid composition of *C. muelleri* in batch culture (Chapter 5).

Chapter 2: Formulation of a live microalgae diet to study the importance of fatty acids for shellfish culture using linear programming

Reis Batista, I., Kamermans, P., Verdegem, M.C.J., Smaal, A.C.

Abstract

Bivalve aquaculture relies on the production of large quantities of live microalgae as live diets. Least cost linear programming is commonly utilized to formulate animal diets, but has not yet been applied to formulate bivalve diets. For bivalve growth, the types of dietary fatty acids seem to be more important than the total lipid content. The objective of this study was to grow monocultures of algae under controlled conditions and to formulate live algal diets for bivalves with specific eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA) and arachidonic acid (ARA) contents using least-cost linear programming. Cultures of marine diatoms *Skeletonema costatum*, *Phaeodactylum tricornutum*, *Thalassiosira pseudonana*, *Chaetoceros muelleri* and flagellated species *Pyramimonas parkeae*, *Tetraselmis suecica* and *Brachiomonas submarina* were used. These cultures were sampled in the exponential phase to determine their dry weight (DW), organic matter (OM), lipid and fatty acids (FA) composition. These constraints were used in a least-cost programming software, with the production effort (necessary to produce 1 g of DW of the microalgae) as an optimizing goal. Three diets were designed, with similar OM, lipid and total FA contents but different EPA, DHA and ARA contents. There are limitations to the use of this technique when dealing with live feed, since each biochemical components cannot be separated within each algae. However, with some flexibility on minimum values for constraints differences in biochemical composition of the microalgae allowed the formulation of three diets with different fatty acid contents. The present approach of using linear programming to develop shellfish diets based on live microalgae has proven to be efficient.

Keywords: Bivalves; live microalgae diet; diet formulation; fatty acids; linear programming

1. Introduction

In commercial bivalve hatcheries and nurseries, larvae and juveniles are fed cultured live algae. The use of alternatives to live algae diets such as liquid concentrates of non-living phytoplankton, live refrigerated laboratory grown mono-species cultures (Pales Espinosa & Allam, 2006), yeast diets (Coutteau *et al.*, 1994) and wheat-germ flour (Fernandez-Reiriz *et al.*, 1998) could only be used as partial live food replacements for some shellfish species (Spencer, 2002). Microalgae production is expensive, accounting for 40 % of the costs to grow 5-mm bivalves (Helm *et al.*, 2004). To reduce expenses, the subsequent grow-out is mostly done relying on natural phytoplankton. Trials to culture bivalves up to commercial size in ponds are ongoing in the Netherlands as part of the development of a commercial land-based integrated multi-trophic aquaculture production system (Stichting Zeeuwse Tong, 2012). One of the aims of the project was the development of land-based grow-out culture of bivalves. By growing bivalves in coastal ponds, farmers have the option to optimize bivalve food quality by composing diets for example rich in essential fatty acids. Microalgae used for bivalve diets need to be non-toxic, digestible, nutritious and of adequate size (Caers *et al.*, 1998). Bivalves are often fed a mixture of diatoms and flagellated species, as these groups (Chlorophyceae, Prymnesiophyceae, Prasinophyceae) compensate deficiencies in nutrient composition that are otherwise commonly found in mono-algae diets (Volkman *et al.* 1989, Brown 1997, Spencer 2002, Helm *et al.* 2004). Mixed diatom-flagellated diets used today in hatcheries are based on trial and error and previous results with similar species.

Fatty acids have been the focus of larvae and post-settled bivalve nutrition for decades (e.g. Chu & Dupuy, 1980, Langdon & Waldock, 1981). For growth of early staged of bivalve growth, the types of dietary fatty acids seem to be more important than the total lipid content (Ackman, 1983, Webb & Chu, 1981). The limited capability of many marine animals to synthesize n-3 polyunsaturated fatty acids (PUFAs) such as eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3) from their precursor 18:3n-3 (De Moreno *et al.*, 1976; Langdon & Waldock, 1981) indicate that EPA and DHA need to be supplied through the diet. These fatty acids are important in the regulation of the membrane fluidity and functionality (Knauer & Southgate, 1999), and are believed to have an impact on shellfish growth. The supply of dietary EPA and DHA has been considered essential for growth and development of bivalves (Chu & Webb, 1984, Enright *et al.*, 1986), although the supply of either EPA or DHA seem to be sufficient to fulfil the n-3 PUFA requirements

of some species (Albentosa *et al.*, 1996b). In addition, n-6 PUFAs are also relevant in bivalve nutrition, especially arachidonic acid (ARA, 20:4n-6), that similarly to EPA is important as a precursor for the production of eicosanoids. However, the amount of specific fatty acid requirements for shellfish are unknown, and are considered species specific (Albentosa *et al.*, 1996a).

Least-cost linear programming is commonly used to formulate shrimp (e.g. Gokulakrishnan & Bandyopadhyay, 1995), fish (e.g. Eguia *et al.*, 2000) and livestock diets (e.g. Peña *et al.*, 2007), but has not yet been applied to formulate bivalve diets. Least-cost linear programming generates solutions that meet minimum requirements established about quality and quantity of dietary ingredients and the overall nutritional quality required by the user at the lowest cost possible. Working with live microalgae cultures complicates diet formulation, as the composition of pure algae cultures varies with irradiance (Blanchemain & Grizeau, 1996), nutrient composition of the culture medium (Wikfors, 1986), length of the light-dark cycle (Brunet *et al.*, 1996), and temperature (Araujo & Garcia, 2005). Nevertheless, when culturing mono-species batches of algae under controlled conditions, the composition should be sufficiently uniform to be able to formulate high quality diets.

The objective of this study was to formulate live microalgae diets to study the importance of specific fatty acids for shellfish culture using linear programming. Monocultures of seven indigenous microalgae of different classes were grown under controlled conditions to determine their dry weight, lipids and fatty acids contents. The results were then used as parameters in linear programming to formulate three live diets with similar organic weight, lipids and PUFA contents but different EPA, DHA and ARA contents.

2. Material and Methods

Microalgae

The marine diatoms *Skeletonema costatum* (Center for Culture of Marine Phytoplankton -CCMP 1332), *Phaeodactylum tricornutum* (Culture Collection Yerseke -CCY 162), *Thalassiosira pseudonana* (CCMP 1013), *Chaetoceros muelleri* (CCMP 1316) and the flagellated Prasinophyceae *Pyramimonas parkeae* (CCMP 724) and Chlorophyceae *Tetraselmis suecica* (CCY 9913) and *Brachiomonas submarina* (CCY 0810) were selected because they can be grown easily, are

indigenous in the study area of the Oosterschelde estuary, SW Netherlands, and of adequate size for bivalves. *T. pseudonana*, *S. costatum*, *C. muelleri*, *P. tricornutum* and *T. suecica* are amongst the commonly used species in bivalve hatcheries (Coutteau & Sorgeloos, 1992) and *B. submarina* and *P. parkeae* are used in fish hatcheries (Becker, 2004; Coutteau, 1996). Species were cultured three 10-L batch cultures per species, starting each with a 1-L inoculum. The culture medium consisted of 0.2 µm filtered seawater (30‰) with modified Walne's medium (Walne, 1970): different concentration of vitamin B12 (10 mg/100mL), B1 (10 mg/100mL) and addition of vitamin H (200 µg/100mL). The medium for the diatoms was further enriched with silicate (4 mL of Na_2SiO_3 (80 g L^{-1}) per litre of culture). The cultures were grown in a climate room at 19 °C, continuously aerated and under 24 hours continuous light (66 µmol photon m^{-2} s^{-1}). Daily aliquots of each 10-L batch culture (7 species, 3 replicates per species) were collected to determine the cellular concentration (cells/mL) of each culture using a Bürker haemocytometer.

Analysis

When the cultures reached the early exponential phase, samples were taken for determination of dry weight, organic matter (OM), lipid and fatty acids (FA). The exponential phase was chosen for the characterization of the cultures since microalgae are collected for feeding at this phase. In this phase the cultures are usually still healthy, the biomass is abundant and there are fewer risks of the culture collapse. For dry weight and organic matter determination samples were filtered through pre-ashed (in the oven at 450ºC for 6 hours) and pre-weighted fibre-glass Whatman GF/C 47mm filters. The filters were subsequently rinsed with 0.5 M ammonium formate to remove residual salts, dried at 70°C until constant weight to calculate dry weight and ashed at 450°C until constant weight to measure ash weight. Organic matter was calculated as the difference between dry weight and ash weight. For lipid and fatty acid composition samples were filtered through fiberglass Whatman GF/F 47mm filters used in each sample. Total lipids were extracted according to Bligh & Dyer, (1959) and quantified with the charring method of Marsh & Weinstein, (1966). For the determination of fatty acids lipids were extracted according to Bligh & Dyer, (1959) to obtain total lipid extracts. Total lipid extracts were derivatized by mild methanolysis to yield fatty acid methyl esters (FAME). Gas chromatograph-flame ionization detection (GC-FID; Thermo GC Ultra) with a 50m*0.32mm*0.25µm BPX70 column was used. The identification of the fatty acids

from peaks of interest was done by comparison of equivalent chain length using FAME 12:0, 16:0 and 19:0 as retention time markers (ECL values, Boschker *et al.*, 1999, Boschker *et al.*, 2005) and confirmed by gas chromatography-mass spectrometry (GC-MS) with reference standards or culture samples. Fatty acid concentrations of total lipids were expressed on dry weight basis as mg of C per g^{-1} of dry weight.

Diet formulation

A software package for solving both linear programming (LP) and mixed integer linear programming (MILP) problems was used (OM Partners computer package). To run this program it is minimally required to define an optimization goal. Constraints are optional and should not be too restrictive so a solution can be found. Costs are of paramount importance in feed formulation. For shellfish, large scale feed production is not applied, as the grow-out phase takes place in the field. Therefore, the optimization goal used was to minimize the diet production effort. Production effort (Peffort) combines time (days in exponential phase of growth), medium (Mfactor; 1 for flagellated species and 1.1 for diatoms) and the culture volume (Vol, (mL)) necessary to produce 1 g of dry weight of algae (Formula 1).

Formula 1: $Peffort = time \times Mfactor \times Vol$

The constraints focused on the lipids, OM, EPA, DHA, ARA and total fatty acid concentrations and the n-6/n-3 ratio. Three diets with similar OM content and n-6/n-3 ratio were formulated, each diet being a mix of two algae species. Additional constraints were used to target differences in EPA, DHA and ARA contents between diets: a high EPA, low ARA and no-DHA diet were formulated. The constraint boundary values were selected based on the microalgae used. Average values of OM, lipids, total PUFA, ARA, EPA and DHA of each microalgae species were calculated. OM and lipids content of the diets were set by a minimum boundary defined as the average value of organic matter and lipid of the all microalgae used. The n6/n3 ratio values were set to vary around the values considered suitable for shellfish growth (0.8>n6/n3 ratio>0.1) (Webb & Chu 1981, Fernandez-Reiriz *et al.* 1998). Minimum and maximum boundaries were set for total PUFA and n6/n3.

Total PUFA boundaries varied between the average of total PUFA of the microalgae and a total PUFA concentration 1.5 times higher than the average. In order to have high values of EPA, DHA and ARA in the high-EPA diet the average values of EPA, DHA and ARA of all the microalgae were multiplied by the factor 2. Whenever a

solution was impossible (problem unfeasible), the constraints for EPA, DHA and ARA were relaxed by 25% until a solution was obtained. Diet no-DHA was bounded by the DHA concentration (DHA=0) and EPA and ARA value higher than the average value of this component. The low-ARA diet was bounded by EPA values higher than its average, DHA values twice as high as the average and ARA values between 25% and 50% of the average value of all microalgae. Whenever the problem was unfeasible the value for EPA constraint was lowered by 25% until a feasible solution was obtained. When working with live microalgae the different constraints are impossible to separate, and priority choices need to be made. It was considered a priority choice to lower only EPA constraint boundaries as a first step, since the average EPA concentration of all microalgae was much higher than the DHA or ARA average concentrations.

Statistical analysis

Significant differences ($P<0.05$) in composition between the microalgae species were determined by One-Way ANOVA (independent samples, normal distribution) with a Games-Howell post-hoc (no homogeneity of variances).

3. Results

Microalgae cultures

The cultures were sampled until the stationary phase at day 14 for all species, except for *S. costatum* (10 days), *T. pseudonana* (12 days) and *T. suecica*, which were still in the late exponential phase when the culture was terminated on day 16 (Table 1). Significantly higher cellular concentration was achieved in *P. tricornutum* cultures ($11*10^6$ cells mL^{-1}), whereas the lowest cellular concentration was registered in *B. submarina* cultures ($3.3*10^5$ cells mL^{-1}. The flagellated species reached the exponential phase later (8 to 10 days) than any diatom, increasing their production effort of producing 1 gram of dry weight to values higher than Peffort = 38862 . The exception of lower production effort was observed for *P. tricornutum*, due to its heavy cells and relatively short time to reach the desired phase (Peffort = 50156). There were significant differences in the lipid content between the different microalgae. Higher values of lipids were found in *C. muelleri* (519.7 mg g^{-1} DW), *B. submarina* (496.2 mg g^{-1} DW) and *P. parkeae* (226.7 mg g^{-1} DW).

Table 1: Mean and standard error (n = 3) of the cellular concentration (10^5 cell mL^{-1}), dry weight (10^{-4} g mL^{-1}), organic matter and lipids (mg g^{-1} DW), arachidonic acid (ARA, 20:4n-6), eicosapentaenoic acid (EPA, 20:5n-3), docosahexaenoic acid (DHA, 22:6n-3), n-3, n-6 fatty acids, total PUFA (T.PUFA) and total fatty acids (T.FA) (mg C g^{-1} of dry weight) and ratio between the total n-6 and n-3 fatty acids (n-6/n-3) of the exponential phase of growth of *Skeletonema costatum* (SC), *Thalassiosira pseudonana* (TP), *Chaetoceros muelleri* (CM), *Phaeodactylum tricornutum* (PT), *Pyramimonas parkeae* (PP), *Brachiomonas submarina* (BS) and *Tetraselmis suecica* (TS). The time (days in the exponential phase) and the production effort (P effort, product of Time with a price factor for the medium and the volume of culture (*Vol*, mL*)* necessary to produce 1 g of dry weight) are shown. The different superscripts represent significant differences (One-Way ANOVA, Games-Howell post-hoc test, P<0.05).

	SC	*TP*	*CM*	*PT*	*PP*	*BS*	*TS*
CellCon	11.4±4.3^{a}	9.6±3.18 ^{a}	40.0±7.5 ^{a}	107.7±35.5 ^{a}	4.3±0.9 ^{a}	3.28±0.9 ^{a}	10.5±1.0 ^{a}
DW	2.0±0.3 ^{a}	1.1±0.6 ^{a}	1.40±0.31 ^{a}	2.7±0.8 ^{a}	0.8±0.2 ^{a}	1.78±0.7 ^{a}	0.8±0.1 ^{a}
OM	467.2±140.8^{ab}	591.3±5.4^{b}	785.0±45.2^{ab}	866.0±23.4^{a}	734.3±57.3^{ab}	806.2±10.4^{a}	797.0±52.4^{ab}
Lipids	177.7±16.1^{b}	165.0±29.1^{b}	519.7±114.0^{a}	226.7±33.4^{b}	405.7±111.7^{a}	496.2±127.3^{a}	147.8±37.7^{b}
ARA	0.0±0.0	0.1±0.0^{c}	1.6±0.9^{a}	0.4±0.6^{bc}	0.0±0.0	0.9±0.1^{ab}	0.11±0.0^{bc}
EPA	0.03±0.04^{d}	4.6±0.6^{b}	9.9±0.8^{a}	4.0±0.6^{b}	0.13±0.0^{cd}	0.3±0.03^{cd}	1.2±0.3^{c}
DHA	0.02±0.03^{b}	0.6±0.1^{b}	1.6±0.3^{a}	0.8±0.1^{b}	1.8±0.8^{a}	0.0±0.0	0.0±0.0
n-3	0.08±0.07^{d}	9.4±1.1^{c}	12.2±1.0^{c}	5.5±0.3^{d}	21.7±6.5^{b}	33.0±1.6^{d}	13.4±4.5^{abc}
n-6	0.01±0.01^{c}	0.2±0.0^{cd}	1.8±1.0^{b}	1.3±0.2^{b}	1.6±0.4^{b}	3.3±0.6^{a}	1.0±0.2^{bc}
n-6/n-3	0.05±0.07^{c}	0.02±0.00^{c}	0.2±0.2^{b}	0.2±0.0^{a}	0.01±0.0^{c}	0.1±0.0^{b}	0.1±0.04^{bc}
T.PUFA	0.3±0.1^{d}	12.2±1.5^{c}	25.7±3.2^{b}	9.4±1.9^{c}	29.5±8.9^{bab}	38.5±2.6^{a}	15.1±4.4^{bc}
T.FA	1.3±0.4^{d}	38.0±6.7^{bc}	70.4±3.9^{a}	34.3±3.5^{c}	41.4±11.2^{abc}	55.2±4.5^{ab}	24.0±4.9^{c}
Time	5.3±0.5^{c}	3.8±0.63^{c}	5.1±0.6^{c}	7.3±0.7^{bc}	9.5±0.7^{b}	8.6±0.8^{b}	10.2±1.6^{ab}
P effort	27643.3	20507.8	27376.4	50155.6	38861.7	55930	46956.4

A similar pattern was observed in the fatty acid contents of the microalgae. *T. suecica* and *B. submarina* cultures had no DHA, whereas *S. costatum* had no ARA. With the expectation of *S costatum*, diatom cultures had higher EPA concentrations than any of the other tested species. Highest values were found in *C. muelleri* cultures (9.9±0.8 mg C g^{-1} of DW), where high DHA concentrations were also present (1.6±0.9 mg C g^{-1} of DW). In terms of DHA concentrations, higher concentration were found in *P. parkeae* (1.6±0.9 mg C g^{-1} of DW) and *C. muelleri*, whereas DHA contents of *P. tricornutum, S. costatum* and *T. pseudonana* were low.

Diet formulation

Minimum boundaries for organic matter (OM>720 mg g^{-1} DW) and lipid content of the diets (Lipids>300 mg g^{-1} DW) were defined as the average value of organic matter and lipid of the all microalgae used. Total PUFA concentrations of all diets designed were within the defined range (Table 2).

Table 2: Constraints used in the formulation of the three diets (high EPA, low ARA and no DHA). Organic matter, lipids, total PUFA, arachidonic acid (20:4n-6), eicosapentaenoic acid (20:5n-3), docosahexaenoic acid (22:6n-3) are expressed in mg C g^{-1} of dry weight.

	OM	Lipids	PUFA	*n*6/ *n*3	20:4n-6	20:5n-3	22:6n-3
High EPA	>720	>300	18.7<x>32.7	0.10<x<0.8	>0.8	>5.1	>1.2
Low ARA	>720	>300	18.7<x>32.7	0.10<x<0.8	0.2<x>0.1	>1.4	>1.4
No DHA	>720	>300	18.7<x>32.7	0.10<x<0.8	>0.4	>0.8	0

However, there were some unfeasible problems, which caused the original boundaries of the constraints EPA, DHA and ARA of the diets to be lowered, in order to find a solution. The solution to the diet high EPA was only found after lowering the boundary values of the EPA, DHA and ARA concentrations by 25% (resulting in 175% of the average EPA, DHA and ARA concentrations of the microalgae). No-DHA diet boundary levels for EPA concentrations (25 % of the average EPA concentration) and low ARA diet (50% of the average EPA concentration) were lowered. The LP solutions retained three flagellated (*P. parkeae*, *T. suecica* and *B. submarina*) and two diatom species (*C. muelleri* and *P. tricornutum*) (Table 3). Diets high EPA and low ARA were composed of the same Prasinophyceae (*P. parkeae*) and different diatoms (*C. muelleri* and *P. tricornutum*, respectively), whereas the no-DHA diet was composed of Chlorophyceae (*T. suecica* and *B. submarina*).

The resulting three diets have 310-466 mg of lipids per gram of dry weight, and 761-801 mg of organic matter per gram of dry weight. The contribution of each microalgae was different for each diet, which explains the differences in the fatty acid composition of each diet. As requested, the EPA content of the no-DHA diet is lower than in the high-EPA diet, whereas the diet with lower DHA content is the no-DHA diet. The low-ARA diet is similar to the balanced diet with the exception of the lower ARA concentration.

4. Discussion

Our results have shown that different microalgae cultures grown using similar methods have different growth performances and unique compositions. There are several reports about the biochemical composition of microalgae (e.g. Fernández-Reiriz *et al.* 1989, Harrison *et al.* 1990, Zhukova & Aizdaicher 1995, Brown 1997), showing that the composition differs widely by cultivation method (light, temperature, mediums).

Table 3: Mean composition of the High EPA, Low ARA and No-DHA diets. The table shows the proportion of each microalgae for each diet (dry weight basis), as well as the organic matter, lipids, ratio between the total n6 and n3 fatty acids (n6/n3), total PUFA, arachidonic acid (20:4n-6), eicosapentaenoic acid (20:5n-3) and docosahexaenoic acid (22:6n-3) contents (mg g^{-1} of dry weight) of three diets.

	High EPA	Low ARA	No DHA
	53.0% *C. muelleri* + 47.0 % *P. parkeae*	72.5% *P. parkeae* + 27.5% *P. tricornutum*	46.5% *B. submarina* + 53.5% *T. suecica*
OM	761.18	770.55	801.28
Lipids	466.12	357.4	309.83
*n*6/*n*3	0.1	0.09	0.09
PUFA	27.48	23.99	26
ARA	0.84	0.11	0.46
EPA	5.31	1.2	0.81
DHA	1.68	1.52	0

Although there is some variation in the lipid contents of the microalgae found in literature, our values are in most cases in the same range (Table 4). This is the case for *S. costatum*, *T. pseudonana*, *P. tricornutum* and *T. suecica*. *C. muelleri* cultured in this study had, however, higher lipid content. Given the similarity between the lipids contents of most of the microalgae used in this study the difference found in *C. muelleri* is unlikely to be attributed to an error in methodology. This suggests that our cultivation techniques (enriched Walne's medium, 24 hours light, 19°C) result in cultures with high lipid content for this species.

When looking at the fatty acid composition of the studied microalgae (Table 1), significantly higher concentrations were found in *C. muelleri*. On the opposite end, low fatty acid contents were found in *S. costatum.* *S. costatum* grown during this studies also had lower organic content than the other cultured species and reached only around 10 million cell mL^{-1}, which indicates that the culture conditions used were not suitable for high biomass production.

Furthermore, the low fatty acid contents also showed these conditions were not appropriate for the accumulation of fatty acids. The fatty acid profile of *P. tricornutum* was similar to previous studies (Patil *et al.*, 2007). Similarly, the high concentration of DHA found in *P. parkeae* was previously recorded for other species of the Prasinophyceae class (Dunstan *et al.*, 1992). EPA but no DHA was found in the Chlorophyceae *B. submarina* and *T. suecica*. Although there are no reports on fatty

acid composition of *B. submarina*, the values obtained with *T. suecica* agreed with several other studies (Chu & Greaves 1991, Caers *et al.* 1998).

Table 4: Variation in the lipid contents of different microalgae (percentage of dry weight), *Skeletonema costatum*, *Thalassiosira pseudonana*, *Chaetoceros muelleri*, *Phaeodactylum tricornutum* and *Tetraselmis suecica* found in literature and in the present study.

	S. costatum	*T. pseudonana*	*C.muelleri*	*P.tricornutum*	*T.suecica*
Literature	10.0[1] – 19.0[2]	8.5[3] – 19.0[1]	8.2[4] -24.0[1,7]	8.2[4] - 24.0[1,7]	6.0[8] -17.4[1,5]
This study	17.7±1.6	16.5±2.9	52.0±11.4	22.7±3.3	14.8±3.7

[1]Brown, 1991; [2]Brown and Jeffrey, 1995; [3] Mansour *et al.*, 2005; [4]Epifanio *et al.*, 1981; [5]D'Souza and Loneragan, 1999; [7]Gatenby *et al.*, 2003, [8]Albentosa *et al.*, 1993

Given the different composition of the indigenous microalgae tested it was possible to design diets with the desired composition. The results also indicate that some species are more suitable to be used in shellfish culture than others. The diatoms *P. tricornutum* and *C. muelleri* seem to be the most interesting options to use in a diet for shellfish, given its high organic matter contents and EPA concentrations. The species of this study with less appealing fatty acid composition were *S. costatum*, *B. submarina*, and *T. suecica*. Although *T. suecica* has a low fatty acid content, the fact that this species is commonly used as food for molluscs (e.g. Volkman *et al.*, 1989) and the high organic matter content achieved indicates there may other factors to consider other than the fatty acid profile alone for shellfish nutrition.

The objective of this study was to demonstrate the usefulness and applicability of linear programming for selecting specific diets of live microalgae for bivalves. There are limitations to the use of this technique when dealing with live feed, as the biochemical components cannot be separated, which affects the liberty to try some combinations. The constraint's boundaries used need to be expressed as fairly wide ranges in order to be able to formulate a diet with the given biochemical composition of the algae. Also, in this study, we allowed lowering of the minimum value of specific fatty acids in order to formulate diets with similar organic matter and lipid compositions and different fatty acid contents. With this approach it was possible to design mixed diets to test the importance of fatty acids that are of interest for bivalve growth (EPA, DHA and ARA) while keeping the organic matter content and n-6/n-3 ratio similar. By combining linear programming with mixed integer programming it was possible to combine several constraints (OM, lipids, total FA, EPA, DHA and ARA) in the formulation of live algae diets, while optimizing the production effort. The linear programming approach can also be used with more strict maximum and

minimum ranges for several constraints, once the nutritional requirements of bivalves are known. The optimizing goal (production effort in our case) can also be changed into any other component of microalgae culture, including production costs. Culturing diets with specific and adequate biochemical composition for growth of bivalves at the least production cost possible will be an advantage for the development of inland aquaculture of these animals.

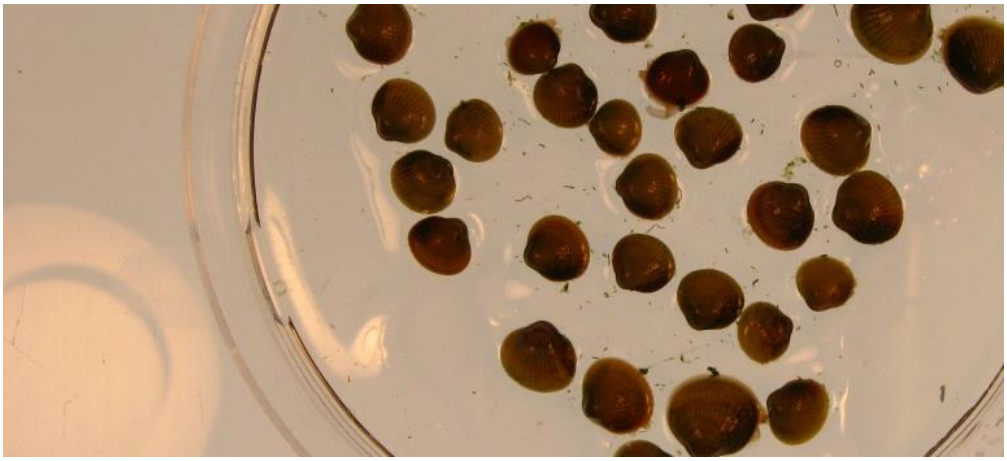
5. Conclusions

Compromises between the important constraints and the allowed variation are more important than when dealing with inert ingredients that can be mixed in any desired combination. The differences between the biochemical composition of *S. costatum*, *P. tricornutum*, *T. pseudonana*, *C. muelleri*, *P. parkeae*, *T. suecica* and *B. submarina* allowed the formulation of three different diets with different fatty acid contents, but similar organic matter content and n-6/n-3 ratio. Compromises between the important constraints and the allowed variation are more important than when dealing with inert ingredients that can be mixed in any desired combination. For this purpose there needs to be some flexibility on the boundaries for each constraint and a ranking of the most important (and therefore not flexible) constraints should be made. The approach of using linear programming to develop shellfish diets based on live microalgae was possible and allowed to consider various constraints simultaneously. The advantage of this approach is that it is possible to determine the relative contribution of different microalgae species to the diet based on actual biochemical composition and keep the different components within pre-defined boundaries.

Acknowledgments

The authors would like to thank Ainhoa Blanco, Emiel Brummelhuis and Ad van Gool for their invaluable help during the sampling moments. This research was funded by the project Zeeuwse Tong.

Chapter 3: Growth and fatty acid composition of juvenile *Cerastoderma edule* (L.) fed live microalgae diets with different fatty acid profiles



Published as

Reis Batista, I., Kamermans, P., Verdegem, M.C.J. Smaal, A.C. (2014) Growth and fatty acid composition of juvenile Cerastoderma edule (L.) fed live microalgae diets with different fatty acid profiles. *Aquaculture Nutrition,* **20**,132-142.

Abstract

The importance of dietary 20:5n-3 (EPA), 22:6n-3 (DHA) and 20:4n-6 (ARA) for growth, survival and fatty acid composition of juvenile cockles (Cerastoderma edule) was investigated. Cockles of 6.24±0.04 mm and 66.14±0.34 mg (live weight) were distributed into three treatments where live microalgae diets were fed constantly below the pseudofaeces production threshold, for three weeks. Diets had distinct fatty acid contents: high EPA (53% *Chaetoceros muelleri* + 47% *Pyramimonas parkeae*), no DHA (47% *Brachiomonas submarina*+ 53% *Tetraselmis suecica*) and low ARA concentrations (73% *P. parkeae* + 27% *Phaeodactylum tricornutum*). Growth was positively affected by high EPA and low ARA diets whereas no significant growth was observed for the no DHA diet. High mortality of cockles fed no DHA diet raises questions about its suitability for cockles. In balanced diets with EPA and DHA, lower concentrations of ARA do not limit growth. The impact of dietary fatty acids was evident in the fatty acids of neutral and polar lipids of cockles. In polar lipids of all cockles there was a decrease of EPA, in contrast with an increase of DHA. The combination of EPA and DHA in a live microalgae diet was beneficial for the growth and survival of juvenile cockles.

Keywords: *Cerastoderma edule*, fatty acids, growth, microalgae, cockle

1. Introduction

The cockle (*Cerastoderma edule*) is a filter feeder bivalve commercially fished from mudflats and intertidal areas across Europe. In addition, cockles are being tested in the framework of a study to develop inland multi-trophic aquaculture (Stichting Zeeuwse Tong, 2012). However, there is limited information about which diets generate fast growth and produce healthy high quality cockles. Extensive research has been done on oysters (e.g. Langdon & Waldock, 1981; Badillo-Salas *et al.*, 2009 for *Crassostrea gigas*; Enright *et al.*, 1986; González-Araya *et al.*, 2011 for *Ostrea edulis*) and clams (e.g. Coutteau *et al.*, 1994; Fernández-Reiriz *et al.*, 2006 for *Ruditapes philippinarum*; Albentosa *et al.*, 1996a; Albentosa *et al*., 1999 for *Ruditapes decussatus*), but the dietary requirement of bivalves is species specific (Albentosa *et al.*, 1996b). One of the most studied topics in bivalve nutrition has been the lipid composition of the diet, with special attention to fatty acids. The importance of essential n-3 polyunsatured fatty acids (PUFA) such as eicosapentaenoic acid (20:5n-3, EPA) and docosahexaenoic acid (22:6n-3, DHA) for the growth and development of bivalves is commonly accepted (Langdon & Waldock, 1981; Chu & Webb, 1984; Enright *et al.*, 1986). Arachidonic acid (20:4n-6, ARA) and n-6 PUFA's are also important for scallops (Milke *et al.*, 2008) and mussels (Pettersen *et al.*, 2010). The importance of these fatty acids for the common cockle remains to be determined. Most commonly used diets consist of specific live microalgae species chosen for their digestibility, easiness of culture and biochemical composition. Based on empirical evidence, a mixture of diatoms and flagellated species is considered an appropriate diet for shellfish (Helm *et al.*, 2004). A quantitative analysis of the fatty acid requirements would allow fine-tuning algae mixtures for shellfish diets appropriate for implementation in a commercial setting. The importance of dietary 20:5n-3, 22:6n-3 and 20:4n-6 for growth, survival and fatty acid composition of juvenile cockles remains to be established. The objective of this experiment was to determine the response (filtration, absorption, growth and survival) and fatty acid composition of polar (structural components) and neutral (energy reserves) lipids in juvenile cockles fed diets with different fatty acid profiles.

2. Material and methods

Juvenile cockles

An initial sample of cockles (*Cerastoderma edule*) of 6.24±0.04 mm shell length and 66.14±0.34 mg live weight were collected in summer (August 2010) from the mudflats of Viane, Oosterschelde estuary, Netherlands (51° 37, 5' 0"N 4° 3, 0' 0"E). A sub-sample of 25 individuals (shell and meat) was used to immediately determine dry weight (70 °C until constant weight) and ash free dry weight (450°C until constant weight), while a second subsample of 30 individuals was stored at -80°C until further processing to determine the biochemical composition. The remaining collected animals were randomly distributed into 9 groups used for 3 treatments executed in triplicate. Each experimental unit consisted of 40 cockles stocked in a 165 x 90 x 100 mm aquarium filled with 400 mL seawater filtered through a 0.5 µm Millipore filter. Per treatment, three aquaria were part of a closed recirculation system connected to a continuously aerated food container. The experiment was conducted at 18°C in a climate controlled room. Algae were fed constantly to the cockles at concentrations below the pseudofaeces production threshold. The pseudofaeces production threshold was determined by direct observation of 5 animals fed each diet at 5, 7, 10, 12 and 15 mg of dry weight algae L^{-1}, for 2 hours or until pseudofaeces production was observed. The highest algae concentration at which no pseudofaeces were produced was 10 mg of dry weight L^{-1} and used for the feeding experiments. Throughout the 3 weeks of experimental period the algae concentration in the food container was maintained using an algae supply machine. The algae supply machine is based on measuring the fluorescence and consists of a photo sensor placed in a measuring chamber. The signal generated by the sensor is relative to the amount of algae. A measuring and control cabinet received the signal level from the photo sensor and operates a peristaltic pump. Whenever the cell density in the food container decreased below the set value new algae were added to the food container until the desired concentration was restored (Kamermans, pers. Comm.). Each aquarium was siphoned daily to remove faeces and every 2 days the aquarium volume was replaced with clean seawater. Biofilm formation was removed weekly. During biofilm removal cockles were removed from the containers, counted and individually measured (shell length, mm) and weighed (live weight, mg). At the end of the experiment all the animals were counted, weighed, measured. A subsample was immediately used to determine the dry weight

and ash free dry weight while the remaining animals were preserved at -80ºC until the biochemical composition was determined (within a year).

Diets

Monoalgal cultures of the flagellated *Pyramimonas parkeae* (Center for Culture of Marine Phytoplankton -CCMP 724), *Brachiomonas submarina* (Culture Collection Yerseke -CCY 0810), *Tetraselmis suecica* (CCY 9913) and the diatoms *Chaetoceros muelleri* (CCMP 1316) and *Phaeodactylum tricornutum* (CCY 162) were used. Monocultures grown under constant light (66 µmol photon m^{-2} s^{-1}) and temperature (19°C) in 10 L batch cultures of filtered seawater (0.2 µm) enriched with Walne's medium (Walne, 1970), supplied with silicate (4 mL of Na_2SiO_3 (80 g L^{-1}) per litre of culture) in the diatom cultures. The fatty acid composition of these microalgae was determined to formulate diets with similar organic matter content but different fatty acid composition (Chapter 2) and is shown in Table 1. Samples from the monoalgal cultures were filtered through fibre-glass filter Whatman GF/F 47mm filters, preserved at -80°C and used for the determination of total fatty acid composition. The microalgae for the growth experiment were collected in the exponential growth phase and mixed (on dry weight basis) to produce 3 diets with distinct fatty acid contents: diet high EPA (53% *C. muelleri* + 47% *P. parkeae*), diet no DHA (47% *B. submarina*+ 53% *T. suecica*) and diet low ARA concentration (73% *P. parkeae* + 27% *P. tricornutum*) (Table 2).

Analysis

Protein contents of cockles were determined following Lowry *et al.*, 1951) after hydrolysis in 1N NaOH for 24h at 30°C. Carbohydrate contents were extracted with TCA 15% and quantified according to Dubois *et al.*, 1956). Total lipids were extracted using Bligh & Dyer, 1959) and quantified according to Marsh & Weinstein, 1966). For the determination of fatty acids in the microalgae and cockles the lipids were extracted according to Bligh & Dyer, 1959) to obtain total lipid extract. For the cockles, the total lipids extract was fractioned on a silicic acid column into different polarity classes (Boschker *et al.*, 1999, Boschker *et al.*, 2005).

Table 1: Dry weight (DW, 10^{-4} g mL^{-1}) and fatty acid composition of the microalgae *Chaetoceros muelleri*, *Pyramimonas parkeae*, *Phaeodactylum tricornutum*, *Tetraselmis suecica*, and *Brachiomonas submarina*, in the exponential phase. Mean values (±SD, n=3) are shown as relative contents (percentage of total fatty acids) and as total fatty acid concentration (Total FA, mg C g^{-1} DW).

	Chaetoceros muelleri	*Pyramimonas parkeae*	*Phaeodactylum tricornutum*	*Tetraselmis suecica*	*Brachiomonas submarina*
DW	1.40±0.31	0.83±0.17	2.70±0.80	0.83±0.14	1.78±0.66
FA	% of total	% of total	% of total	% of total	% of total
12:0	0.32±0.04	0.73±0.18	0.33±0.07	1.38±0.52	0.37±0.10
14:0	10.49±2.88	0.31±0.06	6.81±0.85	0.25±0.01	0.27±0.02
16:0	13.72±0.94	15.20±0.69	18.13±5.77	18.77±3.17	15.46±0.74
18:0	2.28±1.02	0.12±0.11	0.58±0.44	0.35±0.14	0.43±0.04
16:1n-7	37.09±1.85	2.88±0.37	30.10±2.07	1.87±0.75	2.32±0.14
18:1n-9	0.32±0.07	0.30±0.07	1.92±0.54	6.72±2.61	1.63±0.33
18:1n-7	1.30±0.20	4.19±0.46	0.65±0.82	4.01±0.17	6.43±0.14
20:1n-9	n.d.	n.d.	n.d.	1.19±0.18	n.d.
16:2n-7	11.77±0.30	2.18±0.23	4.41±3.76	1.16±0.08	0.44±0.62
16:2n-6	0.17±0.04	5.24±0.14	0.02±0.03	0.63±0.26	0.29±0.41
16:2n-4	3.45±0.55	7.54±0.65	1.31±0.20	0.64±0.17	1.23±0.13
16:3n-4	11.49±1.52	n.d.	8.51±0.98	n.d.	n.d.
16:3n-3	0.02±0.03	1.10±0.11	0.01±0.02	1.70±0.14	1.755±0.08
16:4n-3	n.d.	11.84±1.01	n.d.	17.23±2.88	21.87±0.37
18:2n-6	0.34±0.06	3.84±0.33	2.86±1.45	4.01±1.64	4.44±0.76
18:3n-6	0.70±0.29	0.06±0.05	0.38±0.39	0.39±0.10	1.65±0.33
18:3n-3	n.d.	21.64±0.84	1.50±1.07	17.70±0.41	30.92±1.36
18:4n-3	0.58±0.14	12.13±0.79	0.24±0.06	13.47±3.52	4.74±0.12
20:4n-6	2.21±1.19	n.d.	1.03±1.47	0.49±0.06	1.58±0.03
20:5n-3	14.11±1.09	0.33±0.09	11.76±0.71	5.23±0.22	0.60±0.11
22:5n-3	0.07±0.06	1.09±0.06	n.d.	0.02±0.23	n.d.
22:6n-3	2.27±0.29	4.00±3.46	2.48±0.44	n.d.	n.d.
T. PUFA n-6	2.55±1.25	3.94±0.39	3.89±0.32	4.50±1.70	6.02±0.73
T. PUFA n-3	17.30±0.84	52.28±1.18	16.23±1.21	56.41±6.76	60.12±2.25
Total FA	70.21±3.92	40.94±11.24	34.13±3.47	23.45±4.92	54.94±4.54

n.d.= not detected. Minor fatty acids (contribution of less than 1%) are not shown.

The non-polar (neutral) lipid fraction was retrieved in chloroform and the most polar (polar) fraction in methanol. Total fatty acid extract of microalgae and polar and neutral fractions of cockles were derivatized by mild methanolysis to yield fatty acid methyl esters (FAME). Gas chromatograph-flame ionization detection (GC-FID; Thermo GC Ultra) with a 50m*0.32mm*0.25µm BPX70 column was used.

Qualification from peaks of interest of total, neutral and polar fatty acids was done by gas chromatography-mass spectrometry (GC-MS) and also with reference standards or culture samples. The identification of the fatty acids from peaks of interest was done by comparison of equivalent chain length using FAME 12:0, 16:0 and 19:0 as retention time markers (ECL values, Boschker *et al.*, 1999, Boschker *et al.*, 2005). Fatty acid concentrations of total lipids (for microalgae), and of neutral and polar lipids were expressed on dry weight basis as mg of C per g of dry weight (microalgae or cockle flesh). The fatty acid profiles of total, neutral and polar lipids were expressed as percentage of the total fatty acids in each fraction.

Table 2: Description of the differences between eicosapentaenoic acid (20:5n-3), docosahexaenoic acid (22:6n-3), arachidonic acid (20:4n-6), total polyunsaturated fatty acids (total PUFA) and total fatty acids (total FA) composition of diets high EPA, low ARA and no DHA. Presence (+), low values (-) and absence (/) of the fatty acids in the diets is shown.

	High EPA	No DHA	Low ARA
20:5n-3	++	+	+
22:6n-3	+	/	+
20:4n-6	+	+	-
Total PUFA	+	+	+
Total FA	++	+	+

Bioenergetics

Absorption efficiency (AE, percentage) of cockles fed each diet was estimated following Conover (1966). The organic fraction (ratio between ash free dry-weight and dry-weight) of the diets (F) and egestion (E) were used to calculate the absorption efficiency:

Absorption efficiency $=\frac{F-E}{(1-E)\times F}\times 100.$

For these calculations diets and faeces (collected after a 24 hour feeding period) were filtered through pre-ashed (450ºC for 6 hours) and pre-weighted fibre-glass filter Whatman GF/C 47mm filters using a vacuum pump. The filters were subsequently rinsed with 0.5 M ammonium formate to remove residual salts, dried in the oven (70ºC) until constant weight, weighed (dry weight), and then ashed (450ºC) until constant weight (ash weight). Clearance rate (CR, L h^{-1} ind^{-1}) is the rate

in which a certain volume of water is cleared from all particles. For the purpose of this experiment we assumed that microalgae used (>2 µm) are 100% efficiently retained. Clearance rate was determined at the end of the experiment. Per treatment, a fixed flow rate the decrease in microalgae cells concentration over time was monitored. The flow rate was set so that the cell concentration was at least 10% reduced compared to the animal free control system. Animals were transferred to the containers and left to acclimatize for approximately one hour.

After the acclimatization period the microalgae cell concentration in the control (Cc) and containers with animals (Cf) were measured with a Coulter particle counter 5 to 8 times throughout one-hour period. The flow rate (flow, L h^{-1}) and the number of animals in each container (n) was registered to determine the clearance rate (see Smaal & Vonck, 1997):

Clearance rate = $\left(\frac{Cc-Cf}{Cf} \times flow\right)/n.$

The specific growth rate (SGR, %wet weight day^{-1}) was calculated (see Dolmer *et al.*, 2001) using the individual wet weight gain:

SGR = $\frac{\ln w_f - \ln w_i}{time} \times 100$

Where w_f represents mean final wet weight (g), w_i mean initial wet weight (shell and body, g) and *time* (days) of the feeding period.

Statistics

Significant differences between the initial and final weight (live, dry and ash-free dry) and shell length of each treatment were determined using a One-Way ANOVA test with Least Significant Difference (LSD) post-hoc test or Games-Howell post-hoc (when there was no homogeneity of variances). The same design was used to determine the differences in clearance rate, absorption efficiency, final shell length, individual weight, survival, specific growth rate, protein, lipid and carbohydrate contents, as well as fatty acid composition of the cockles between treatments. Percentage data were transformed by angular transformation (arcsin of square root) prior to analysis. When the ANOVA assumptions were not met the non-parametric Kruskal-Wallis test was used. Significance value of 5% ($P<0.05$) was used in all statistical analysis.

3. Results

Random repartition of cockles was controlled between 9 groups at the beginning of the experiment and there were no significant differences between dry weight (27.38 mg ind^{-1}), ash-free dry weight (3.06 mg ind^{-1}), shell length (6.24 mm) or live weight (66.14 mg ind^{-1}) (One-Way ANOVA, $P<0.05$) (Table 3).

Table 3: Mean and standard error values of individual live weight (mg), shell length (size, mm), dry weight (DW, mg ind^{-1}), ash-free dry weight (AFDW, mg ind^{-1}), at the beginning (initial) and end of the experimental period, for treatment high EPA, low ARA and no DHA. Also shown are the survival (%), total shell increase (shell increase, mm/day) and specific growth rate (SGR, %wet weigh day^{-1}) for the totality of the experimental period (21 days). For each parameter different superscript represent significant differences.

	Initial	High EPA diet	Low ARA diet	No DHA diet
Live Weight	66.14 ± 1.19^a	108.85 ± 2.19^b	110.81 ± 1.21^b	66.87 ± 1.98^a
Size	6.24 ± 0.04^a	7.84 ± 0.10^b	7.81 ± 0.07^b	6.38 ± 0.15^a
DW	27.38 ± 0.87^a	48.45 ± 1.60^b	46.00 ± 1.46^b	27.51 ± 1.22^a
ADFW	3.06 ± 0.10^a	6.97 ± 0.51^b	6.95 ± 0.31^b	2.60 ± 0.13^a
Survival	-	$90\% \pm 0.0^a$	$95\% \pm 2.0^a$	$27\% \pm 13^b$
Shell Increase	-	0.08 ± 0.002^a	0.08 ± 0.002^a	0.004 ± 0.008^b
SGR	-	$2.23\% \pm 0.25^a$	$2.62\% \pm 0.05^a$	$0.02\% \pm 0.25^b$

Clearance rates of the diets used were different (Figure 1). Highest values (L h^{-1} ind^{-1}) were recorded for the low ARA diet (0.11) , followed by high EPA (0.05) and no DHA diet (0.03) whereas there were no differences between the absorption efficiency of the different diets low ARA (47.2%), high EPA (69.1%) and no DHA (70.5%).

Diet composition had a significant effect on the final size and weight reached by the cockles (Figure 2). Cockles fed high EPA and low ARA diets increased in weight (wet, dry and ash-free dry weight) and shell length (7.84 mm and 7.81 mm, respectively) over time ($P < 0.01$). This was not observed in animals fed no DHA diet, where weight and size remained similar over time.

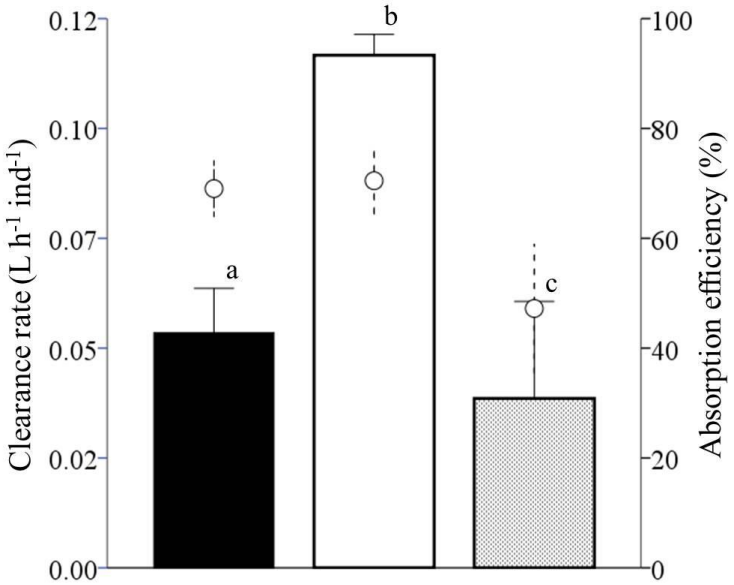


Figure 1: Mean absorption efficiency (%, circles) and clearance rate (L h-1 ind-1, bars) of the animals fed high EPA diet (black), low ARA diet (white) and no DHA diet (grey). Error bars represent standard deviation (n=3). Different superscripts represent significant differences (P<0.05) in the clearance rates of the animals fed different diets. Absorption efficiencies of the diets were not significantly different.

Final weight (live, dry and ash-free dry weight) and shell length of cockles fed high EPA and low ARA diets were higher than for cockles fed no DHA diet (P<0.001). However, these parameters were similar for treatments high EPA and low ARA. Lower survival was observed when cockles were fed no DHA diet than when cockles were fed high EPA and low ARA diets (P<0.0001) (Figure 3). A similar pattern was observed for the specific growth rate (Table 3).

Treatment high EPA and low ARA had no significant effect on the total protein, lipid and carbohydrate contents of the animals (Figure 4). However, fatty acid composition of the cockles was affected by diet (Tables 4 and 5).

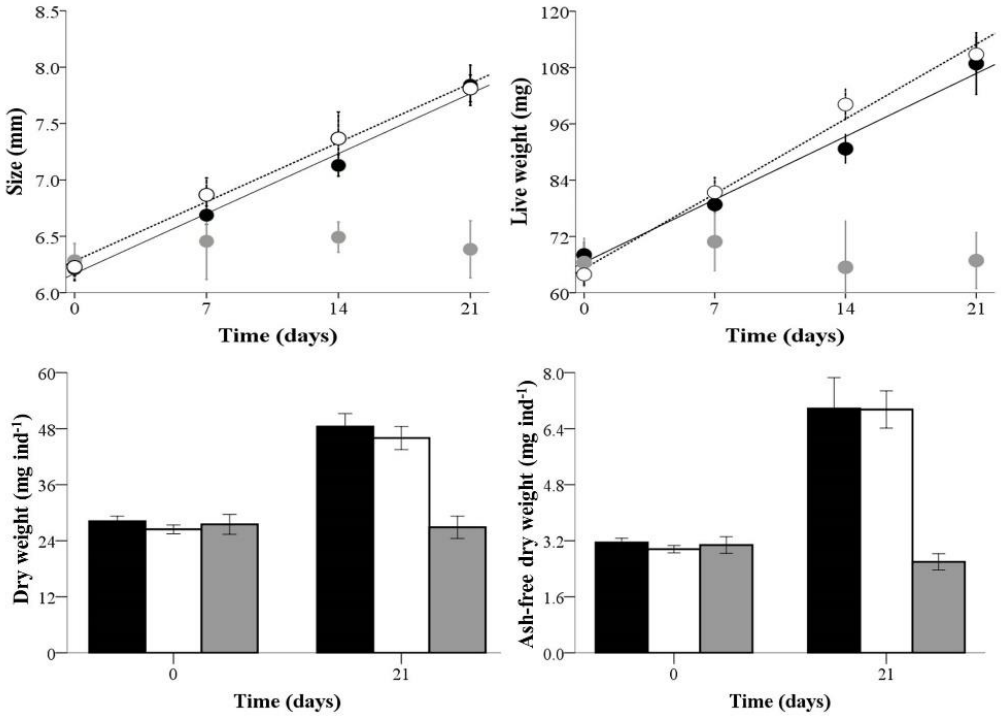


Figure 2: Mean values of size (mm), individual live weight (mg), dry weight (mg ind^{-1}) and ash-free dry weight (mg ind^{-1}) of cockles *Cerastoderma edule* fed high EPA diet (black bars/circles), low ARA diet (white bars/circles) and no DHA diet (grey bars/circles) during the experimental period (21 days). Error bars represent the standard deviation (n=3).

Higher concentrations of fatty acids were present in the polar rather than in the neutral lipids and there was an increase of the concentration of total fatty acids in polar and neutral lipids of the cockles in treatment low ARA compared to the initial values. Feeding low ARA diet resulted in a decrease in the percentage of 20:4n-6 and an increase of 20:5n-3, 22:6n-3 and total PUFA n-3 percentages of the neutral lipids of the cockles. Feeding high EPA diet resulted in an increase of 22:6n-3 and total PUFA n-3 percentage in the neutral lipids. Comparing the composition of neutral lipids of cockles fed high EPA and low ARA diets, higher percentages of total PUFA, total PUFA n-3 and 20:5n-3 were found in cockles fed low ARA diet, whereas higher percentages of total PUFA n-6 and 20:4n-6 were found in cockles fed high EPA diet.

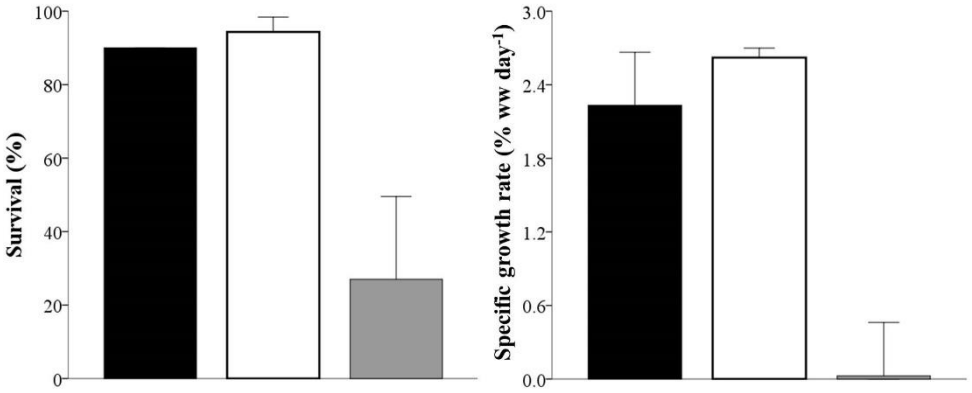


Figure 3: Mean cumulative survival (%) and specific growth rate (% wet weight day^{-1}) of the cockles *Cerastoderma edule* fed high EPA diet (black bars), low ARA diet (white bars) and no DHA diet (grey bars) during the experimental period (21 days). Error bars represent the standard deviation (n=3).

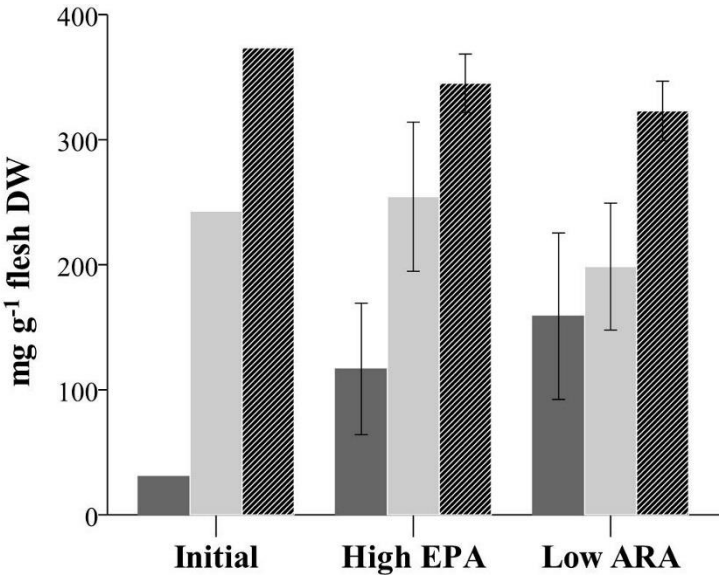


Figure 4: Mean carbohydrates (dark grey), lipid (light grey) and protein (hatched) contents (mg g^{-1} flesh dry weight) of the cockles at the beginning of the experiment (initial) and at the end of the experimental period of treatment high EPA diet and low ARA diet. Due to low survival the carbohydrate, lipid and protein contents of cockles fed no DHA diet were not determined. Error bars represent standard deviation (n=3).

The differences between diet effects were less pronounced when comparing fatty acid concentrations of the neutral lipids, expressed as mg C g^{-1} dry weight of cockles. There was however an increase in the concentration of monounsaturated fatty acids, total PUFA n-6 and total fatty acids in the neutral lipids of cockles fed low ARA diet. Fatty acid composition of polar lipids of cockles was also affected by the diets (Table 5). Feeding low ARA diet resulted in a decrease in the percentage of 20:4n-6 and 20:5n-3 and an increase in the percentage of total monounsaturated fatty acids, alpha-linolenic acid (ALA, 18:3n-3**)** and 22:6n-3 in the polar lipids of the cockles ($P< 0.0001$), when compared with the initial values. When compared with the initial values, high EPA diet resulted in a decrease in the percentage 20:5n-3 and an increase in the percentage of total monounsaturated fatty acids, 20:4n-6, 18:3n-3 and 22:6n-3 in the polar lipids of the cockles.

Between polar lipids of cockles fed diets high EPA and low ARA, higher percentages of total monounsaturated fatty acids, 18:3n-3 and 22:6n-3 were found in cockles fed low ARA diet, whereas higher percentages of 20:4n-6, 20:5n-3 and total PUFA n-6 were found in cockles fed high EPA diet . Significant differences were also detected when comparing fatty acid concentrations (mg C g^{-1} dry weight) of the polar lipids. There was an increase of total saturated and monounsaturated fatty acid, 22:6n-3 and total fatty acids in the polar lipids of cockles fed low ARA diet. The high EPA diet resulted in a significant increase of 18:3n-3. Differences were found between the total monounsaturated fatty acid, 18:3n-3 and 22:6n-3 of concentrations of cockles fed high EPA and low ARA diets. Cockles of treatment fed no DHA diet showed an increase of 18:3n-3 and 20:4n-6 into the polar lipids compared to the initial value. On the contrary, the concentration and relative content of 20:5n-3 and 22:6n-3 were reduced in these animals.

4. Discussion

Cerastoderma edule responded differently to the diets used in this study. Growth was positively affected by low ARA and high EPA diets whereas no significant growth was observed with the no DHA diet. Absorption efficiency and clearance rates can be related to apparent digestibility and uptake of the algae, respectively, by the cockles. Diets used were successfully digested by the animals, as shown by similar absorption efficiencies, although at different clearance rates.

Table 4: Neutral fatty acid composition of *Cerastoderma edule* initially (n=2) and fed high EPA (n=3) and low ARA diets (n=3). Mean values (±SD) are shown as relative contents (percentage of total neutral fatty acids) and as concentration (mg C g^{-1} flesh DW). Fatty acid composition of the neutral lipids of cockles fed no DHA diet was not determined due to low survival in this treatment.

	Initial *C. edule* (n = 2)		*C. edule* fed high EPA Diet (n = 3)		*C. edule* fed low ARA Diet (n = 3)	
	% of total	mgC g^{-1}DW	% of total	mgC g^{-1}DW	% of total	mgC g^{-1}DW
14:0	1.51±0.14^{3}	0.01±0.00	1.88±0.21^{1}	0.03±0.01	1.28±0.20^{2}	0.04±0.03
16:0	10.58±0.64	0.05±0.01^{2}	12.56±1.89	0.17±0.04^{1,2}	8.55±0.06	0.28±0.14^{1}
18:0	9.51±0.65^{a}	0.05±0.01^{2*}	8.56±1.23^{a}	0.12±0.02^{1*}	5.20±0.61^{b}	0.17±0.07^{1*}
20:0	1.25±0.12^{a}	0.01±0.00	0.23±0.21^{b}	0.00±0.00	0.10±0.08^{b}	0.00±0.00
24:0	3.98±0.87^{a}	0.02±0.00	0.91±0.09^{b}	0.01±0.00	0.53±0.37^{b}	0.01±0.00
T Sat	28.54±2.69^{1}	0.19±0.04	25.73±3.20^{1}	0.41±0.09	16.68±82^{2}	0.62±0.28
16:1n-7	2.54±0.00^{b}	0.01±0.00^{2}	8.83±1.23^{a}	0.12±0.03^{1,2}	9.25±1.27^{a}	0.32±0.19^{1}
16:1n-5	3.27±0.87	0.02±0.01	0.87±0.05	0.01±0.00	0.47±0.15	0.01±0.01
18:1n-9	2.29±0.59	0.01±0.00^{2}	1.22±0.02	0.02±0.00^{2}	1.82±0.26	0.06±0.02^{1}
18:1n-7	3.28±0.04^{c}	0.02±0.00^{2}	9.93±0.92^{b}	0.14±0.03^{2}	12.92±0.91^{a}	0.42±0.18^{1}
20:1n-9	0.94±0.25	0.00±0.00	0.32±0.29	0.00±0.00	0.43±0.37	0.02±0.02
T Mono	12.32±0.61^{c}	0.06±0.02^{2}	21.16±1.79^{b}	0.30±0.08^{1,2}	24.88±1.66^{a}	0.83±0.41^{1}
T Mono n-9	3.23±0.34^{a}	0.02±0.00^{2}	1.54±0.31^{b}	0.02±0.01^{2}	2.25±0.41^{b}	0.07±0.04^{1}
T Mono n-7	5.82±0.07^{b}	0.03±0.01^{2}	18.76±2.14^{a}	0.26±0.07^{2}	22.17±1.40^{a}	0.74±0.37^{1}
18:2n-6	n.d.	n.d.	0.58±0.05^{b*}	0.01±0.00^{2*}	1.72±0.42^{a*}	0.05±0.02^{1*}
18:3n-3	n.d.	n.d.	1.23±1.07	0.02±0.02	1.03±1.79	0.05±0.09
20:2n-6	0.34±0.47^{b*}	0.00±0.00	0.99±0.04^{b*}	0.01±0.00	2.07±0.31^{a*}	0.07±0.02
20:3n-3	0.33±0.47^{b*}	0.00±0.00^{2}	0.68±0.11^{b*}	0.01±0.00^{2}	1.31±0.22^{a*}	0.04±0.02^{1}
20:4n-6	6.76±0.69^{a}	0.03±0.01	5.59±0.38^{a}	0.08±0.01	2.21±0.32^{b}	0.07±0.03
20:5n-3	12.15±0.35^{b}	0.06±0.01	10.86±0.68^{b}	0.15±0.04	19.33±1.52^{a}	0.63±0.26
22:5n-3	1.29±0.55	0.01±0.00	1.55±0.12	0.02±0.01	1.97±0.43	0.06±0.02
22:6n-3	4.0±0.24^{b*}	0.02±0.00	5.89±0.26^{a*}	0.08±0.02	6.72±1.14^{a*}	0.21±0.09
T PUFA	28.00±1.04^{3}	0.14±0.04	32.35±0.76^{2}	0.46±0.13	40.21±2.42^{1}	1.32±0.62
T PUFA n-6	7.09±1.16^{ab*}	0.04±0.01^{2}	7.23±0.45^{a*}	0.10±0.02^{1,2}	4.57±0.35^{b*}	0.15±0.06^{1}
T PUFA n-3	19.66±0.95^{c}	0.10±0.02	22.65±1.18^{b}	0.32±0.10	32.91±2.14^{a}	1.08±0.52
Total FA		0.49±0.11^{2}		1.40±0.37^{1,2}		3.32±1.64^{1}

n.d..= not detected. Minor fatty acids (contribution of less than 1% for the neutral fraction) are not shown. Numbers represent significant differences ($p<0.05$, One-Way ANOVA, LSD post-hoc) between concentrations of fatty acids in neutral fatty acid of cockles; Letters represent significant differences ($p<0.05$, One-Way ANOVA, LSD post-hoc) between percentages of fatty acids;* represent significant differences ($p<0.05$, One-Way ANOVA, Games-Howell post-hoc).

Table 5: Polar fatty acid composition of *Cerastoderma edule* initially (n=2), fed high EPA (n=3), low ARA (n=3) and no DHA diets (n=3). Mean values (±SD) are shown as relative contents (percentage of total polar fatty acids) and as concentration (mg C g^{-1} flesh DW). Due to low survival in treatment fed no DHA diet only one sample of *C. edule* was analysed for polar fatty acids in this treatment.

	Initial *C. edule* (n=2)		*C. edule* fed high EPA Diet (n=3)		*C. edule* fed low ARA Diet (n=3)		*C. edule* fed no DHA Diet	
	% of total	mgC g^{-1}DW	% of total	mgC g^{-1}DW	% of total	mgC g^{-1}DW	% of total	mgC g^{-1}DW
14:0	1.21±0.03^{a}	0.21±0.03	0.88±0.08^{b}	0.19±0.02	0.72±0.10^{b}	0.21±0.06	0.56	0.09
16:0	8.62±0.07	1.52±0.28^{1}	8.66±0.31	1.85±0.29^{1,2}	8.75±0.28	2.52±0.49^{2}	9.03	1.46
18:0	9.46±0.06^{b}	1.67±0.31^{2}	10.43±0.42^{a}	2.23±0.34^{1,2}	10.19±0.35^{ab}	2.92±0.56^{1}	10.41	1.68
24:0	n.d.	n.d.	1.90±0.09	0.41±0.10^{2}	2.27±0.11	0.65±0.13^{1}	1.01	0.16
T Sat	21.64±0.47	3.92±0.73^{2}	23.32±0.77	5.05±0.82^{1,2}	23.31±0.75	6.78±1.33^{1}	22.89	3.85
16:1n-7	1.89±0.14	0.33±0.07^{2}	3.18±0.02	0.67±0.12^{1,2}	3.32±0.18	0.95±0.23^{1}	1.22	0.2
18:1n-9	0.57±0.02^{c}	0.09±0.02	0.75±0.05^{b}	0.15±0.02	1.24±0.12^{a}	0.36±0.09	1.11	0.18
18:1n-7	2.44±0.01	0.43±0.07^{c}	4.76±0.14	1.02±0.18^{2}	5.45±0.10	1.56±0.32^{1}	4.35	0.7
T Mono	5.68±0.21^{c}	1.01±0.21^{2}	9.26±0.19^{b}	1.98±0.35^{2}	10.90±0.24^{a}	3.14±0.68^{1}	7.74	1.25
T Mono n-9	1.18±0.04	0.21±0.03^{2}	1.27±0.07	0.27±0.04^{2}	2.11±0.08	0.61±0.13^{1}	2.17	0.35
T Mono n-7	4.42±0.12	0.78±0.16^{2}	7.96±0.16	1.71±0.30^{1,2}	8.76±0.24	2.52±0.55^{1}	5.57	0.9
18:3n-3	0.13±0.00^{c}	0.02±0.00^{2}	1.14±0.07^{b}	0.24±0.04^{1}	1.92±0.11^{a}	0.56±0.13^{3}	6.66	1.08
20:4n-6	4.17±0.07^{c}	0.74±0.14	5.18±0.12^{b}	1.11±0.21	2.99±0.12^{a}	0.86±0.14	8.12	1.31
20:5n-3	22.55±0.16^{a*}	3.98±0.68	14.78±0.20^{b*}	3.17±0.59	12.57±0.64^{c*}	3.61±0.74	12.17	1.97
22:4n-6	1.20±1.70	0.24±0.34	1.30±0.05	0.28±0.07	0.97±0.05	0.28±0.04	2.94	0.48
22:5n-3	4.53±0.10^{a}	0.80±0.12^{1,2}	3.04±0.03^{b}	0.65±0.13^{2}	3.50±0.06^{c}	1.00±0.18^{1}	2.97	0.46
22:6n-3	8.64±0.10^{c}	1.53±0.29^{2}	10.46±0.19^{b}	2.24±0.40^{2}	12.22±0.31^{a}	3.51±0.72^{1}	6.38	1.03
T PUFA	44.60±1.41^{ab}	7.89±1.64	41.33±0.39^{a}	8.86±1.63	39.49±0.37^{a}	11.32±2.17	45.82	7.41
T PUFA n-6	5.94±1.77^{ab*}	1.08±0.50	7.51±0.12^{a*}	1.61±0.31	4.83±0.20^{b*}	1.38±0.23	11.86	1.92
T PUFA n-3	37.32±0.35	6.58±1.10	32.57±0.41	6.98±1.26	32.76±0.17	9.39±1.82	32.86	5.32
total FA		17.64±3.12^{2}		21.47±4.16^{1,2}		28.67±5.53^{1}		16.18

n.d. = not detected. Minor fatty acids (contribution of less than 1% for the polar fraction) are not shown. Numbers represent significant differences ($p<0.05$, One-Way ANOVA, LSD post-hoc) between concentrations of fatty acids in neutral fatty acid of cockles; Letters represent significant differences ($p<0.05$, One-Way ANOVA, LSD post-hoc) between percentages of fatty acids;* represent significant differences ($p<0.05$, One-Way ANOVA, Games-Howell post-hoc).

Higher clearance rates of low ARA and high EPA diets with similar absorption efficiency are consistent with the growth results. Lower clearance rates of no DHA diet indicate a reduced uptake of this diet, when compared with the other treatments. The significantly smaller size of the animals fed no DHA diet at the end of the experiment could have contributed for these differences, but the observed periods of clearance rate inactivity indicate other explanations are needed. The differences in clearances rates can be explained by the different microalgae used in each treatment. To be an adequate diet for shellfish microalgae should be digestible, non-toxic, of adequate size and nutritional values (Pauw *et al.* 1984). All the microalgae used are non-toxic and of adequate size for young juveniles (<15 μm).

Lipids and in particular polyunsaturated fatty acids of the n-3 class such as 20:5n-3 and 22:6n-3 are important in shellfish diets targeting fast growth (e.g. Waldock & Holland 1984, Albentosa *et al.* 1999, Alkanani *et al.* 2007, Badillo-Salas *et al.* 2009). Low values of 20:4n-6 did not seem to influence the growth of the cockles.20:4n-6 is a precursor for the production of eicosanoids (Caers *et al.*, 1998) and is considered essential because it cannot be synthesised from 18:2n-6 (Langdon & Waldock, 1981).

The role of 20:4n-6 in maturation and immune response of oysters (*C. gigas* and *C. corteziensis*) has been investigated (Delaporte *et al.*, 2006; Hurtado *et al*., 2009), but no impact on growth was found (Hurtado *et al.*, 2009). However, 20:4n-6 has been recognized as an important n-6 PUFA for post-larval growth of *Placopecten magellanicus* (Milke *et al.*, 2008).Our results indicate that in a balanced diet with 20:5n-3 and 22:6n-3 , lower concentrations of 20:4n-6 do not limit growth. The combination of 20:5n-3 and 22:6n-3 in the high EPA and low ARA diets was beneficial for the growth of *C. edule.*

Higher 20:5n-3 concentrations of diet high EPA did not result in higher growth rates when compared with low ARA diet. A possible explanation for this result is the difference between the clearances rates of the diets, leading to lower food uptake of diet high EPA. A combination of 20:5n-3 and 22:6n-3 is regarded as more beneficial for growth, but it is possible that the presence of only one of these fatty acids can fulfil the n-3 PUFA requirements of bivalves (Langdon & Waldock, 1981). Our growth results of cockles fed no DHA diet suggest that 20:5n-3 alone can sustain growth for this species. However, high mortality observed with no DHA diet raises questions about the suitability of the diet for this species. Cultures of *Tetraselmis suecica* and

Brachiomonas submarina are adequate for spat and they are commonly used as food in hatcheries (*T. suecica,* Coutteau & Sorgeloos, 1992b; *B. submarina,* Becker, 2004). Nevertheless, the combination of these two species (no DHA diet) was not an adequate (poor growth and high mortality), and therefore interpretations on the essentiality of 22:6n-3 for this bivalve based on the present results needs to be done with care and how essential 22:6n-3 is in the diet of juvenile cockles need further.

The impact of dietary fatty acids was less evident in the fatty acids of neutral lipids than in polar lipids of the cockles, which disagrees with previous studies on early spat (Caers *et al.*, 1999) and adult clams (Beninger & Stephan, 1985) and *C. gigas* (Pazos *et al.*, 1996). In these studies a more conservative nature of the polar lipids was observed, but the different development staged need to be considered. Neutral lipids constitute the lipid reserves of the animals and are more susceptible to dietary and seasonal changes (Beninger & Stephan, 1985; Pazos *et al*., 1996). Under field condition, neutral lipids are mainly constituted by saturated fatty acids stored as energy, but can also be a temporary reservoir of PUFA (Ackman, 1983). However, as a response to the different diets an increase in the saturation degree of the fatty acids of the cockles was observed, which reflects the higher degree of saturation of the diets fed. This was further shown by the increases of 20:5n-3 and 22:6n-3 and decrease of 20:4n-6 in the neutral lipids of cockles fed low ARA diet, demonstrating a clear imprint of this diet onto the neutral lipids of the animals.

The composition of the polar lipids was also influenced by the dietary fatty acids, contrary to previous studies, where the polar lipids composition has been shown to be very conservative (Beninger & Stephan, 1985; Pazos *et al.*, 1996; Caers *et al.*, 1999). The polar lipids composition of the animals reflected the fatty acid profiles (mono-unsaturated fatty acids and 18:3n-3) of the diets used. However, there was a decrease in the 20:5n-3, in contrast with the increase in 22:6n-3, in all cockles. Preferential incorporation of 22:6n-3 over 20:5n-3 in the polar lipids can be explained by a higher requirement of 22:6n-3 for *C. edule*. Higher affinity for 22:6n-3 has been shown for starved *Crassostrea gigas* (Thompson & Harrison, 1992) and *Ruditapes philippinarum* fed a diet with no PUFA (Caers *et al.*, 1998), where the preferential retention of this fatty acid indicates its importance for these animals. For 20:5n-3, the utilization of this fatty acid instead of incorporation when provided by the diet above the requirements of the animals has been suggested (Caers *et al.*, 1999).

In conclusion, growth of *Cerastoderma edule* was influenced by the 20:5n-3 and 22:6n-3 composition of the diets used. Other than influencing growth, diets with the

different fatty acid profiles impacted the fatty acid composition of the polar and neutral lipids. Preferential accumulation of 22:6n-6 in polar lipids indicates the importance of this fatty acid for structural functions. However, the importance of 20:5n-3 or 22:6n-3 for growth of cockles should be investigated.

Acknowledgments

The authors gratefully acknowledge Dr. Philippe Soudant for his help with the chromatograms, Marco Houtekamer, Pieter van Rijswijk (NIOZ) and Dr. Lara Pozzato for their assistance with the fatty acid analysis and Emiel Brummelhuis, Ad van Gool and Ainhoa Blanco for their technical support. This research was funded by the project Zeeuwse Tong.

Chapter 4: Dietary study on *Cerastoderma edule* juveniles: growth and fatty acids metabolism

Reis Batista, I., Kamermans, P., Verdegem, M.C.J., Soudant, P., Smaal, A.C.

Abstract

The development of a successful land-based grow-out system for bivalves requires the understanding of fatty acid metabolism and pathways. Although these parameters have been described to some extent for some bivalves, the importance of essential fatty acids and especially non-methylene-interrupted fatty acids (NMID FA) for juveniles is not fully known and determined. To assess the degree by which 20:5n-3 and 22:6n-3 , on their own or combined, are essential for growth of juvenile cockles *Cerastoderma edule* we compared growth responses and fatty acid profiles with treatments feeding diets: 1) with20:5n-3 (*Tetraselmis suecica* -Tetra); 2) with 22:6n-3 (*Pyramimonas parkeae* - Pyra); 3)with 20:5n-3 and 22:6n-3 from a mixture of the diatom *Chaetoceros muelleri* with green algae *P. parkeae* - Chaeto+Pyra); 4) with 20:5n-3 and 22:6n-3 from a mixture of the green algae *T. suecica* with *P. parkeae* - Tetra+Pyra) and 5) without long-chain (>C20) fatty acids (*Dunaliella tertiolecta* - Duna). An unfed treatment was included to gain insight in the use of fatty acid reserves when no food was supplied. Furthermore, the treatment of a diet with no long-chain fatty acids and rich in NMI fatty acids precursors was included to prove possible biosynthesis of fatty acids. The fatty acid composition of the polar (structure compounds) and neutral lipids (reserves) were analysed. Shell length increase was observed in all fed treatments. Fed treatments had significantly higher survival rates than unfed animals. Specific growth rate was correlated with the amount of dry weight and total fatty acids fed. Significantly higher growth rate was observed with treatment Tetra (rich in 20:5n-3), while the lowest growth rates were found in treatments Duna and Pyra. Unfed animals maintained the initial overall fatty acid profile of the neutral lipids, whereas fed animal showed a decrease of 20:5n-3 and 22:6n-3 when these fatty acids were not provided by the diets. In the present study, the fatty acid profile of the polar lipids confirmed that, like most marine invertebrates, juvenile *C. edule* was not capable to biosynthesize 20:5n-3 and 22:6n-3 from 18:3n-3 but were able to biosynthesize diene and triene NMI fatty acids. The occurrence of NMI fatty acids was diet related, thus refuting the possibility of production of NMI as replacement for 20:5n-3 and 22:6n-3.

Keywords: *Cerastoderma edule* juvenile; fatty acids; EPA; DHA; growth; polar and neutral lipids; biosynthesis.

1. Introduction

Grow-out of bivalve juveniles mainly occurs under natural conditions, after transferring wild or reared animals to food-rich areas. *Cerastoderma edule* is an important natural resource in Europe and may be used for land-based aquaculture. In land-based aquaculture, grow-out is done in outdoor-ponds, under controlled water quality and food supply. Little research has been done on the growth response and changes in biochemical body composition of juvenile bivalves during the grow-out phase. The n-3 polyunsaturated fatty acids (PUFA) are considered important for shellfish larvae and spat (Webb & Chu, 1982; Albentosa *et al.*, 1996a; Albentosa *et al.*, 1996b; Fernandez-Reiriz *et al.*, 1998; Soudant *et al.*, 1998), since most marine bivalves have a limited capability to synthesize eicosapentaenoic acid (20:5 n-3, EPA) and docosahexaenoic acid (22:6 n-3, DHA) (Langdon & Waldock, 1981; Chu & Greaves, 1991; Fernandez-Reiriz *et al.*, 1998). Chain elongation and desaturation of alpha-linolenic acid (18:3n-3, ALA) into EPA and/or DHA has been described in spat of *Crassostrea gigas* and *C. virginica*, but at rates too low to sustain growth (Langdon & Waldock, 1981; Chu& Greaves, 1991, respectively). No elongation and formation of EPA and DHA was observed in spat of *Venerupis pullastra* and *Ruditapes decussatus* (Albentosa *et al.*, 1996a; Albentosa *et al.*, 1996b; Fernandez-Reiriz *et al.*, 1998).

De novo fatty acid synthesis has been described in marine bivalves, by elongation and desaturation of 16:1n-7 and 18:1n-9 into non-methylene-interrupted diene (NMID) fatty acids 20:2Δ5,13 and 20:2Δ5,11 and their chain elongation products 22:2Δ7,15 and 22:2Δ7,13 (Zhukova, 1986). NMI triene fatty acids (NMIT) 20:3Δ5,11,14 and 22:3Δ7,13,16 have also been described in marine mussels and cockles, resulting from desaturation of 20:2n-6 into 20:3Δ5,11,13 and elongation to 22:3Δ7,13,16 (Garrido & Medina, 2002; Le Grand *et al*., 2013). It has been suggested that NMI fatty acids synthesis can be correlated with periods of starvation or lack of essential fatty acids (Klingensmith, 1982), and that the synthesis can be impacted by dietary fatty acid composition (Delaporte *et al.*, 2005). The presence of NMI fatty acids in cell membranes has been proposed to protect against oxidation processes (Barnathan, 2009), and their synthesis has also been shown to decrease in cockles affected by disseminated neoplasia (Le Grand *et al.*, 2013). However, the role of these fatty acids for bivalves is still not very well understood (Delaporte *et al.*, 2005; Barnathan, 2009; Le Grand *et al.*, 2013).

EPA and DHA are considered essential nutrients for oysters and clams since higher intake stimulates growth (Webb & Chu, 1981; Ackman, 1983; Laing *et al*., 1987;Fernandez-Reiriz *et al.*, 1998).The combination of EPA and DHA in live microalgae diets enhances the growth of the clam *C. edule* (Reis Batista *et al.*, 2013), although studies with *Ruditapes decussatus* have demonstrated that either 20:5n-3 or 22:6n-3 can fulfil the n-3 growth requirement of this clam (Albentosa *et al.*, 1996b; Fernandez-Reiriz *et al.*, 1998). For the development of a successful land-based grow-out system for bivalves, fatty acid metabolism and pathways need to be understood. Although these parameters have been described to some extent for some bivalves, the importance of essential and NMI fatty acids for juvenile cockles is still not fully understood.

To assess the degree by which EPA and DHA, alone or combined, are essential for growth of juvenile cockles, we compared growth and fatty acid profiles of animals fed diets with different fatty acids contents. The diets used provided: 1) 20:5n-3 (*Tetraselmis suecica* -Tetra); 2) 22:6n-3 (*Pyramimonas parkeae* - Pyra); 3) 20:5n-3 and 22:6n-3from a mixture of the diatom *Chaetoceros muelleri* and green algae *P. parkeae* (Chaeto+Pyra); 4), 20:5n-3 and 22:6n-3 from a mixture of the green algae *T. suecica* with *P. parkeae* (Tetra+Pyra) and from two green algae (*T. suecica* and *P. parkeae* -Tetra+Pyra); 5) no long-chain (>C20) fatty acids (*Dunaliella tertiolecta* -Duna). The experiment was also designed to allow the identification of possible biosynthesis of essential and NMI fatty acids.

2. Material and methods

Juvenile cockles

Cockles (*Cerastoderma edule*) of 6.85±0.62 mm shell length and 77.60±0.37 mg live weight were collected in July 2011 on the mudflats in Viane, Oosterschelde estuary, The Netherlands (51° 37, 5’ 0”N; 4° 3, 0’ 0”E). A subsample of 30 wild cockles was used to determine the dry weight (100° C until constant weight) and ash-free dry weight (450° C until constant weight), and another subsample of 50 individuals was stored at -80° C for latter determination of fatty acid profile of polar and neutral lipids. The remaining animals were randomly distributed into 6 treatments with 3 replicate tanks (165mm x 90mm x 100mm) each, stocking 60 cockles per tank. Each treatment consisted of a closed recirculation system of three experimental tanks

filled with 400 mL of filtered 0.2 µm seawater (salinity of 30), connected to a constantly aerated food container. The experiment was conducted at 18ºC in a climate controlled room.

Five different diets were supplied to the animals at concentrations below the pseudofaeces production threshold. The latter was determined by direct observation of duplicate groups of 5 animals fed each one of the experimental diets at 5, 7, 10, 12 and 15 mg of dry weight L^{-1} until the first faeces were produced. The lowest concentration at which no pseudofaeces were produced of the five diets was 10 mg dry weight L^{-1}, and was set as feeding level for the experiment. Throughout the experimental period the diet concentration in the food container was maintained at 10 mg of dry weight L^{-1} using an algae dosing machine, as described by Reis Batista *et al.* (2013). The amount of algae provided to the food container depended on the feeding activity of the animals. Food consumption was recorded. One treatment did not receive a diet (unfed). Experimental tanks were siphoned daily to remove faeces. Water quality (pH, dissolved oxygen, and nitrite, nitrate and dissolved ammonia) was monitored every third day and maintained by partial refreshment of the water every other day. Attached biofilm on sides and bottom of the experimental tanks was removed weekly, at which point the animals were taken out of the tanks and counted (for survival determination), measured (shell length, mm) and weighed (live weight, mg).

At the end of the experiment all the animals were counted, weighed, and measured. One sub-sample was used to determine the dry and ash-free dry weight immediately and another sub-sample was preserved at -80ºC for the later determination of fatty acid profile of polar and neutral lipids.

Treatments

Monoalgal cultures of green algae *Pyramimonas parkeae* (CCMP 724), *Tetraselmis suecica* (CCY 9913), *Dunaliella tertiolecta* (CCY) and diatom *Chaetoceros muelleri* (CCMP 1316) were grown under constant light (3500 Lux) and temperature (19°C) in 10 L batch cultures of filtered seawater (0.2 µm, salinity of 30) enriched with modified Walne's medium (Walne, 1970). Modifications concerned changed concentrations of vitamin B12 (10 mg/100 mL) and B1 (10 mg/100 mL) and the addition of vitamin H (200 µg/100 mL). For diatom culture, 4 mL of Na_2SiO_3(80 g L^{-1}) was added per litre of culture. When cultures reached the exponential growth phase they were fed to the cockles. Three monoalgal diets were fed: *T. suecica*

(Tetra), *P. parkeae* (Pyra) and *D. Tertiolecta* (Duna). Two mixed diets were fed: diatom *C. muelleri* with green algae *P. parkeae* (Chaeto+Pyra) and green algae *T. suecica* and *P. parkeae* (Tetra+Pyra), both mixed 50-50 on a dry-weight basis. These diets were chosen for their different fatty acid profiles (Table 1). Throughout the experimental period samples of the monoalgal cultures were taken, filtered through pre-ashed fiberglass Whatman GF/F 47mm filters and stored at -80°C until processed for the determination of total fatty acids. The amount of dietary fatty acids fed during the experiment was calculated based on the total dry weight fed and the fatty acid composition of the algae used.

Table 1: Presence (more than 1% of total fatty acids, +) or absence (less than 1% of total fatty acids,-) of specific fatty acids in the monoalgal diets Tetra (*T. suecica*), Duna (*D. tertiolecta*) and Pyra (*P. parkeae*) and bi-algal diets (50-50 dry weight basis) Chaeto+Pyra (*C. muelleri* and *P. parkeae*) and Tetra+Pyra (*T. suecica* and *P. parkeae*).

	Tetra	Duna	Pyra	Tetra+Pyra	Chaeto+Pyra
16:1n-7	+	+	+	+	+
18:1n-9	+	+	-	+	-
20:5n-3	+	-	-	+	+
22:6n-3	-	-	+	+	+

Fatty acid analysis

Total lipids were extracted using Bligh & Dyer (1959) and quantified according to Marsh & Weinstein (1966). For the determination of the fatty acids in the microalgae and cockles, lipids were extracted according to Bligh & Dyer (1959). For the cockles, the total lipid extract was fractioned on a silicic-acid (0.5 gr) column into different polarity classes (Boschker *et al.*, 1999, Boschker *et al.*, 2005) The non-polar fraction (neutral lipids) was retrieved in chloroform (10 mL) and the mostly polar fraction (polar lipids) in methanol (15 mL). The total lipid extract of the microalgae and the neutral and polar lipids of the cockles were derivatized by mild methanolysis to yield fatty acid methyl esters (FAME). Gas chromatograph-flame ionization detection (GC-FID; Thermo GC Ultra) with a 50m*0.32mm*0.25µm BPX70 column was used to determine fatty acids concentrations of total lipids (microalgae) and of neutral and polar lipids (cockles). The identification of the total fatty acids, and of the fatty acids of the neutral and polar lipids was done by comparison of equivalent chain length using FAME 12:0, 16:0 and 19:0 as retention time markers (ECL values,

Boschker *et al.*, 1999, Boschker *et al.*, 2005) and confirmed by gas chromatography-mass spectrometry (GC-MS) on reference standards and culture samples. Non-methylene-interrupted fatty acids (NMI FA) found in cockles were 20:2Δ5,11, 20:2Δ5,13, 20:3Δ5,11,14, 22:2Δ7,13, 22:2Δ7,15 and 22:3Δ7,13,16. Fatty acid concentrations of total lipids (for microalgae), and of neutral and polar lipids were expressed on dry weight basis as mg of C per g of dry weight (microalgae or cockle flesh). The fatty acid profiles of total, neutral and polar lipids were expressed as percentage of the total fatty acids in each fraction.

Growth and survival

The specific growth rate (SGR, %wet weight day^{-1}) was calculated (see Dolmer *et al.*, 2001) using the individual wet weight gain:

$$\text{SGR} = \frac{\ln w_f - \ln w_i}{time} \times 100,$$

Where w_f represents mean final wet weight (g), w_i mean initial wet weight (shell and body, g) and *time* (days) of the feeding period.

The experimental tanks were monitored daily and any dead animals were removed immediately. Survival was calculated as the number of live animals at the end of the experiment divided by the number stocked.

Statistics

Significant differences between weight (live, dry and ash-free dry), shell length, survival, specific growth rate, and the fatty acid profile of the polar and neutral lipids of cockles initially (initial) and at the end of the experimental period were determined using One-Way ANOVA. Post-hoc Tukey's honest significant difference test was used to identify the significant differences between treatments (Tukey's HSD, homogeneous subsets using significance value $P<0.05$). Percentage data were transformed (arcsin of square root) prior to analysis.

Relationship between specific growth rate and the amount of dietary fatty acids fed to the cockles were tested using a Pearson's r correlation test ($P<0.05$). Treatment effects on the overall fatty acid profile of the cockles were assessed using multivariate analysis. Per sample, standardized percentages of polar and neutral fatty acids were used to calculate a Bray Curtis resemblance matrix on which one-way permutational analyses of variance MANOVA (PERMANOVA, 9999 permutations, significance value p <0.05) was performed. This was followed by pairwise tests with Monte Carlo. Distance based linear models (DistLM, adjusted R^2,

9999 permutations, *p*Y<0.05) were used to explore correlations between fatty acid fed (unfed, Tetra, Tetra+Pyra, Chaeto+Pyra and Pyra) and the fatty acid profile in the cockles.

3. Results

Diets

C. muelleri had the highest 16:1n-7, 20:4n-6 and 20:5n-3 contents. *P. parkeae* had high 16:1n-7, 18:3n-3 and 22:6n-3 contents, whereas *D. tertiolecta* and *T. suecica* had no 22:6n-3. *T. suecica* also had the highest percentage of 18:1n-9 of the used microalgae (Table 2). *D. Tertiolecta* had high total n-3 polyunsaturated fatty acids, but no long chain fatty acids with more than 20 carbons.

Growth

The microalgae diets resulted in differences in specific growth rate (Fig. 1). Unfed cockles showed the lowest specific growth rate, while higher specific growth rates were found for cockles fed Tetra, followed by cockles fed Chaeto+Pyra. Survival was similar between all fed treatments and 40%lower for the unfed cockles (P < 0.05).

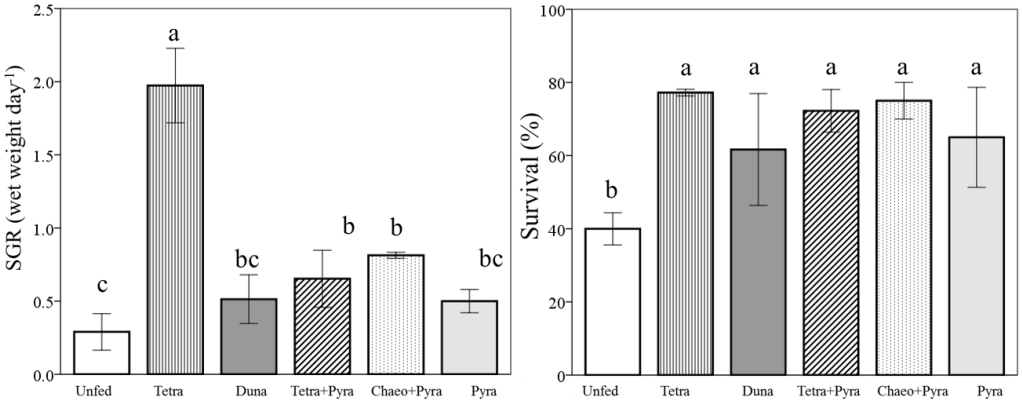


Figure 1: Mean specific growth rate (% of wet weight gain day-1) and survival (%) of the cockles *C. edule* unfed and fed monoalgal diets Tetra (*T. suecica*), Duna (*D. tertiolecta*) and Pyra (*P. parkeae*), bi-algal diets (50-50 dry weight basis) Chaeto+Pyra (*C. muelleri* and *P. parkeae*) and Tetra+Pyra (*T. suecica* and *P. parkeae*) at harvest (experimental period of 28 days). Error bars represent the standard deviation (n=3). Different superscripts represent significant differences (One-Way ANOVA, Tukey's HSD homogeneous subsets, P<0.05).

Table 2: Fatty acids profile of total lipids of the microalgae *Chaetoceros muelleri*, *Pyramimonas parkeae*, *Dunaliella tertiolecta* and *Tetraselmis suecica*. Mean values (±SD, n=4, except *P. parkeae* n=2) are shown as relative contents (percentage of total fatty acids).

	Chaetoceros muelleri	*Pyramimonas parkeae*	*Dunaliella tertiolecta*	*Tetraselmis suecica*
14:0	9.3±1.2	0.3±0.0	0.3±0.0	0.3±0.0
18:0	12.2±1.7	14.6±0.2	21.1±1.1	19.0±1.5
20:0	1.5±0.2	0.1±0.0	0.6±0.2	0.2±0.0
T Sat	24.6±3.5	15.4±0.2	22.2±1.2	20.0±1.4
16:1n-7	33.3±1.1	3.6±0.0	1.5±0.3	2.7±0.3
18:1n-9	0.6±0.4	0.4±0.0	5.6±2.9	9.8±2.9
18:1n-7	2.6±0.6	3.3±0.1	1.8±0.3	3.2±0.1
16:2n-7	8.3±1.3	2.6±0.1	1.0±0.7	0.6±0.0
16:2n-6	0.1±0.1	3.7±0.0	0.3±0.6	0.4±0.1
16:2n-4	1.6±0.5	5.0±0.0	2.5±0.7	2.1±0.3
16:3n-4	6.4±1.1	n.d.	n.d.	n.d.
16:3n-3	n.d.	1.4±0.0	2.8±0.1	0.9±0.0
16:4n-3	n.d.	11.3±4.0	14.8±2.4	15.7±1.2
18:2n-6	0.5±0.2	2.0±0.0	6.0±1.4	7.4±0.6
18:3n-6	0.9±0.1	0.1±0.0	3.9±1.0	0.7±0.1
18:3n-3	0.0±0.0	21.9±0.6	33.0±0.5	15.3±0.7
18:4n-3	0.4±0.1	13.6±0.0	1.0±0.1	8.3±1.7
20:4n-6	3.8±0.3	n.d.	n.d.	0.5±0.0
20:5n-3	10.7±2.5	0.4±0.0	n.d.	7.7±0.0
22:5n-3	n.d.	1.4±0.0	n.d.	n.d.
22:6n-3	1.6±0.4	6.4±0.1	n.d.	n.d.
T.PUFA	38.9±4.6	72.9±0.4	65.6±3.0	60.5±4.6
T. PUFA n-6	5.5±0.2	5.8±0.0	10.2±0.9	9.0±0.7
T.PUFA n-3	13.0±2.8	59.5±0.5	51.9±2.7	48.7±3.7
T.FA^{+}	68.6±14.4	40.7±10.3	35.8±7.8	26.3±1.2

n.d.= not detected. Minor fatty acids (contribution of less than 1% for the total fatty acid concentrations) are not shown; $^{+}$ Total FA – total fatty acid concentrations (mg C g^{-1} of dry weight).

Total amount of dry weight fed (mg per individual over 28 days) was highest for Tetra (24.6±0.3), followed by Duna (13.7±3.7), Tetra+Pyra (10.0±0.8), Chaeto+Pyra (9.8±0.5) and Pyra (9.5±2.2). The specific growth rate of the cockles increased with the dry weight of algae fed ($r=0.886$, $n=14$, $P<0.000$) and the total amount of fatty acids given ($r=0.719$, $n=14$, $P<0.005$).

Shell length at the end of the experimental period was significantly higher than initial shell length, except for the unfed cockles (Figure 2). Considering the initial ash-free dry weight (AFDW) a slight increase was observed in all fed treatments (122-199% of initial) and a decrease was observed in the unfed animals (60 % of initial AFDW) (Figure 2).

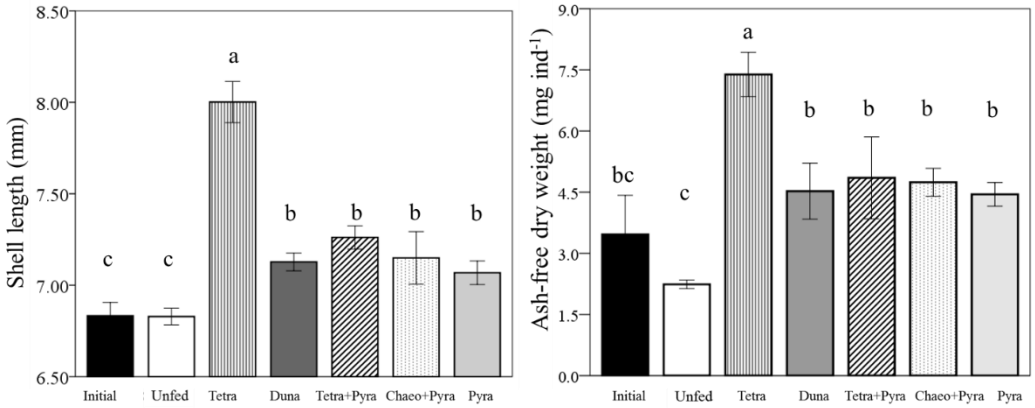


Figure 2: Mean of size (mm) and ash-free dry weight (mg ind^{-1}) of cockles *C. edule* unfed and fed monoalgal diets Tetra (*T. suecica*), Duna (*D. tertiolecta*) and Pyra (*P. parkeae*), bi-algal diets (50-50 dry weight basis) Chaeto+Pyra (*C. muelleri* and *P. parkeae*) and Tetra+Pyra (*T. suecica* and *P. parkeae*) at harvest (experimental period 28 days) during the experimental period (28 days). Black bars represent initial values. Error bars represent the standard deviation (n=3). Different superscripts represent significant differences (One-Way ANOVA, Tukey's HSD homogeneous subsets, P<0.05).

Fatty acid profile of cockles

Fatty acids of neutral lipids

The fatty acids profile of neutral lipids in cockles was affected by the treatments (PERMANOVA Pseudo – $F_{6, 12}$= 12.026, *p*Y<0.0001) (Table 3). Pairwise testing showed that fatty acid profile of neutral lipids of unfed cockles did not significantly differ from the initial condition (Monte Carlo Tests, *p*Y> 0.05), whereas in fed cockles the overall neutral lipid fatty acid profile changed (Monte Carlo tests, *p*Y<0.05). No differences were observed between the overall fatty acid profile of cockles fed Chaeto+Pyra and Pyra; or between cockles fed Pyra and Tetra+Pyra; or between cockles fed Pyra and Tetra; or between cockles fed Tetra and Tetra+Pyra (Monte Carlo Tests, *p*Y> 0.05).

Table 3: Fatty acids profile of neutral lipids of *Cerastoderma edule* initially (initial) and at harvest Mean values (±SD, n=3) are shown as relative contents (percentage of total fatty acids in neutral lipids).

	Initial	*Unfed*	Tetra	Duna	Tetra+Pyra	Chaeto+Pyra	Pyra
14:0	1.8±0.6^{ab}	0.6±0.6^{c}	0.4±0.0^{c}	0.6±0.1^{bc}	0.4±0.0^{c}	2.5±0.2^{a}	0.7±0.3^{bc}
16:0	13.3±1.6^{ab}	6.7±2.4^{b}	14.0±0.8^{a}	13.3±0.1^{ab}	13.0±3.7^{ab}	11.6±0.3^{ab}	8.9±3.1^{ab}
18:0	13.3±1.6^{a}	6.7±2.4 ^{a}	14.0±0.8 ^{a}	13.3±0.1^{a}	13.0±3.7 ^{a}	11.6±0.3 ^{a}	8.9±3.1 ^{a}
T. Sat	26.8±1.5^{a}	16.3±4.0^{a}	24.7±2.1^{a}	20.9±0.9^{a}	21.4±3.1^{a}	25.4±1.6^{a}	25.7±3.9^{a}
16:1n-7	1.9±0.1^{c}	3.4±0.7^{b}	1.3±0.2^{c}	1.8±0.3^{c}	1.2±0.3^{c}	8.8±0.8^{a}	1.4±0.3^{c}
16:1n-5	1.8±0.3^{a}	n.d.	1.2±0.3^{a}	1.1±0.3^{a}	1.5±0.1^{a}	1.6±0.2^{a}	2.1±0.8^{a}
18:1n-9	3.7±0.3^{bc}	5.0±1.5^{ab}	7.0±0.7^{a}	5.9±0.3^{ab}	4.0±0.6^{bc}	1.6±0.3^{c}	1.4±0.7^{c}
18:1n-7	3.2±0.3^{bc}	2.2±0.7^{c}	5.1±0.7^{ab}	3.8±0.5^{bc}	5.7±1.4^{ab}	7.3±0.6^{a}	4.2±0.5^{abc}
20:1n-9	0.9±0.8^{bc}	n.d.	3.5±0.8^{a}	2.1±0.1^{ab}	2.7±0.1^{ab}	0.3±0.5^{c}	n.d
20:1n-7	7.5±1.4^{a}	5.9±2.3^{ab}	2.1±1.0^{ab}	1.4±0.1^{b}	2.5±0.2^{ab}	7.1±1.3^{ab}	2.6±3.7^{ab}
T. Mono	19.7±3.1^{ab}	17.6±0.5^{bc}	20.4±0.7^{ab}	16.6±0.9^{bc}	17.5±2.3^{bc}	27.1±0.9^{a}	11.6±6.2^{c}
T. Mono n-9	4.6±1.0^{c}	5.0±1.5^{bc}	10.5±0.1^{a}	8.0±0.4^{ab}	6.7±0.8^{bc}	1.9±0.6^{d}	1.4±0.7^{d}
T. Mono n-7	12.6±2.0^{b}	11.5±2.3^{bc}	8.5±0.7^{bc}	7.1±0.6^{c}	9.4±1.5^{bc}	23.2±1.8^{a}	8.3±4.6^{bc}
16:4n-3	n.d.	n.d.	1.0±0.5^{a}	1.8±0.3^{a}	1.0±0.6^{ab}	0.1±0.1^{b}	0.1±0.1^{b}
18:2n-6	0.3±0.6^{b}	0.1±0.2^{b}	4.5±1.0^{a}	4.1±0.2^{a}	1.6±2.2^{b}	0.4±0.3^{b}	0.8±0.3^{b}
18:3n-6	n.d.	n.d.	0.4±0.1^{b}	1.7±0.0^{a}	0.6±0.5^{b}	0.1±0.1^{b}	0.1±0.1^{b}
18:3n-3	n.d.	n.d.	6.4±5.8^{ab}	21.2±0.6^{a}	16.7±0.4^{a}	1.0±1.7^{b}	n.d.
20:2n-6	n.d.	0.2±0.4^{c}	1.6±0.2^{ab}	2.7±0.1^{a}	2.2±0.2^{a}	0.7±0.4^{bc}	1.4±0.0^{abc}
20:2Δ5, 11	n.d.	0.6±0.5^{a}	0.2±0.1^{a}	n.d.	n.d.	0.4±0.4^{a}	0.5±0.7^{a}
20:2Δ5, 13	n.d.	2.3±0.9^{a}	1.3±0.8^{a}	0.70±0.5^{a}	1.2±0.2^{a}	1.0±0.9^{a}	2.7±0.3^{a}
20:3n-3	n.d.	n.d.	1.1±0.3^{ab}	2.9±0.0^{a}	1.7±0.5^{ab}	0.6±0.4^{b}	1.3±0.4^{ab}
20:4n-6	9.9±1.8^{a}	7.4±2.6^{ab}	5.1±2.6^{ab}	3.2±0.3^{b}	3.3±0.0^{ab}	8.2±1.3^{ab}	6.5±2.9^{ab}
20:5n-3	9.8±2.6^{a}	6.5±2.1^{ab}	10.4±2.4^{a}	3.0±0.6^{b}	4.9±1.0^{ab}	10.2±0.5^{a}	6.2±2.0^{ab}
22:2Δ7, 13	1.6±0.6^{a}	2.7±0.7^{a}	1.9±1.2^{a}	1.0±0.3^{a}	2.2±2.3^{a}	0.5±0.5^{a}	2.0±1.5^{a}
22:2Δ7, 15	2.3±0.7^{a}	7.1±4.8^{a}	3.6±3.1^{a}	2.0±2.8^{a}	1.4±1.7^{a}	1.4±1.2^{a}	3.2±2.5^{a}
C22:3Δ7,13,16	1.2±0.2^{a}	2.6±0.6^{a}	1.6±0.7^{a}	1.7±0.4^{a}	1.9±1.1 ^{a}	1.1±0.1^{a}	2.2±0.4^{a}
22:4n-6	1.3±0.3^{a}	1.6±0.4^{a}	0.3±0.1^{a}	0.4±0.1^{a}	0.2±0.2^{a}	0.5±0.5^{a}	0.7±0.1^{a}
22:5n-3	2.2±0.7^{abc}	4.5±2.2^{a}	0.7±0.1^{bc}	0.6±0.4^{d}	1.2±0.2^{bcd}	1.6±0.0^{bcd}	2.9±0.7^{ab}
22:5n-6	1.8±0.4^{ab}	2.7±1.0^{a}	0.4±0.1^{c}	0.4±0.2^{c}	0.6±0.2^{c}	0.9±0.2^{bc}	1.0±0.1^{bc}
22:6n-3	5.3±0.4^{b}	6.2±1.4^{b}	1.2±0.5^{c}	1.5±0.2^{c}	4.0±0.7^{b}	5.8±0.2^{b}	11.5±1.4^{a}
T. PUFA	43.5±3.5^{b}	49.5±1.0^{ab}	45.7±2.3^{ab}	52.7±3.3^{a}	54.0±5.2^{a}	42.3±7.3^{b}	48.8±5.1^{ab}
T. PUFA n-6	14.6±1.3^{a}	12.5±3.6^{a}	13.0±1.9^{a}	13.6±0.2^{a}	10.2±2.0^{a}	11.7±1.0^{a}	11.2±2.5^{a}
T. PUFA n-3	18.6±3.7^{c}	19.4±1.6^{c}	21.7±3.5^{c}	31.8±0.2^{a}	30.2±3.3^{ab}	20.0±2.3^{c}	22.8±0.5^{bc}
T. NMI	5.1±1.4^{b}	15.2±3.9^{a}	8.6±3.2^{ab}	5.4±3.1^{b}	6.8±5.0^{ab}	4.6±1.0^{b}	10.9±3.4^{ab}
22:2Δ7,15/22:2Δ7,13	1.4±0.1^{a}	3.1±2.9 ^{a}	2.7±2.7 ^{a}	2.3±3.6 ^{a}	0.5±0.1 ^{a}	2.6±0.3 ^{a}	1.7±0.0 ^{a}
TotalFA^{+}	0.6±0.1^{a}	0.9±0.4^{a}	2.1±1.1^{a}	2.3±0.5^{a}	2.2±0.8^{a}	1.0±0.4^{a}	1.5±0.6^{a}

*n.d. = not detected; +Total FA – total fatty acid concentrations of neutral lipids (mg C g^{-1} of flesh dry weight); Different superscript letter signal significantly different means for each fatty acid (One-Way ANOVA, Tukey's HSD homogeneous subsets, P<0.05).

Within the fatty acids profile, the increase in the total mono-unsaturated fatty acids of cockles fed Chaeto+Pyra reflects the composition of the diet. This dietary influence was further shown by the increase of polyunsaturated fatty acids in animals fed high PUFA content diets such as Duna and Tetra+Pyra, mainly due to the increase of 18:3n-3. Animals fed Pyra, however, did not incorporate18:3n-3 into the neutral lipids and the PUFA percentage did not change during the experiment.
In the unfed, Tetra, Tetra+Pyra, Chaeto+Pyra and Pyra treatments, the level of 20:5n-3was maintained. A significant reduction of this fatty acid in the neutral lipids was only observed in animals of treatment Duna. Animals of Pyra showed an accumulation of 22:6n-3 in the neutral lipids, whereas a decrease of this fatty acid was found in animals fed treatments without this fatty acid (Tetra and Duna).
A significant increase of the percentage of 16:1n-7 (present in all diets) in the neutral lipids of the cockles was only found in treatments Chaeto+Pyra and unfed. The increase of 18:1n-9 was a direct result of the treatments, with higher values of this fatty acid in animals fed Tetra and Duna, as well as unfed. The total NMI fatty acids in neutral lipids remained similar to the initial condition in all treatments except for an increase of these fatty acids in the unfed animals.

Fatty acids of polar lipids

The fatty acids profile of polar lipids in cockles was also affected by the diet (Pseudo – $F_{6, 14}$=62.5, pY<0.0001) (Table 4). Pairwise testing revealed that the fatty acids of polar lipids changed during the experiment in all treatments, including the unfed control (Monte Carlo Tests, pY< 0.05).
Differences were also observed for some fatty acids of the polar lipids (Table 4). 20:5n-3 accumulated in the polar lipids of cockles fed Tetra and decreased in all other treatments, including Tetra+Pyra and Chaeto+Pyra. The increase of 22:6n-3 was only registered in cockles fed Pyra, and significantly decreased in unfed animals or when fed Tetra and Duna, which had no 22:6n-3 (Figure 3).
Total NMI fatty acid percentage was highest in the unfed animals, and similar between the remaining treatments. However, differences in specific NMI fatty acids were recorded. An increase of 18:2n-6, 20:2n-6, 20:3Δ5, 11, 14 and 22:3Δ7, 13, 16 was observed in animals fed Tetra, Duna, Tetra+Pyra and Pyra, which had a higher dietary 18:2n-6 supply (Figure 3).

Table 4: Fatty acids profile of polar lipids of *Cerastoderma edule* initially (initial) and at harvest Mean values (±SD, n=3) are shown as relative contents (percentage of total fatty acids of polar lipids).

	Initial	Unfed	Tetra	Duna	Tetra+Pyra	Chaeto+Pyra	Pyra
14:0	0.9±0.1^{b}	0.6±0.0^{c}	0.4±0.0^{e}	0.6±0.1^{cd}	0.5±0.1^{d}	1.1±0.0^{a}	0.6±0.0^{cd}
16:0	7.7±0.2^{c}	4.5±0.2^{d}	12.6±0.6^{a}	9.9±0.2^{b}	10.9±1.6^{ab}	8.8±0.4^{bc}	9.3±0.5^{bc}
18:0	10.1±0.4^{b}	11.6±0.1^{a}	8.2±0.5^{d}	8.8±0.5^{cd}	9.0±0.5^{bcd}	10.0±0.0^{bc}	9.8±0.6^{bc}
24:0	n.d.	n.d.	1.0±0.1^{b}	n.d.	1.3±0.1^{a}	1.2±0.1^{a}	1.4±0.0^{a}
T. Sat	20.6±0.7^{bc}	18.9±0.2^{c}	23.2±0.6^{a}	21.0±0.9^{abc}	23.1±1.1^{a}	22.8±0.6^{ab}	22.8±0.1^{ab}
16:1n-7	1.6±0.2^{b}	1.4±0.1^{b}	1.0±0.1^{c}	1.4±0.0^{b}	1.3±0.1^{bc}	4.3±0.2^{a}	1.5±0.1^{b}
18:1n-9	1.1±0.5^{c}	0.8±0.0^{c}	4.0±0.4^{a}	2.9±0.2^{ab}	2.3±0.3^{b}	0.7±0.1^{c}	1.0±0.0^{c}
18:1n-7	2.6±0.1^{d}	3.4±0.4^{c}	3.5±0.1^{c}	3.2±0.1^{c}	4.2±0.3^{b}	5.0±0.2^{a}	4.6±0.3^{ab}
20:1n-11	2.4±0.0^{b}	3.0±0.2^{a}	1.1±0.1^{d}	1.5±0.1^{cd}	1.3±0.3^{cd}	1.6±0.1^{c}	1.7±0.1^{c}
20:1n-9	0.8±0.0^{d}	0.6±0.0^{de}	2.6±0.0^{a}	2.1±0.1^{b}	1.8±0.2^{c}	0.5±0.0^{e}	0.6±0.0^{de}
20:1n-7	3.3±0.1^{b}	4.0 ±0.1^{a}	1.6±0.1^{d}	2.1±0.1^{c}	2.4±0.2^{c}	4.0±0.1^{a}	3.2±0.1^{b}
T. Mono	11.9±0.5^{c}	13.2±0.5^{b}	13.9±0.6^{b}	13.2±0.1^{b}	13.3±0.5^{b}	16.6±0.4^{a}	12.6±0.1^{bc}
T. Mono n-9	1.9±0.5^{c}	1.4±0.1^{c}	6.6±0.5^{a}	5.1±0.3^{b}	4.1±0.5^{b}	1.3±0.01^{c}	1.6±0.0^{c}
T. Mono n-7	7.5±0.1^{c}	8.8±0.4^{b}	6.2±0.3^{d}	6.6±0.1^{d}	7.9±0.2^{c}	13.5±0.3^{a}	9.4±0.2^{b}
18:2n-6	0.3±0.0^{de}	0.2±0.1^{e}	3.1±0.3^{a}	2.2±0.3^{b}	1.7±0.2^{b}	0.4±0.0^{cd}	0.7±0.0^{c}
18:3n-3	0.6±0.1^{e}	n.d.	5.8±0.3^{b}	10.6±0.7^{a}	4.9±0.4^{bc}	1.1±0.1^{d}	4.7±0.2^{c}
18:4n-3	0.6±0.1^{a}	n.d.	1.0±0.1^{a}	0.2±0.3^{b}	1.0±0.1^{a}	0.4±0.0^{ab}	1.2±0.0^{a}
20:2n-6	1.0±0.0^{a}	0.7±0.0^{a}	1.9±0.1^{a}	2.5±0.1^{a}	2.2±0.3^{a}	0.9±0.0^{a}	1.4±0.0^{a}
20:2Δ5, 11	0.3±0.0^{c}	0.4±0.0^{bc}	0.5±0.0^{a}	0.5±0.0^{ab}	0.3±0.1^{c}	0.3±0.0^{c}	0.4±0.0^{bc}
20:2Δ5, 13	1.2±0.1^{ab}	2.0±0.0^{a}	0.8±0.0^{b}	0.8±0.0^{b}	0.8±0.2^{b}	1.9±0.0^{a}	1.5±0.0^{ab}
20:3n-3	0.2±0.0^{de}	0.1±0.1^{e}	0.6±0.0^{bc}	1.4±0.1^{a}	0.9±0.1^{b}	0.3±0.0^{cd}	0.4±0.0^{c}
20:3Δ5, 11, 14	0.3±0.1^{c}	n.d.	0.9±0.0^{a}	1.4±0.0^{a}	1.1±0.1^{a}	0.3±0.0^{b}	1.0±0.2^{a}
20:4n-6	5.3±0.1^{b}	8.0±0.2^{a}	5.4±0.4^{b}	5.5±0.2^{b}	4.2±0.5^{c}	7.4±0.2^{a}	3.2±0.0^{d}
20:5n-3	15.8±0.7^{b}	12.8±0.2^{c}	18.0±0.3^{a}	8.9±0.5^{e}	11.0±0.5^{d}	13.9±0.2^{c}	7.6±0.2^{f}
22:2Δ7, 13	4.0±0.1^{ab}	4.7±0.0^{a}	5.4±0.5^{a}	5.1±0.2^{a}	4.8±0.3^{a}	2.5±0.1^{c}	2.8±0.1^{bc}
22:2Δ7, 15	6.5±0.4^{a}	7.2±0.2^{a}	2.3±0.3^{b}	2.5±0.1^{b}	3.1±0.6^{b}	6.9±1.2^{a}	4.5±0.0^{ab}
C22:3Δ7,13,16	3.1±0.1^{d}	3.6±0.0^{c}	5.2±0.5^{ab}	5.5±0.1^{a}	4.7±0.1^{b}	2.9±0.0^{d}	3.9±0.1^{c}
22:4n-6	2.3±0.1^{a}	3.8±0.1^{a}	1.2±0.2^{a}	1.5±0.1^{a}	1.3±0.2^{a}	1.9±0.0^{a}	1.3±0.0^{a}
22:5n-3	3.1±0.2^{b}	3.5±0.1^{b}	1.8±0.2^{c}	1.7±0.1^{c}	3.0±0.1^{b}	2.8±0.1^{b}	4.1±0.2^{a}
22:5n-6	2.6±0.1^{b}	3.5±0.1^{a}	n.d.	1.3±0.2^{c}	n.d.	0.2±0.2^{d}	n.d.
22:6n-3	10.8±0.4^{b}	8.6±0.4^{c}	3.5±0.3^{e}	5.2±0.6^{d}	9.7±0.1^{bc}	10.1±0.2^{b}	15.1±0.3^{a}
T. PUFA	60.6±0.8^{a}	62.1±0.5^{a}	56.3±1.2^{a}	59.9±2.5^{a}	55.2±4.3^{a}	55.4±1.5^{a}	58.4±0.1^{a}
T. PUFA n-6	11.8±0.2^{ab}	16.8±0.2^{a}	12.1±0.6^{ab}	14.0±0.1^{a}	9.8±0.3^{bc}	10.7±0.3^{bc3}	6.8±0.0^{c}
T. PUFA n-3	31.7±1.0^{b}	25.1±0.6^{d}	31.4±0.7^{b}	28.4±0.6^{c}	31.4±0.6^{b}	29.3±0.2^{c}	34.0±0.3^{a}
T. NMI	15.6±0.4^{ab}	17.8±0.1^{a}	13.8±2.5^{b}	17.0±0.4^{ab}	14.6±1.5^{ab}	13.9±0.4^{ab}	15.6±0.5^{ab}
22:2Δ7,15/22:2Δ7,13	1.6±0.1^{ab}	1.5±0.0^{bc}	0.4±0.0^{bc}	0.4±0.0^{c}	0.7±0.1^{bc}	2.5±0.1^{a}	1.6±0.1^{abc}
TotalFA^{+}	18.0±1.0^{ab}	12.2±0.1^{b}	19.8±7.4^{ab}	17.6±2.3^{ab}	22.4±4.6^{a}	16.22±1.45^{ab}	21.9±1.3^{ab}

n.d. = not detected; +Total FA – total fatty acid concentrations of polar lipids (mg C g^{-1} of flesh dry weight); Different superscript letter signal significantly different means for each fatty acid (One-Way ANOVA, Tukey's HSD homogeneous subsets, P<0.05).

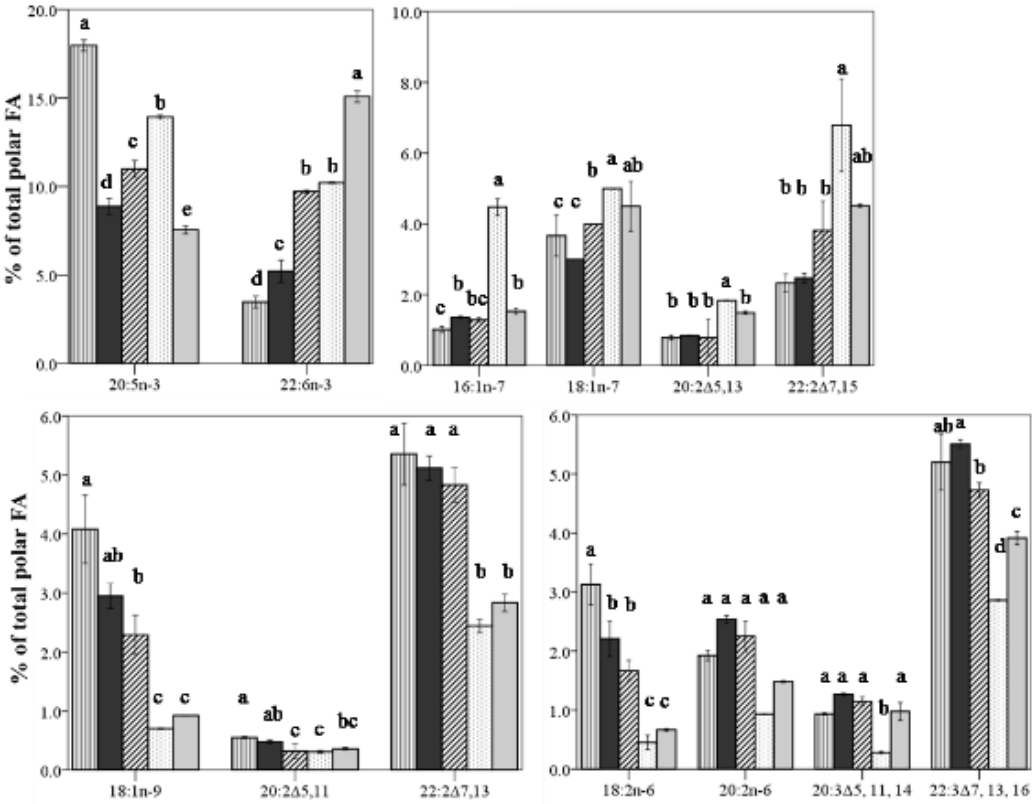


Figure 3: Fatty acid percentage (% of total polar lipid fatty acids) in harvested cockles fed Tetra (white with vertical stripe bars), Duna (dark grey bars), Tetra+Pyra (side-stripped white bars), Chaeto+Pyra (dotted white bars), Pyra (light grey bars). Error bars represent standard deviation (n = 3). Different superscripts represent significant difference for each fatty acid (One-Way ANOVA, Tukey's HSD homogeneous subsets, P<0.05).

Likewise, the dietary influence was observed as significant increase of 16:1n-7 and 18:1n-7 in animals fed Chaeto+Pyra and higher 20:2Δ5,13 and 22:2Δ7,15 percentages in polar lipids of animals fed Chaeto+Pyra and Pyra. A similar pattern occurred in animals with treatments Tetra and Duna, where the supply of dietary 18:1n-9 resulted in an increase of 18:1n-9, 20:2Δ 5,11 and 22:7,13 in the polar lipids of the animals.

4. Discussion

Growth

Growth of *Cerastoderma edule* juveniles in this study was significantly influenced by the applied treatments. Animals fed live microalgae diets had higher shell length and ash-free dry weight than unfed animals. Differences in the specific growth rates of animals fed the different microalgae diets show the relevance of certain fatty acids for growth of juvenile cockles.

Survival was similar between all fed treatments, indicating no major detrimental effect of the diets for the animals. The amount of dry weight fed is an important parameter in determining growth, as shown by a positive correlation with specific growth rate ($r=0.886$, $n=14$, $P<0.000$). This is probably one of the reasons for the underperformance, in terms of growth rate, of the animals fed diets containing *P. parkeae*. When the treatments contained *P. parkeae*, growth was observed, indicating that the animals could take up and use the microalgae.

It remains unclear, however, why the animals consumed less of these treatments, although the reduced consumption could be related to problems digesting the algae.

Cockles fed Tetra showed the highest specific growth rate, demonstrating that the presence of high concentrations of 20:5n-3 provided by the diet had a positive effect on live weight increase, as previously recorded for the juveniles of this species (Reis Batista *et al.*, 2013).

The hypothesis that either 20:5n-3 or 22:6n-3 can fulfil the n-3 PUFA requirements for growth of bivalves (Langdon & Waldock, 1981, Albentosa *et al.*, 1996b; Fernandez-Reiriz *et al.*, 1998) was partially confirmed for the juvenile cockles of this study fed with high concentrations of 20:5n-3 (Tetra), which grew significantly faster than the other animals. Previous work on juvenile cockles had shown that diets with 20:5n-3 and 22:6n-3 (such as mixed diets of *C. muelleri* and *P. parkeae*) resulted in higher growth rates than diets without 22:6n-3 (no DHA diet, *Brachiomonas submarina* and *T. suecica*, Reis Batista *et al.*, 2013). However, the authors highlighted that the lack of this fatty acid was unlikely of being the only factor responsible for the slow growth rates, as the low clearance rates and high mortality observed when feeding the no-DHA diet point to the unsuitability of the diet. The present study confirms that 22:6n-3 is not essential for fast short-term growth, since feeding diets containing no 22:6n-3 (Tetra) over a four week period resulted in the highest observed growth rates and high survival of the animals. 20:5n-3 has been

described to be essential for growth of bivalves (Webb & Chu, 1982; Albentosa *et al*., 1996a; Albentosa *et al.*, 1996b; Fernandez-Reiriz *et al.*, 1998), mainly due to its energetic role (Soudant *et al.*, 1998; Delaporte *et al.*, 2003). This is in line with our results for treatment Tetra and may explain the success of these animals over the positive control treatment Chaeto+Pyra, given the higher amounts of Tetra fed.
Cockles fed diets containing 20:5n-3 and 22:6n-3 (Chaeto+Pyra and Tetra+Pyra) in this study grew at 0.8 and 0.7 % of wet weight per day, respectively, which was lower than previously recorded for juvenile cockles (2.2% of wet weight per day for a mixed diet of *C. muelleri* and *P. parkeae*, Reis Batista *et al.*, 2013). However, the period analysed was different (3 weeks vs. 4 weeks in the present study) and in the present study animals requested less food throughout the entire study (animals were fed based on the decrease of the concentration of food particles).
T. suecica has previously been described to be good for growth of *Ruditapes decussatus* seed (Albentosa *et al.*, 1996a), moderate for growth of *Venerupis pullastra* (Albentosa *et al.*, 1993), and poor for growth of *Ostrea edulis* (Laing & Millican, 1986). Mono-algal diets of *T. suecica* seem to be adequate for growth of juvenile cockles, when supplied in sufficient quantity. However, mixing *T. suecica* with other green algae such as *P. parkeae* (Tetra+Pyra, present study) or with *B. submarina* (Reis Batista *et al.*, 2013) resulted in low growth rates.

Cockle fatty acids

Observing the responses to differences in dietary composition can help to identify fatty acid *de novo* synthesis pathways and fatty acid metabolism. Fatty acids profile of the neutral lipids (reserve function) and polar lipids (structural function) changed significantly during the four weeks experiment.

Neutral lipids

The overall fatty acids profile of the unfed cockles was similar to the initial cockles, whereas significant differences were observed in the treatments fed microalgae diets. These differences are due to dietary changes, to which neutral lipids, the lipid reserves for animals, are known to be susceptible (Beninger & Stephan, 1985; Pazos *et al.*, 1996).Animals fed the different treatments revealed that neutral lipids can also function as a diet regulated reservoir of PUFA, as exemplified by the decrease of 20:5n-3 and 22:6n-6 in neutral lipids of animals fed diets without these fatty acids

(Duna and Pyra; Tetra and Duna, respectively). Animals fed *P. parkeae* as a mono-algal species either did not incorporate 18:3n-3 into their neutral lipids, or they incorporated it at a very slow rate, resulting in lower PUFA of these animals in comparison to the other treatments where similarly high PUFA diets (Duna, Tetra+Pyra) were used. This could be a result of preferential use of 18:3n-3 for elongation into 20:3n-3 in neutral lipids (and not storage) or accumulation in the polar lipids by the cockles fed Pyra.

The NMI fatty acids have been found in several marine invertebrates and were first described for molluscs by Zhukova & Svetashev, 1986) and for adult cockles by Le Grand *et al* (2013). The present study shows that the percentage of NMI fatty acids of neutral lipids of diet fed juvenile cockles remained similar to the initial condition, with the exception of animals fed Chaeto+Pyra. The increase of NMI fatty acids in the unfed animals supports the link between increasing NMI and starvation as previously suggested by Klingensmith (1982).

Polar lipids

Fatty acids profile of polar lipids is considered to be conservative (Beninger & Stephan, 1985; Pazos *et al.*, 1996), but is also subject to changes due to dietary fatty acids, (see Reis Batista *et al.*, 2014 and the present study). The treatments showed a clear relationship in the contribution of the diets to the fatty acids profile of the polar lipids in cockles. Unlike in neutral lipids, the overall fatty acids profile of the polar lipids of the initial animals was different from all treatments (animals unfed and fed microalgae diets). Changes in the fatty acids profile of the unfed cockles were evident in the decrease in the PUFA n-3, mainly due to the decrease of 20:5n-3 and 22:6n-3.

The preferential accumulation of 20:5n-3 in the polar lipids was only observed in animals of the Tetra treatment, although this fatty acid was also supplied in high concentrations to animals in Tetra+Pyra and Chaeto+Pyra treatments. However, the maintenance of 20:5n-3 in the neutral lipids of these animals and the concomitant significant decrease (in comparison with initial composition) in the polar lipids of the animals fed Chaeto+Pyra (exposed to high concentrations of this fatty acid) indicates a possible requirement for 20:5n-3 in the polar lipids. Once this requirement is met, incorporation of dietary 20:5n-3 into the neutral lipids occurs, as reserve, resulting in a reduced accumulation of this fatty acid in the polar lipids. In animals of treatment

Tetra, we found a maintenance and accumulation of 20:5n-3 in both neutral and polar lipids, reflecting the fatty acid content of the diet used and possibly a compensation mechanism, given the lack of 22:6n-3 of this diet. Moreover, the 22:6n-3 composition of the animals was impacted by the diet. High 22:6n-3 levels in the polar lipids of the unfed cockles indicate their importance in the phospholipids, given their major role in the regulation of the membrane fluidity (Knauer & Southgate, 1999). Higher affinity for 22:6n-3 has been shown for unfed *Crassostrea gigas* (Thompson & Harrison, 1992) and *C. edule* juveniles fed diets with 20:5n-3 and 22:6n-3 (Reis Batista *et al.*, 2013). The 22:6n-3 fatty acid in animals of treatments without this fatty acid (Duna, Tetra), reached values significantly lower than the ones observed in initial and unfed cockles. This suggests that when animals are fed diets without 22:6n-3, the animals are not able to maintain the initial condition and this fatty acid is greatly reduced. However, the lack of dietary 22:6n-3 may have impacted the survival chances of the animals due to the very low percentages of this fatty acid in animals fed Tetra at the end of the experimental period, considering the more structural role of this fatty acid (Soudant *et al.*, 1998; Delaporte *et al.*, 2003). This result also shows the inability of juvenile cockles to synthesize this fatty acid from its dietary precursors 18:3n-3(Duna) or 20:5n-3 (Tetra).

The higher percentages of NMI fatty acids in polar lipids, when compared to their contribution to the neutral lipids, demonstrate a structural role of these fatty acids for cockles, as previously suggested for other molluscs (Paradis & Ackman, 1977; Irazu *et al.*, 1984; Zhukova, 1986; Pirini *et al.*, 2007). In marine invertebrates, NMI fatty acids can be *de novo* synthesized from dietary fatty acids 16:1n-7, 18:1n-9 (Zhukova, 1986) and 20:2n-6 (Garrido & Medina, 2002). In the present study we demonstrate these synthesis pathways due to the higher recorded values of 20:2Δ5,11 and 22:2Δ7,13 in polar lipids of animals of treatments Tetra and Duna (high in 18:1n-9) and of 20:2Δ5,13 and 22:2Δ7,15 in polar lipids of animals of treatment Chaeto+Pyra (rich in 16:1n-7). Similarly, the increase of 20:3Δ5, 11, 14and 22:3Δ7, 13, 16 was observed in animals of treatments with high 18:2n-6 (Tetra, Duna, Tetra+Pyra). The increase of these NMI has been previously described in adult *Mytilus galloprovincialis* due to a decrease in n-3 PUFA (Pirini*et al*, 2007). However, in the present study the increase of 20:3Δ5,11,14 and 22:3Δ7,13,16 indicates that cockles are able to elongate the latter into 20:2n-6, which in turn can be further elongated and desaturated into the referred NMI (Garrido & Medina, 2002). High percentages of NMI in unfed bivalves have already been described as

a consequence of starvation (Klingensmith, 1982). Although the function of NMI is not fully understood (Delaporte *et al.*, 2005; Barnathan, 2009; Le Grand *et al.*, 2013), it has been hypothesized that 20 and 22 NMI could have similar functions for the stability and fluidity of the membranes as 20:5n-3 and 22:6n-3 and can be used as replacement for these fatty acids when they are not provided by the diet (Klingensmith, 1982; da Costa *et al.*, 2011).The latter hypothesis was not confirmed by our study. Overall, in our study, NMI composition of the polar lipids of *C. edule* juveniles fed different microalgae diets was a result of the dietary precursors and no evidence of replacement of n-3 PUFA by NMI for growth or survival was found.

We observed clear linkages between the growth of *C. edule* juveniles and the quantity and fatty acid contents in the diet. Fast short-term growth was obtained when feeding high concentrations of a diet rich in 20:5n-3. The diets also impacted the cockle's fatty acids profile. We conclude that *C. edule* cannot *de novo* synthesize 20:5n-3 and 22:6n-3, which was shown by the decrease of these fatty acids in the polar lipids of animals fed diets lacking them. We observed *de novo* synthesis of NMID and NMIT as a direct result of the different precursors supplied by each diet, whereas no evidence of replacement of n-3 PUFA by NMI for growth was observed.

Acknowledgements

The authors gratefully acknowledge Marco Houtekamer and Pieter van Rijswijk (NIOZ) for their assistance in fatty acid analysis. This research was funded by the project Zeeuwse Tong.

Chapter 5: Culturing *Chaetoceros muelleri* using simplified mediums: effects on production and biochemical quality

Published as

Reis Batista, I., Blanco, A. G., van Dalen, P., Kamermans, P., Verdegem, M., Smaal, A. (2015) Culturing *Chaetoceros muelleri* using simplified media with different N sources: effects on production and lipid content, *European Journal of Phycology*, **50 (1),** 92-99

Abstract:
Land-based bivalve aquaculture depends on large-scale cultures of live microalgae for food. The intensity of large-scale microalgae production is important for cost-effectiveness. Using Walne's medium as control, simplified media containing nitrogen, phosphorus, silica, iron, manganese and vitamins were designed to determine the impact of nitrogen source and molar N:P ratio (sodium nitrate, $NO_3$9:1 and ammonium chloride, NH_4 9:1 and NH_4 25:1) on growth, dry-weigh biomass, culture duration and lipid contents of *Chaetoceros muelleri*, a commonly used diatom in shellfish aquaculture.
During exponential phase (day 6), dry-weight production was similar between control and simplified media, indicating that this microalga can successfully grow on simplified media and use ammonium as nitrogen source. Duration of the cultures was also different between cultures grown on nitrate or ammonium. Low nitrogen concentration in cultures grown with nitrate caused the collapse of these cultures within 11-13 days, after a short stationary phase. Cultures grown with ammonium had a longer stationary phase and were still alive on day 20, in spite of the low nitrogen concentrations observed after day 13 in cultures grown with NH_4 9:1. During stationary phase (day 18) there was an increase of lipid content in algae under conditions of low nitrogen availability (NH_4 9:1) and extended low phosphorus availability (NH_4 25:1).
Considering dry-weight production, culture duration, nutrient efficiency and lipid composition, the simplified media containing ammonium, phosphorus, silica, iron, manganese and vitamins are a viable and profitable choice for batch culture of C. muelleri. At the exponential phase, the simplified medium NH_4 9:1 was as effective as the control. Regarding the overall cultivation period, both simplified media with ammonium are effective and suitable, depending on the purpose of the cultures and whether lipid contents ($NH_4$9:1), dry weight biomass ($NH_4$25:1) or nitrogen input and output ($NH_4$9:1) are desired.

Keywords: *Chaetoceros muelleri*; simplified culture media; nutrient ratios; productivity: lipids; duration of culture

1. Introduction

Land-based bivalve aquaculture depends on the large-scale cultures of live microalgae as food source. Live microalgae partial replacements in food have been developed (e.g. Nevejan *et al.*, 2007), but live microalgae are still required for bivalve growth. The quantity, quality and duration of batch cultures are situation specific and influenced by several aspects such as irradiance, nutrient composition of the medium (e.g. Harrison *et al.*, 1990), pH (e.g. Hansen, 2002), length of the light-dark cycle (e.g. Nielsen & Sakshaug, 1993) and temperature (e.g. Goldman & Mann, 1980).The duration of a microalgal culture is important for shellfish production, since batch cultures that can be maintained for longer periods can be partially harvested for food for a longer period, thus reducing the amount of cultures needed at any given point. Furthermore, longer transition between exponential and stationary phase reduce the risk of sudden collapse and allow for a better use of the cultures. Changes in the nutrient concentration in the medium during cultivation alter the biochemical composition of the algae (Harrison *et al.*, 1990; Zhukova & Aizdaicher, 1995; Reitan *et al.*, 1997), which impacts the culture quality. In cultures grown under nitrogen limitation there is a shift from protein synthesis into synthesis of reserve compounds such as lipid or carbohydrates, which is species dependent (Hu, 2004).

Apart from carbon, nitrogen and phosphorus are the most important nutrients for microalgal nutrition (Grobbelaar, 2004). For marine diatoms, silica is also of great importance, since it is a component of the frustules (e.g. Martin-Jézéquel *et al.*, 2000). Although minerals and metals are important for microalgal growth (see Grobbelaar, 2004),the use of simplified media (i.e., media with a reduced number of minerals, metals and vitamins and therefore requiring less resources and preparation time) might reduce costs of microalgal production for large-scale microalgae culture. Research has been done on this topic, either as outdoor microalgal production by fertilization of seawater with inorganic fertilizers (e.g. De Pauw *et al.*, 1983) or as production of microalgae as part of water treatment processes (e.g. Hammouda *et al.*, 1995). However, studies on the coupling of simplified media on pure cultures of microalgae with interest for shellfish aquaculture are scarce, which means the efficiency of these media for single species cultures needs to be addressed. Given the reduction of nutrients in simplified media, defining an adequate molar N:P ratio supporting high algal productivity while reducing costs is important. Nutrient ratios have received a lot of attention in microalgae cultures studies. The Redfield N:P ratio of 16 is considered as an optimum for phytoplankton

(Redfield, 1958), although lower N:P ratios have been described as suitable for large-scale growth of diatoms, e.g. N:P ratio of 10 (Hussenot *et al.*, 1997) and 12 (Lefebvre *et al.*, 2004).A higher N:P ratio of simplified media could however have a beneficial effect on the duration and productivity of the cultures.

For culture media, nitrogen is usually supplied in the form of nitrate (Guillard & Ryther, 1962; Walne, 1970; Keller *et al.*, 1987), although algae are also able to use ammonia, urea and even nitrite, with similar production results (Becker, 1995; Fidalgo *et al.*, 1998; Lourenço *et al.*, 2002; Grobbelaar, 2004; Liang *et al.*, 2006). When using ammonium as nitrogen source of f/2 medium on cultures of *Chaetoceros muelleri* Liang *et al.* (2006) found no differences in growth; however Lourenço *et al.* (2002) reported toxicity and lower yield for some microalgal species grown using ammonium as the nitrogen source of Walne's medium. The benefits of supplying either nitrate or ammonium are not clear (Grobbelaar, 2004), and to our knowledge the effects of the type of nitrogen sources on duration of batch cultures have not been addressed so far.

Production, quality and duration of the cultures are crucial for cost-effective large-scale microalgae cultures for land-based shellfish aquaculture. For the purpose of a land-based microalgae and shellfish aquaculture we studied the impact of simplified culture media on the culture performance of *C. muelleri*, a marine diatom. This marine diatom is widely used in shellfish hatcheries for its high lipid content (Helm *et al.*, 2004). In order to determine the impact of simplified media (containing only nitrogen, phosphorus, silica, iron, manganese and vitamins) on growth, productivity (dry weight biomass), lipid composition and duration of *C. muelleri* cultures, a simplified medium (composed only of nitrogen, phosphorus, silica, iron, manganese and vitamins) was designed to be compared to the control medium Walne's (1970) (nitrate, N:P ratio 9:1). Furthermore, two other simplified media were design to determine the impact of a cheaper nitrogen source (ammonium) and different N:P ratios (N:P ratios 9:1 and 25:1) on the cultures of *C. muelleri*. Nutrient compositions of the media throughout the cultivation period were determined to characterize the nutrient dynamics of the cultures and their impacts.

2. Materials and Methods

Cultures and media

Cultures of *Chaetoceros muelleri* (Center for Culture of Marine Phytoplankton - CCMP 1316) were grown at 19°C under constant light (66 µmol m^{-2} s^{-1} photon). Cultures were acclimatized to the control and to three experimental culture media for a period of three weeks and sequentially up-scaled to 100 mL and 250 mL un-aerated cultures and 3Lcontinuously aerated cultures. After one week, these 3L cultures were used as inoculum for the experimental cultures. Experimental cultures consisted of three replicate 10L bottles per treatment, at initial cellular concentration of $40 \bullet 10^4$ cells mL^{-1}. All cultures were continuously aerated and no additional carbon dioxide was supplied. Walne's medium (Walne, 1970) enriched with silica was used as control. A preliminary test in 1 ml volumes showed that, apart from nitrogen, phosphorus and silica, supplementing the media with vitamins, iron and manganese increased growth of *C. muelleri* (data not shown). Thus, three experimental culture media were created as simplifications of Walne's medium. These simplified experimental culture media contained the same concentrations and sources of silica, vitamins, iron, manganese and phosphorus and different nitrogen sources/concentrations (Table 1).

The phosphorus concentration was maintained constant in all media, resulting in different N:P ratios of the media. Two media were made using ammonium chloride as nitrogen source, at two different concentrations in order to obtain N:P molar ratios of 9:1 (NH_4 9:1) and 25:1 (NH_4 25:1), respectively. The other media (Walne's and NO_3 9:1) had an N:P ratio of 9:1 but sodium nitrate as a nitrogen source.

Culture sampling

Daily, an aliquot from each culture was used to determine the cellular concentration (cells/mL) using a Bürker haemocytometer. Every second day a sample from each culture was taken to determine dry weight, temperature, pH and nutrient concentration. To determine the lipid contents of the microalgae in exponential and stationary phase samples were taken on day 6 and 18, respectively.

Table 1: Medium composition (mmol L^{-1} of culture) of the different treatments, Walne's, NO_3 9:1, NH_4 9:1, and NH_4 25:1.

	Walne	NO_3 9:1	NH_4 9:1	NH_4 25:1
$NaNO_3$-N	01-01-18	01-01-18	-	-
NH_4Cl-N	-	-	01-01-18	01-03-20
$NaH_2PO_4 \cdot 2H_2O$-P	0.13	0.13	0.13	0.13
Na_2SiO_3-Si	0.42	0.42	0.42	0.42
$MnCl_2.4H_20$-Mn	2	2	2	2
$FeCl_3.6H_20$-Fe	5	5	5	5
H_3BO_3[1]	+	-	-	-
EDTA[1]	+	-	-	-
Trace metal solution[2]	+	-	-	-
Vitamin solution[3]	+	+	+	+

[1]As in Walne's medium; [2] Trace metal solution= $ZnCl_2$ (21 mg L^{-1} of culture), $CoCl_2.6H_2O$ (20 mg L^{-1} of culture) $(NH_4)_6Mo_7O_{24}.4H_2O$ (9 mg L^{-1} of culture) and $CuSO_4.5H_2O$ (20 mg L^{-1} of culture); [3] Vitamin solution= Vitamin H (0.2 µg L^{-1} of culture), Vitamin B12 and Vitamin B1 (0.01 mg L^{-1} of culture)

Dry weight and lipid determination

Microalgal cultures were sampled every second day for the determination of dry weight. A known volume of the culture was filtered through pre-ashed (in the oven at 450ºC for 6 hours) and pre-weighted fibre-glass filter Whatman GF/C 47mm filters. The filters were subsequently rinsed with 0.5 M ammonium formate to remove residual salts, dried at 70°C until constant weight to calculate dry weight. To determine the dry weight production (mg L^{-1}) at exponential and stationary phases the values of dry weight recorded at day 6 (exponential) and 18 (stationary) were used. For the lipid determination, a known volume of culture was filtered through pre-ashed (in the oven at 450ºC for 6 hours) and pre-weighed fibre-glass filter Whatman GF/F 47mm filters. The filters were stored at -80 °C until analysis. Total lipids were extracted using Bligh & Dyer (1959) and quantified according to Marsh & Weistein (1966).

Nutrient analysis

Culture media were sampled for nutrient concentrations throughout the cultivation period, in the exponential and stationary phase. Culture samples were filtered using pre-ashed fibre-glass filter Whatman GF/C 47mm filters. The filtrate was collected and stored at -20°C for the nutrient analysis. Nutrient analyses were performed with

a Skalar auto analyser following the standard procedures. Nitrogen was measured (µmol L^{1}) as ammonia (NH_3, protocol number 155-006), nitrite (NO_2 protocol 467-033) and nitrite+nitrate(NO_2+ NO_3, protocol 461-032), silica as silicate (SiO_2, protocol 563-051), whereas phosphorus concentrations were determined from ortho-phosphate (protocol 503-011 analysis).Apparent nutrient uptake (mmol $g^{-1}$of dry weight),was calculated as the amount of nutrients consumed to produce 1 gr of dry weight, for the exponential phase and stationary phase as follows:

Apparent nutrient uptake = $(C_0 - C_t)/ DW$

Where C_o and C_t represent the initial nutrient concentrations and concentrations at time *t* and DW represents the dry weight (g) production at time *t* (day 4-6 for exponential phase, day 16-18 for stationary phase). The sum of concentrations of ammonia, nitrate and nitrite was used in the calculation of the apparent nitrogen uptake. The lower the apparent nutrient uptake, the higher the conversion of the nutrient into dry weight biomass.

Statistical analysis

Normality of the cellular concentration, dry weight and lipid composition of the different cultures after 6 and 18 days of culture was tested using Shapiro-Wilk test. One-Way ANOVA with Least-Square Difference post-hoc tests (or Games-Howell when homogeneity of variances was not met) were used to determine differences between the treatments (day 18 was considered as a different treatment). The same procedures were used to determine differences between apparent nutrient uptake at exponential (day 4 for phosphorus, day 6 for nitrogen and silica) and stationary (day 16 for nitrogen and silica and day 18 for phosphorus) phases. To determine differences between the nutrient concentrations of the different treatments at specific sampling moments, One-Way ANOVA with Least-Square Difference post-hoc tests were performed.

3. Results

Cultures grown in media NH_4 9:1 and NH_4 25:1 lasted longer (>21 days), had longer stationary phases and achieved higher maximum cellular concentration ($9.6 \cdot 10^6$ cells/mL and $12 \cdot 10^6$ cells/mL, respectively) than cultures grown in Walne's medium (10 days, $8.2 \cdot 10^6$ cells/mL) and NO_3 9:1 medium (12 days, $6.0 \cdot 10^6$ cells/mL) (Figure 1).

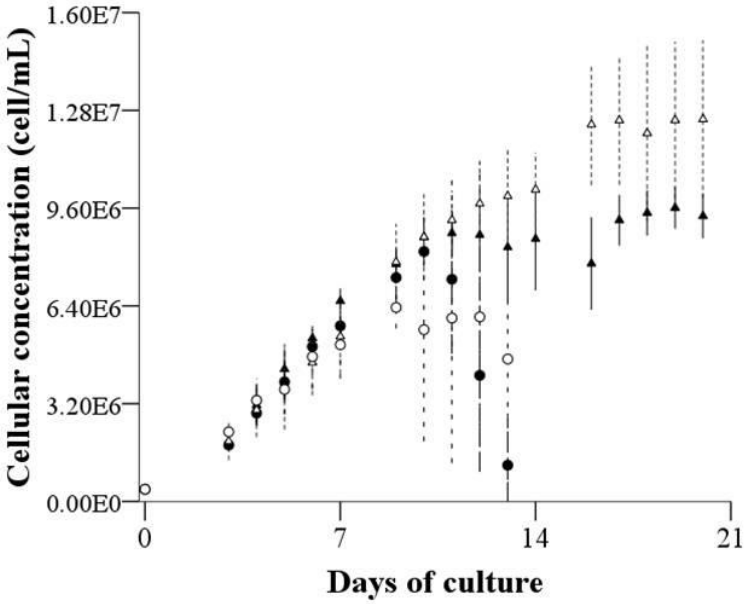


Figure 1: Cellular concentration of the cultures of *Chaetoceros muelleri* grown using Walne's medium (black circle) and media NH_4 9:1 (black triangles), NH_4 25:1 (white triangles) and NO_3 9:1 (white circles) through the cultivation period. Error bars of the cultures grown with Walne's (dashed black) and media NH_4 9:1 (solid black), NH_4 25:1 (dotted black) and NO_3 9:1 (long dashed black) represent standard deviation (n=3).

During the exponential phase (day 6), there were no significant differences between the cellular concentrations of the different media (ANOVA, P>0.05). Throughout the cultivation period, the pH of the cultures ranged between 8.0 and 9.1 (Figure 2). Higher values of pH were recorded on cultures grown in nitrate media after 11(Walne's) and 13 (NO_3 9:1) days of culture, reaching values of 8.8±0.3 and 9.1±0.2, respectively. The pH of the cultures grown on ammonium as nitrogen source varied between 8.2±0.1 and 8.6±0.3 (day 13, NH_4 9:1).

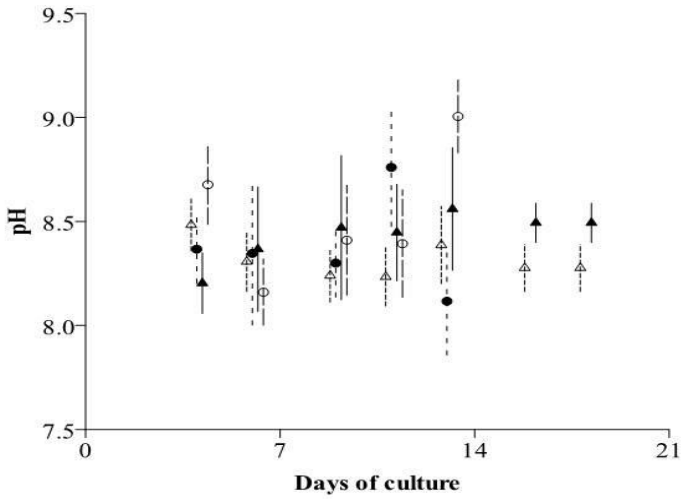


Figure 2: pH of the cultures of *Chaetoceros muelleri* grown using Walne's medium (black circle) and media NH_4 9:1 (black triangles), NH_4 25:1 (white triangles) and NO_3 9:1 (white circles) through the cultivation period. Error bars of the cultures grown with Walne's (dashed black) and media NH_4 9:1 (solid black), NH_4 25:1 (dotted black) and NO_3 9:1 (long dashed black) represent standard deviation (n=3).

Dry weight production of the cultures during exponential phase (Figure3a) was significantly different from the dry weight production during stationary phase ($F(5, 12) = 313.139$, $P<0.0001$). The post hoc Fisher LSD test showed that neither the amount nor the source of nitrogen affected the dry weight production in the exponential phase (6 days). In the stationary phase, significantly higher dry weight production was achieved in cultures grown with NH_4 25:1 than in cultures grown with NH4 9:1.

Lipid contents of the microalgae grown in different media were significantly different ($F(5, 12) =24.988$, $P<0.0001$) (Figure3b). Games-Howell post hoc comparisons showed that the lipid contents recorded in the exponential phase of cultures grown in NO_3 9:1and NH_4 25:1 were significantly lower than in cultures grown in Walne's medium. In the stationary phase the highest lipid content was observed in the cultures grown in the medium NH_4 9:1. The collapse of the microalgal cultures grown on Walne's (after day 11) and NO_3 9:1 (after day 13) was accompanied by a sharp

decrease of nitrate concentration in the cultures (Figure4). Although the nitrogen concentrations of the cultures grown in NH_4 9:1 were low after day 13, the cellular concentration slightly increased and remained stable (in stationary phase) for another 7 days.

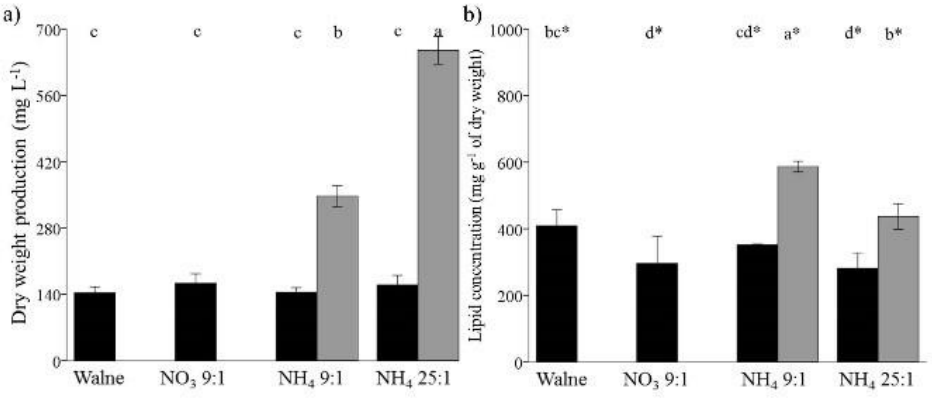


Figure 3: (a) Dry weight production (mg L^{-1}) and (b) lipid concentration (mg g^{-1} of dry weight) of the cultures of *C. muelleri* grown in media Walne's, NO_3 9:1, NH_4 9:1 and NH_4 25:1, in the exponential phase (black bars) and stationary phase (grey bars). Error bars represent standard deviation (n=3) and different superscripts represent significant differences (ANOVA, LSD or Games-Howell (*) post-hoc $p<0.05$).

All nutrient concentrations were reduced throughout the cultivation period (Figure 4). Phosphorus concentrations at day 4 were significantly different between treatments ($F(3, 12) = 41.045$, $P<0.0001$). Significantly higher phosphorus concentrations were found in the ammonium treatments (NH_4 9:1 and NH_4 25:1) and the lowest concentrations were observed in the cultures grown with NO_3 9:1(LSD post-hoc test, $P<0.05$).The sharpest nutrient concentration decrease was observed in the silica concentrations of all treatments. At day 6, silica concentrations were reduced to less than 100 µmol L^{-1} in all cultures, with higher concentrations registered in cultures grown in NH_4 9:1 and very low concentrations in culture grown in NO_3 9:1. After day 13 silica concentrations in ammonium treatments were reduced even further to very low values (<100 µmol L^{-1}). Nitrogen consumption was observed in all treatments. Nitrate was similarly used in Walne's and $NO_3$9:1 treatment and it was depleted in both treatments by day 13. Ammonia concentrations in the NH_4 9:1 cultures were

greatly reduced by day 13, whereas ammonia concentrations in theNH_4 25:1 cultures never reached values below 75 µmol L^{-1}, even after 20 days of culture.

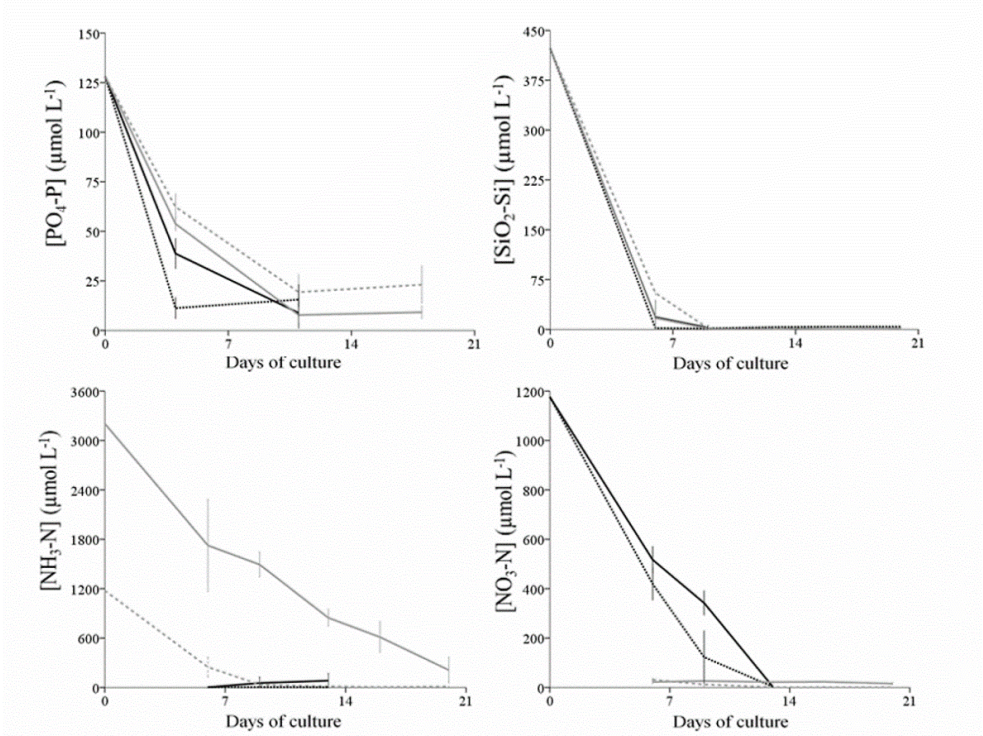


Figure 4: Nutrient concentrations (µmol L^{-1}) of phosphorus (PO_4-P), silica (SiO_2-Si) and nitrogen (NH_3-N and NO_3-N) of the cultures of *C. muelleri* using media Walne's (black line), NO_3 9:1 (dashed black line), NH_4 9:1 (dashed grey line) and NH_4 25:1 (grey line) throughout the cultivation period. Error bars represent standard deviation (n=3).

Apparent nutrient uptake to produce 1 g of dry weight (mmol g^{-1} dry weight) is shown in Figure 5. For all tested nutrients, there were no significant differences between the apparent nitrogen, silica or phosphorus uptake for each media at the exponential phase. However, the expected decrease in the apparent nutrient uptake between exponential and stationary phase was observed. Apparent nitrogen uptake at the beginning of stationary phase was lower than in the exponential phase but similar between the media NH_4 9:1 and NH_4 25:1. The same was observed for the apparent uptake of phosphorus and silica.

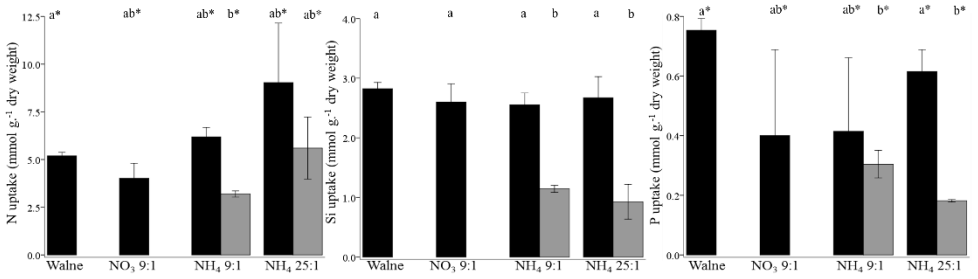


Figure 5: Mean apparent nutrient uptake (nitrogen, N; silica, Si; and phosphorus, P) (mmol $g^{-1}$of dry weight) of the cultures of *Chaetoceros muelleri* grown in batch culture using media Walne's, NH_4 9:1, NH_4 25:1 and NO_3 9:1 at the exponential phase (day 4-6, black bars) and stationary phase (day 16-18, grey bars). Error bars represent standard deviation (n=3) and different superscripts represent significant differences (ANOVA, LSD or Games-Howell (*) post-hoc $p<0.05$).

4. Discussion

In our experiments, *C. muelleri* was successfully grown on simplified media (using only nitrogen, phosphorus, silica, manganese, iron and vitamins) and the microalgae could use ammonium as nitrogen source, as previously reported (Lourenço *et al.*, 2002; Liang *et al.*, 2006). Liang *et al.* (2006) used f/2 medium with nitrate, ammonium or urea as nitrogen source and found that cellular density of *C. muelleri* was not affected by the different nitrogen sources in a complete medium, which agrees with our findings in the exponential phase, using simplified media. An excess of nitrogen in NH_4 25:1 did not result in an increase in dry weight production at the exponential phase. In contrast with other reports on complete culture media with alternative nitrogen sources (Lourenço *et al.*, 2002; Liang *et al.*, 2006) where cultures reached stationary phase at the latest by day 14 depending on the species, the duration of the cultures in our experiment was influenced by the different nitrogen sources and concentrations.

Several factors influence the duration and productivity of a batch culture, such as nutrients, light and pH (Hansen, 2002). pH is known to affect the availability of nutrients, mainly of carbon (Chen & Durbin, 1994), an essential nutrient in algal nutrition (Grobbelaar, 2004). In the present study the recorded pH values of all

treatments were within the acceptable values for diatom growth (Taraldsvik & Myklestad, 2000). Higher pH values were recorded in the cultures grown using nitrate as nitrogen source. This is a known result of using nitrate as nitrogen source (Grobbelaar, 2004). In the cultures grown with nitrate, pH values higher than 8.5 were only registered after the cultures had started to collapse (11 days for Walne's and 13 days for NO_3 9:1 cultures) and after a sharp reduction of nitrogen and phosphorus in the media. This punctual high pH values could have resulted in carbon limitation (as found for *Skeletonema costatum* (Taraldsvik & Myklestad, 2000) and *Thalassiosira pseudonana* and *Thalassiosira oceanic*a (Chen & Durbin, 1994)), at a point where the cultures were already nutrient deficient. However, the observed differences in growth and culture duration cannot only be attributed to the observed pH, suggesting that the different nitrogen sources and concentrations had an effect on the cultures duration. In a batch culture, the stationary phase is reached due to decreased nutrient or light availability in the medium. In this phase, cellular concentration remains constant and the cells use stored energy reserves (Fogg & Thake, 1987), until there is a collapse, due to nutrient depletion or light limitation (Hoff & Snell, 1987). When nitrogen was supplied as nitrate, the quick nitrogen consumption by the microalgae in combination with a reduction of phosphorus concentration, increasing pH and high cellular concentration (increasing the possibility of light limitation) contributed to the collapse of the cultures between days 11 to 13. The stationary phase of the cultures grown with Walne's and NO_3 9:1 was very short or inexistent, which indicates that the cells could not cope with the nutrient shortage and light limitation, possibly due to low stored reserves at that stage.

Nitrogen concentrations in the cultures of treatment NH_4 9:1 were also very low after day 13, but the cultures were alive, in the stationary phase for another 7 days. The maintenance of a healthy culture after reduction of nitrogen could be the result of no carbon limitation, high energy reserves during the exponential phase, or even a fast turnover of nitrogen in the medium when ammonium is used, coupled with sufficient phosphorus concentrations. At stationary phase, phosphorus concentrations were similar to the ones observed at exponential phase of the control. Silica is considered an essential nutrient for diatoms (e.g. Martin-Jézéquel *et al.*, 2000) and its concentrations were highly reduced after only 6 days of culture, indicating fast consumption of this nutrient. Silica supplied as sodium silicate is difficult to dissolve in seawater, raising the possibility that the actual concentration of silica in the media

was lower than expected. Although silica limitation can have an impact on growth (Martin-Jézéquel *et al.*, 2000), in the present experiment this would have been less relevant given the observed nitrogen and/or phosphorus limitations. Higher silica concentrations found in medium NH_4 9:1 in the exponential phase did not result in significantly higher dry weight production, indication that the low values observed for most of the cultivation period in all treatments did not seem to negatively affect growth of this species.

Cumulative dry-weight production at exponential phase was not significantly different between control and simplified media. Cumulative dry-weight production at the stationary phase was significantly higher than in the exponential phase due to a longer cultivation period observed when using ammonium as nitrogen source in the simplified media. Higher dry-weight production in the overall cultivation period was achieved when a surplus of nitrogen was supplied (NH_4 25:1), due to an increase of the maximum cellular concentration achieved.

The lipid contents of *C. muelleri* at exponential phase were similar between the control (Walne's medium) and the simplified medium NH_4 9:1, indicating that for lipid production this simplified medium can be successfully used. In the stationary phase higher lipid contents were also observed in cultures grown in NH_4 9:1 The use of simplified media containing nitrate and high concentrations of ammonium resulted in a significantly lower lipid content in the exponential phase, possible due to the lack of micronutrients in the media (for NO_3 9:1 and NH_4 25:1) and excess of nitrogen (NH_4 25:1), which could have affected lipid production. At the stationary phase, an increase of lipids under nitrogen limitation was observed for the microalgae grown in NH_4 9:1, this agrees with previous reports of an up to seven-fold lipid increase under nitrogen limitation for this species (McGinnis *et al.*, 1997). Under nitrogen-limited growth conditions, there is a shift from synthesis of proteins into synthesis of carbohydrates or lipids, depending on the microalgae species (Hu, 2004). The increase of the observed lipid content in the stationary phase of cultures grown in NH_4 25:1, which was still nitrogen-sufficient, was related to the phosphorus-limited medium observed during this period (Figure 4), as previously recorded for Bacillariophyceae (Reitan *et al.*, 1994).

The difference in the apparent nutrient uptake (defined as the amount of nutrient required to produce 1 g of dry weight algae, Figure 5) is an important indicator to

determine which medium is more suitable for the large-scale microalgal production, while maintaining high dry-weight production and quality. At the exponential phase, there were no significant differences between nitrogen, silica and phosphorus apparent uptake, indicating that the microalgae in the simplified media were as capable of using the nutrients as the microalgae in the control. However, the high apparent nitrogen uptake observed in the exponential phase of cultures grown in NH_4 25:1 indicates that nitrogen was less effectively used to produce biomass. At the stationary phase, no difference in apparent nutrient uptake was found between media NH_4 25:1 and NH_4 9:1, which indicated that the simplified media could effectively use the nutrients provided for growth.

In order to determine which simplified medium is more interesting and profitable for the large-scale production of *C. muelleri* the duration, dry-weight production, lipid content of the microalgae and apparent nutrient uptake efficiency should be considered. Considering these factors, at the exponential phase, the most effective simplified medium was NH_4 9:1, with comparable values to the control. If deciding to harvest in the stationary phase, the choice between NH_4 9:1 and NH_4 25:1 media will depend on the purpose of the culture: dry-weight production (e.g. for biomass production for shellfish maintenance) or lipid production (e.g. for high quality food for shellfish). However, the amount of nitrogen used should be taken into account when deciding between the two media. In the present study cultures of NH_4 25:1 were inoculated with more nitrogen and on day 16 the nitrogen values were still 611±187 μmol L^{-1}, which displays an important loss of nitrogen by harvesting time. If the medium needs to be further treated before being discarded or used as food for shellfish, costs of the operation would be increased.

5. Conclusions

Considering dry-weight production, duration, nutrient efficiency and lipid contents of the microalgae, ammonium media enriched with phosphorus, silica, iron, manganese and vitamins are a viable and profitable choice for the batch culture of *C. muelleri*.

Dry-weight production, lipid content and apparent nutrient uptake of the cultures at exponential phase were not significantly different between control and simplified media. Therefore we conclude, if harvesting occurs during the exponential phase,

the less expensive simplified media (with only 6 components) could be used for more cost-efficient production of *C. muelleri*. Regarding the overall cultivation period the most effective culture media were the simplified NH_4 9:1 and NH_4 25:1. The choice between these two media should be based on the final use of the cultures and whether high lipid contents of the microalgae (for high quality cultures for aquaculture or biofuels - NH_4 9:1), dry weight production (for biomass production - NH_4 25:1) or low nitrogen input and output (for recirculation systems - NH_4 9:1) are desired.

Acknowledgements

The authors would like to thank Angelo Hofman and Noortje Ros for their analysis of the nutrient concentrations. The authors would also like to thank the anonymous reviewers which helped improve this manuscript. This research was funded by the project Zeeuwse Tong.

Chapter 6: General discussion

This thesis investigated the importance of dietary fatty acids for *C. edule* growth, survival and fatty acid body composition during grow-out of juveniles (Chapters 3 and 4). Live microalgae were used to formulate diets with distinctive fatty acid profiles (Chapter 2 and 3), that were fed as mono-algal or mixed-algal diets (Chapter 4). Options to reduce costs of algal culture media while maintaining algal productivity for a large-scale setting were also explored (Chapter 5). The relevance of other nutritional factors other than fatty acids as well as differences in the animal's response to different diets (growth, composition, food conversion ratio) will be discussed here.

1. How do live microalgal diets with similar total lipids and total PUFA contents and different composition in terms of fatty acid profiles (amounts EPA, DHA and ARA) influence growth, survival and fatty acid composition of *C. edule* juveniles?

Linear programming was used to formulate mixed live microalgal diets with different fatty acid profiles (Chapter 2). Linear programming is commonly used to formulate inert feeds for a range of animals, but had yet to be used to design microalgal diets. Batch cultures of seven local algae species were monitored and tested for dry weight, organic matter content, lipid and fatty acid contents. The data were then used as parameters used for the linear programming. Diet constraints (range of values allowed for each parameter) were based on literature values and on the observed composition of the species and used to formulate diets of similar organic matter content but different fatty acids profiles. By using this method it was possible to formulate live microalgal diets with the required composition. Three diets were formulated: i) containing a high EPA level and DHA and ARA (High-EPA); ii) containing EPA and DHA, but little ARA (Low-ARA; iii) and a diet containing no-DHA. Linear programming has been used in diet formulation of pigs (e.g. Peña *et al.*, 2007), cattle (e.g. Tozer, 2000), poultry (e.g. D' Alfonso *et al.*, 1992) prawns (e.g. Das *et al.*, 1996) and several fishes (e.g. Ghosh *et al.*, 2011). This method can be very useful in designing high quality shellfish diets for each development phase, since numerous constraints can be combined leading to an optimal solution. Nevertheless, the use of linear programming has also limitations, especially when using live microalgae cultures. Each microalgae cell contains a mix of macro and micro-nutrients, many of them unknown, which are added to a diet as a package, when fed as live cultures. This can reduce the number of possible outcomes.

However, the main advantage of linear programming (see Chapter 2) is the ability to design diets meeting multiple requirements with specific composition of several different parameters still outweighs the limitations.

An important factor to consider when using live microalgae diets is the variability in product quality resulting from batch cultures. Although batch culture phases are well described (e.g. Fogg & Thake, 1987), the duration of each phase differs between species. It is therefore important to know the properties and behaviours of microalgae species during batch culture. Microalgae cultures in the exponential phase have high cell concentration, cells are reproducing, there is no nutrient limitation in the medium and there is less variability in the cellular contents (Helm *et al.*, 2004). When the cultures enter the stationary phase, nutrient and light limitations might impact the lipid, protein and carbohydrate contents and size of the microalgae. Furthermore, the stationary phase is usually short, and there is a constant risk of collapse of the cultures. This explains the preference for using algae harvested in exponential growth phase when feeding live microalgae diets to shellfish.

In order to increase the effectiveness of linear programming for designing live microalgal diets it may be necessary to expand the information available on the live microalgae. Depending on the life-stage of the animals being fed by the microalgae, other parameters such as proximate composition may be considered due to changes in their nutritional requirements. For example, lipids are considered to be of great importance for larvae (Marshall *et al.*, 2010) and spawning animals (Sühnel *et al.*, 2012), whereas dietary proteins (*Mytilus trossulus* Kreeger & Langdon, 1993; *C. gigas* Knuckey *et al.*, 2002) and dietary proteins and lipids (*M. mercenaria,* Wikfors *et al.*, 1992) have been correlated with growth of juveniles. The inclusion of information on protein, amino-acids, sterols and carbohydrate content, in addition to lipid and fatty acid contents, will allow matching of the composition of live microalgae diets with the nutritional requirements of each life stage. Another important factor to consider during diet formulation is microalgae size (e.g. de Pauw *et al.*, 1984). In the present work only species of appropriate individual size for filter feeding juveniles were used. If a broader spectrum of algae were to be used, size should be included as parameter, to ensure that animals can feed on the algae. Information on microalgae digestibility could also be included as a constraint. This can be done either with specific values for clearance rate and absorption efficiency of a particular microalgae species, or by relying on reported digestibility values.

Diets formulated in Chapter 2 were used to determine the role of specific dietary fatty acids for the growth and survival of juvenile cockles (Chapter 3). Diets were formulated to determine (1) whether diets with EPA and DHA result in higher growth when compared with diets without DHA, and (2) whether low ARA concentrations in a diet with EPA and DHA will affect growth.

Growth and survival was higher when the juveniles were fed diets with EPA and DHA, at any ARA concentration, whereas high mortality and lack of growth were observed in juvenile cockles fed a diet without DHA (*T. suecica* and *B. submarina*). A similar pattern was observed in the clearance rates and absorption efficiencies of the diets. The lowest values for both parameters were found in animals fed the no-DHA diet. The no-DHA diet seems to indicate the unsuitability of this diet for juvenile cockles, since the cockles could not obtain sufficient energy from it to remain alive. Therefore no conclusion on how essential DHA may be for growth of juvenile cockles, could be made in Chapter 3.

The amount of ARA in the diet did not influence growth and survival of juveniles, when the diets contained EPA and DHA. Similarly, literature data suggests that the feeding of ARA enrichment diets had no impact on survival and growth of the adult oyster *C. corteziensis* (Hurtado *et al.*, 2009). In contrast, dietary ARA positively influenced post larval growth of *Placopecten magellanicus* (Milke *et al.*, 2008), indicating ARA might be required for growth during the early development stages. Although dietary ARA is important for maturation and immune response of the oysters *C. gigas* and *C. corteziensis* (Delaporte *et al.* 2006, Hurtado *et al.* 2009), low values of this fatty acid are sufficient for growth and survival of juvenile cockles.

It was concluded that when juvenile cockles were fed diets with similar total lipids and total PUFA contents, higher growth rates and survival were observed when animals were fed diets containing EPA and DHA. Furthermore, the increase of dietary ARA in the diet containing EPA and DHA showed no added positive effect on growth and survival.

2. **a)** Is EPA or DHA or the combination of both essential for juvenile cockles?

To determine whether EPA or DHA alone could fulfil the polyunsaturated (PUFA) needs of the juvenile cockles, mono-algal and mixed-algal diets were fed to juvenile cockles (Chapter 4). In this experiment, highest growth was recorded in animals fed a diet with EPA only, followed by animals fed EPA and DHA in mixed-algal diets.

The lowest growth rates were observed in animals which were fed diets without EPA and without long-chain fatty acids (treatments Duna and Pyra). It was also found that specific growth rate was correlated with the total dry algal weight fed and total dietary fatty acids provided. These results show that access to high quantities of EPA positively impacted growth of juvenile cockles in short-term experiments. A similar positive effect of microalgal diets (composed of different microalgae species and with different fatty acid compositions) containing EPA on growth of different clams, oysters and scallops in different life stages has also been reported by other studies (Table 1). Out of 12 examples only one best performing diet did not contain EPA.

Table 1: Growth response of best performing microalgae diets from the literature (SGR in % of wet weight (WW) day^{-1} or % of dry weight (DW) day^{-1}) of different bivalve species (post-larvae<1mm, spat <1 mm, seed 1-5 mm and juvenile >5mm) fed microalgae diets. The presence (+) or absence (-) of eicosapentaenoic acid (20:5n-3, EPA) and docosahexaenoic acid (22:6n-3, DHA) in diets composition is shown.

Species	Phase	Growth[1]	Diet composition		References
			EPA	DHA	
Cerastoderma edule	juvenile	1,97% WW 1,33% DW	+	-	Chapter 4
		2,23% WW 2,72% DW	++	+	Reis Batista *et al.* , 2014 (Chapter 3)
		2,62%, WW 2,47% DW	+	+	Reis Batista *et al.* , 2014 (Chapter 3)
Ruditapes decussatus	spat	8,03%* WW 7,26% DW	+	-	Albentosa *et al.* , 1996a; Albentosa *et al.* , 1996b;
	seed	7,81% DW	-	+	Fernandez-Reiriz *et al.* , 1998
Ruditapes philippinarum	spat	8,06% WW	+	+	Caers *et al.* , 1998
Crassostrea gigas	spat	4,20%* LW	+	+	Langdon & Waldock, 1981
	seed	2,73%* DW	+	-	Knauer & Southgate, 1997
Crassostrea corteziensis	seed	11,21% * LW	+	+	Rivero-Rodriguez *et al.* , 2007
Argopecten irradians	postlarvae	8,7% Shell height day^{-1}	+	+	Milke *et al.* , 2006
	juvenile	2.9 % shell height day^{-1}	+	+	
Placopecten magellanicus	juvenile		+	+	Parrish *et al.* , 1999

* Indicates values calculated from the data presented in the reference.

As with juvenile cockles (Chapter 3 and 4), highest growth rates were registered when animals (*R. philippinarum* spat, *C. gigas* spat, *C. corteziensis* seed, bay scallop *Argopecten irradians* post-larvae and juveniles and scallop *Placopecten magellanicus* juveniles) were fed diets containing EPA and DHA. Juvenile cockles were also capable to grow at high rates when fed only EPA (Chapter 4), similarly to

R. decussatus spat (Albentosa *et al.*, 1996a, Albentosa *et al.*, 1996b) and *C. gigas* seed (Knauer & Southgate, 1997). In all the referred examples, and contrarily to juvenile cockles, *R. decussatus* seed (Fernandez-Reiriz *et al.*, 1998) grew at the highest rates when fed diets without EPA.

The literature review shows, that the large majority of the best performing diets in the reported studies contained EPA, indicating that this fatty acid seems necessary for the growth of the majority of species. However, the supply of EPA is not a guarantee for good growth and other factors, such as digestibility, diet acceptance and feeding behaviour, may limit growth despite the presence of EPA in the diet. This is exemplified in the studies of Albentosa *et al*. (1996a, 1996b), where a diet containing EPA and no DHA resulted in the highest growth rate of *R. decussatus* spat (7.26 % dry weight day^{-1}) whereas a similar monoalgal diet with EPA composed of *P. tricornutum* resulted in the lowest growth rate (5,47 % dry weight day^{-1}).

The growth of batches of juvenile cockles fed similar mixed diets of *C. muelleri* and *P. parkeae* was high in the experiments carried out in August 2010 (2,2 % wet weight day^{-1} in Chapter 3) and lower in the experiment carried out in July 2011 (0,8 % wet weight day^{-1} Chapter 4). This reflects the differences observed in the diet consumption, since in both experiments animals were fed *ad libitum* using an algal dosing machine. In Chapter 3, cockles consumed more Chaeto+Pyra diet than in Chapter 4. However, the food conversion ratio (FCR= (total diet fed in mg dry weight) / (mean live weight gain of whole animal including shell per average individual in mg)), of these diets was similar in both experiments, being 0.59 and 0.57 in Chapter 3 and Chapter 4, respectively. Hence in both experiments the animals were able to use the diet as energy for growth. The experiments also showed that the animals collected in 2011 fed less on *C. muelleri* and *P. parkeae diet.* The reasons for the difference in feeding behaviour remain unclear. Cockle's feeding behaviour is known to be affected by the organic content of the diet (e.g. Ibarrola *et al.*, 1996), but given the similarity between the diets used (and the microalgae composition), a possible explanation for the differences observed in the uptake of the similar diet is the difference of initial conditions between cockle batches. Apart from the difference in the growth and collection dates, the initial fatty acid composition of animals was also different (Figure 1). The percentage of EPA found in the cockles collected in 2010 and used in Chapter 3 was significantly higher in polar lipids than in the cockles used in Chapter 4 (15.8% and 9.8 %, respectively). On the contrary, the cockles used in

Chapter 4 showed a significantly higher percentage of DHA than the cockles used in the previous experiment.

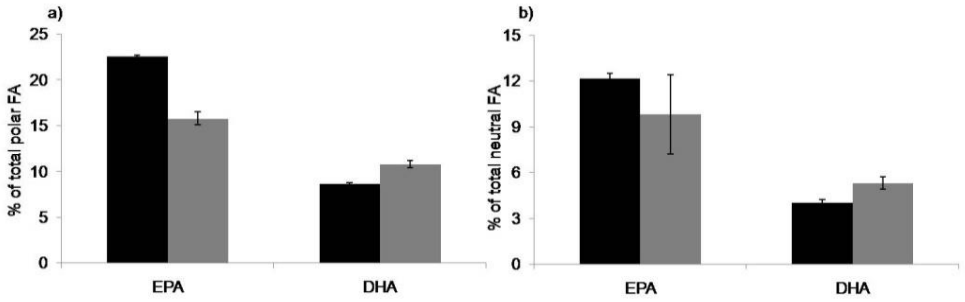


Figure 1: Initial EPA (20:5n-3) and DHA (22:6n-3) contribution for the total fatty acids of polar (a) and neutral (b) lipids of the juveniles *C. edule* collected in August 2010 (Chapter 3, black bars) and in July 2011 (Chapter 4, grey bars). Error bars represent standard deviation.

The different fatty acid composition observed show the differences in the dietary conditioning that the animals were subjected to in the field. Although the animals were collected in the same season and at the same location in the Oosterschelde estuary, their nutritional background was clearly different, which may have affected the acceptance of the diet provided thus affecting food intake during the different experiments.

2 **b)** What is the impact of dietary fatty acids on fatty acid profiles of the cockles?

The fatty acid profile of the polar and neutral lipids of the animals was influenced by the fatty acid composition in the diet (Chapters 3 and 4). An increase in the total fatty acid fraction of the neutral lipids was observed in all cockles fed microalgae diets. Neutral lipids are the lipid reserves of the animals that can be mobilized for energy or used as building blocks; they are known to be susceptible to dietary changes (Beninger & Stephan, 1985; Pazos *et al.*, 1996). The observed increase in percentages of unsaturated and PUFA fatty acids of neutral lipids in animals which were fed microalgae diets revealed a dietary imprint and the storage function of these lipids for PUFA (Ackman, 1983).

Although the composition of polar lipids is more conservative (e.g. Caers *et al.*, 1999), the fatty acid profile of these lipids was also impacted by the different diets. When the animals were supplied with high EPA and DHA concentrations (Chapter 3) a preferential incorporation of DHA over EPA was observed. This indicates the structural importance of this fatty acid and its importance for juvenile *C. edule*, which was already demonstrated for oysters (Thompson & Harrison, 1992; Caers *et al.*, 1999; Caers *et al.*, 2000) and scallops (Milke *et al.*, 2006). The importance of DHA for the cockles was further confirmed by the maintenance of DHA levels in unfed animals (Chapter 4). However, high growth in juvenile cockles fed *T. suecica* (with no DHA) indicated that either EPA or DHA can fulfil the n-3 PUFA requirement, as previously reported for other bivalve species (Albentosa *et al.*, 1996a, Coutteau *et al.*, 1994b, Fernandez-Reiriz *et al.*, 1998, Langdon & Waldock, 1981), as long as minimal amounts of DHA are present in the animals.

The growth results and fatty acid profiles of the cockles reinforce the conclusion about the importance of dietary EPA for the fast short-term growth of juvenile cockles. The decrease of DHA in the polar lipids of animals fed diets without this fatty acid also reflected the dietary link. The observed requirements depend on the function of each fatty acid, including the structural role of DHA (Soudant *et al.*, 1998, Delaporte *et al.*, 2003). The preferential retention of DHA in starved larvae of *C. gigas* (Thompson & Harrison, 1992) was also found in the unfed juvenile cockles in this study, as well as in early spat of *R. philippinarum* fed a diet with no PUFA (Caers *et al.*, 1998). Although our experimental results are not fully conclusive, the inclusion of EPA (due to its energetic role) and DHA (due to its structural role) in a live microalgal diet is advantageous for the successful land-based grow out operation of juvenile cockles. Given EPA and DHA importance in the short-term growth experiments shown, long-term growth studies on the effects of EPA and DHA deprivation should be carried out in a land-based culture setting.

3. Are mixed live microalgae diets combining different taxonomic classes better than single class diets?

Microalgae diets containing both diatoms and flagellates (e.g. Chlorophyceae and Prasinophyceae) are considered suitable for shellfish (Helm *et al.*, 2004) because the composition is better balanced compared to mono-algal diets, especially regarding total PUFA content. Prasinophyceae are also frequently used with varying

success depending on the chosen species. In the present short-term experiments the uptake of the diet (and consequently the dry weight fed) seemed to be as important as diet composition. The reason behind the unsuitability of the no-DHA diet (Chapter 3) constituted only by Chlorophyceae can therefore not be explained by the lack of diatoms, but is probably related with reduced intake and digestibility, as well as with the dietary condition of the wild cockles. In Chapter 4 growth of juvenile cockles on mono-algal and mixed-algal diets was monitored. The FCR of the mono-algae diets varied between 0.43 (*T. suecica*, indicating excellent nutrient retention) and 1.08 (*D. tertiolecta,* indicating more metabolic loss). By mixing the Chlorophyceae *T. suecica* with the Prasinophyceae *P. parkeae* we saw an increase of the FCR to 0.63 as well as lower growth rates, when compared with animals fed only *T. suecica*, due to lower food consume by the animals fed the mixed diet. The reduction of the acceptability of the mixed diet was possibly due to with difficulties in the digestibility of *P. parkeae* when compared with *T. suecica* was added to the diet. When comparing the FCR of the animals fed the two mixed-algal diets containing both EPA and DHA, the Chlorophyceae and Prasinophyceae diet (*T. suecica* and *P. parkeae*) and the Bacillariophyceae and Prasinophyceae diet (*C. muelleri* and *P. parkeae*, FCR 0.59), it was shown that the beneficial effect of a mixed diet of diatoms and Prasinophyceae was minimal, indicating that there may be no positive effect of mixing a diatom and a flagellated species within the short term growth period.

4 **a)** Can cockles produce EPA and DHA from the precursor 18:3n-3?

In this thesis it was shown that juvenile cockles, like other bivalves (e.g. clams *Mesodesma mactroides* (De Moreno *et al.*, 1976), and *Ruditapes philippinarum* (Caers *et al.*, 1999), *Scapharca broughtoni* (Zhukova, 1986), mussels *Mytilus galloprovincialis* (Garrido & Medina, 2002), and oysters *Crassostrea gigas* (Delaporte *et al.*, 2005) are not able to biosynthesize EPA, DHA or ARA from the precursors 18:3n-3 and 18:3n-6. Results of Chapter 3 indicated that cockles could not synthesize DHA (low DHA found in polar lipids of cockles fed no-DHA diet). In Chapter 4, using diets with high fatty acid content and no >20-C long-chain fatty acids (*D. tertiolecta*), no biosynthesis was observed (by maintenance or increase in EPA or DHA content). Therefore, dietary EPA and DHA needs to be provided.

4 **b)** Can cockles, like other marine bivalves, *de novo* produce NMI fatty acids?

The biosynthesis pathways to produce dienoic and trienoic NMI found in marine invertebrates, including molluscs (Zhukova&Svetashev 1986) were also found in cockles (Chapter 4). Sequential elongation and desaturation of 16:1n-7 and 18:1n-9 resulted in the production of 20:2Δ5,13, 20:2Δ5,11, 22:2Δ7,15 and 22:2Δ7,13 (Zhukova, 1986). The desaturation of 20:2n-6 into 20:3Δ5,11,14 and further elongation it into 22:3Δ7,13,16 (Garrido & Medina, 2002; Le Grand *et al.*, 2013) was also observed, demonstrating the ability of juvenile cockles to produce NMI fatty acids.

4 **c)** Is the NMI synthesis a direct result of the dietary fatty acid composition or a replacement for lacking EPA or DHA in the diet?

The function of NMI is not fully understood (e.g. Delaporte *et al.*, 2005), but it has been hypothesized that 20 and 22 NMI could have similar functions for the stability and fluidity of the membranes as EPA and DHA. NMI fatty acids were present in high percentages in the polar lipids, demonstrating the more structural role of these fatty acids for cockles, as previously suggested for other molluscs (e.g. Zhukova & Svetashev, 1986). The NMI can be used in the razor clam *Solen marginatus* as a replacement for EPA and DHA when they are not provided by the diet (da Costa *et al.*, 2011).This was not observed in our study with juvenile cockles. Changes in the NMI composition were a direct result of biosynthesis from different dietary fatty acids used as precursors (16:1n-7, 18:2n-9 and 20:2n-6). No evidence of NMI fatty acid synthesis as replacement for lacking dietary EPA or DHA was found. Their role in the juvenile cockles could therefore be correlated with the structural role and prevention of membrane oxidation (Barnathan, 2009).

5. Is the production and the duration of a batch culture of *C. muelleri* different between the standard Walne's medium and a simplified medium?

There are several different growth media recipes for different microalgae and culture targets. The differences between the growth media are usually small, related to source of nitrogen, carbon and phosphorus, nutrient concentrations and presence of some micro-nutrients. Some of these widely used media are Walne's (Walne, 1970), Guillard (Guillard & Ryther, 1962) and f/2 (a reduction of Guillard media, Guillard, 1975) and Bold´s basal (Bischoff & Bold, 1963). All media have a similar

supply of macro nutrients (such as nitrogen, carbon and phosphorus) and a solution of trace metals, in order to optimize growth. A simplified culture medium that has less ingredients, is easier to prepare and results in microalgae growth will be an advantage for the development of land-based aquaculture of shellfish. To this end, the focus of the study was the impact of simplified media on dry weight production and lipid composition of the diatom *C. muelleri*. The simplified media designed were enriched with nitrogen, phosphorus, silica, manganese, iron and vitamins. Different nitrogen sources (nitrate as in Walne's and ammonium as an alternative) and N/P ratios were also used (9:1 and 25:1).

The results presented in Chapter 5 show that cultures grown on seawater enriched with these simplified media achieved similar cellular concentration as the control medium at the exponential phase. The lipid and dry matter production was also similar between all media used, indicating that the simplified media can be used as an alternative to Walne, for *C. muelleri.* Microalgae can successfully use nitrate or ammonium as nitrogen (Kaplan *et al.*, 1986) as shown in this study. It was observed that the use of ammonium as nitrogen source resulted in a longer, more stable stationary phase, which has not been previously reported for this species (Liang *et al.*, 2006, Pacheco-Vega & Sánchez-Saavedra, 2009). The shorter stationary phase observed in the cultures grown with nitrate seemed to be related to carbon and nitrogen limitations, as well as the pH registered in these cultures. The use of nitrate as nitrogen source is known to increase the pH of cultures (Grobbelaar, 2004), as was observed in towards the end of the exponential phase of cultures grown using nitrate. The pH of the cultures has a strong impact on cellular growth, with the growth of diatoms being negatively affected by pH values higher than 8.4 (Taraldsvik & Myklestad, 2000, Chen & Durbin, 1994), which were related with the collapse of the nitrate cultures after the exponential phase. The use of ammonium may lead to the acidification of the culture medium (Grobbelaar, 2004) and thus negatively affect the growth rate if the pH is too low (Eustance *et al.*, 2013). This may have helped keep the pH of the cultures at levels that allow the survival of the cells, thus increasing cultivation period. In the stationary phase of the cultures grown on ammonium, the higher dry matter production was achieved when nitrogen was supplied (N/P 25:1) in excess, whereas lipids increased in cultures where nitrogen was low (N/P 9:1), as previously reported for several species, such as *C. muelleri* and *Isochrysis* sp. (Guschina & Harwood, 2006, Hu, 2004, McGinnis *et al.*, 1997, Pernet *et al.*, 2003).The longer and stable stationary phase observed in the cultures grown on

ammonium, in combination with biochemical composition of the cells, indicate that these cultures can be harvested for feed for a longer period of time. It is common practice to harvest microalgal cultures (for shellfish feeding) while the cultures are in the exponential phase, as a way to assure quality and not to risk losing the culture due to a short stationary phase, followed by the collapse of the culture. Furthermore, the systematic harvest of microalgae at the same development stage will reduce the differences in the biochemical composition of the microalgae, thus assuring a more stable and reproducible diet supply for shellfish. As long as the quality of the microalgae is known, adequate and similar between different batches, microalgae in either the exponential or stationary phase can be used as diets for shellfish. In the cultures of *C. muelleri* grown on ammonium, the increase in the duration of the culture with high dry matter and lipid contents allows for a longer period of harvest, without the risk of a sudden crash.

Simplified media containing seawater enriched with ammonium, phosphorus, silica, iron, manganese and vitamins are a viable choice for the batch culture of *C. muelleri*. These simplified media are also a profitable choice when compared with Walne medium. Using the price of the nutrients for Walne medium, 12,40 €, (calculated using laboratory grade chemicals at a laboratory scale extrapolated into large scale culturing systems of 1000L), the nutrients for simplified media cost only 36,5% (4,54 € for N:P 25:1) and 34,8% (4,32 € N:P 9:1) of that total, resulting in significant savings. The choice between the two simplified media should be based on the final use of the cultures and whether lipid contents of the microalgae (N:P ratio 9:1), dry matter production (N:P ratio 25:1) or low nitrogen input (N:P ratio 9:1) are desired.

Conclusions and future perspectives

Conclusions

The results of this thesis indicate that land-based aquaculture of cockles and microalgae is a promising venture, with a high potential for expansion.

Growth

The present work demonstrated that mono-algal diets with high EPA, as well as mixed live microalgae diets with high EPA and DHA contents proved to be suitable diets for *C. edule* juveniles. Growth and survival of juvenile cockles was observed, with no apparent detrimental effect, provided the

amount fed of these diets was sufficient. The grow-out of juvenile *C. edule* on microalgae diets has already been reported once. While the impact of dietary fatty acids on juveniles of cockles has not been determined, cockles of 4,9mm were successfully grown into juveniles in a hatchery setting, after being fed *ad libitum* a mixture of *D. tertiolecta*, *P. tricornutum*, *T. suecica*, *S. costatum*, *C. muelleri* and I*sochrysis galbana* affinis Tahiti (Pronker *et al.*, 2015). It was observed that animals kept in a mesh over sand, at lower density (500 individuals per m^2), grew at a rate of 168µm day^{-y} over a period of 77 days. When the animals were kept at higher densities (1000 individuals per m^2), the growth rates rapidly decreased in animals after 60 days after fertilization, to 59 µm day^{-y} over the following 40 days. The animals used in this thesis were stocked at 2693 individuals per m^2 and 4040 individuals per m^2 and grew at the highest growth rates of 76 (Chapter 3) and 41 (Chapter 4) µm day^{-y}, respectively over the different experimental periods. These values are similar with the growth rates previously recorded, for animals grown on high densities (Pronker, 2015). The growth rates of the cultured cockles in this thesis are in accordance with the rates expected under natural conditions for these animals. Using field data of the overall growth period May-September (Kamermans *et al.*, 2003) and the initial size of the cockles used in the thesis, growth rates under natural conditions would be very similar to the ones observed in Chapter 3 (2.2 % in field vs. 2.2% for High EPA diet) and Chapter 4 (2.1 % in field vs. 2.0% for Tetra). In spite of the fact that these were only short-term growth experiments, considering the growth rates observed in this study, juvenile cockles (shell length>5mm) could be grown into commercial size (shell length of 3 cm, calculated fresh weight of 14.40 g (Stralen, 1990)) within 242-262 days, assuming a constant growth rate. Although growth rates are expected to be reduced with the increase in size of the animals, these results are in accordance to pilot results have shown that land-based shellfish production can be achievable in a one-year period (see Land-based shellfish production).

Fatty acids

While there are no studies on growth of cockles during grow-out, studies on spat of different species of clams are available (the grooved carpet shell *R. decussatus* (Albentosa *et al.*, 1999, Pérez Camacho *et al.*, 1998, M.

Albentosa, 1996, Albentosa *et al.*, 1996b), hard clam *M. mercenaria* (Pales Espinosa & Allam, 2006, Caers *et al.*, 1995, Coutteau *et al.*, 1994b) and the Manila clam (*R. philippinarum* Fernández-Reiriz *et al.*, 2006, Piveteau *et al.*, 1999, Caers *et al.*, 1999, Coutteau *et al.*, 1994a). The nutritional requirements for these species are still not completely understood, but the role of EPA and DHA has been addressed. However, there is no consensus on whether EPA or DHA are crucial for the growth of these species, indicating that either EPA or DHA can fulfil the n-3 fatty acid requirements (Albentosa *et al.*, 1996a, Coutteau *et al.*, 1994b, Fernandez-Reiriz *et al.*, 1998, Langdon & Waldock, 1981). In the present study it is suggested that EPA has a role providing energy for growth of the juvenile cockles, whereas DHA is important due to its structural role.

The results on the fatty acid metabolism of cockles indicated that EPA and DHA cannot be synthesized by cockles, and that the 20 and 22 NMI fatty acids can be produced by the cockles, but not sufficiently to overcome EPA or DHA deficiencies. Supply of dietary EPA and DHA is therefore necessary for the animals as a way to maintain and enrich their n-3 fatty acid content, which is important for the animal and also for human nutrition.

Microalgae

Large-scale microalgae production needs to be developed to support land-based bivalve culture. Simplified media have been successfully designed for batch cultures of *C. muelleri*, a marine diatom with a high EPA content that is frequently used as food for shellfish. The medium (simplified with ammonium as nitrogen source) represents a significant decrease in the cost and preparation of the medium. Using the new medium the culture lasted longer, allowing for a longer period of production and resulting in a higher total cellular production per batch. Depending on the specific needs of the shellfish, media can be chosen targeting a high dry matter production or high lipid content in the algae.

Land-based shellfish production

The viability of land-based shellfish aquaculture was accessed during the Zeeuwse Tong project. Although *C. edule* was chosen as primary species for research, high cockle mortality observed during the first winter of the

project led to the decision to use *R. philippinarum* as bivalve species in the fish – shellfish – ragworms integrated farming project concept. Results obtained in the pilot land-based cultures of microalgae and bivalves, show that *R. philippinarum* (shell length 1cm) could be cultured to commercial size (>3cm) in 228 days (Smaal *et al.*, 2014). Not only was the growth of juveniles determined in these pilots. In addition, the effects of fattening of oysters, introducing of adult oysters into ponds for a final fattening period of some weeks of feeding specific diet, on size and fatty acid contents were also accessed. As shown in Table 2 it was found that after six weeks of fattening with live microalgae the oysters *C. gigas* had a significant increase in wet weight, as well as an increase in total fatty acid concentration (Jansen, 2014).

The Zeeuwse Tong's pilot land-based aquaculture operations have therefore shown that land-based aquaculture of shellfish in the Netherlands is possible. A harvestable yield can be reached within a year and the accumulation of fatty acids during short term feeding has shown that the approach is also promising for improving the quality of the product.

Table 2. Fatty acid concentrations (mg C g^{-1} DW) of selected fatty acids in oysters *Crassostrea gigas* prior and post a fattening period of approximately 6 weeks in semi-intensive pond system and fed with high quality phytoplankton diets (Retrieved from Jansen, 2014).

	Prior fattening	Post fattening
20:5n-3*	4.27 ± 1.21	11.00 ± 2.03
22:6n-3*	2.87 ± 0.70	5.05 ± 1.04
T. PUFA*	12.17 ± 2.91	24.63 ± 4.43
T. PUFA n-3*	10.33 ± 2.47	21.60 ± 3.92
Total FA*	27.02 ± 6.24	47.87 ± 9.10

*Asterisk indicates significant differences in each fatty acid concentration prior and after the fattening period (t-test, ά=0.05)

Future Perspectives

The viability of land-based aquaculture of cockles depends on its profitability that is highly regulated by costs of diet production and survival and growth rates of the animals. Future developments in the microalgae culture should aim to reduce production costs while increasing productivity in a year-round production cycle. This

will require the development of simplified media for other microalgae species (such as *T. suecica*) and further extensive research on semi-continuous or continuous outdoor algal cultures throughout different seasons, preferably be executed at commercial pilot scale. When working in large scale it may become difficult to ensure that microalgae are always harvested and fed to bivalves during only the exponential phase of growth. Considering the changes in lipid composition reported in Chapter 5 as the culture develops, the knowledge of the microalgae quality throughout all the cultivation is therefore crucial to be able to provide diets with the necessary quality for each development stage of the bivalves. In addition, efficient methods for microalgae harvest and supply to the animals still need to be developed.

Combining large-scale production of high quality microalgae with bivalve culture in a land-based aquaculture system is possible, as was shown by the Zeeuwse Tong project, with estimated yield of 10 Kg per m^2 of the Manila clam (Smaal *et al.* 2014). However, there are still some bottlenecks to be overcome. For land-based shellfish culture, seed used in the grow-out operation should be obtained from hatcheries and not rely on animals collected from the wild. The reduction of variability of feeding history will assure a more coherent initial condition of the animals and will help reduce differences in growth responses caused such factors. Furthermore, this would provide opportunities for improved performance through selective breeding. Regarding nutritional aspects, several points should be taken under consideration. Given the high reduction of DHA and EPA fraction in the polar lipids of cockles fed mono-algal diets without DHA and EPA in the short-term experiments shown here, the long-term effects of DHA and EPA deprivation on growth and survival need to be addressed. Moreover, differences in uptake rate between similar diets by juvenile cockles should be addressed to elucidate causes for variations in feeding behaviour. The importance of dietary ingredients other than lipids and fatty acids for growth cannot be excluded. Specific growth rate was found to be positively correlated with total algal dry weight fed to the animals, even though the proximate composition of the diets differed. Further research should be carried out to determine the contribution of dietary proteins and carbohydrates to growth of juvenile cockles using a similar approach for formulating diets with similar lipid and organic matter composition while varying the amount of proteins and carbohydrates.

In summary, although there are several challenges to be solved, the use of land-based grow-out of cockles as an alternative to wild fishery is promising.

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Summary

Land-based shellfish culture as a part of a multi-trophic aquaculture systems has yet to be implemented in Europe. Recently the pilot project Zeeuwse Tong (The Netherlands) evaluated the feasibility of a system of fish (Dover sole), ragworms, phytoplankton and bivalves. This thesis focused on the dietary fatty acids impact on growth, survival and fatty acid composition of juveniles (shell length >5mm) of the common cockle *Cerastoderma edule*, for land-based culture. Dietary fatty acids were chosen as the main nutritional research subject given the existing literature indicating their importance for reproduction, and growth and survival of larvae, post-larvae, spat and seed of most bivalves. However, since grow-out of juvenile cockles in land-based aquaculture is not common, no information is available on the dietary fatty acid requirements of juveniles. Furthermore, in order to develop land-based culture of cockles it is crucial to have a supply of high quality live microalgal diets produced with minimum effort. Therefore, the use of simplified microalgae media were also investigated.

To determine if the presence of specific fatty acids is more important for growth and survival of juvenile cockles than the total amount of dietary fatty acids supplied, least cost linear programming was used to design live microalgae diets. Monocultures of indigenous algae were grown under controlled conditions and sampled to determine their quality. Cultures of marine diatoms *Skeletonema costatum*, *Phaeodactylum tricornutum*, *Thalassiosira pseudonana*, *Chaetoceros muelleri* and flagellated species *Pyramimonas parkeae*, *Tetraselmis suecica* and *Brachiomonas submarina* were sampled in the exponential phase to determine their dry weight (DW), organic matter (OM), lipid and fatty acids (FA) composition. These constraints were used in a least-cost programming software, using the production effort (necessary to produce 1 g of DW of the microalgae) as an optimization goal. Three algal diets were designed with specific eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA) and arachidonic acid (ARA) contents but with similar OM, lipid and total FA contents. There are limitations to the use of this technique when dealing with live feed, since each biochemical component cannot be separated within each algae. However, with some flexibility on minimum values for constraints, the differences in biochemical composition of the microalgae allowed the formulation of three diets with different fatty acid contents. The present approach using linear programming to develop shellfish diets based on live microalgae was proven to be efficient in designing diets with specific characteristics.

The formulated diets were then used to determine the importance of dietary 20:5n-3 (EPA), 22:6n-3 (DHA) and 20:4n-6 (ARA) fatty acids for growth, survival and fatty acid composition of juvenile cockles (*Cerastoderma edule*). Juvenile cockles (6.24±0.04 mm and 66.14±0.34 mg (live weight)) were constantly fed live microalgal diets, below the pseudofaeces production threshold, during a 21 day period. The diets previously designed had contrasting fatty acid contents: high EPA (53% *Chaetoceros muelleri* + 47% *Pyramimonas parkeae*), no DHA (47% *Brachiomonas submarina*+ 53% *Tetraselmis suecica*) and low ARA concentrations (73% *P. parkeae* + 27% *Phaeodactylum tricornutum*).

Growth was positively affected by high EPA and low ARA diets, whereas no significant growth was observed for the no DHA diet. High mortality of cockles fed no DHA diet and the low absorption efficiency and clearance rate of this diet, however, indicate that this diet is unsuitable for juvenile cockles. In balanced diets with EPA and DHA, lower concentrations of ARA did not limit growth. The combination of EPA and DHA in a live microalgae diet was beneficial for the growth and survival of juvenile cockles.

Since it was not possible to determine the importance of EPA versus DHA for growth and survival of juvenile cockles, an additional growth experiment was designed. Growth and fatty acid profiles of juveniles cockles were determined after 28 days of feeding diets:1) with EPA (*Tetraselmis suecica*-Tetra); 2) with DHA (*Pyramimonas parkeae*-Pyra); 3) with EPA and DHA from a mixture of the diatom *Chaetoceros muelleri* with green algae *P. parkeae* -Chaeto+Pyra); 4) with EPA and DHA from a mixture of the green algae *T. suecica* with *P. parkeae* -Tetra+Pyra) and 5) without long-chain (>C20) fatty acids (*Dunaliella tertiolecta* -Duna). A control treatment of unfed animals was also kept for the same period.

Growth occurred in all fed treatments, and survival was significantly higher than in unfed animals. Specific growth rate was correlated with the amount of dry weight and total fatty acids fed. Significantly higher growth rate was observed with treatment Tetra (rich in EPA), while the lowest growth rates were found in treatments Duna and Pyra treatments without EPA. These results indicate that EPA is necessary for the short-term growth of cockles. Furthermore, the fatty acid profile of the polar lipids confirmed that, like most marine invertebrates, juvenile *C. edule* are not able to biosynthesize EPA and DHA from 18:3n-3 but are capable of biosynthesizing diene and triene NMI fatty acids. The occurrence of the NMI fatty acids was diet related, thus refuting the possibility of production of NMI as replacement for EPA and DHA.

In order to develop large-scale microalgae cultures as feed for shellfish, the development of a cost-effective large-scale microalgae culture is of highest importance. Using Walne´s medium as a positive control, simplified media containing nitrogen, phosphorus, silica, iron, manganese and vitamins were formulated to determine the impact of nitrogen source and molar N:P ratio (sodium nitrate, $NO_3$9:1 and ammonium chloride, NH_4 9:1 and NH_4 25:1) on growth, dry-weigh biomass, culture duration and lipid contents of *C. muelleri*. This marine diatom was chosen given its robustness and quality as food for juvenile cockles.

C. muelleri reacted positively to all simplified media. At the exponential phase, all cultures had reached similar cellular concentrations and dry weight productions, although highest lipid contents were found in cultures grown using Walne's and NH_4 25:1 media. The rapid decrease of nitrogen concentration in cultures grown with nitrate caused the collapse of these cultures within 11-13 days, after a short stationary phase. However, cultures grown on ammonium media had a longer cultivation period, 20 days. During the stationary phase (day 18) there was an increase of lipid content in algae under conditions of low nitrogen availability (NH_4 9:1) and extended low phosphorus availability (NH_4 25:1). Considering dry-weight production, culture duration, nutrient efficiency and lipid composition, the simplified media containing ammonium, phosphorus, silica, iron, manganese and vitamins proved to be a viable choice for batch culture of *C. muelleri.* At the exponential phase, the simplified medium NH_4 9:1 was as effective as the control. When looking at the overall cultivation period (18 days), both simplified media with ammonium were effective and suitable for diatom production. The choice between these two media depends on the final purpose of the microalgae cultures and whether lipid contents ($NH_4$9:1), dry weight biomass ($NH_4$25:1) or nitrogen input and output ($NH_4$9:1) are more important.

In this thesis it was demonstrated that mono-algal diets with high EPA, as well as mixed live microalgae diets with high EPA and DHA contents proved to be suitable diets for *C. edule* juveniles, provided the amount fed was sufficient. The growth rates of the cultured cockles in this thesis are in accordance with the rates expected under natural conditions. Juvenile cockles (shell length > 5mm) could be grown into commercial size (shell length of 3 cm, calculated fresh weight of 14.40 g (Stralen, 1990)) within 242-262 days, less than a year, assuming a constant growth rate. Although growth rate may be reduced with the increase in size of the animals, pilot results have shown that land-based shellfish production can be achieved in a one-

year period. The use of the simplified microalgae culture medium tested represents a significant decrease in the cost and preparation of the medium, as well as longer production and harvest periods, while maintaining microalgae quality. These results indicate that land-based aquaculture of cockles and microalgae is therefore a promising venture, with potential for expansion.

Acknowledgements

In order to finish this chapter, many thanks are needed. Without further ado, here it goes. First I would like to thank the people who gave me the opportunity to do this PhD and being involved in the Zeeuwse Tong project, my promotor Prof. Dr. Aad Smaal, my daily supervisor Dr. Pauline Kamermans and Dr.ir. Jan Ketelaars. I would also like to acknowledge the support, time, expertise and dedication of my supervising committee that was completed by the addition of Dr. ir. Marc Verdegem as my second copromoter. Aad, thank you for letting me try to find my way, while guiding me into this new shellfish world and sharing your vast expertize. This was a long run, and I appreciate your patience and dedication. Marc thank you for all the suggestions, discussion and teaching moments, for your dedication and time you spent on skype calls! A special thank you is needed Pauline, the one person without which this thesis would have not seen the light of day. Thank you for all the daily supervision, reminders and suggestions, knowledge, tips and extra hours of work, long skype meetings, countless pep talks and an unwavering conviction this could be done. I also want to thank Dr. Philippe Soudant for his help with the fatty acid identification, at a time where I was somewhat lost. It was a pleasure to be able to learn so much from you, and I am very grateful for all your investment in this work.

I was very lucky to have the chance to work at IMARES in Yerseke and to have the help of Ainhoa, Emiel, Ad, Pauline, Hans, Noortje and Angelo during the preparation and sometimes duration and analysis of my experiments. Pauline, Brenda and Antonio, thank you for your assistance in collecting cockles. I would also like to thank Marco, Anton, Peter, Lara and Michele, colleagues from the neighbouring institute, now NIOZ, for all the help, useful talks and support. A special thank you is due to the two brilliant, enthusiastic and very helpful students I had the pleasure of supervising during my PhD, Anne Marie Bosman and Pim van Dalen.

Living 4 years in Yerseke was definitively something remarkable for many reasons, one of which was to get to know this group of people. I had the pleasure of spending my working hours with you all and you made my stay there easier, funnier and enjoyable. Thank you Ainhoa, Emiel, Ad, Joke, Marnix, Ilse, Jeanet, Anneke, Carola, Jack, Johan, Hans Z., Karin, Wout, Eugene, Tim, Mascha, Hans v., Martine, Jeroen W., Douwe and Eva.

But a PhD is not only made of work, and so I also have to thank the people who made my life in Zeeland possible. Ainhoa and Anton, I can never thank you enough for letting me into your family and making me feel like a part of it. Thank you for everything, you were my home away from home, and I am so grateful for that. To

these and all the other friends I made during this period...it was a blast!! Ina, Sander, Saskia, Vanessa, Chris, Francesc, Erika, thank you for everything. I would also like to thank the friends I had a chance to work along with, my PhD colleagues. A special thank you for Stephan, Jacob, Michel and André for the many (many!) great times and support! To Jeroen, Antonio, Brenda and Wouter, you made the group complete. Thank you all for the company, jokes, dinners and drinks, courses and life. Again, it was a blast!
To the Den Haag duo, Katherine, João and Pico, thanks for the all the walks, talks and life lived!
Apesar do contacto não ser diário e complicado por horários, trabalhos e distâncias, quero também agradecer à Ana Catarina e Maria Ana, por me manterem ligada à planície dourada e por serem uma constante lembrança de que há coisas que são para sempre.
Às pessegas Gisela e Marina, Mahaut e Paula, obrigado pela companhia, apoio e gargalhadas, ainda que muitas vezes por mail. FCMA girls, power on!!

This time spent in the Netherlands doing my PhD has brought me also one great joy personally. I am so happy that our paths crossed, Thorsten. You are the one good thing to come out of this, and for that I will be forever grateful. Thank you for holding me up, for being there for me and for being you. Looking forward to our next adventure, mein Liebling!
E claro, não posso deixar de agradecer à minha família. Quando tudo isto começou, já sabiamos que ia ser difícil, mas nenhum de nós sabia bem o quanto. Obrigado por tudo. Por me manterem sã, terem sempre palavras motivadoras, braços abertos e apoio, muito apoio. Pai, obrigado pelos mimos, força, colo e palavras confortantes. Ana, a ti devo-te um agradecimento especial, porque para além de me teres acompanhado a cada passo e estares sempre a torcer por mim, de me teres dados os sobrinhos mais lindos do mundo e de me teres visitado, ainda tiveste o bom senso de me teres mandando parar com coisas e acabar isto de uma vez por todas! Alexandra, obrigado pela motivação e tudo o resto, e especialmente pela capa linda. Eurico cunhadinho, obrigado pelos petiscos e carinho. Carolina e António, obrigado por existirem e fazerem de mim uma tia babada e muito feliz. Obrigado a todos. Mãe, apesar de me ter dito para vir, sair daí e ir tentar a minha sorte por estes lados, não há dia em que não me pergunte se valeu a pena. Tenho tantas saudades. Mas já está, mãe. Obrigada por tudo.

About the author

Isabel C. dos Reis Batista was born on the 13th of October 1983 in Beja, Portugal. She went on to study Marine Biology at the University of Algarve, were she soon discovered her interest in Integrated Aquaculture. She did her first thesis internship at IFREMER Bouin, under the supervision of Dr, Jérôme Hussenot and Prof. Dr. Maria Teresa Dinis, on microalgae growth on saline groundwater. She then went on to do a second internship at IMARES Yerseke investigating the possibility of using local groundwater for diatom production, with Dr. Pauline Kamermans. She finished her MSc on Marine Biology, with a specialization in Aquaculture and Fisheries in December 2007. On the 1st of September 2008 the author started her PhD on microalgae and shellfish culture, in a land-based integrated aquaculture setting. The author has since published two scientific papers and has been developing a commercial project to produce microalgae. On a lighter note, she has started to learn German, developed her skills as a baker, continues to dance on her living room and is now preparing for new challenges in yet another country.

WIAS Graduate School Training and Supervision Plan

	year	ECTS
The Basic Package		**3**
WIAS Introduction Course	2009	
Course on philosophy of science and/or ethics	2012	
Scientific Exposure		12
International conferences		
European Aquaculture Society	2010	
European Aquaculture Society	2011	
World Aquaculture Society	2012	
Seminars and workshops		
WIAS Science Day	2010	
WIAS Science Day	2011	
IMARES PhD Day	2011	
Presentations		
Oral presentation at the European Aquaculture Society Conference	2010	
Poster presentation at the WIAS Science Day	2010/2011	
Oral presentation at the European Aquaculture Society Conference	2011	
Oral presentation at the IMARES PhD Day	2011	
Oral presentation at the World Aquaculture Society Conference	2012	
Oral presentation at the World Aquaculture Society Conference	2012	
In-Depth Studies		6
Marine algae culture: techniques, uses and development perspectives	2010	
Design of Experiments (14-16 October)	2009	
Statistics for the Life Sciences	2011	
PhD students' discussion groups	2010-2011	
Professional Skills Support Courses		5
Techniques for Writing and Presenting a Scientific Paper	2012	
Language course	2009/2010	
Insight Discovery Day	2010	
Research Skills Training		6
Preparing own PhD research proposal	2008-2009	
Didactic Skills Training		3
Supervising theses		
MSc major	2009-2010	
BSc thesis	2010-2011	
Education and Training Total		**34**

* one ECTS credit equals a study load of approximately 28 hours

Colophon

The research described in this thesis was financially supported by the Zeeuwse Tong project (Zeeland Sole Project, co-funded by the European Fisheries Fund).

Financial support from Wageningen UR for printing this thesis is gratefully acknowledged.
Cover art by Alexandra Reis Batista
Cover design by Thorsten Balke
Printed by CPI Koninklijke Wöhrmann

EAT AND BE EATEN

PORPOISE DIET STUDIES

Maarten Frederik Leopold

Thesis committee

Promotor
Prof. dr. ir. P.J.H. Reijnders
Professor of Ecology and Management of Marine Mammals
Wageningen University

Other members
Prof. dr. A.D. Rijnsdorp, Wageningen University
Prof. dr. U. Siebert, University of Veterinary Medicine, Hannover, Germany
Prof. dr. M. Naguib, Wageningen University
Mr M.L. Tasker, Joint Nature Conservation Committee, Peterborough, United Kingdom

This research was conducted under the auspices of the Netherlands Research School for the Socio-Economic and Natural Sciences of the Environment (SENSE).

EAT AND BE EATEN

PORPOISE DIET STUDIES

Maarten Frederik Leopold

Thesis

submitted in fulfilment of the requirements for the degree of doctor
at Wageningen University
by the authority of the Rector Magnificus
Prof. dr. ir. A.P.J. Mol,
in the presence of the
Thesis Committee appointed by the Academic Board
to be defended in public
on Friday 20 November 2015
at 4 p.m. in the Aula.

Maarten Frederik Leopold
Eat or be eaten: porpoise diet studies
239 pages

PhD thesis, Wageningen University, Wageningen, NL (2015)

With references, with summaries in Dutch and English
ISBN 978-94-6257-558-5

There is a crack
a crack
in everything...
that's how the
light gets in

Leonard Cohen (1992) Anthem

Contents

Names and addresses of authors

Geert Aarts[1]
Lineke Begeman[2,3]
Judith D. L. van Bleijswijk[4]
Sophie M. J. M. Brasseur[1]
Elze Dijkman[1]
Andrea Gröne[2]
Jan Haelters[5]
Eileen Heße[1,6]
Jaap van der Hiele[7,8]
Sjoukje Hiemstra[2]
Lonneke L. IJsseldijk[2]
Thierry Jauniaux[9,10]
Guido O. Keijl[1,11]
Mardik F. Leopold[1,12]
Jaap van der Meer[13,14]
Erik H.W.G Meesters[1]
Lara Mielke[1,6]
Peter J.H. Reijnders[1,12]
Tara Schelling[1,6]
Liliane Solé[1,15]
Lizzy van der Steeg[1,6]
Carolien H. Strating[1,16]
Dorien Verheyen[1]
Harry J. Witte[4]

[1] Wageningen-IMARES, PO Box 57, 1780 AB Den Helder, The Netherlands
[2] Department of Pathology, Faculty of Veterinary Medicine, Utrecht University, Yalelaan 1, 3584 CL Utrecht, The Netherlands
[3](current address): Viroscience Department, Erasmus MC, Wytemaweg 80, 3015 CN Rotterdam, The Netherlands
[4]Molecular Biology Laboratory, Department of Biological Oceanography, Royal Netherlands Institute for Sea Research, PO Box 59, 1790 AB Den Burg, The Netherlands
[5]Royal Belgian Institute of Natural Sciences (RBINS), Operational Directorate Natural Environment (OD Nature), 3de en 23ste Linieregimentsplein, B-8400 Oostende, Belgium
[6]University of Applied Sciences van Hall Larenstein, PO Box 1528, 8901 BV Leeuwarden, The Netherlands
[7]EHBZ Regio zuidwest, c/o Seal Rehabilitation and Research Centre, Hoofdstraat 94a, 9968 AG Pieterburen, The Netherlands
[8]Present address: Stichting A Seal for Sea Mammal Rescue, Haringvlietplein 3, 3251 LD Stellendam, The Netherlands
[9]Department of Pathology, Fundamental and Applied Research for Animals & Health (FARAH), Faculty of Veterinary Medicine, B43 Liège University, 4000 Liège, Belgium
[10]Royal Belgian Institute of Natural Sciences (RBINS), Operational Directorate Natural Environment (OD Nature), Gulledelle 100, 1200 Brussels, Belgium
[11]Naturalis Biodiversity Center, PO Box 9517, 2300 RA Leiden, The Netherlands
[12]Department of Aquatic Ecology and Water Quality Management, PO Box 47, 6700 AA Wageningen University, The Netherlands
[13]Royal Netherlands Institute for Sea Research, PO BOX 59, 1790 AB Den Burg, The Netherlands
[14]Vrije Universiteit, Faculty of Earth & Life Sciences, Department of Theoretical Biology, de Boelelaan 1085, 1081 HV Amsterdam, The Netherlands
[15]HZ University of Applied Sciences, Edisonweg 4, 4382 NW Vlissingen, The Netherlands
[16]CAH Vilentum Applied University, Stadhuisplein 40, 1315 XA Almere, The Netherlands

Introduction:
Being small, living on the edge

1

1 Introduction: Being small, living on the edge

The harbour porpoise *Phocoena phocoena* (Linnaeus, 1758), is one of the smallest cetaceans, in an order that holds less than 100 living species (Rice 1998; Committee on Taxonomy 2014; Wilson & Mittermeier 2014). The taxonomy of cetaceans is not yet fully resolved, and many "species" may in fact be clusters of very similar, or cryptic species that are not easily distinguished from each other. With the rapidly advancing work on genetics, existing species are increasingly being split into several species (e.g. LeDuc *et al*. 2008; Jackson *et al*. 2014). The same may be said about the skills of field observers, who also provide new evidence that known species should probably be split into several new species (e.g. Pitman *et al*. 2010).

The size range within the limited number of cetacean species is rather staggering. The order has the largest animal species that ever lived on Earth at one end of the size spectrum, the blue whale *Balaenoptera musculus*, and several smaller-than-man sized species at the other end. The largest recorded blue whales measured more than 30 meters and must have weighed over 175 tons (although none were ever weighed, for obvious reasons). Such gigantism has been made possible by the abundant, reliable, and high-quality food resources in the world's oceans (Sibly & Brown 2007), and the zero-gravity environment that these animals live in (Gaskin 1982). Some species evolved in exactly the opposite direction, however, and became dwarfs, at least among the cetaceans. The harbour porpoise is one of these "dwarfs", at less than 2 meters long and less than 100 kg, ca 0.05% of the mass of a blue whale.

Harbour porpoise taxonomy is still being debated, as the species has many populations, or "subspecies" that effectively live in isolation from each other. One may wonder if distant and diagnosable populations are still joined by a cline, and should thus be treated as subspecies, or not, and should be seen as full species

(see Helbig *et al*. 2002 for a discussion on speciation). The Committee on Taxonomy (2014) currently recognises four subspecies of *Phocoena phocoena: P. p. phocoena*, the Atlantic harbour porpoise; *P. p. vomerina*, the Eastern Pacific harbour porpoise; *P. p. relicta*, the Black Sea harbour porpoise; and a yet unnamed subspecies (scientifically), the Western Pacific harbour porpoise. Even in the NE Atlantic, where different populations appear to be well-connected, harbour porpoises may be evolving towards forming several new species (Andersen 2003; Fontaine *et al*. 2007, 2014). The largest population in the Atlantic probably lives in and around the North Sea, and is over 300,000 individuals strong (Hammond *et al*. 2013). North Sea animals are connected to animals living further south (Biscay and possibly further), east (Baltic), north (Norwegian Sea and Barents Sea), and west (Faroe Islands, Island and possibly still further west). Even though porpoises are small for cetaceans, they are larger than most (remaining) fish in the North Sea, and with their comparatively large numbers and high metabolic rates (see below) they are important predators within the North Sea ecosystem.

One might speculate that the removal of most large (>1 m or so) fish from the North Sea should have benefitted the harbour porpoise. Along similar lines, it has been suggested that the large-scale removal of krill-eating whales from the Antarctic would have benefitted other krill-eaters, such as penguins and seals (Gaskin 1982; Fraser *et al*. 1992), but such correlations are difficult to substantiate as other factors have also had major impact on the population developments of the latter (Croxall *et al*. 1992). Likewise, it has been suggested that North Sea seabirds have profited at large from the removal of large predatory fish from the system, which significantly released the predation pressure on small fish (Daan *et al*. 2005), the main prey of small cetaceans and seabirds. However, a critical review of the carry-on effects on seabird populations has shown that again, other factors were probably more influential (Camphuysen & Garthe 2000). Population trajectories of North Sea seabirds (Mitchell *et al*. 2004) are much better known than those of harbour porpoises, with data from only two recent, North Sea-wide surveys available (Hammond *et al*. 2002, 2013). Moreover, while seabirds generally may have benefitted from the removal of large fish, some species have certainly benefitted from the vast amounts of discards and offal provided to them by modern fisheries. In contrast, porpoises are not known to take fishery waste and must thus have profited less, if at all. In any case, even though harbour porpoises may have benefitted slightly from fisheries by a relaxation of competition, they have also suffered directly, as thousands of porpoises have been accidentally bycaught in fishing nets, particularly bottom-set gillnets (Vinther 1995, 1999; Vinther & Larsen 2004).

Being small has its merits...

Evolving into being smaller than close relatives probably reduces intra-order competition, among other resources, for food. Becoming small also opens up new habitats, most notably the productive shelf seas and shallow nearshore waters where food is relatively abundant. Furthermore, for a fish-eating cetacean, the sea holds more little fish than large fish, so in all likelihood there is more food available for a small cetacean than for mid-sized piscivorous cetaceans, such as killer whales (*Orcinus orca*) or minke whales (*Balaenoptera acutorostrata*). All these, in combination with a high metabolic rate ("living fast"; Read & Hohn 1995; Spitz *et al*. 2012) ensures relatively rapid reproduction and potentially a large population size, at least compared to the larger whales, when corrected for the size of the ranges of distribution. These factors combined may have made the harbour porpoise numerically very successful, and by far the most abundant cetacean in the North Sea (Hammond *et al*. 2002, 2013).

...and its drawbacks

Harbour porpoises may be small cetaceans, their size is comparable to large fish in the North Sea, such as Atlantic cod *Gadus morhua*, several species of elasmobranchs, and the Atlantic bluefin tuna *Thunnus thynnus*. All these are now greatly reduced in numbers, or even extinct in the North Sea (e.g. Census of Marine Life 2007), but in recent evolutionary sense, competition between porpoises and large fish was probably much more severe in the North Sea than it is today. Another disadvantage of becoming small is, that the risk of becoming prey increases. Harbour porpoises are known victims of large sharks, such as the great white *Carcharodon carcharias* (Arnold 1972; Long & Jones 1996; Lucas & Natanson 2010; De Maddalena & Heim 2012); the Greenland shark *Somniosus mirocephalus* (Williamson 1963) and the mako shark *Isurus oxyrinchus* (The Idependent 2014); or killer whales (van Dieren 1931; Bondesen 1977; Saulitis *et al*. 2000; Morisaka & Connor 2007; Dahlheim & White 2010; Williams *et al*. 2014) and of grey seals (Vodden 1995; Haelters *et al*. 2012a, 2015; Bouveroux *et al*. 2014; van Bleijswijk *et al*. 2014; Jauniaux *et al*. 2014; Leopold *et al*. 2015a,b; Stringell *et al*. 2015). Locally, they are also frequent victims of lethal aggression from bottlenose dolphins *Tursiops truncatus* in Scotland (MacLeod *et al*. 2007a; Deaville *et al*. 2014). Predation or inter-species aggression may take a serious toll on the population of porpoises, at least locally, but the consequences of being small on everyday life are probably even more severe in terms of energy management.

Porpoise energetics

Kleiber (1961) has pointed out that being small may have serious consequences for thermoregulation. Gaskin (1978, 1982) has elaborated on this in terms of the energy balance of a warm blooded mammal living permanently in the sea. All marine mammals are insulated, many by a thick pelage (polar bear *Ursus maritimus*; sea otters (mustelids) and pinnipeds), but all by a subcutaneous blubber layer. As the smallest of cetaceans, the harbour porpoise has the largest surface to volume ratio and per kg of body mass, will lose the most heat, all other things being equal. While an exercising large whale may see its activity limited by the risk of overheating, the small harbour porpoise is rather in danger of hypothermia. This risk is greatest in the smallest porpoises and this is probably why juveniles generally have a thicker blubber layer than adults (Hokkanen 1990; Lockyer 1995; Koopman 1998; McLellan *et al*. 2002). Other evolutionary adaptations of harbour porpoises are reduced size of the flukes, dorsal fin and flippers (Worthy & Edwards 1990), a relatively thick blubber layer that also has higher insulative quality (compared to tropical dolphins: Worthy & Edwards 1990), a high metabolic rate, which in itself ensures a high rate of heat production (Parry 1949; Read & Hohn 1995; Spitz *et al*. 2012). One of the most vivid

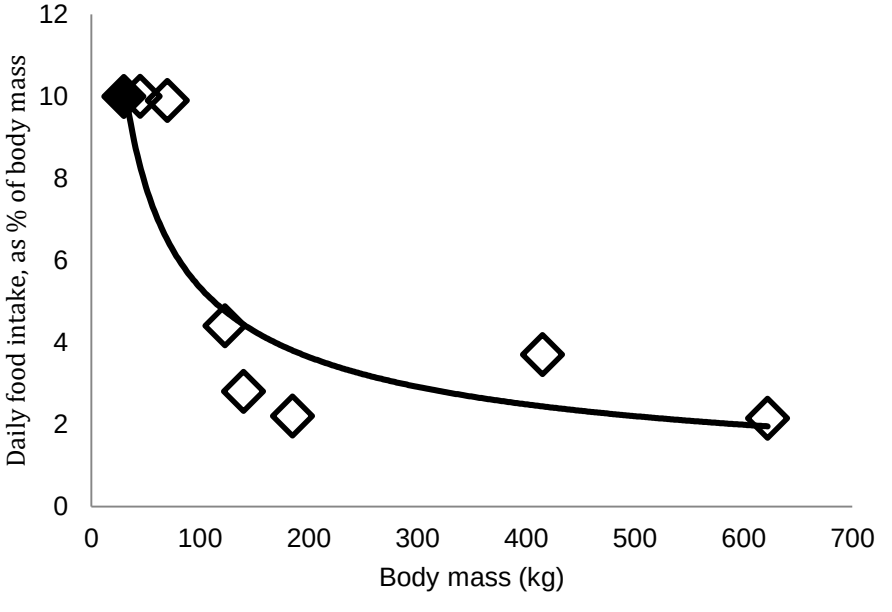

Figure 1. *Daily rations needed to keep various cetaceans healthy in captivity. After Kastelein (1998). The black dot signifies the harbour porpoise.*

references to the metabolic functioning of harbour porpoises is that they could be seen as "aquatic shrews". This was proposed by Kanwisher and Sundnes (1965), who reported that the daily energy expenditure of porpoises is three times as high as that of a similar sized terrestrial mammal.

Harbour porpoises need large amounts of food per day relative to their body mass to sustain themselves, and are quite intolerable to starvation (Kanwisher & Sundnes 1965; Yasui & Gaskin 1986; Kastelein *et al*. 1997; Koopman *et al*. 2002; Bjørge 2003; Lockyer *et al*. 2003). Of all cetaceans that are being kept in captivity, harbour porpoises need the most food per kg of body mass (Figure 1).

It has been suggested that harbour porpoises should eat prey with a high energy density (Spitz *et al*. 2012, 2014). Indeed, diet studies have shown that harbour porpoises worldwide tend to have fatty schooling roundfish species as an important component of their diets (Figure 2).

However, diet studies have also shown that harbour porpoises take many more prey species, including many of a much lower energy density, than these schooling, fatty fish. Rather than targeting only prey with the highest possible energetic return ($kJ \bullet gram^{-1}$), they seem to take a mixture of energy-rich and lean prey (Fink 1959; Sergeant & Fisher 1957; Neave & Wright 1968; Smith & Gaskin 1974; Recchia & Read 1989; Smith & Read 1992; Gaskin *et al*. 1993; Fontaine *et al*. 1994; Aarefjord *et al*. 1995; Raum-Suryan 1995; Sekiguchi 1995; Kenney *et al*. 1996; Read *et al*. 1996; Malinga *et al*. 1997; Gannon *et al*. 1998; Walker *et al*. 1998; Birkun 2002; Börjesson *et al*. 2003; Lockyer & Kinze 2003; Lockyer *et al*. 2003; Víkingsson *et al*. 2003; Santos & Pierce 2003; Santos *et al*. 2004; Spitz *et al*. 2006; Haelters *et al*. 2012b; Koponen 2013; Leopold *et al*. 2015a).

The situation in the North Sea is probably little different, but several of the available diet studies do not indicate energy-rich prey to be of prime importance here, hence the question mark in Figure 2. In much of Denmark the diet appears to be dominated by cod and whiting *Merlangius merlangus* (Källquist 1974; Aarefjord *et al*. 1995; Santos 1998), but Lockyer & Kinze (2003) report that sandeels, gadoids, clupeids and sole *Solea solea* (in that order) were the dominant food species in 24 by-caught animals in autumn 1997. Sveegaard (2010) finds that herring may now be of prime importance. For Germany (North Sea) Lick (1991, 1993) and Benke *et al*. (1998) report a remarkable diet, with some 40% of prey mass consisting of flatfishes (27% sole, 11% dab *Limanda limanda* and 2% flounder *Platichthys flesus*); in the largest adult porpoises flatfishes even contributed ca. 50%. Overall, sandeels (36.6%) were the most important single prey species, while gadoids (cod and whiting, together 15%) were of secondary

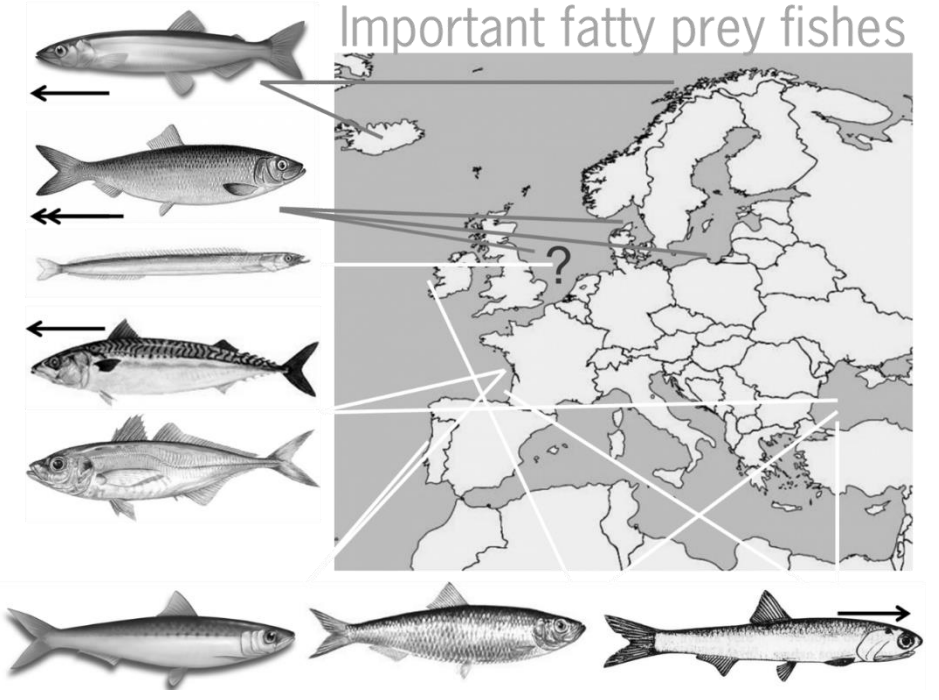

Figure 2. *Overview of important, fatty prey fish found in porpoise diet studies. From top left to bottom right: capelin* Mallotus villosus *(NE America, Iceland, Norway); herring* Clupea harengus *(NE America, North Sea, Baltic); sandeels Ammodytidae (NE America, North Sea); mackerel* Scomber scombrus*, and horse mackerel* Trachurus trachurus *(NE America, Biscay, Black Sea); pilchard* Sardina pilchardus *(Biscay); Sprat* Sprattus sprattus *(Ireland, Black Sea); anchovies* Engraulis *spp. (Biscay, Black Sea, Japan). Extra-limital and not in this picture: Pacific sardine* Sardinops sagax *(west coast USA) and pearlsides* Maurolicus *spp. (east coast USA).*

importance. In Belgium gobies and sandeels, and to a lesser extent, gadoids, were found to be the most important prey groups (Haelters *et al*. 2011, 2012b). In northeast Scotland, the stomach of a single by-caught juvenile (114 cm long, mid-June) porpoise yielded no less than 240 whiting otoliths and about two dozens of sandeel otoliths (Scott 1903). Whiting otoliths were no more than 10 mm long, corresponding to maximum fish lengths of 22 cm maximum (cf. Leopold *et al*. 2001). In a more comprehensive study in east Scotland, Rae (1965, 1973) found herring and whiting as the main prey of by-caught porpoises. Earlier sources (cited by Rae 1965) indicate a larger contribution of whiting to the diet. Remarkably, Rae did not find sandeels, that were the second-most (after whiting) prey along the Scottish east coast in later years (Santos *et al*. 2004). However, Martin *et al*. (1990) also did not report sandeels in porpoises stranded in Shetland

(n=14), Orkney (1) and on the east (1) and south coasts (2) of England. Various gadoids dominated the stomach contents, followed by herring and, in Shetland, greater argentine *Argentinus silus*. Evans & Scanlan (1989), finally, spotted a match in time and place in the occurrence of porpoises and herring around the UK and considered that herring must be a very important prey. Still, MacLeod *et al*. (2007b,c) speculated that sandeels were the main prey in the north-western North Sea, and that the large-scale, southward shift in range of harbour porpoises, observed around the turn of this century would be caused by a crash in the North Sea populations of sandeels.

In general, energy-rich fish, such as herring and sandeels are indeed found as prey in diet studies on North Sea porpoises, but with few exceptions, these fish do not stand out as being of prime importance. This may seem remarkable in a region where porpoises appear to be thriving, at least occur in relatively high numbers over a large area. However, many of the diet studies available for the North Sea were based on rather small sample sizes, or have become quite dated. With mass-movements of porpoises occurring in the North Sea (Hammond *et al*. 2013), resulting, among other things, in a return of the species into Dutch waters after decades of near-absence (Camphuysen 2004, 2011; Leopold & Camphuysen 2006), there is a need to strengthen our understanding of porpoise ecology. Several aspects of this wider theme have been identified as being in need of scrutiny: porpoise feeding ecology, the position of the species in the food web and its relation with other species (including man), and the problem of incidental bycatch (Reijnders *et al*. 2009; Camphuysen & Siemensma 2011; CBS, PBL, 2014). To help develop our understanding of porpoise functioning in the Dutch part of the North Sea, the Dutch Ministry of Economic Affairs has funded research on the species, focused on pathology of animals found dead on Dutch beaches, diet and the identification and quantification of bycatch. This thesis is one of the fruits of this this investment. Its primary aim was to get a better understanding of what harbour porpoises eat, and for what reasons. This goal was approached by working together closely with the veterinary pathologists, nationally and internationally, responsible for the necropsies that produced the stomachs for this study, and with the keeper of the national porpoise strandings database (www.walvisstrandingen.nl). All different lines of information were linked in the largest existing database on harbour porpoise stomach contents, and multivariate analyses were used to assess porpoise diet in the light of a range of co-variates that might push individual diets away from the population average. This approach was chosen to gain a much better understanding of how individual porpoises function, and what the problems are that they face every day. Incidentally, this study has also helped unravelling the mystery of porpoise mutilation that affected many dozens of porpoises every year, and, one might say as another bycatch, has

provided new insights into the problem of correctly inferring bycatch, on carcasses washing up on shore.

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Not all harbour porpoises are equal: Which factors determine what individual animals should, and can eat?

Mardik F. Leopold & Erik H.W.G Meesters

2

Abstract

Diet studies of marine mammals typically summarise prey composition across all individuals studied. Variation in individual diets is usually ignored, but may be more than just "noise" around an optimal foraging strategy that should be the same across the entire population. Instead, different individuals may have both different needs, and different skills to fulfil their requirements and diets may differ structurally between different groups of individuals within a population. Here we show that diets of harbour porpoises differ with age and nutritional condition of individuals, as well as seasonally. Even though all porpoises should probably strive to feed, at least partially, on energy-rich prey, such as clupeids or sandeels, the diet of juveniles is dominated by small, lean, gobies, and that of adults by larger, but also lean gadoids. Prey with a relatively high energy density was found in only a third of the porpoises with non-empty stomachs, and in about one quarter of all porpoises. In a multivariate assessment of prey composition against factors such as porpoise size, season and porpoise body condition, we found the highest proportion of empty stomachs, the lowest reconstructed prey masses in non-empty stomachs, and the lowest proportion of energy-rich prey in summer. We also found lower reconstructed prey masses in porpoises in poorer condition. Our results show that individual differences matter, in that porpoise diet develops with porpoise size (as a proxy for age) and that this development may be affected by the change of the seasons, and by individual mishap, leading to starvation.

Keywords: *Phocoena phocoena*, diet, individual differences, energy-rich prey, starvation, multivariate analysis

Not all harbour porpoises are equal: which factors determine what individual animals should, and can eat?

2

Mardik F. Leopold & Erik H.W.G Meesters

Introduction

Studies on the diets of marine mammals that rely on stomach contents analyses typically consider the population level, rather than individuals. Diets usually contain several different prey species and the relative importance of each prey, across all animals examined is often described by three statistics: the aggregate percentage of animals (with non-empty stomachs) in which a certain prey was found (known as the percentage frequency of occurrence, %FO); the relative numerical abundance: the total number of a certain prey as the percentage of total prey numbers found (%N); or the summed mass (using proxies like prey volume or reconstructed weights) of a certain prey as the percentage of the total prey mass found (%M). Each index has its strengths and weaknesses. %FO, as a presence/absence measure, describes the relative number of predators that has eaten a certain prey, but is not sensitive to the amounts eaten. %N is probably indicative for the amount of effort put into foraging for a given prey type, but is mostly dominated by small prey species that are abundantly taken, while possibly contributing relatively little to ingested prey mass or energy. In contrast, %M puts more weight onto large prey, even if these are only rarely taken. Overall diets are therefore also described by giving all three indices next to each other, or by combining them, in e.g. the index of relative importance, IRI = %FO•(%N+%M). This index was first proposed and used by fishery biologists, who used prey volume for M (Pinkas *et al*. 1971; Hyslop 1980). Although the unit of this index (%^{2}) would seem questionable, and the absolute IRI values are meaningless, the rationale behind this IRI is, that the biases of each term would more or less cancel each other out and that within-study IRI values would be useful (Bigg & Perez 1985; Duffy & Jackson 1986). Nevertheless, it would probably make more sense to use data from individuals, rather than unweighted population averages as the sampling unit for analysing diet (Thompson *et al*. 2007).

A further point of criticism on generic diet studies is that predators will rarely be sampled randomly (Pierce & Boyle 1991). Prey composition is likely to vary between individuals and if this variation is non-random, but dependent on factors such as predator age, gender, body condition, location, time, the outcome of a diet study presented as a generic diet or population average, will be biased.

Here, we examine the diet of one of the smallest cetaceans, the harbour porpoise *Phocoena phocoena*, using a large number of stomach contents (n=829). Santos & Pierce (2003) reviewed diet studies on harbour porpoises globally and found ample evidence of geographic, seasonal and interannual variation in diet. These can all largely be explained by the fact that predators need to feed on prey that are available where and when they are feeding. More interesting, therefore, are differences in prey composition, between individual animals that are feeding in the same waters at the same time, as such differences must be due to prey selection. For instance, individual body condition of porpoises has been found to be correlated to diet, with well-nourished individuals feeding relatively more on high-energy prey such as clupeids and sandeels, and emaciated porpoises having a diet largely restricted to lean prey: gadoids and gobies (see also Chapter 3).

Santos & Pierce (2003) noted that the prey base is often poorly known, hampering studies of prey selection. However, diet studies of different porpoises feeding concurrently, can ignore this as the prey on offer may, although unknown, be assumed to be the same across individuals. We used both univariate and multivariate statistical techniques to explore differences in prey composition between various groups of harbour porpoises exploiting the same waters. To this end we used stomach contents of animals stranded along the Dutch shoreline (SE North Sea and adjacent estuarine waters), between 2006 and 2014.

Material and Methods

Necropsies

Harbour porpoises stranded anywhere on the Dutch coast (ca. 650 km, including the North Sea, Wadden Sea, Eastern and Western Scheldt) were used in this study. For each carcass, stranding date and location was noted. In the analyses, year, month and region were used. For the latter, we grouped the strandings into five stretches of North Sea coastline and the three estuarine waters mentioned above (Figure 1). Collected carcasses went through a standard necropsy (Jauniaux *et al*. 2008, Begeman *et al*. 2013), from which the following information was used:

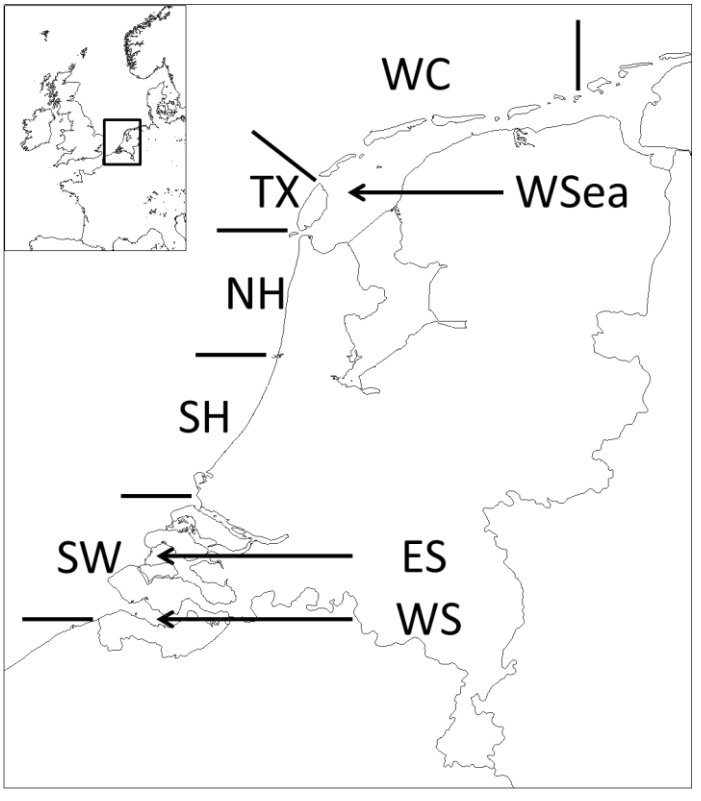


Figure 1. *Regions used in this study. Five stretches of North Sea coastline: Eastern Wadden Sea Islands North Sea coast (WC); Texel North Sea coast (TX), North-Holland NH), South-Holland (SH) and South-West (SW), and the Wadden Sea (WSea), Eastern Scheldt (ES) and Western Scheldt (WS).*

- Porpoise length, from the tip of the snout to the notch in the tail fluke (cm);
- Gender (male, female);
- Porpoise nutritional condition code (NCC) on a six-point scale, from 1 (very fat and muscular) to 6 (extremely emaciated), as defined by Kuiken & García Hartmann (1991).
- Carcass freshness, the decomposition condition code (DCC), on a five-point scale, from very fresh to very old carcasses. This assessment was only used to select animals suitable for NCC assessment: NCC was only assigned to animals that were very fresh, fresh, or starting to putrefy (DCC 1-3). For a further explanation of NCC and DCC, see Chapter 3.

Porpoise age class, blubber thickness and cause of death were also determined in each case, but these were excluded from the analyses presented here. Age

correlates strongly with body length and blubber thickness is closely related to NCC, while cause of death included various levels of uncertainty. Blubber thickness and cause of death are treated separately, elsewhere (Leopold *et al*. 2015a, and Chapters 3, 4 & 8).

Prey composition

Preliminary analyses revealed that larger porpoises tended to have the remains of fewer, but larger prey in their stomach. Therefore, we used reconstructed prey biomass, rather than prey numbers for analysis. Every effort was made to estimate prey biomass, from remaining prey hard parts, as correctly as possible. As ingested prey are quickly digested, and also their hard parts get affected in the grinding, acid environment of a porpoise's stomach, sagittal otoliths, which were the main items for prey identification, were all graded for wear (according to Leopold *et al*. 2015a) and their sizes corrected, before estimating fish size. Numbers of prey still present, as represented by prey hard parts were determined by pairing left and right otoliths, taking prey number as the number of pairs, plus the number of remaining single left or right otoliths. Other items found, e.g., bones (particularly from fish skulls, or vertebrae) were also used, if these added to the number of prey species or individuals (cf. Tollit *et al.* 1997). Fish mass was estimated from otolith size, after correction for wear, by using otolith size-fish mass relationships derived for specimen caught locally (Leopold *et al*. 2001). In cases where fish mass needed to be estimated from bones, we used Watt *et al*. (1997), or our own reference collection. The latter was also used for cephalopods, crustaceans and polychaetes.

Statistical analysis

Univariate models were constructed using generalized additive models (GAMs), in R (R Core Team 2015), version 3.2.1 ('World-Famous Astronaut'), with the package 'mgcv' (Wood 2006). Simulations of one linear model in order to calculate confidence limits for combinations of explanatory variables were run using the package 'arm' (Gelman & Su 2015). Multivariate analyses were mostly done using Primer (Clarke & Gorley, 2015) version 7.0.7. Multivariate analyses included Permanova (Anderson 2001; McArdle & Anderson 2001), Principal Component Analysis (PCA), Non-metric multidimensional scaling (nMDS) and cluster analysis (unconstrained divisive clustering, Primer 7). Because of large differences in prey masses across prey species and across individual harbour porpoises, prey biomass data were 4^{th} root transformed to conform to model assumptions. Further details are given in the Results section, under the various analyses.

Results

Empty stomachs

A total of 829 stomachs was examined, of which 158 were empty (19%). The distribution of stomachs over the years and month, with the monthly percentage of empty stomachs indicated, is given in Table 1. No relationship was found between the percentage of empty stomachs and either year or region (Pearson's Chi-squared test with simulated p-value >0.1 (based on 2000 replicates).We fitted a GAM to the data (stomachs being empty or not) using a binomial error distribution and a logit link function. After a backward selection procedure in which the least significant variable was consecutively removed, the final model, including only significant terms, was:

Probability of an empty stomach = Intercept + s(Month) + s(TL),

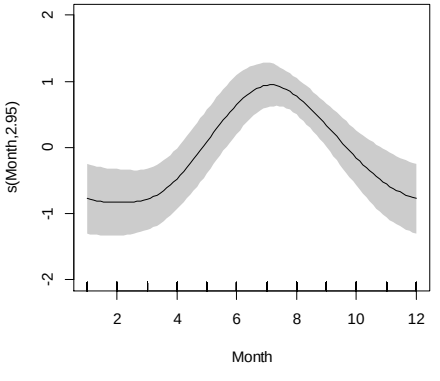
Table 1. *Distribution of available stomachs over the months and years, with shading indicating the percentage of empty stomachs.*

n stomachs with % empty:			No data		>0,..,≤20%		>20,..,≤50%		≥50%	
Month/Year	**2006**	**2007**	**2008**	**2009**	**2010**	**2011**	**2012**	**2012**	**2014**	**Average % empty**
1	0	2	2	7	4	4	7	7	6	**8.1**
2	3	4	6	19	2	10	4	7	2	**12.3**
3	17	8	9	7	10	16	17	29	3	**6.9**
4	10	8	1	6	5	14	9	20	0	**5.5**
5	2	3	0	3	2	11	4	12	0	**21.6**
6	3	0	6	0	2	5	4	5	2	**22.2**
7	2	9	9	0	3	25	24	4	6	**43.9**
8	13	3	13	0	13	85	22	11	8	**30.4**
9	4	7	5	3	2	28	25	10	1	**21.2**
10	0	2	13	2	5	38	17	3	3	**15.7**
11	1	4	10	3	5	4	0	4	0	**6.5**
12	2	0	10	3	3	6	4	4	1	**6.1**
Average % empty	**12.3**	**10.0**	**17.1**	**5.7**	**21.4**	**23.6**	**26.3**	**13.8**	**21.9**	**19.1**

Table 2. *Parametric coefficients (upper panel) and approximate significance of the selected smooth terms (lower panel) for the model on the probability that stomachs were empty. edf: estimated degrees of freedom.*

	Estimate	**SE**	**z value**	**p-value**
Intercept	-1.6578	0.1045	-15.87	2.00E-16
	edf		**Chi-sq**	**p-value**
s(Month)	2.955		47.35	2.72E-12
s(TL)	7.581		31.21	2.01E-04

in which s denotes the smoothing function. The smoother for month used a cyclic cubic regression splines in which months 12 and 1 match for obvious reasons. Although the deviance explained was only 13%, both Month and Porpoise length (TL) contributed significantly (Table 2). Animals appeared to have a higher chance of dying with an empty stomach in the summer months (June, July, August, September; Figure 2a), and both very small and very large porpoises had an elevated probability of having empty stomachs (Figure 2b). Concerning the latter, however, we note that some of the smallest animals may have been still nursing neonates, while sample sizes for both very small and very large animals were relatively low, resulting in wide confidence limits here.



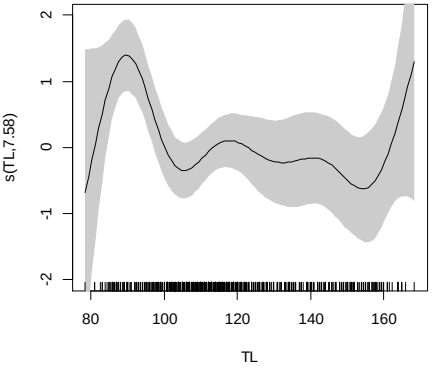


Figure 2a (left). *Smoother for Month, indicating the probabilities that stomachs were empty (line). Grey area indicates approximate 95% confidence limits.*

Figure 2b (right). *Smoother for Porpoise length (TL, cm). Distribution of lengths is given by vertical marks along the X-axis.*

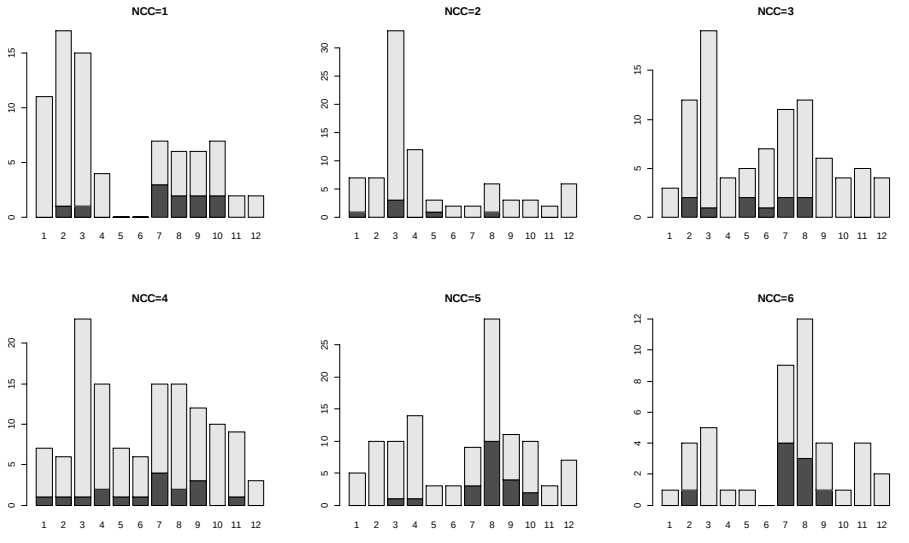


Figure 3. *Distribution of carcasses with NCC 1 to 6 over the months (all years combined) for non-empty (grey) and empty stomachs (black).*

NCC

Given that the incidence of empty stomachs was highest in summer, we expect that porpoises may also be in poorer body condition in summer. NCC, as a measure of body condition, was available for 467 carcasses.

Irrespective of the year we examined the distribution of NCC values over the months, in relation to the relative numbers of non-empty and empty stomachs. Indeed, somewhat more NCC 3-6 animals with empty stomachs were found from June through August, while the NCC 5 and 6 cases showed clear peaks in July/August (Figure 3). Note, however, that animals in very good body condition (NCC 1) with empty stomachs were predominantly found from July through September.

Interactions between NCC, TL en Month

During the necropsies, it was noted that very fat animals were mostly juveniles. There may thus be an interaction between NCC and porpoise age (or size), and since a new generation of porpoises enters the population in summer (Lockyer 2003), there may also be an interaction between NCC and Month.

To test whether necropsied animals were smaller in the summer, or in particular NCC classes, or whether NCC was different for porpoises that died with an empty stomach (as compared to those with non-empty stomachs) we ran a full linear model (including all the interactions) with TL as dependent variable and NCC, summer period (months June, July, August, September classified as 1, other months as 0), and whether stomachs were empty as explanatory variables. The model was run through a forward-backward selection procedure that used AIC as selection criterion (Bozdogan 1987). The final model indicated a highly significant interaction between summer and empty stomachs ($F_{1,454}$=13.47, p=0.0003). A second interaction between NCC and empty stomachs was almost significant at the 5% level ($F_{5,458}$=2.15, p=0.057). Given these interactions, p-values for main effect are rather useless here, so we used simulation to generate 95% confidence limits for the model estimates of the various levels of the 3 main factors. Using 1000 simulations 95% confidence limits were calculated (Figure 4).

Two interactions are clear from Figure 4: the interaction between summer and empty stomach, meaning that the average length of animals with non-empty and empty stomachs differs between the summer months and the other months. Animals with empty stomachs in the summer are smaller than those in the other months, whether their stomachs are empty or not. Moreover, animals with empty stomachs are smaller in summer than animals with non-empty stomachs in the same period, while the opposite was found in winter. The second interaction between NCC and TL is also apparent: for animals with empty stomachs, both in summer and in "winter" (October through May) the larger animals with empty stomachs were in poorer body condition than smaller conspecifics. There appears to be a linear relation between NCC and TL. This is absent in animals with non-empty stomachs.

The most likely interpretation of these interactions is, that in summer starving individuals are mostly the smallest porpoises, while in winter animals that die with an empty stomach and that have a very poor body condition are mostly the larger (older) animals. Inexperience, or community rank may thus affect young animals in summer, when they must switch from nursing to taking solid food, while old animals are most likely to starve in winter, or die from disease which prevents them from eating properly.

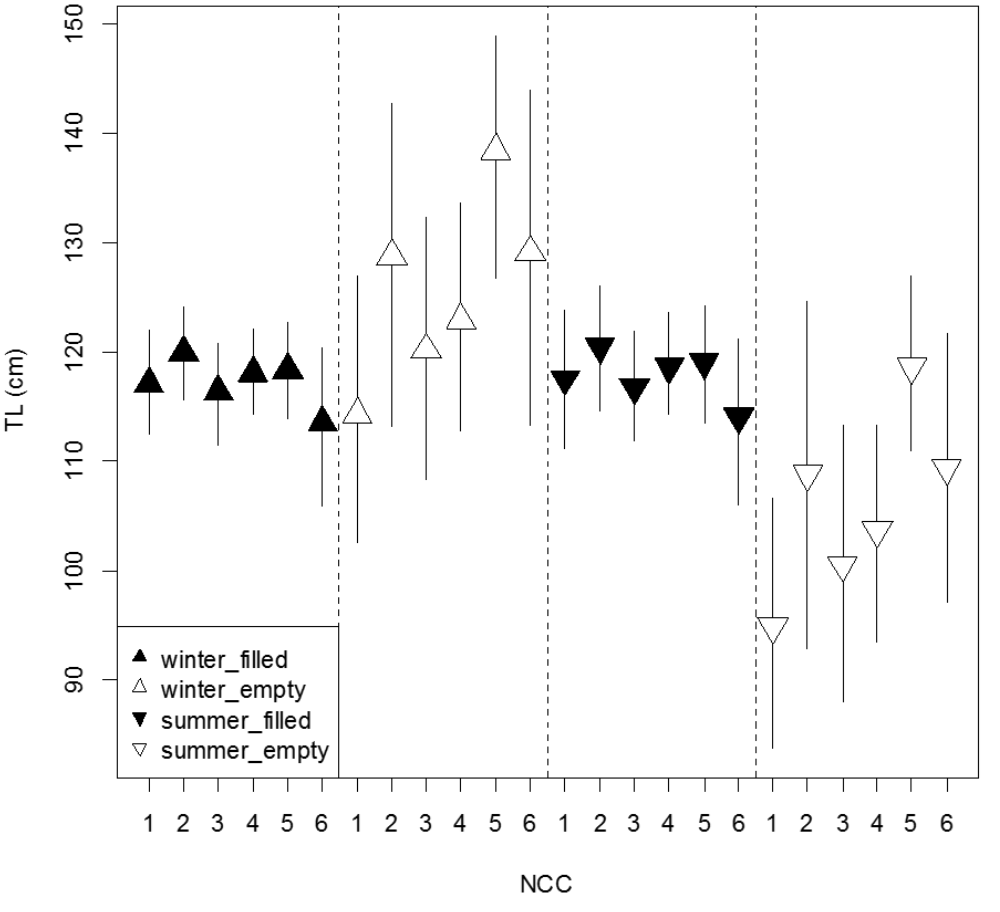


Figure 4. *Average porpoise body length (TL, cm: symbols) with 95% confidence limits (vertical lines through symbols) for carcasses with NCC 1 to 6, for summer (June-September) and "winter" (October-May) (all years combined) with non-empty (filled symbols) and empty stomachs (open symbols).*

Non-empty stomachs

In mid-summer (June-August) the non-empty stomachs contained the lowest amounts of (reconstructed) prey mass (Figure 5). The overall average reconstructed prey mass for non-empty stomachs was 224 gram (CL 190, 263).

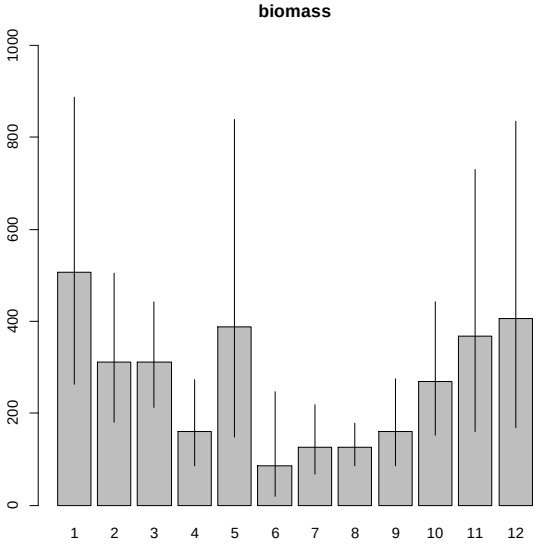


Figure 5. *Average reconstructed prey mass (gram) per porpoise with 95% confidence limits (lines), per month.*

A GAM for total biomass (with Gaussian error distribution) was constructed to investigate the observed patterns further (Full model: Reconstructed prey biomass = intercept + s(Year) + s(Month) + Region + Age + Sex + DCC + s(TL)). A backward selection in which the variable with the highest p-value was sequentially dropped from the model resulted in a model where all remaining variables (Year, Month and TL) were significant.

Figure 6 shows the partial plots for the effects of Year, Month and Porpoise length on the amount of reconstructed prey mass found. Effects of other variables were not significant and are not shown. Average reconstructed prey mass was comparatively low in the early years of this study, and showed a strong seasonal pattern with high values in winter and low values in summer.

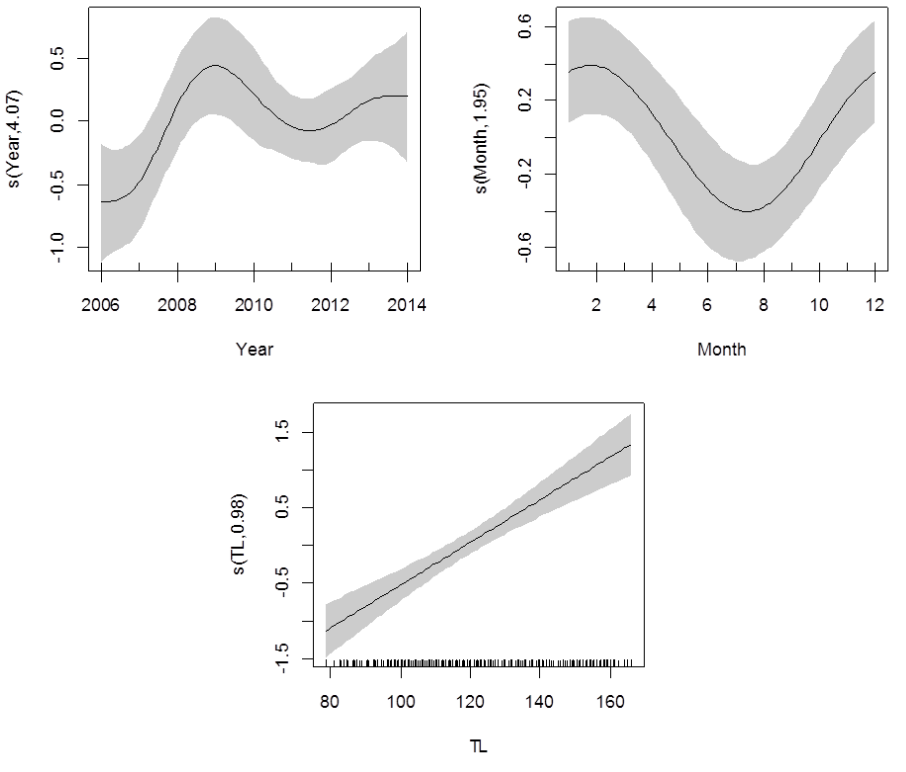


Figure 6. *Partial plots of smooth components of the fitted GAM-model on reconstructed prey mass (with Gaussian error distributions). With these three significant variables (Year, Month and TL)* $R^2_{(adj)}$*=0.12; Deviance explained=13% (n=659).*

Table 3. *Parametric coefficients (upper panel) and approximate significance of the selected smooth terms (lower panel) for the model on reconstructed prey mass. edf: estimated degrees of freedom.*

	Estimate	**SE**	**t value**	**p-value**
Intercept	3.86951	0.07461	51.86	2.00E-16
	edf		**F**	**p-value**
s(Year)	4.0745		2.194	0.00174
s(Month)	1.9481		2.153	1.26E-05
s(TL)	0.9806		49.441	2.27E-12

Higher reconstructed prey masses were found in larger animals. Table 3 gives the parametric coefficients for the model and the approximate significance of the selected smooth terms: year, month and TL. Note that the smooth function for TL is basically a linear relationship (edf close to 1).

NCC

The average reconstructed prey mass in non-empty stomachs, per NCC class is depicted in Figure 7. Clearly, the higher NCC classes have less prey mass in the stomachs.

Overall prey composition

Ignoring all variables that might affect prey selection of individual porpoises, the overall prey composition across all animals included in this study is given in Table 4. Gobies dominate the diet in terms of prey numbers, gadoids in terms of prey mass. Prey guilds that are relatively lean (having less than 5 $kJ \bullet g^{-1}$ wet mass (Chapter 3) comprise the majority of all prey found, both by number (78%) and by

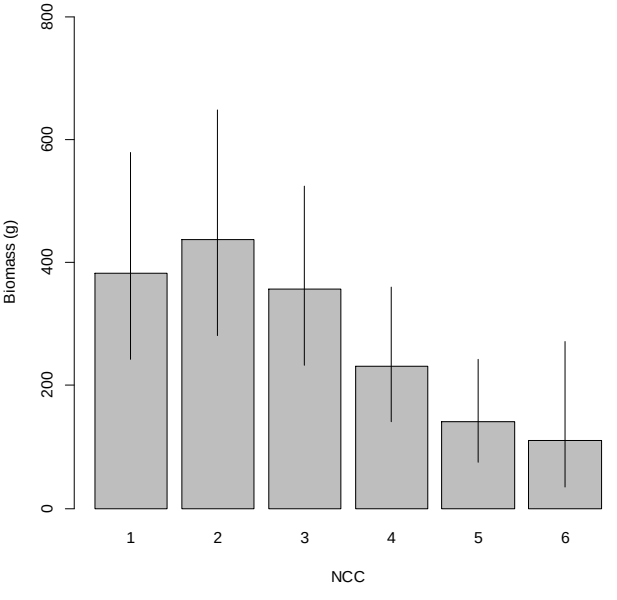


Figure 7. *Average reconstructed prey mass with 95% confidence limits (lines) per porpoise, per NCC class.*

mass (58%). The IRI indicates that gobies, gadoids and sandeels are of primary importance; clupeids, estuarine roundfish, pelagic roundfish and squid of secondary importance, and all other fish and invertebrates of minor importance. Looking at how much the different prey guilds contribute to the diet by their relative mass, gadoids, gobies, sandeels and clupeids are of primary importance; estuarine and pelagic roundfish of secondary importance and all other prey of minor importance. If we take the energy densities of the various prey guilds into accounts, the importance of gadoids and gobies decreases somewhat, relative to that of sandeels and clupeids. These four prey guilds together may be considered as the "big four" in harbour porpoise feeding ecology in Dutch waters.

Table 4. *The overall, relative importance of ten prey guilds for harbour porpoises in The Netherlands. Shaded rows give prey of relative high energy density.*

Prey guild	Number of stomachs	Number of prey	Summed prey mass	%FO	%N	%M	IRI
Clupeids	191	3274	51 461	28.5	2.8	10.9	390
Sandeels	278	9009	85 701	41.4	7.8	18.1	1073
Estuarine roundfish	130	3511	22 002	19.4	3.0	4.7	149
Pelagic roundfish	60	355	28 660	8.94	0.3	6.1	57
Gobies	419	95 322	96 728	62.4	82.2	20.5	6411
Gadoids	249	2178	172 341	37.1	1.9	36.5	1423
Other demersal roundfish	32	113	2253	4.77	0.1	0.5	3
Flatfish	37	122	1388	5.51	0.1	0.3	2
Squid	135	1341	10 873	20.1	1.2	2.3	70
Other invertebrates	176	729	1257	26.2	0.6	0.3	23
Totals	**671**	**115 954**	**472 665**		**100**	**100**	

A clear, though simplified pattern emerges if we visualize the reconstructed prey biomass data against TL, in a PCA, with TL grouped into 10 cm classes (Figure 8). Apparently, smaller animals take predominantly gobies, while sandeels and clupeids are mostly found in mid-sized porpoises and gadoids and pelagic roundfish are most typical of very large porpoises.

Energy-rich prey

Given the mixture of energy-rich and lean prey in the diet, and the presumption that energy-rich prey should be preferred (Whelan & Brown 2005; Rosen *et al.* 2007; this thesis, Chapter 3), we tested whether energy-rich prey was randomly distributed over animals of different sizes, and over the years, months, and regions.

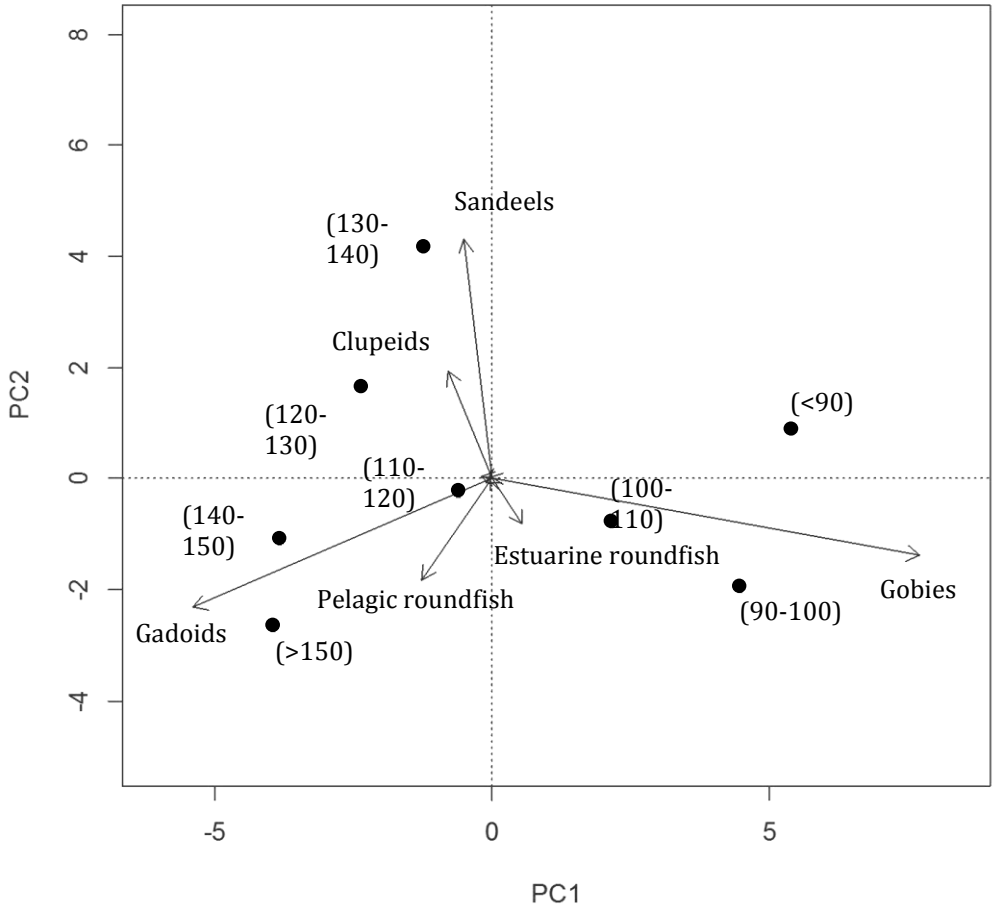


Figure 8. *PCA-plot showing relative reconstructed biomass for each prey guild and porpoise size in 10 cm TL-classes (dots).*

The GAM model used was:

$(RPM_{H\text{-}E})^{0.25}$ = s(Year) + s(Month, bs = "cc") + Region + s(TL), where:

$(RPM_{H\text{-}E})^{0.25}$ is the fourth-root transformed reconstructed prey mass of all high-energy prey combined, per individual (empty stomachs excluded). The same model was run on the relative amount of high-energy prey.

Breaking up the available data (671 non-empty stomachs) into a three-dimensional matrix (nine years x 12 months x 9 regions, and assessing the effect of TL simultaneously is challenging and many cells end up with no data. For this reason, interactions between the terms were left out and only the main factors could be tested (Table 5). The results show that both for absolute high-energy prey mass and for the proportion of these prey, all terms contribute significantly. Larger animals eat more high-energy prey, both in absolute and in relative terms; there was an increase in the consumption of high-energy prey over the length of the study period and consumption of high-energy prey, again both in absolute and in relative terms, was highest in winter. Some regional differences were also apparent (Figure 9), with the highest average energy density of prey in the Western Scheldt, due to a high proportion of smelt being eaten here (Chapter 5).

Table 5. *Parametric coefficients (upper panel) and approximate significance of the selected smooth terms (lower panel) for the models on reconstructed prey mass.*

Absolute prey mass high-energy prey						**Relative prey mass high-energy prey**				
	df	**F**		**p-value**			**df**	**F**		**p-value**
Region	7	3.646		7.21E-04		**Region**	7	3.646		7.21E-04
	edf	**Ref.df**	**F**	**p-value**			**edf**	**Ref.df**	**F**	**p-value**
s(Year)	1.000	1.000	22.75	2.27E-06		**s(Year)**	1.658	7.000	2.264	4.30E-05
s(Month)	4.408	5.411	12.44	4.47E-12		**s(Month)**	5.863	8.000	6.369	4.45E-10
s(TL)	4.205	5.199	11.48	7.22E-11		**s(TL)**	1.782	9.000	3.431	7.38E-09

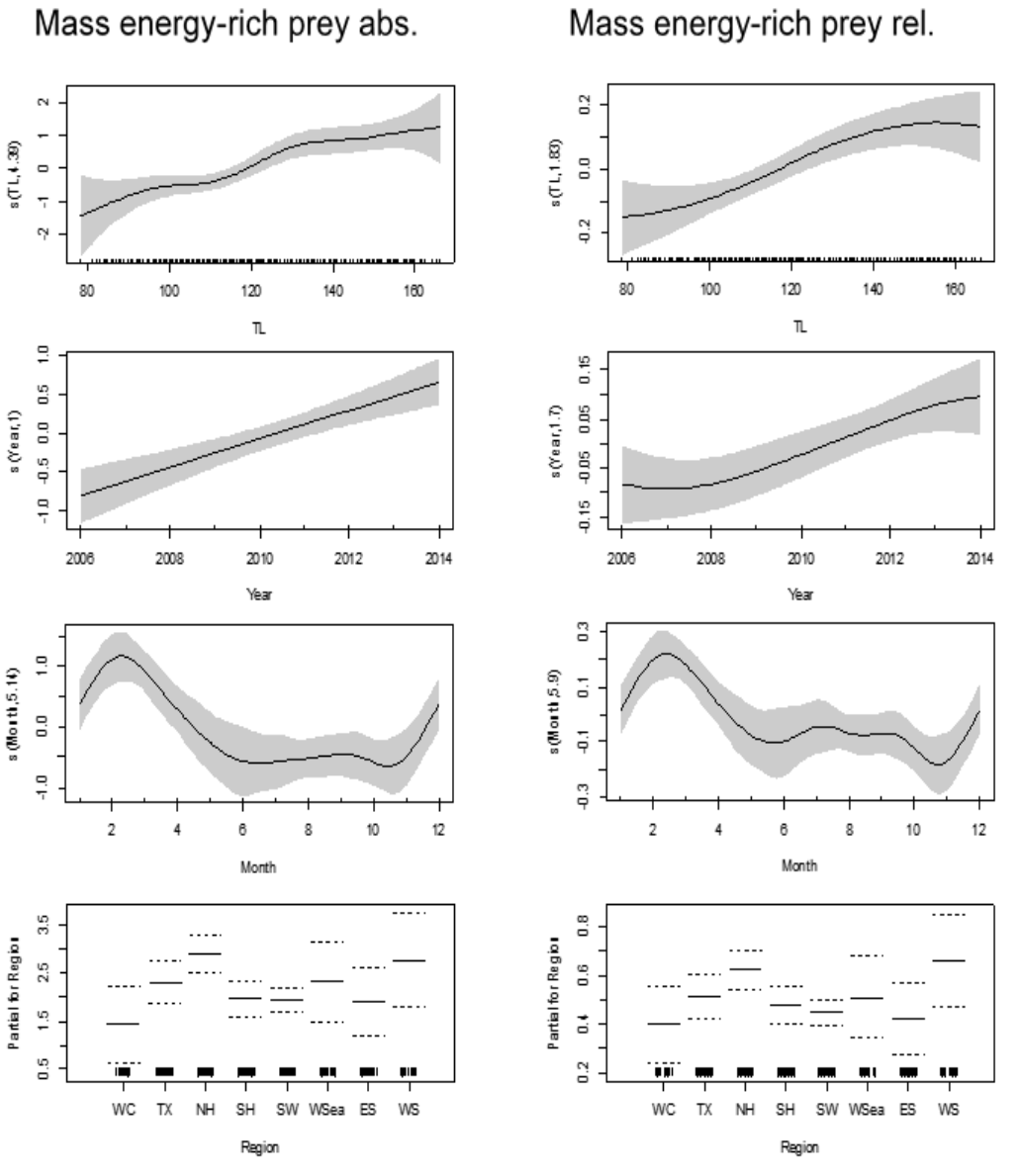


Figure 9. *Partial plots of smooth components of the fitted GAM-model on reconstructed high-energy prey mass (with Gaussian error distributions). Left panel: in absolute terms, right panel, in relative terms, to total reconstructed prey mass. With these three significant variables* $R^2_{(adj)}$*=0.12; Deviance explained=13% (n=659). Regions: see Figure 1.*

Multivariate analyses

nMDS

Non-metric multidimensional scaling of the reconstructed prey biomass data for non-empty stomachs (after 4th root transformation and using Bray-Curtis dissimilarity) teases apart the porpoises with various diets. Animals with remains of just one prey guild stand out most, as these are most dissimilar from all other animals. Because reconstructed prey mass is also important in this ordination, porpoises in which only one type of prey was found are not placed directly on top of each other, but somewhat separated (Figure 10), as reconstructed prey masses will differ between individuals. Most animals are placed more or less centrally, as they have a mixed diet. There is one animal containing only demersal roundfish (vertebrae of a dragonet *Callionymus lyra*. There was only one such animal and therefore it is positioned away from all the other samples. Several data transformation were tried but we felt that the ordination shown in Figure 10 was the most accurate in terms of the similarity of samples taking also into account the estimated biomass. Note, that the 4th root transformation of prey mass data keeps the spread of markers in check. Using untransformed values would result in extreme dominance of single-prey samples, with large prey mass.

Cluster analysis

The available prey composition data and guild-specific prey mass data were analysed for the presence of groups of similar contents using clustering. Both hierarchical agglomerative clustering and hierarchical divisive clustering indicated a separation in groups mostly based on the dominance of the different prey categories. Both resulted in similar groupings. Divisive clustering gave a slightly better separation of the samples and this is further used. All groups were tested using the simprof method (Clarke *et al*. 2008) and significant divisions are presented in Figures 11 and 12, showing 13 distinct clusters.

The 13 clusters had different numbers of porpoises, varying from 4 in cluster i, to 112 in cluster e2 (Table 6). Prey remains of all prey guilds were found in clusters a1, a2 and b, but some average prey masses per guild were very low in cluster b (Table 7). From cluster c onward, prey from increasing numbers of guilds were lacking. 13 animals (<2%) were not placed in any of the 13 clusters.

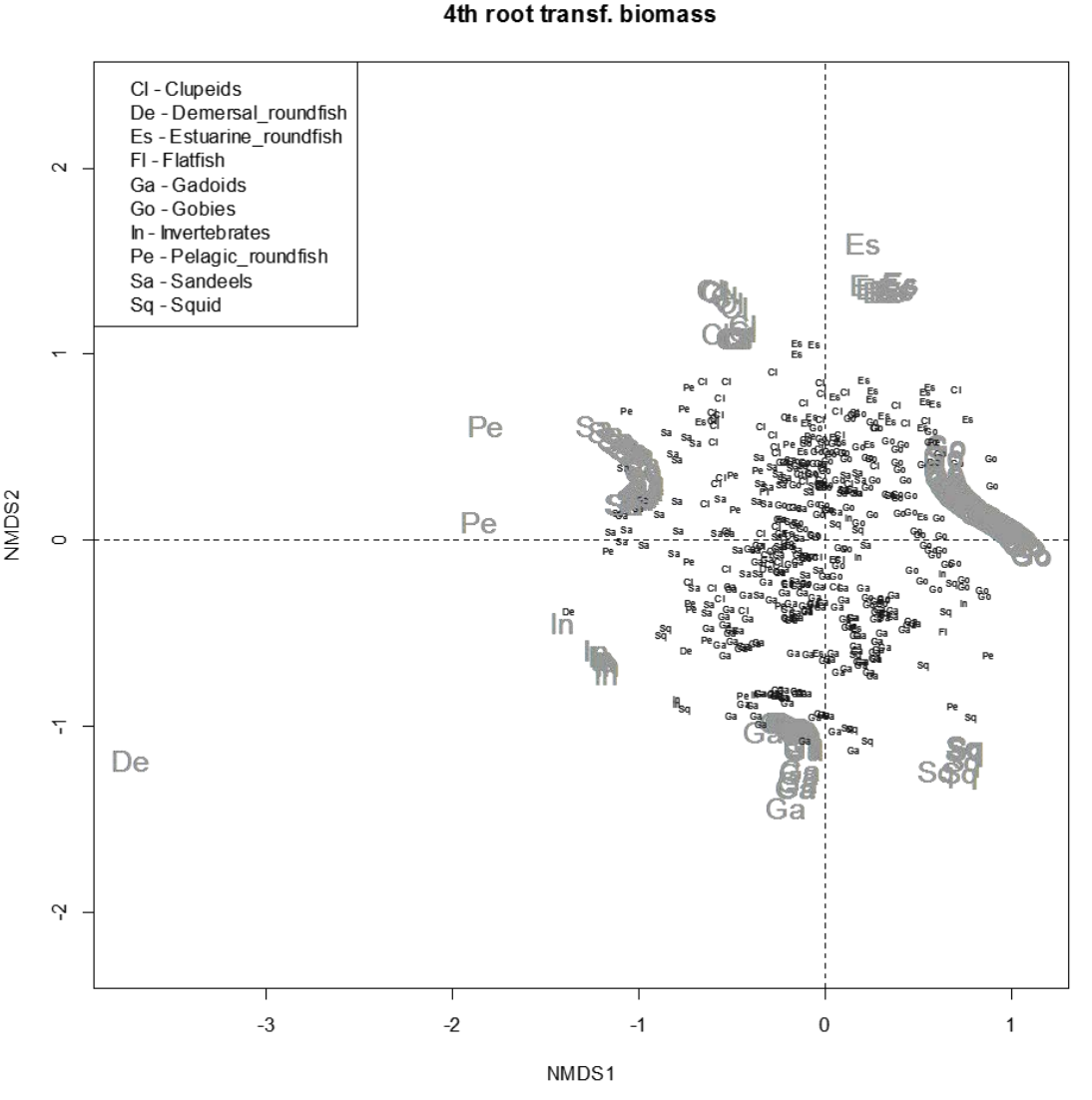


***Figure 10**. nMDS plot of individual stomach contents. Large labels denote samples that have only one prey guild. Smaller labels indicate mixed diets, dominated by the prey guild given. The closer a mixed sample to one of the dominant samples the more it is dominated by that prey type.*

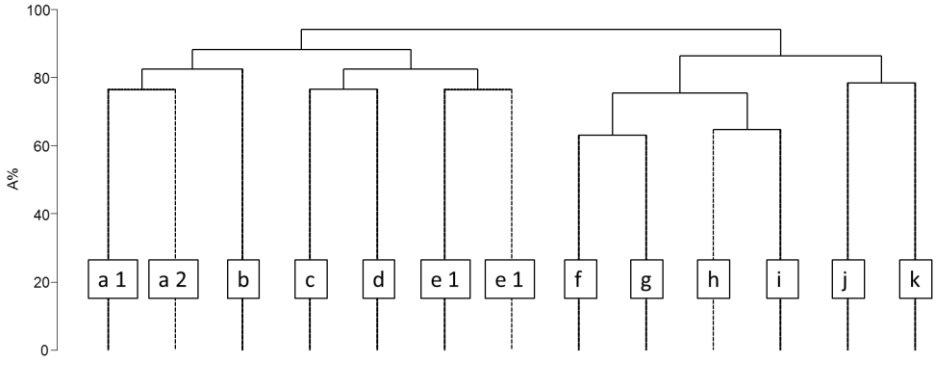


Figure 11. *Results of hierarchical unconstrained divisive clustering (UNCTEE) of the 671 non-empty stomach contents. Clusters are significantly different at the 1% level (indicated by single letters under the branches) or at the 5% level (letter-number combination). Cluster names in boxes over the braches.*

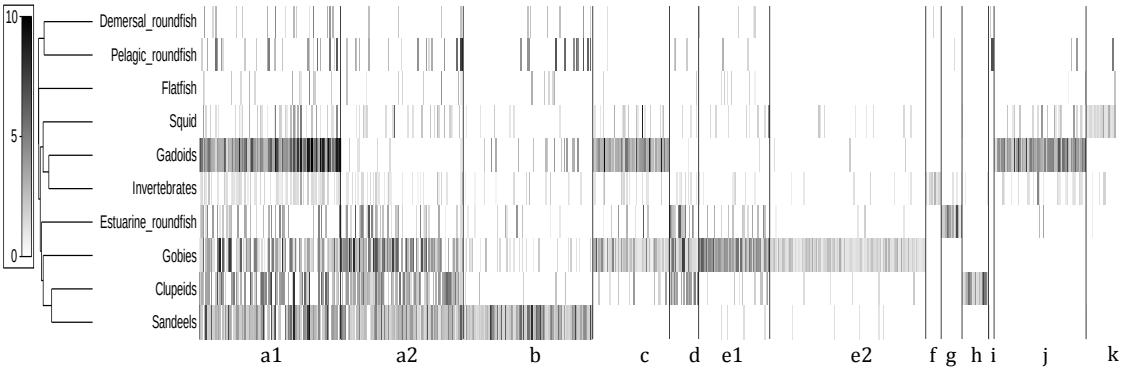


Figure 12. *The 13 clusters of porpoises with significantly different prey composition and mass, with the relative importance of prey indicated. Each small vertical line indicates the presence of a particular prey guild in one stomach. Cluster names, indicated at the bottom are the same as in Figure 9.*

Table 6. *The numbers of porpoises in each cluster (top row) and the numbers of porpoises within each cluster (a1,...,k) in which remains of each prey guild were found (presence/absence).*

n porpoises	**102**	**88**	**93**	**55**	**21**	**51**	**112**	**11**	**15**	**19**	**4**	**66**	**21**
Guild/cluster	**a1**	**a2**	**b**	**c**	**d**	**e1**	**e2**	**f**	**g**	**h**	**i**	**j**	**k**
Clupeids	60	69	5	9	15	9	5	0	0	19	0	0	0
Demersal roundfish	15	4	6	1	0	4	0	1	0	0	1	0	0
Estuarine roundfish	31	35	9	7	14	12	4	0	15	0	0	2	1
Flatfish	13	8	5	1	1	6	0	0	0	0	0	2	1
Gadoids	102	7	14	55	1	1	3	0	0	0	0	66	0
Gobies	80	77	23	55	21	51	112	0	0	0	0	0	0
Invertebrates	58	29	12	13	7	7	7	11	3	2	0	21	4
Pelagic roundfish	21	15	12	1	3	2	0	0	1	0	2	2	1
Sandeels	89	84	93	0	0	4	5	0	0	0	1	0	1
Squid	33	22	6	15	3	8	8	0	0	1	0	18	21

Table 7. *Back-transformed prey mass data, averaged over the individual porpoises per cluster and per prey guild.*

Guild/cluster	**a1**	**a2**	**b**	**c**	**d**	**e1**	**e2**	**f**	**g**	**h**	**i**	**j**	**k**
Clupeids	26.3	34.4	0	0	38.1	0	0	0	0	94.3	0	0	0
Demersal roundfish	0	0	0	0	0	0	0	0	0	0	0.1	0	0
Estuarine roundfish	0.7	1.1	0	0	24.7	0.1	0	0	81.3	0	0	0	0
Flatfish	0	0	0	0	0	0	0	0	0	0	0	0	0
Gadoids	490.2	0	0	157.4	0	0	0	0	0	0	0	201.1	0
Gobies	29.0	107.0	0	18.3	58.2	185.2	6.5	0	0	0	0	0	0
Invertebrates	0.3	0	0	0	0	0	0	3.2	0	0	0	0	0
Pelagic roundfish	0.2	0.2	0.2	0	0	0	0	0	0	0	79.6	0	0
Sandeels	74.3	52.1	91.4	0	0	0	0	0	0	0	0	0	0
Squid	0.2	0	0	0.1	0	0	0	0	0	0	0	0.1	4.6

Although the samples are very skewed, back-transformed (from 4th root transformation) data for prey mass give a good impression of the dominance of the various prey guilds in the 13 clusters (Table 7).

The results from the clustering in the nMDS provide an image of how the various clusters are positioned relative to each other and which carry the most weight in terms of reconstructed prey biomass (Figure 11). In this ordination, stomachs with high reconstructed prey biomass, dominated by gadoids (cluster a) are put slightly left of the centre, in the direction of the TL vector. In other words, large animals with full stomachs tend to be in this cluster. In contrast, the animals in clusters e1 and k will mostly be small, starving individuals (high correlation with NCC) that have only a few goby, or squid remains in their stomach. Clusters a1 and a2 are

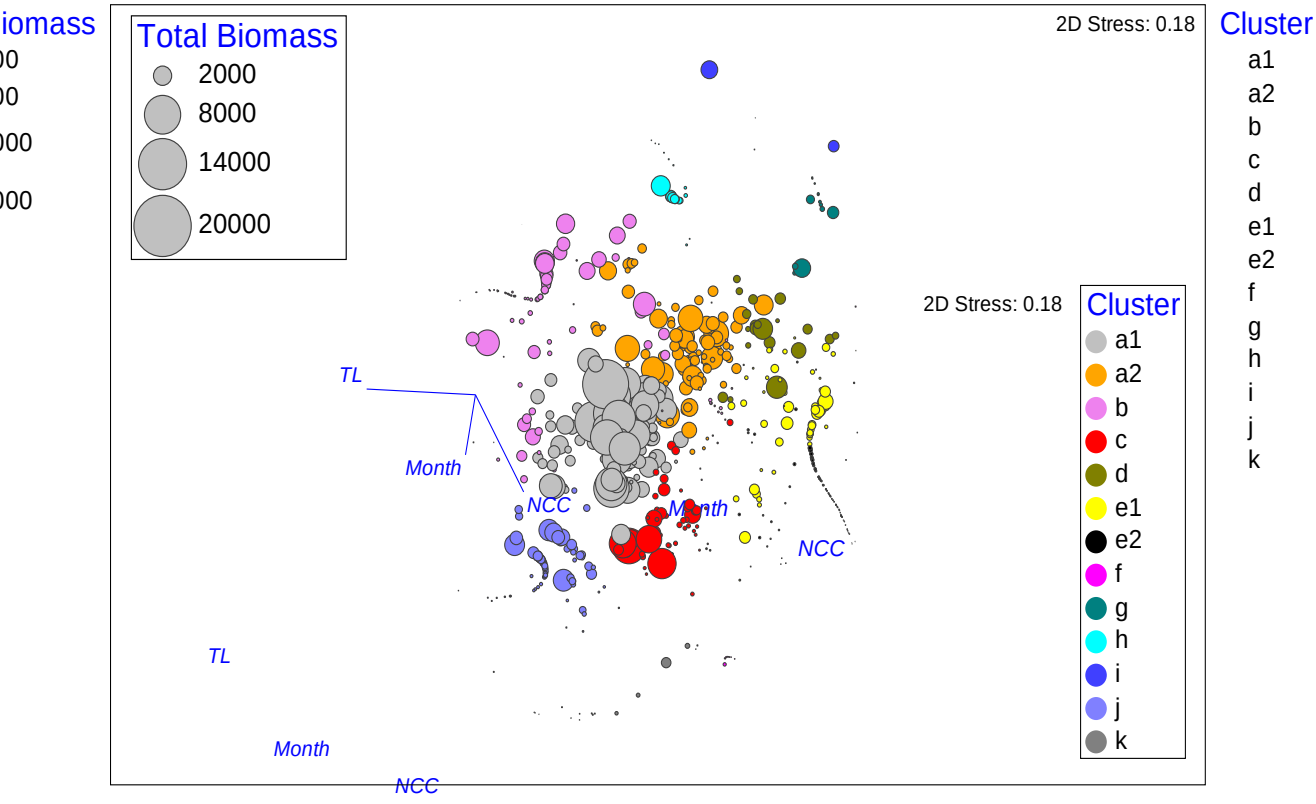


Figure 13. *The 13 clusters of porpoises with significantly different prey composition in an nMDS ordination. Reconstructed prey mass indicated by symbol size (see key to the left); cluster identity by colour (see key to the right: same cluster names as in Figures 11 and 12). Vectors for TL, Month, and NCC indicate the direction and strength of a positive correlation with the data.*

separated along the TL-axis and differ by a dominance of gadoids in the diet (Tables 6 and 7). Animals in cluster J (nearly only gadoids) have a strong correlation with month, so must appear with a narrow time window, when conditions are probably poor (high NCC). Animals in cluster b (dominated by sandeels) also correlate with month (but negatively) and NCC (negatively), so must also occur within a certain time of year, and are in relative good body condition.

A Permanova analysis was done with TL as a co-variable. This ensures that the effect of the other variables is tested after the effect of TL is removed from the data. The results show that the inclusion of TL as a co-variable is very much justified as

it has the highest F-value (Table 8). However, this final analysis also indicates that the variability in prey composition between individual porpoises is not only related to Porpoise length, but that the variables NCC and Month also explain a significant amount of variation.

Table 8. *Permanova table of results, on the data for all non-empty stomachs (reconstructed prey masses per prey guild, per porpoise, 4th root transformed).*

(co)variable	df	Pseudo-F	P(perm)	Unique perms
TL	1	38.156	0.001	997
NCC	6	4.9812	0.001	997
Month	11	3.6367	0.001	997

Discussion

Spitz *et al*. (2014) have argued that in diet studies, prey should be grouped in ecological guilds, rather than taxonomically, if we are to understand the foraging decisions that animals make while selecting certain prey. Although their study aims at understanding dietary differences between several related predatory species, this recommendation is also relevant for intra-specific differences in prey choice. In the present study on harbour porpoise diets, we grouped prey species in 10 guilds that are a compromise between ecological similarity and taxonomical relatedness. This is partly caused by limitations in our ability to correctly identify all otoliths to the species level (otoliths of most goby species are notoriously similar in shape, as are those of the various species of sandeels), our lack of ecological knowledge of fish behaviour, as well as porpoise behaviour and feeding skills. We do not know how e.g. different species of gobies differ in their availability to a foraging porpoise, but given their very similar appearances, sizes, and energy densities, we assume that one goby is as good as another, for a foraging harbour porpoise. Similarly, we lump herring and sprat as clupeids; smelt and sand smelt as estuarine roundfish; all different flatfishes; cod, whiting and pouts as (schooling) gadoids and other, more solitary gadoids such as rocklings with other bottom-dwelling fish as demersal roundfish; all gobies as gobies; all sandeels as sandeels; mackerel, horse mackerel and seabass as (large) pelagic roundfish; all cephalopods as squids; and all crustaceans, worms, and other remaining invertebrates as other invertebrates. These “guilds” are thus put together mostly on ecological grounds, but are still rather taxonomical and any such clustering may be challenged. However, we feel that the “big four”: gadoids, gobies, sandeels and clupeids contain prey fishes that, within each of these groups, would seem very similar to a foraging

harbour porpoise. The remaining guilds are of marginal importance and any changes of species in these from one guild to another would make very little difference on the outcome of the analyses.

Most porpoise diet studies to date have worked on a taxonomical basis when concerning prey, and have lumped stomach contents of all individual porpoises, much as we did in Table 4. In the present study however, we went several steps further. First, we included empty stomachs in the analysis, while these are usually discarded. We found that empty stomachs also carry information: the probability of dying with an empty stomach was found to be much higher in summer. While summer is often regarded a time of plenty, harbour porpoises apparently find this a particular difficult time. This is not only shown by relatively high percentages of empty stomachs, but also by lower reconstructed prey masses in non-empty stomachs, and by a lower percentage of high-energy fish in the diet in summer.

Another important result of this study is that porpoise age significantly affects diet. Weaning is probably a gradual process in harbour porpoises (Camphuysen & Krop 2011), young animals starting on solid prey mostly take gobies. Apparently, these are abundant and easy to catch for these still inexperienced piscivores. However, the energetic return of a goby is low, as these fish are small (the average goby taken was estimated to be circa 1 gram), and low in mass-specific energy, and hence in energy per individual prey. For very young porpoises, learning to eat fish is probably more important than the energetic return per prey caught, as they are still largely dependent on their mother's milk.

Note that a harbour porpoise, unlike a large rorqual, needs to find, catch and handle each individual prey in succession. As the amount of food needed to sustain a porpoise will increase with porpoise size, growing porpoises "outgrow" these small, lean prey. If we consider that a porpoise needs to eat about 10% of its own body mass per day (Leopold *et al*., submitted-a), a young, 20 kg animal would need to eat 2000 gobies if it was to eat these solely. An adult porpoise of e.g. 50 kg would need to eat 5000 gobies and would seem to have every reason to target larger prey, of higher energy density, and this is exactly what we find in this study.

This has implications for studies that use generic diets of predators to predict predation pressure (e.g. Temming & Hufnagl 2014). Such modelling should include demographic data on the predator and correct for age-related differences in diet, as well as for seasonal variation in diet. The existence of seasonality in the prey spectrum taken is hardly surprising, given that various fish species have their own life cycles that will change their availability, and profitability, as harbour porpoise prey through the year. Some preferred prey, e.g. clupeids, will be available at other

times of year than others, e.g. sandeels, but with four different high-energy prey guilds available in Dutch waters, porpoises have a choice and may nearly always find some preferred prey. Still, one of the high-energy prey guilds, pelagic roundfish, is probably only available to the largest, most experienced porpoises, while estuarine roundfish is only locally available. Pelagic roundfish was found as main prey in only four of all animals examined (cluster i) but occurred as secondary prey in some animals in clusters a and b. This leaves only two high-energy guilds to most porpoises: clupeids and sandeels, and these were both found abundantly in less than a third of the examined porpoises with non-empty stomachs (the 190 animals in clusters a1 and a2), and in less than a quarter of all porpoises. 93 more porpoises (cluster b) had taken mainly sandeels; 19 animals (cluster h) mainly clupeids; and another 21 animals (cluster d) had taken a mixture of clupeids and estuarine roundfish. This leaves about half of the animals with non-empty stomachs, and over 60% of all porpoises examined, without high-energy prey. This may be the reason why considerable numbers of stranded porpoises had a poor body condition: there is also a good correlation between body condition (NCC) and the relative amount of high-energy prey found in the stomach (this Chapter and Chapter 3). Even though we must be careful with making inferences from stranded, dead animals, as this may very well be a biased part of the total population in terms of body condition, it is clear that considerable numbers of porpoises fail to find sufficient amounts of suitable, high-energy prey, and get emaciated. According to our results, this affects mostly young, inexperienced animals in summer, and old porpoises in winter.

Other than dietary constraints, harbour porpoises face predatory grey seals in Dutch waters (Chapters 6-9) and drowning in bottom-set nets (Chapter 4). In these studies, that focus on these particular problems, specific causes of death were also found to be correlated with diet, showing that animals that die in specific micro-habitats, were feeding on specific prey. These studies show that porpoises feeding near the sea floor target different prey than animals foraging higher in the water column.

Given the interactions found between porpoise body condition, size, and season, this may be partly governed by the amount of blubber carried by individual porpoises, at different life stages and at different times of year. Blubber thickness affects buoyancy and through this, locomotor costs and diving capabilities (Adachi *et al*. 2014). Porpoise size primarily dictates what they can eat (small, lean gobies when they are small themselves; fast, fat pelagic roundfish when they are large), what they should eat when they grow larger (high-energy clupeids and sandeels) if they are to maintain good nutritional condition. Season, however, also dictates what they can eat, through varying availabilities of preferred prey, while losing body

condition, for whatever reason, appears to have grave consequences on what a porpoise still is able to eat. If losing weight means that a porpoise is becoming less able to catch the best prey, returning to a good state of heath will become increasingly difficult for leaner animals. Such a "positive" feedback could force individuals into a downward spiral, underlining the problems these small cetaceans face, should they find themselves in a situation of reduced availability of high-quality prey.

Acknowledgments

The necropsies were carried out on Texel in 2006, under supervision of Dr Thierry Jauniaux and from 2007 onwards at the Faculty of Veterinary Pathology Medicine, Department of Pathobiology of Utrecht University, led by, successively: Lidewij Wiersma, Lineke Begeman and Lonneke IJsseldijk, under supervision of Prof. Dr. Andrea Gröne, and with help of numerous other veterinary pathologists, students and volunteers. None of this would have been possible in the first place, without the enormous effort of volunteers on the Dutch beaches, who collected the hundreds of –often leaky and smelly- porpoise carcasses. Much of the work with the stomach contents was carried out by BSc and MSc students, at IMARES-Texel. A very big "Thank you!" is in order here!

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Are starving harbour porpoises (*Phocoena phocoena*) sentenced to eat junk food?

Mardik F. Leopold, Eileen Heße, Lonneke L. IJsseldijk, Lineke Begeman, Lara Mielke, Tara Schelling, Lizzy van der Steeg, Erik Meesters, Guido O. Keijl, Thierry Jauniaux, Sjoukje Hiemstra, Andrea Gröne & Jaap van der Meer

3

Abstract

The distribution of harbour porpoises *Phocoena phocoena* in the North Sea has shifted southwards in recent years. Apparently, many animals left areas previously rich in sandeels and moved to a region where much leaner gobies and gadoids are important prey. This shift in range, and presumably in diet, does not seem to have affected the body condition of all porpoises in the South. Body condition varies in stranded specimen found in The Netherlands, from very good to very poor. Emaciation is a common cause of death in this species, indicating that periods of decreased quantity or quality of prey can be detrimental to the species. The question thus arises whether emaciated harbour porpoises could not find sufficient food or whether their food was of insufficient quality. Stomachs of emaciated animals are not necessarily empty but, in fact, often contained food remains. In this study we examine these remains and compare the prey composition of well-nourished porpoises to that of progressively leaner specimens, collected between 2006 and 2014. We hypothesize that porpoises might starve by eating relatively too much prey with a low fat content that has a low energy density. Such food may be referred to as junk food: prey that is too lean for maintaining a good body condition. Results show that there is a significant difference in prey composition between animals in a good body condition and animals in a poor body condition, that starving animals have fewer prey remains in their stomachs, and that these prey, on average, are of lower quality. Healthy harbour porpoises take a mixture of fatty fish and leaner prey: the "big four" in dietary terms are clupeids and sandeels with a relatively high fat content, and gadoids and gobies, which are leaner prey. Our findings show that there is a negative correlation between the loss of body mass and the ingestion of fatty fish. This indicates that the emaciation is likely due to a lack of energy-rich prey, and that harbour porpoises need these prey in their diet to prevent starvation.

Keywords: diet, prey composition, prey quality, stomach content analysis, nutritional condition, body mass

Are starving harbour porpoises (*Phocoena phocoena*) sentenced to eat junk food?

3

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Introduction

With more than 200,000 individuals, harbour porpoises *Phocoena phocoena* are the most numerous cetaceans in the North Sea (Hammond *et al.* 2013), but local abundances have varied considerably over time. In the second half of the 20th century, porpoise densities were relatively low in the southern parts of the North Sea, but have recently shown a steep increase here (Camphuysen 2004; 2011; Witte *et al.* 1998; Thomsen *et al.* 2006; MacLeod *et al.* 2009; Haelters *et al.* 2011; Wenger & Koschinski 2012; Peschko *et al.* 2016), possibly at the expense of more western and north-western parts (Hammond *et al.*, 2013; Peltier *et al.* 2013). Apparently, many animals shifted from areas where they could feed on sandeels (MacLeod *et al.* 2007a,b) or herring (Evans & Scanlan 1989) to a region where much leaner gobies, gadoids and even flatfishes are important prey (Lick 1991;1993; Benke *et al.* 1998; Siebert *et al.* 2001; Santos *et al.* 2005; Leopold & Camphuysen 2006; Haelters *et al.* 2012).

Harbour porpoises have a relatively small body mass to body surface ratio, and as a result, a high rate of heat loss (Kanwisher & Sundnes 1965; Spitz *et al.* 2012). Therefore, porpoises need large amounts of food per day relative to their body mass to sustain themselves, which leaves them quite intolerable to starvation (Kanwisher & Sundnes 1965; Yasui & Gaskin 1986; Kastelein *et al.* 1997a; Koopman *et al.* 2002; Bjørge 2003; Lockyer 2003). Harbour porpoises should thus eat prey with a high energy density (Spitz *et al.* 2012; 2014). Indeed, diet studies have shown that harbour porpoises worldwide tend to have fatty schooling roundfish species as an important component of their diets. However, mixtures of several dozens of different prey species are generally found in single studies, suggesting that porpoises are generalist predators, taking a broad prey spectrum. Still, in each part of their range, one to four prey species tend to dominate the prey composition of the diet (expressed as percentage of total reconstructed prey mass) and at least one major prey species has a high energy content. Such key prey species include: herring *Clupea* spp., sprat *Sprattus sprattus*, pilchard *Sardina pilchardus*, Pacific sardine *Sardinops sagax*, anchovies *Engraulis* spp., capelin *Mallotus villosus*, pearlsides *Maurolicus*

spp., scad *Trachurus trachurus*, mackerel *Scomber scombrus*, and sandeels Ammodytidae (Fink 1959; Sergeant & Fisher 1957; Neave & Wright 1968; Smith & Gaskin 1974; Recchia & Read 1989; Smith & Read 1992; Gaskin *et al.* 1993; Fontaine *et al.* 1994; Aarefjord *et al.* 1995; Raum-Suryan 1995; Sekiguchi 1995; Kenney *et al.* 1996; Read *et al.* 1996; Malinga *et al.* 1997; Gannon *et al.* 1998; Walker *et al.* 1998; Birkun 2002; Börjesson *et al.* 2003; Lockyer & Kinze 2003; Lockyer *et al.* 2003a; Víkingsson *et al.* 2003; Santos & Pierce 2003; Santos *et al.* 2004; Spitz *et al.* 2006; Haelters *et al.* 2012; Koponen 2013; Leopold *et al.* 2015a). Diet studies have shown that harbour porpoises do not restrict themselves to such energy-rich prey. Considerable proportions of their intake may consist of prey types that have rather low energy contents, such as gadoids, gobies, or squid.

Presumably, however, a diet with a high proportion of lean prey could be detrimental to porpoise health (MacLeod *et al.* 2007a,b; Spitz *et al.* 2012, 2014). Of porpoises that washed up dead in the southern North Sea, a considerable proportion of non-neonates were emaciated. The body condition in stranded animals was found to vary, from very good to very poor, indicating that at least some animals had thrived on the prey locally available (Jauniaux *et al.* 2002; 2008; Siebert *et al.* 2006; Deaville *et al.* 2010; Gröne *et al.* 2012; Haelters *et al.* 2012). The question thus arises whether emaciated animals had a different prey composition than animals in good condition, and if so, if the diet of emaciated animals specifically lacked fatty fish species. Stomachs of emaciated animals were not always empty. In this study we examine the stomach contents and compare the prey composition of emaciated porpoises to that of individuals in good condition. We hypothesise that porpoises might starve by eating relatively too much lean prey and too little energy-rich prey.

Lean prey has been described as junk food. The junk food hypothesis was formulated in the early 1990's for marine predators (Piatt & Anderson 1996), stating that when preferred prey is replaced by less nutritious prey, the consumer faces reduced fitness (Whitfield 2008), even when animals can feed *ad libitum* on such prey (Rosen & Trites 2000, Donnelly *et al.* 2003, Wanless *et al.* 2005, van Gils *et al.* 2006). Such a change in diet might result from an ecosystem shift that reduces the availability of preferred prey (Rosen & Trites 2000; Litzow *et al.* 2002; Jodice *et al.* 2006; MacLeod *et al.* 2007ab; Österblom *et al.* 2008), or the energy content of preferred prey (Wanless *et al.* 2005) or from easy access to low quality food, such as fishery waste (Pichegru *et al.* 2007). Slightly confusing, junk food for wild animals is exactly the opposite of human junk food. While human junk food is fatty fare, the opposite applies to animals in the wild - food without enough fat and energy to sustain them (Whitfield 2008).

Material and Methods

Assessing the nutritional status of the harbour porpoises

The nutritional state of stranded porpoises was assessed for each carcass during standard necropsies (Jauniaux *et al.* 2008, Begeman *et al.* 2013), using the Nutritional Condition Condition Codes (NCC) as defined by Kuiken & García Hartmann (1991, see ES-1). Animals were assessed as NCC=1 (very good); 2 (good); 3 (slightly emaciated); 4 (bad), 5 (very bad) and 6 (extremely bad). NCC was only assessed in porpoises that were reasonably fresh, i.e. that had Decomposition Codes (DCC) 1-3 (ES-1). A total of 510 intact carcasses of DCC1-3 were measured and weighed and their NCC was scored. These carcasses ranged from a length of 77 cm, the smallest porpoise which had hard prey remains in its stomach, to 168.5 cm long, and from 6.0-62.0 kg (Table 1). Note that sample sizes presented in the following tables may differ slightly due to unknown variables (1 with gender unknown, 5 without stranding date, 4 without stranding location).

***Table 1**. Number, length (L, in cm, measured from the tip of the snout to the notch in the tail fluke) and body mass (kg) of examined harbour porpoises, per NCC-class.*

NCC	n	avg L	min(L)	max(L)	SD(L)	avg Mass	min(Mass)	max(Mass)	SD(Mass)
1	65	112.8	78.0	161.5	19.4	26.2	6.8	57.5	11.6
2	82	120.7	78.0	162.0	22.0	29.6	6.0	62.0	13.3
3	97	116.5	77.0	161.0	22.1	24.8	6.3	57.5	12.2
4	109	119.0	78.0	166.0	22.2	23.3	7.9	57.5	11.8
5	110	121.4	77.0	168.5	22.7	23.4	6.9	51.0	12.0
6	47	112.9	79.5	157.5	18.9	18.5	7.3	43.0	8.3

Next to NCC, which is an assessment rather than a true measurement, blubber thickness (mm) was measured at three standard locations along the left side of the body: dorsally, laterally and ventrally, just anterior of the dorsal fin. The average of these three values was compared to NCC, to evaluate the merits of the latter. We have shown earlier (Leopold *et al.* 2015b) that NCC can be used as a continuous variable. We assumed a non-linear relationship (general model: $M=a(L)^b$) to the data for each NCC class, where L is porpoise length in cm, and M is porpoise body mass in kg. This was transformed to: $Y=\ln(a) + bX$, where $X=\ln(L)$ and $Y=\ln(M)$.

We fitted four models to the data for the different NCC classes i=0,...,6:

Model 1: $Y = \ln(a_i) + b_i X$
Model 2: $Y = \ln(a_i) + bX$
Model 3: $Y = \ln(a_i) + 3X$
Model 4: $Y = \ln(a) + bX$

These models are nested and may be represented as a nest plot (Figure 1):

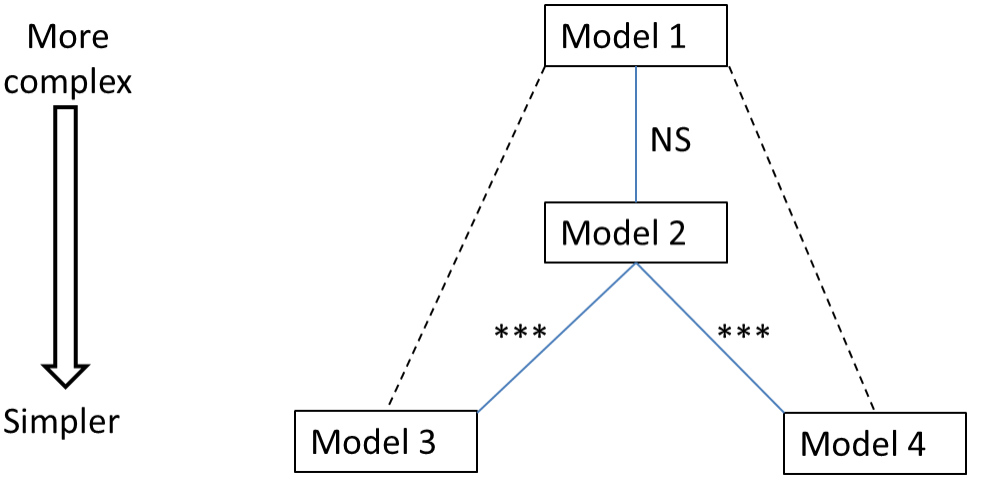


Figure 1. *Nest plot of the four models tested to the Length-Mass per NCC data.*

Model 1 did not perform significantly better than Model 2 (F-test, P>0.05). Neither Model 3 nor model 4 differed significantly from Model 2 (F-test, P<0.001 for both comparisons). Model 2 was therefore chosen as the best model to fit the data.

Stomach content analysis and assigning energy density of prey guilds

Prey remains, first and foremost fish sagittal otoliths, were used to identify fish species and to estimate fish length and weight. In addition to the otoliths, fish bones, eye lenses, scales, cephalopod and annelid jaws, crustacean exoskeleton parts, and copepoditic parasites of gadoids and clupeids were used to identify as many different prey as possible (cf. Tollit *et al.* 2003). Prey were identified and prey sizes back-calculated, using our reference collection, and Härkönen (1986), Clarke (1986) and Leopold *et al.* (2001), following the methods outlined in Leopold *et al.* (2015a). A total of some 70 different prey species were found, that were subsequently grouped into ten prey guilds: small schooling clupeids, sandeels, estuarine roundfish, pelagic roundfish, schooling gadoids, gobies, flatfish, (other) demersal roundfish, squid and other invertebrates (ES-2). We consider clupeids, sandeels, estuarine roundfish and pelagic roundfish to be energy-rich prey (>5 $kJ \bullet g^{-1}$ wet weight) and prey in the other guilds to be low in energy (<5 $kJ \bullet g^{-1}$ wet weight; MacLeod *et al.* 2007a, Spitz *et al.* 2014) acknowledging that species-specific energy densities might vary, between seasons, years, and prey size (Pedersen & Hislop 2001, Wanless *et al.* 2005).

Statistical analysis

Stomach contents were studied in 381 harbour porpoises for which at least NCC and body length were also known. For each porpoise the number of prey (minimum number of individuals per species) was estimated, and for each prey the length and mass. The importance of energy-rich, versus lean prey across the various NCC groups was assessed using four standard indices. Within each group the percentage of animals with empty stomachs was determined, and among the animals with non-empty stomachs (n=301) we determined the frequency of occurrence of energy-rich and lean prey (%FO); the percentage of energy-rich prey by number (%N); and the percentage of energy-rich prey by reconstructed mass (%M). The latter three indices were also combined in the 'Index of Relative Importance (IRI)' (Pinkas *et al.* 1971; Hyslop 1980) as: (%N+ %M) × %FO, where %N is the (number of energy-rich prey•100) divided by the total number of prey items found; %M is the combined (mass of all energy-rich prey•100) divided by total prey mass; and %FO is the percentage frequency of occurrence of each prey group. Note that we used reconstructed prey mass rather than prey volume, as used by Hyslop (1980).

All four indices are presented in ES-2. We used the prey biomass data for further analysis. These showed a large range in values, both between prey guilds and between individual porpoises, and were therefore fourth root transformed before calculating Bray-Curtis dissimilarities between individuals (Bray & Curtis 1957). The resulting distance matrices were analysed using the PERMANOVA routine included in the Primer 6+ software package (Anderson 2001, McArdle & Anderson 2001, Anderson *et al.* 2008), to test for differences between the six NCC groups.

Several co-variables which might have an effect on prey composition were tested, also with PERMANOVA. We considered porpoise gender, age and the season and location of strandings. Gender might influence prey composition if males and females have different energetic requirements or a different distribution at sea. This seems unlikely for young animals, even though there is a slight size difference between the sexes (Lockyer 2003b; Olafsdóttir *et al.* 2003). However, adult females may have higher energy requirements than adult males, during pregnancy and lactation (Smith & Gaskin 1983; Aarefjord *et al.* 1995; Santos & Pierce 2003; Das *et al.* 2004) and must accompany neonates in summer and autumn, while adult males do not face these constraints. Gender was therefore examined in concert with age. Age itself is also likely to influence prey composition, as older animals are larger and also more experienced predators. They may thus have both the need and the skills to catch larger prey or prey with a higher energy density. On the other hand, juveniles need extra energy for growth. Within our samples, we considered three age classes. Animals <100 cm long, that had stranded between 1 May and 31 December were considered calves; animals <100 cm that stranded after 31 December and animals between 100 cm and 130 cm long were considered juveniles. Animals >130 cm were considered adult (cf. Lockyer 2003b, for North Sea harbour porpoises), unless gonad

inspection revealed otherwise. Animals that were clearly neonates were excluded. The distinction between neonates and calves was not always clear however and we set the division at a body length of 77 cm, the smallest animals in our samples that had solid food remains in its stomach.

Diet is likely to vary with season as many fish species are migratory to some extent and show different behaviours during the year that will affect their availability as prey. We considered four seasons: winter (December-February), spring (March-May), summer (June-August) and autumn (September-November).

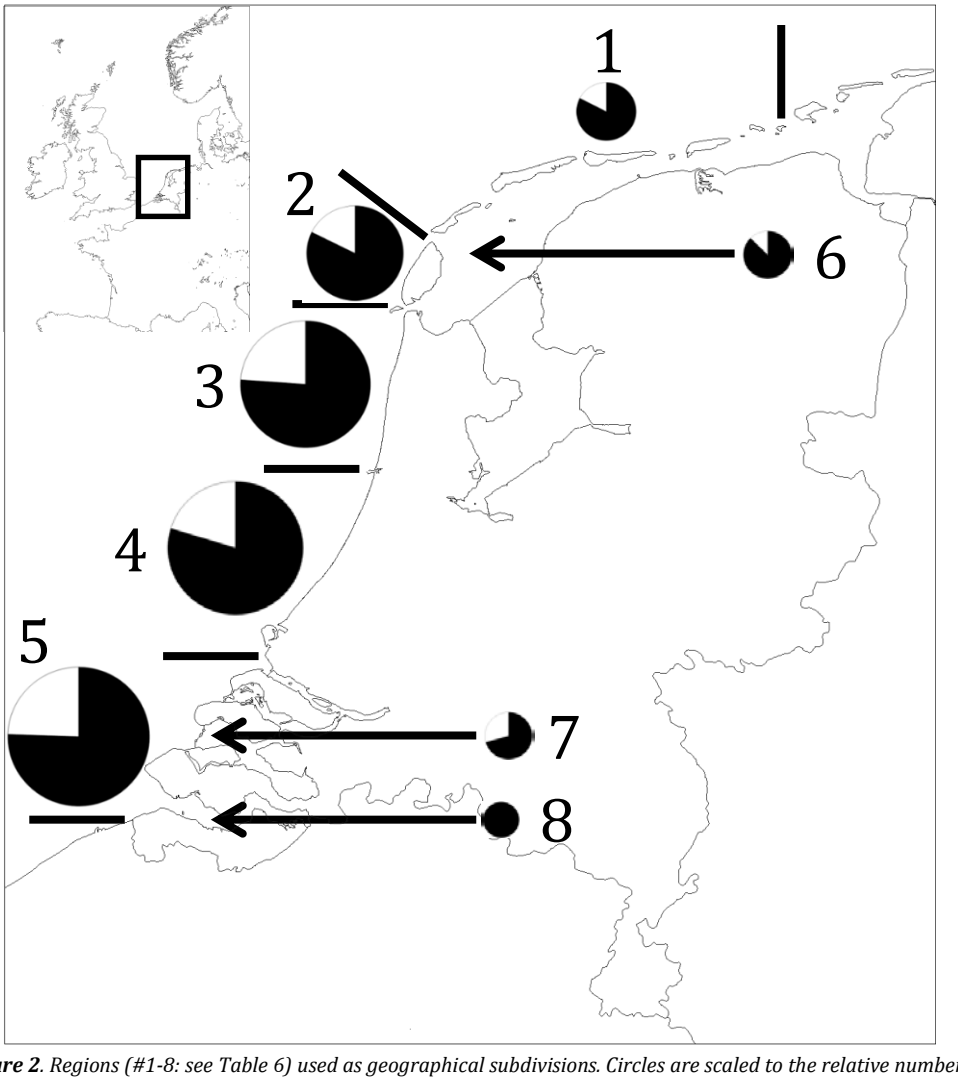


***Figure 2**. Regions (#1-8: see Table 6) used as geographical subdivisions. Circles are scaled to the relative numbers of porpoises for which the stomach content was studied; black: with prey; white: empty. Estuarine waters indicated by arrows.*

We also considered possible regional differences in diet, even though the Dutch part of the North Sea constitutes only a small part of the distribution range of the harbour porpoise in NW Europe. We consider five regions along the Dutch North Sea coast: the Eastern Wadden Islands (Rottum-Vlieland), Texel, North-Holland (mainland coast from Den Helder to IJmuiden), South-Holland (mainland coast between IJmuiden and Hook of Holland), and the Voordelta in the SW of the county; as well as three estuarine waters: the Wadden Sea, the Eastern Scheldt, and the Western Scheldt (Figure 2). The latter three regions are all connected with the North Sea: the Wadden Sea via tidal inlets between the various barrier islands, the Eastern Scheldt by openings in the storm surge barrier separating this former estuary from the North Sea, while the Western Scheldt is an open river. Prey composition of porpoises found in the Eastern and Western Scheldt has been found to be slightly different from animals found along the North Sea coastline (Jansen *et al.* 2013, Chapter 5); no specific study has yet been done on porpoises found in the Wadden Sea.

Prey composition may also vary with year, as fish stocks show large year to year variations (cf. MacLeod *et al.* 2007a). However, porpoises were only available for a few different years, with considerable differences in numbers per month and per year, precluding a meaningful analysis of year to year variation of diets. A full multi-variate analysis of the effects of the various covariates on porpoise diet is presented elsewhere (Chapter 2). Here, we evaluate the effects of each co-variate in turn and consider if any of these co-variates would seriously hamper the analysis of the effects of NCC.

Results

Co-variables

Gender and age

Juveniles, of both sexes, were the most numerous age class within our samples. Adult females were more numerous than adult males, while males were more numerous among the younger ages. Age and gender categories were distributed slightly differently between porpoises in good and poor body condition (Table 2). Most cells are filled, but very lean adults (NCC 6) were very rare.

Average NCC was very similar across age classes and between males and females (T-test: $P>0.1$ for all comparisons; Table 3) and we conclude that any interaction between gender or age with NCC will not seriously hamper an analysis of the effect of NCC on prey composition.

***Table 2**. Distribution of harbour porpoises for which the stomach contents were analysed over the different age and gender categories, per NCC class. Shading indicates sample size: empty cells (white), relatively low sample size (1-5; light grey), medium sample size (6-10, medium grey) and large sample size (>10, dark grey).*

Age,Gender/NCC	**1**	**2**	**3**	**4**	**5**	**6**	**Totals**
Calf-Male	8	3	7	9	4	7	**38**
Calf-Female	1	1	5	3	6	2	**18**
Juvenile-Male	18	15	23	42	28	10	**136**
Juvenile-Female	8	20	19	18	20	14	**99**
Adult-Male	7	6	11	4	10	0	**38**
Adult-Female	4	9	7	17	12	2	**51**
Juvenile-(gender ?)	1						**1**
Totals	**47**	**54**	**72**	**93**	**80**	**35**	**381**

***Table 3**. Average NCC (with Standard Deviation and sample size) for each age group and gender. Differences between groups are tested by Student's T-test.*

Age/NCC	**Avg-NCC**	**SD-NCC**	**n**		**Comparison**	**t**	**df**	**p**
Calf	3.66	1.64	56		Calf/Juvenile	0.292	290	>0.1
Juvenile	3.59	1.50	236		Calf/Adult	0.982	143	>0.1
Adult	3.38	1.40	89		Juvenile/Adult	1.070	323	>0.1
Gender								
Male	3.47	1.52	212		Male/Female	-1.297	378	>0.1
Female	3.67	1.47	168					

Season

Relatively many animals were available for spring and summer (Table 4). In summer, NCC values were significantly higher than in winter and spring, but not statistically different from the values found in autumn. Autumn values were intermediate between those in summer and in winter and spring (Table 5). The sampled harbour porpoises tended to be leaner in summer than in spring and winter. There is thus an interaction between season and NCC, and possibly prey composition.

***Table 4**. Distribution of harbour porpoises for which the stomach contents were analysed over the different seasons, per NCC class. Shading as in Table 2.*

Season/NCC	1	2	3	4	5	6	Totals
winter	15	10	15	12	16	4	**72**
spring	14	26	21	34	22	7	**124**
summer	6	10	23	26	25	17	**107**
autumn	10	7	12	21	16	7	**73**
Totals	**45**	**53**	**71**	**93**	**79**	**35**	**376**

***Table 5**. Average (with Standard Deviation and sample size) NCC for each season. Differences between groups were tested by Student's T-test.*

Season/NCC	Avg-NCC	SD-NCC	n		Comparison	t	df	p
Winter	3.22	1.59	72		Winter/Spring	-0.616	194	>0.1
Spring	3.36	1.43	124		Winter/Summer	-3.280	177	**<0.01**
Summer	3.98	1.41	107		Winter/Autumn	-1.631	143	>0.1
Autumn	3.64	1.51	73		Spring/Summer	-3.311	229	**<0.001**
					Spring/Autumn	-1.282	195	>0.1
					Summer/Autumn	-1.523	178	>0.1

Location

Most animals were collected along the various stretches of North Sea coastline (regions 1-5), with increasing sample size from NE to SW. Lower sample sizes were available from the estuarine waters (Table 6, Figure 2). Overall, numbers of animals were rather equal across NCC-classes. Animals found on the North Sea coasts of the eastern Wadden Sea Islands were somewhat leaner ($p<0.1$) than those found along other stretches of North Sea coastline, while those found South-Holland were marginally fatter (Table 7). There was no clear NE-SW trend in NCC along the North Sea coastline, as animals in the SW had NCC values not different from the other stretches of North Sea coastline combined and were, in fact, slightly leaner than those in South-Holland (with borderline significance: $t=1.811$, $df=171$, $p=0.075$). Animals from the Wadden Sea (region 6) had similar NCC values compared to those found on the seaward sides of the Wadden Sea Islands (regions 1 & 2 combined; $p>0.1$). Animals found in the Eastern Scheldt (region 7) were marginally fatter ($p<0.1$) than those in the adjoining Voordelta (region 5), while those found in the Western Scheldt (region 8) were leaner ($p<0.05$), but note relatively small sample sizes for the estuarine waters. We conclude that regional differences in NCC were slight, or related to small sample sizes and that region is unlikely to affect an analysis of the effect of NCC on prey composition.

Table 6*. Distribution of harbour porpoises for which the stomach contents were analysed over the different regions, per NCC class. Shading as in Table 2.*

Stranding location/NCC	**1**	**2**	**3**	**4**	**5**	**6**	**Totals**
1. Eastern Wadden Sea Islands North Sea coast	1	3	4	3	9	3	**23**
2. Texel North Sea coast	6	5	10	15	12	3	**51**
3. North Holland North Sea coast (DH-IJM)	9	15	20	19	13	12	**88**
4. South Holland coast (HoH-IJM)	14	14	14	17	16	4	**79**
5. NL-SW (Voordelta)	12	9	18	24	22	9	**94**
6. Wadden Sea	2	2	1	6	4	1	**16**
7. Eastern Scheldt	1	4	4	8	0	0	**17**
8. Western Scheldt		1		1	3	3	**8**
9. Unknown	2	1	1	0	1	0	**5**
Totals	**47**	**54**	**72**	**93**	**80**	**35**	**381**

NCC and body mass

Model 2 has a fixed slope parameter b = 2.56778, that is equal for all NCC classes, but a different intercept, a. We also added porpoise gender as a factor to Model 2, but this did not improve the model, so even though male and female porpoises reach different asymptotic lengths and also reach maturity at different lengths (Lockyer 2003b, Learmonth *et al.* 2014), this difference in growth apparently did not significantly impact their length-mass relationships. The values for the intercept a_i are given for each NCC class in Table 8. Predicted body masses for NCC 1,...,6 become progressively smaller. Relative body mass compared to animals with NCC_i equals $\exp(a_i-a_1)$ and is, for example, for NCC 6 animals 30.6% lighter.

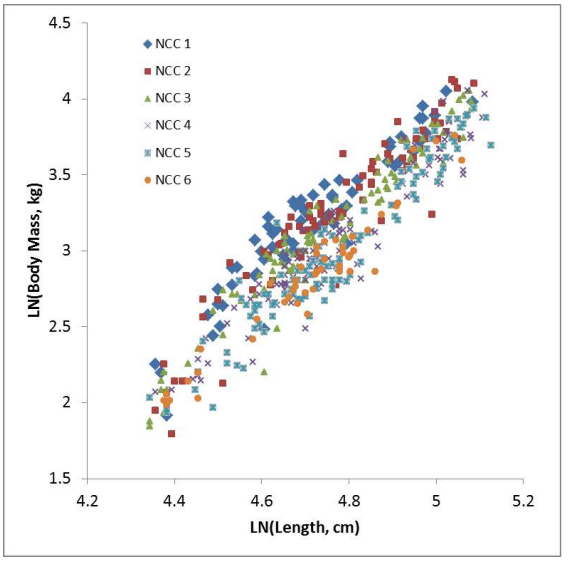
The available data are plotted in Figure 3a (ln-transformed) and the predicted L-M curves for each NCC class are plotted in Figure 3b.

***Table 7**. Average (with Standard Deviation and sample size) NCC for each region. Differences between groups are tested by Student's T-test.*

Stranding location/NCC	**Avg-NCC**	**SD-NCC**	**n**		**Comparison**	**t**	**df**	**p**
1. E-Wadden Islands North Sea coast	4.09	1.44	**23**		1 vs (2,3,4,5)	1.859	333	**<0.1**
2. Texel North Sea coast	3.61	1.42	**51**		2 vs (1,3,4,5)	-0.321	333	>0.1
3. North Holland (DH-IJM)	3.55	1.53	**88**		3 vs (1,2,4,5)	0.049	333	>0.1
4. South Holland (HoH-IJM)	3.24	1.53	**79**		4 vs (1,2,3,5)	-2.095	333	**<0.05**
5. Voordelta	3.66	1.5	**94**		5 vs (1,2,3,4)	-0.817	333	>0.1
6. Wadden Sea	3.69	1.49	**16**		6 vs (1,2)	-0.172	88	>0.1
7. Eastern Scheldt	3.12	0.99	**17**		7 vs 5	-1.891	109	**<0.1**
8. Western Scheldt	4.88	1.36	**8**		8 vs 5	2.415	100	**<0.05**
9. Unknown	2.40	1.67	**5**					

***Table 8**. Model 2 parameters for each NCC class.*

NCC	a_i	% of M_{NCC1}-predicted
1	-8.91101	100
2	-8.99337	92.1
3	-9.08421	84.1
4	-9.19976	74.9
5	-9.25373	71.0
6	-9.27690	69.4



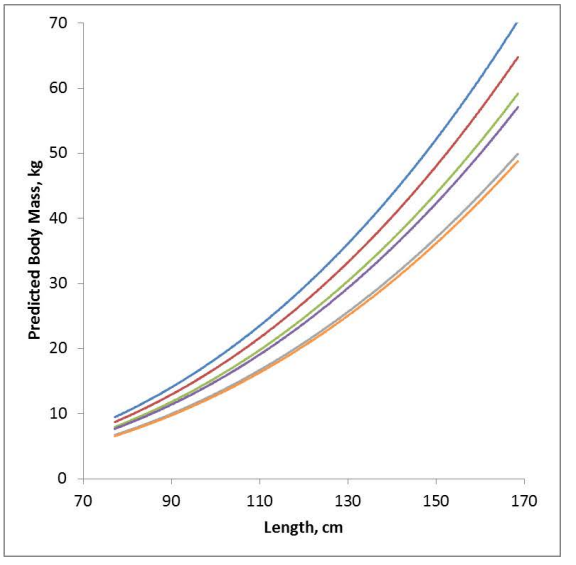


Figure 3a (left). *Body mass as a function of body length, for NCC1...6 (ln-transformed).*

Figure 3b (right). *Predicted porpoise length-mass curves for NCC1...6 (top to bottom; parameters: Table NCC).*

NCC and blubber thickness

NCC closely mirrors the blubber thickness of harbour porpoises and both measures vary with month (Figure 4). The porpoises were fattest from January through March, and leanest in August, with animals gradually losing blubber from April through August and regaining a thicker blubber layer from September through December. NCC thus appears to be a good proxy for the body condition of harbour porpoises.

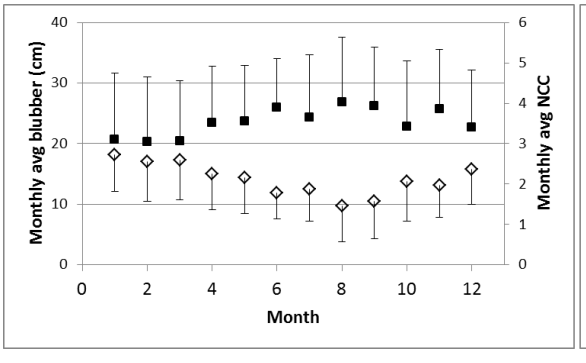
Prey composition

Empty stomachs

Around 17% of all porpoises of NCC1,..,4 did not have any hard prey remains in their stomachs, without a clear trend in this percentage across NCC classes. The leanest porpoises, however, had higher percentages of empty stomachs (Table 9).

***Table 9**. Numbers of harbour porpoises with non-empty and with empty stomachs, per NCC class.*

NCC	non-empty	empty	% empty
1	38	9	19.1
2	46	8	14.8
3	60	12	16.7
4	76	17	18.3
5	58	22	27.5
6	23	12	34.3



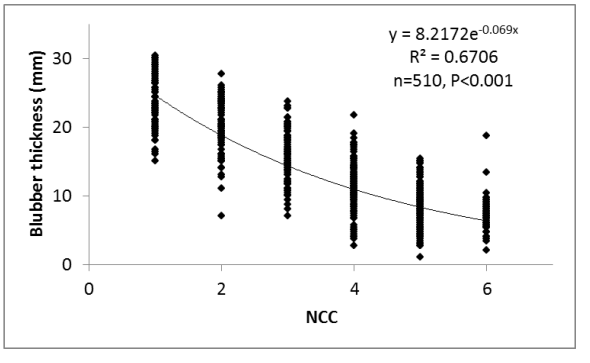


***Figure 4a (left)**. Monthly average (with SD) blubber thickness (open symbols; left Y-axis) and NCC (closed symbols; right Y-axis).*

***Figure 4b (right).** Relationship between NCC and blubber thickness.*

Gender and age

The overall difference in prey composition of males (n=169 non-empty stomachs) and females (n=131; Table 10) was significant, but very small (only 1.2% of the variance explained; Table 11). Considering adults only, however, this percentage increased to 5.6% (p=0.015). For neither juveniles (p=0.245), nor calves (p=0.344) were the diets significantly different between the sexes. Adult males had taken relatively more clupeids and sandeels and less pelagic roundfish and gadoids than adult females. Similar differences were found among the juveniles, but their prey compositions were not significantly different.

Adults had generally taken more gadoids and fewer gobies than juveniles. Prey compositions of adults, juveniles and calves all differed significantly from each other (Table 11). The diet of calves was dominated by gobies, but the contribution of gobies

declined as porpoises got older. Adult diet was dominated by gadoids, irrespective of gender (Table 10).

***Table 10**. Percentages of total prey mass in male and female porpoises per age class. N-values represent numbers of porpoises with non-empty stomachs. The figures at the bottom rows give the average reconstructed prey mass per individual porpoise (for respectively all animals and with empty stomachs excluded).*

Prey guild/Porpoise group	Adult ♂ (n=32)	Adult ♀ (n=42)	Juvenile ♂ (n=121)	Juvenile ♀ (n=84)	Calf ♂ (16)	Calf ♀ (5)
Clupeids	13.93	3.51	14.10	11.01	0.07	1.58
Sandeels	18.83	8.32	18.02	8.47	3.99	0.00
Est. roundfish	1.03	0.67	3.07	7.02	0.00	2.11
Pel. roundfish	0.26	12.25	0.15	3.76	0.00	0.00
Gobies	2.19	2.91	36.73	20.37	94.21	95.92
Gadoids	59.96	70.50	25.30	48.61	1.51	0.00
Dem. roundfish	0.04	0.64	0.68	0.23	0.15	0.00
Flatfish	1.49	0.09	0.44	0.05	0.00	0.00
Squid	1.92	0.93	1.36	0.19	0.03	0.39
Other invert.	0.35	0.18	0.16	0.28	0.04	0.00
per individual (all stomachs)	**1006**	**1293**	**598**	**547**	**172**	**83**
per individual (non-empty)	**1194**	**1570**	**672**	**645**	**407**	**298**

***Table 11**. Pair-wise comparison of the prey composition of calves, juveniles and adults.*

Age, Gender	%-expl.	P(perm)	Unique perms
Calves - Juveniles	12.2	**0.001**	999
Calves - Adults	38.5	**0.001**	999
Juveniles - Adults	8.3	**0.001**	999
Males - Females	1.2	**0.022**	998
Ad-male – Ad-female	5.6	**0.015**	998
Juv-male – Juv-female	-	0.245	997
Calf-male – Calf-female	-	0.344	979

Season

Diets were dominated by gadoids, in all seasons. In summer, overall stomach filling was considerably lower than in the other seasons and porpoises relied more on sandeels and estuarine roundfish than in other seasons. High-energy prey (clupeids, sandeels, estuarine and pelagic roundfish) were relatively scarce in autumn. Conversely, leaner prey, mostly gadoids and gobies, had the highest contribution in autumn and the lowest in spring.

Among these lean prey, the relative contribution of gobies was highest in spring, that of gadoids in autumn (Table 12). With nearly twice as many animals studied in spring as compared to the other seasons and with animals in spring having a relatively good condition (Table 5), overall results for the lower NCC classes will be slightly biased towards gobies, relative to gadoids. However, animals found in winter were in even better condition and their gadoids-dominated diet will balance this out to some extent.

Significant differences were found between seasonal diets, except between summer and autumn (Table 13), which are both characterised by high contributions of gadoids and low contributions of gobies (Table 12).

***Table 12**. Percentages of total prey mass found per season. N-values represent numbers of porpoises with non-empty stomachs. The figures at the bottom rows give the average reconstructed prey mass per animal (for respectively all individuals and with empty stomachs excluded).*

Prey group/Season	Winter (=66)	Spring (n=109)	Summer (n=61)	Autumn (n=60)
Clupeids	14.00	12.33	1.77	4.68
Sandeels	12.20	16.12	22.82	8.25
Estuarine roundfish	0.58	3.80	4.82	2.12
Pelagic roundfish	3.09	8.34	0.01	1.98
Gobies	18.36	33.06	14.01	11.73
Gadoids	48.40	24.44	55.51	69.92
Demersal roundfish	0.85	0.12	0.04	0.56
Flatfish	0.18	0.96	0.42	0.04
Squid	2.09	0.60	0.38	0.53
Other invertebrates	0.23	0.24	0.21	0.19
per capita (all stomachs)	**1123**	**642**	**188**	**820**
per capita (non-empty)	**1225**	**730**	**330**	**997**

***Table 13**. Pair-wise comparison of seasonal prey composition.*

Age	%-expl.	P(perm)	Unique perms
Winter – Spring	4.2	**0.001**	999
Winter - Summer	8.9	**0.001**	998
Winter - Autumn	3.9	**0.006**	999
Spring- Summer	7.4	**0.001**	999
Spring - Autumn	4.2	**0.001**	998
Summer - Autumn	-	0.195	999

Region

Considering all available stomach contents (n=376 porpoises of known location (Table 6), of which 296 contained hard prey remains (Table 14), only a few near-significant differences were found between the North Sea regions 2 to 5, with the largest sample sizes. All other regional prey compositions were not significantly different (Table 15). Because the prey composition along the Eastern Wadden Islands North Sea coasts (region 1) was similar to that in the Voordelta (region 5: p=0.9194), there was no clear NE-SW gradient.

Table 14. *Percentages of total prey mass found per region (1-9: see Figure 2 and Table 6). N-values represent numbers of porpoises with non-empty stomachs. Prey guilds (abbreviated) are, respectively: Clupeids, Sandeels, Pelagic roundfish, Estuarine roundfish, Demersal roundfish, Flatfish, Gadoids, Gobies, Squid and Other invertebrates.*

	N	CL	SE	PRF	ERF	DRF	FF	GA	GO	SQ	O
1.	19	10.14	17.41	0.00	3.99	0.25	0.08	31.18	36.22	0.42	0.32
2.	42	3.46	7.13	5.11	0.04	0.72	0.03	62.82	17.97	2.40	0.32
3.	67	10.15	8.48	0.38	1.72	0.09	0.26	56.45	21.73	0.52	0.21
4.	63	16.43	31.31	9.04	0.46	0.20	1.09	21.82	19.12	0.34	0.20
5.	71	11.23	11.23	3.82	4.37	0.93	0.51	49.39	17.19	1.18	0.15
6.	14	15.62	7.11	0.00	13.65	0.00	0.00	3.39	59.32	0.29	0.62
7.	12	6.06	4.82	0.21	5.15	0.00	0.00	20.56	62.46	0.51	0.22
8.	8	12.59	2.82	15.01	26.62	0.00	0.00	17.09	25.65	0.18	0.04
9.	5	10.76	0.80	0.00	19.07	0.00	0.00	64.08	5.10	0.00	0.18

Table 15. *Pair-wise comparison of regional prey composition.*

Region	%-expl.	P(perm)	Unique perms
Region 1 - 2	-	0.252	997
Region 1 – 3	-	0.788	998
Region 1 – 4	-	0.190	999
Region 1 - 5	-	0.919	998
Region 2 - 3	-	0.210	999
Region 2 - 4	-	0.242	998
Region 2 - 5	1.7	**0.075**	999
Region 3 - 4	1.8	**0.054**	999
Region 3 - 5	-	0.36	999
Region 4 – 5	1.5	**0.072**	999
Regions 1&2 - 6	-	0.209	998
Region 5 - 7	-	0.203	998
Region 5-8	-	0.763	999

NCC and prey composition

The prey compositions of animals of NCC 1,..,6 are shown in Figure 5 in so-called modified Costello diagrams (Amundsen *et al.* 1996, Ringelstein *et al.* 2006). These graphs combine the information on the proportions of porpoises that had taken certain prey(group), irrespective of the amount of prey (%FO along the X-axis) with the contribution of that prey to the diet in terms of mass (%Mass along the Y-axis). Prey that are eaten by the majority of animals and that are also important in terms of mass contribution show up in the upper right corner of these graphs; prey that are found only rarely and constitute little mass are placed near the origin.

Animals in good condition had four main prey groups that constituted a mix of two energy-rich prey types (Spitz *et al.* 2012): sandeels and clupeids, and two leaner prey types: gobies and gadoids. All other prey types were marginally important, particularly in terms of mass-contribution. As porpoises got leaner (higher NCCs), the fatter prey disappeared from the diet: first the clupeids (from NCC 3) and next the sandeels (from NCC 4). In very lean porpoises (NCC 5 and 6) this trend continued as fewer animals still took clupeids or sandeels (Figure 5).

Gadoids and gobies remained important prey as NCC increased, with gadoids always dominating total prey mass, while gobies were always found most frequently. The loss of sandeels and clupeids from the diet in starving porpoises was not compensated by other energy-rich prey, so overall, starving porpoises had a much leaner diet than porpoises in a good body condition. There is no indication that alternative food sources (e.g., invertebrates) were more prominently included in the diet of starving porpoises: they rather stopped eating energy-rich prey.

Differences in prey composition were not significant between successive NCC classes, but were significant, or near-significant, between successive pairs of NCC classes (NCC 1&2; 3&4; 5&6; Table 16).

Table 16*. Pair-wise comparisons of prey composition for different combinations of NCC classes.*

NCC	%-expl.	P(perm)	Unique perms
NCC 1 - 2	-	0.746	997
NCC 1,2 - 3	-	0.134	998
NCC 3 - 4	-	0.532	999
NCC1,2 - 3,4	2.2	**0.01**	998
NCC 4 - 5	-	0.444	998
NCC 3,4 - 5	0.9	**0.099**	998
NCC 5 - 6	-	0.635	996
NCC 3,4 – 5,6	2.2	**0.088**	998

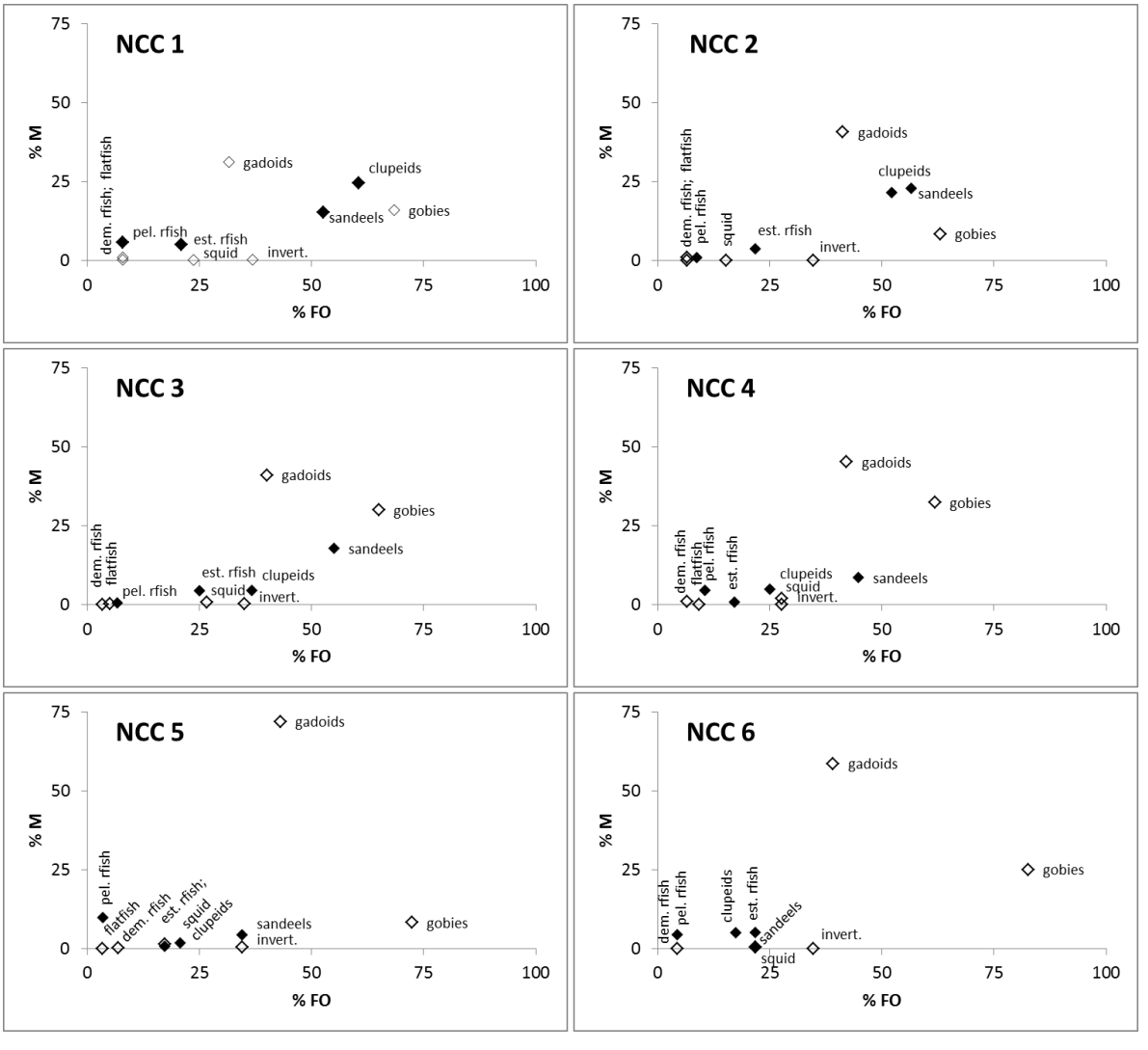


Figure 5. *Costello diagrams, scatterplots of all prey groups according to their % of occurrence and their importance by mass for porpoises at body conditions going from good (NCC 1; upper left) to extremely bad (NCC 6; bottom right). Open symbols: lean prey. Closed symbols: energy-rich prey.*

Discussion and Conclusion

Marine top predators with high metabolic rates are evolutionary geared towards having energy-rich diets (Österblom *et al.* 2008). Spitz *et al.* (2012) suggested that harbour porpoises are such predators, that need to eat prey with a high energy content. However, diet studies have shown that although energy-rich prey are often an important component of their diet, porpoises also eat considerable amounts of prey of a relatively low energy content. The reason for this is unknown but may be that low-quality prey are easier to catch than high-quality prey or are necessary for growth as these are relatively rich in protein or other essential components. Should, however, energy-rich prey be essential for porpoise fitness, porpoises should not be able to stay fit on a diet without such prey, no matter how easy to catch and how abundant low-quality prey may be. In this sense, lean prey could be seen as junk food, if taken in too large amounts. For porpoises in the southern North Sea, this would imply that they can only be successful here, if they manage to find and eat sufficient amounts of fatty fish, such as sandeels or clupeids, in addition to staple foods such as gadoids and gobies.

From the range of prey types available, predators should always harvest prey that have a high energy to mass ratio (Whelan & Brown 2005). Harbour porpoises should therefore always eat at least some fish with a high fat content, that is, fish with a high energy density. It is unknown how long a porpoise can stay fit on a low-energy diet, but in all likelihood, they should strive to, at least periodically, have meals of energy-rich prey. Finding no remains of energy-rich prey in a porpoise's stomach is not necessarily a sign of starvation, as these cetaceans have short gut-residence times (Gaskin 1978; Kastelein *et al.* 1997b) and may alternate meals of lean prey with meals of energy-rich prey and may not feed continuously. Indeed, 19% of the NCC 1 and 2 animals that had non-empty stomachs had no remains of energy-rich prey in their stomachs, while another 17% of these animals had empty stomachs. For the animals of NCC 3 and 4 these figures were 29% and 18%, and for the animals of NCC 5 and 6 these were 38% and 30%, respectively. Leaner animals thus had a higher probability of dying with empty stomachs and had on average eaten prey of lower energy density.

Another constraint for predators, however, is the amount of time (searching time plus handling time) needed to catch different prey, offset against the energy gain of that prey. Prey that are rare may need a long searching time and should probably only be taken when encountered incidentally, e.g., while searching for more common prey. Also, prey that are very fast swimmers, such as mackerel, may not be worth pursuing for less able porpoises, such as younger individuals. This may be the reason why porpoises, when faced with a poor availability of clupeids and sandeels, cannot switch to other high-energy fish, such as mackerel or horse mackerel. In the end, porpoises should only take foods that yield more energy than they require for catching, and they should acquire sufficient energy to sustain themselves.

Given their large surface to volume ratio, harbour porpoises need large amounts of energy to maintain their body temperature. Healthy porpoises have an insulating subcutaneous blubber layer that is at least 1.5 cm thick, the thickness depending on porpoise age and on season (Kastelein *et al.* 1997a, Lockyer *et al.* 2003b, this paper). Maintaining this also requires energy, which is probably the reason that porpoises reduce blubber thickness in summer, at relatively high ambient temperatures. Harbour porpoises are also leaner in the presence of predators/aggressors, supposedly because they need to be more mobile to evade these (MacLeod *et al.* 2007c). Blubber thickness also varies seasonally in captive animals (Kastelein *et al.* 1997a, Lockyer *et al.* 2003b) that are safe both from starvation and predation. Apparently, porpoises balance the costs for maintaining their blubber layer against the cost for thermoregulation and the risk of predation. Apart from insulation, blubber also functions as a safeguard against starvation and porpoises should not deplete their blubber too much, as this will increase heat loss and costs for thermoregulation, and decrease hydrodynamics. Consequently, blubber loss needs to be compensated later by an increased energy intake (Rosen *et al.* 2007), and will decrease the buffer against fatal emaciation.

The fact that NCC values were highest in summer, and blubber thickness was at its lowest, might simply be a response to higher water temperatures. However, stomach filling was also found to be lowest in summer, while the diet lacked (fatty) clupeids and pelagic roundfish. The probability of dying with an empty stomach was much higher in summer (43%) than in the other seasons (8-18%), while the percentage of energy-rich prey was low in both summer (28%) and autumn (25%), compared to winter (38%) and spring (39%). Rather than being a time of plenty and easy living, summer appears, for harbour porpoises, to be a time of scarcity, particularly of energy-rich prey. In addition, gobies, the prey most frequently taken by the porpoises, also seem in short supply (Table 12), both in summer and in autumn. In summer, porpoises must rely heavily on gadoids and with their already thin blubber layer, appear to run a relatively high risk of starvation, if they find these in insufficient quantities, or find too few sandeels, the main energy-rich additional prey in this season.

The gradual decrease of the contributions of clupeids and sandeels from the diet with increasing NCC values (Figure 5) may be seen as support for the junk food hypothesis. This can be interpreted in three different ways:

1. New, relatively low-quality food types are added to the diet, or
2. Prey types are kept constant, but the quality of key prey is reduced, or
3. Relatively high-quality food types are dropped from the diet.

We can rule out the possibility that new, unsuitable prey types had been taken by the starving porpoises, unless such prey would have no identifiable hard parts (e.g. jellyfish). None of the alternative prey groups found in this study was of increased importance in the

emaciated groups. It seems unlikely that the porpoises had been consuming large masses of prey without hard parts, such as jellyfish. We have no records of jellyfish or similar soft-bodied prey (freshly ingested) in porpoise stomachs, in animals that died suddenly, with full stomachs, e.g., in fishing nets (Chapter 4) or from predation by grey seals (Leopold *et al.* 2015a).

In this study, it is important to note that the second possibility cannot be directly assessed from stomach contents analysis. Prey masses, and their quality are assessed from remaining prey hard parts and not measured directly. Should the energy density of certain prey fish be particularly low in the SE North Sea as compared to other parts of the distribution range of porpoises, or should energy density of prey be comparatively low in certain years or seasons (cf. Wanless *et al.* 2005), this cannot be inferred from e.g. otoliths. Estimated relative prey masses therefore take no account of possible regional, year-to-year or seasonal variation within fish species. Prey fish may show dramatic changes in energy density (Wanless *et al.* 2005), and in case e.g. clupeids or sandeels would be exceptionally lean, they prey would probably not be worth pursuing. Such a situation would probably be linked to a certain year (cf. Wanless *et al.* 2005) and although a weak correlation is present in our data between the yearly average NCC and the overall percentage of energy-rich prey, this is not significant ($R^2 = 0.3064$, n=9, p>0.1).

Harbour porpoises should feed on high quality food which corresponds with high metabolic costs of living (Spitz *et al.* 2012), or at least on a sufficient mix of lean and fatty prey (this study). Therefore, the SE North Sea might only be a suitable habitat for harbour porpoises if they can find sufficient amounts of such high-quality prey, next to more lean prey types (gobies, whiting, flatfish) that seem to be abundantly available here. Sandeels (summer), alternating with clupeids (winter and spring), and to a lesser extent pelagic and estuarine roundfish are probably critical dietary components. With increased porpoise densities in the southern North Sea and no evidence of overall decreasing availability of prey fish here (Tulp *et al.* 2008; Tulp 2015) the most likely cause of starvation is temporary shortage of energy-rich prey, particularly clupeids and sandeels. Such shortages may be short-term (days or weeks), given the low tolerance to starvation in harbour porpoises, or seasonal, as indicated by the yearly peak in summer strandings in The Netherlands.

Interestingly, many starving porpoises had still been able to find, catch and eat gobies and gadoids, but failed to consume sufficient amounts of fatty fish. It is impossible to determine which came first: failure to find suitable prey, resulting in starvation, or a reduced body condition leading to a loss of ability to catch high-energy prey. In any case, porpoises that fail to eat sufficient amounts of high-quality fish appear to be at a serious risk of starvation. Starving animals apparently could not compensate for the lack of high-energy prey by consuming more lean prey. In summer, when efficient foraging seems to be most difficult, both a lower mass of both energy-rich and energy-poor prey was found in the stomachs.

A similar pattern was found by MacLeod *et al.* (2007a,b) for starving porpoises in Scotland. Apparently, compensating for loss of energy-rich prey by ingesting more low-energy bulk, is not an option for starving porpoises (cf. Whelan & Brown 2005): quantity of food cannot always replace its quality (Spitz *et al.* 2012).

Acknowledgments

We would like to thank all those people who reported and collected stranded harbour porpoises for this study, and all who helped us with the necropsies over the years. This research was funded by the Dutch Ministry of Economic Affairs (140000353).

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Electronic supplement

ES-1. NCC and DCC codes used, following Kuiken & Garcia Hartmann (1991):

NCC (Nutritive condition code):

The nutritive state of the animal should was evaluated immediately before and during the necropsy, as a general impression based on several details, both externally (the general body shape) and internally (fat and muscular condition).

NCC1: Very good nutritive condition, very well nourished, abundant blubber, significant other subcutaneous fat present in the dorsal neck and -sometimes- on the lateral thorax, pleural fat present, *Longissimus dorsi* and neck are convex, the whole animal shows a "round, barrel-like" body shape.

NCC2: Good nutritive condition, well nourished, abundant blubber, some subcutaneous fat, *Longissimus dorsi* and neck are straight or slightly convex.

NCC3: Normal nutritive condition, blubber is normal thickness, no subcutaneous fat present, neck and *Longissimus dorsi* are straight, on movement of the animal sometimes slightly convex.

NCC4: Bad nutritive condition, blubber is on the thin side, sometimes skin thickness increased, neck and *Longissimus dorsi* visibly concave.

NCC5: Very bad nutritive condition, blubber is thin, skin thickness most often increased, *Longissimus dorsi* and neck clearly concave.

NCC6: Extremely bad nutritive condition, severely emaciated, blubber is very thin, neck and *Longissimus dorsi* are severely concave, the contour of the scapula (especially the *Spina scapulae*) may be visible externally.

DCC (Decomposition condition code):

The decomposition condition code (DCC) is based on the external and internal decomposition signs of the carcass.

DCC 1: Very fresh, less than 48 hours dead, may show signs of rigor mortis (<24h), blood still separates serum (24-48h), rigidity of eyes is diminished but not very flaccid, cornea is not cloudy.

DCC 2: Fresh, first signs of decomposition visible, eyes and surface quality of the skin reveal decomposition, otherwise good state, organs look intact, blood does not separate from serum, no smell of decomposition.

DCC3: Putrefied, skin peeling, moderate but clear signs of decomposition (changes in colour and consistency) of skin and organs, not suitable for bacteriology because of overgrowth, moderate smell of decomposition.

DCC4: Very putrefied, advanced decomposition, skin and organs clearly altered, the loss of consistency changes the organ‘s shape, clear smell of decomposition, not suitable for any tissue analysis, even gross pathology is very unclear and can hardly be interpreted at all.

DCC5: Remains, organs are beyond clear recognition or absent, may be mummified or reduced to mere bones.

ES-2: Base data:

A second ES will be supplied with this paper, once published. In an Excel table, the total numbers, mass, and frequencies of occurrence, as well as %N, %M, %FO and IRI will be supplied for each prey guild and each prey species. This (lengthy and wide) table is not reproduced here.

Stomach contents analysis as an aid to identify bycatch in stranded harbour porpoises *Phocoena phocoena*

Mardik F. Leopold, Carolien H. Strating, Lonneke L. IJsseldijk, Lineke Begeman, Peter J.H. Reijnders & Geert Aarts

4

Abstract

Fisheries bycatch, particularly in bottom-set nets, is an important cause of death in harbour porpoises *Phocoena phocoena*. Identifying bycatch from post-mortem studies on stranded porpoise carcasses is often difficult and relies on a combination of features. One characteristic considered consistent with bycatch is a full stomach, as this signals an acute death. Here we show that when porpoises are mostly bycaught in bottom-set nets, prey species composition, rather than the quantity of prey remains in their stomachs is the most informative characteristic to identify bycatch. Certain and highly probable bycatches (i.e., those porpoise carcasses brought in by fishers or with net marks and other evidence of bycatch) had a high proportion of demersal fish prey in their stomachs, usually >94% by mass of all fishes identified. Less certain cases, so-called probable and possible bycatches, included progressively more animals with lower percentages of demersal fish prey mass. The certain and highly probable bycatches also tended to have higher proportions of demersal prey in their stomachs, compared to animals that had died from other causes of death (e.g., emaciation, infectious disease, grey seal predation or unknown causes). This relationship was used to improve the classification of those porpoises classified as probable or possible bycatch. Prey species composition may thus be used as an additional bycatch criterion during post-mortem studies of stranded cetaceans, if the type of fishery responsible for the bycatches is known.

Keywords: diet, stomach content, prey types, bottom set gill nets, bycatch

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4

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Introduction

Incidental catches in fishing gear, or bycatch, is a main cause of death in harbour porpoises *Phocoena phocoena* throughout their distribution range (Jefferson & Curry 1994; Birkun 2002; Kaschner 2003; Stenson 2003; Reeves & Notarbartolo-di-Sciara 2006; Moore *et al.* 2009; Bjørge *et al*. 2013; Geijer & Read 2013; Reeves *et al*. 2013). Whether or not such bycatches are sustainable is difficult to assess (Lewison *et al*. 2004; Bisack & Magnusson 2014), as this requires information on population size, vital rates, the part of the population that is affected, and the number of individuals removed as bycatch. All these tend to be poorly known, and difficult and costly to measure. Only in rare cases has it been possible to identify a level of sustainable bycatch (IWC 2000; Reijnders *et al*. 2009).

Bycatch can be identified based on information from fishermen (Jepson *et al*. 2005; Wright *et al*. 2013; Vishnyakova & Gol'din 2015), independent on-board observers (Vinther & Larsen 2004; Tregenza *et al*. 1997; Bjørge *et al*. 2013), or necropsies of stranded animals (Baker & Martin 1992; Kirkwood *et al*. 1997; Cox *et al*. 1998; Jepson *et al*. 2000; Siebert et al. 2001; Jauniaux et al. 2002, 2009; Leeney *et al*. 2008; Osinga *et al*. 2008; Hohn *et al*. 2009; Vishnyakova & Gol'din 2015). All three methods are sensitive to bias. Even direct information from fishermen or on-board observers is unlikely to be representative of the entire fishery and some types of fishery with bycatch may not be sampled altogether. The probability that bycaught porpoises wash ashore depends on the distance from shore at which they were discarded (Peltier *et al*. 2013), while the probability that stranded cetaceans are found and used for post-mortem investigation will depend on sampling effort and on coastline accessibility (de Boer *et al*. 2012). Once retrieved, however, carcasses can be examined for signs of bycatch. The criteria for the diagnosis of bycatch among stranded harbour porpoises have been reviewed earlier (Kuiken *et al*. 1991; Kuiken 1994; Jepson *et*

al. 2000; García Hartmann *et al*. 2004; Soulsbury *et al*. 2008). Entanglement in fishing gear may cause both external trauma, such as net marks, line imprints, abrasions, penetrating wounds, extracted teeth; and internal lesions, such as fractured mandibles, ribs and other bones, haemorrhages, pneumothorax, congestion, and severe lung oedema; while handling by the fishers may result in additional post-mortem defects, such as gaff marks, amputations (aiding disentanglement from the netting) or skull fractures from being hauled on board and dropped down onto the deck of the vessel. Other characteristics that are consistent with bycatch are a good nutritional body condition and a full stomach, because these point to an acute death. Finally, the absence of clues for other causes of death is seen as supportive for the possibility of bycatch.

None of the injuries mentioned are specific for fishery bycatch, however. Only fresh animals that are directly retrieved from nets are certain bycatches, while bycatch in animals found dead elsewhere is never certain. Even net marks do not provide certainty of bycatch, as carcasses of animals that died from other causes may also drift into a net and receive such lesions post mortem. Both blunt and sharp trauma may result from reasons other than bycatch (e.g. lethal interactions with predatory seals (Jauniaux *et al*. 2014; Leopold *et al.* 2015a,b) or aggressive dolphins (MacLeod *et al*. 2007). Lung oedema apart from drowning, may result from other reasons of vascular damage, such as increased blood pressure, or decreased drainage of lymph. A good nutritional body condition or a full stomach is no prerequisite for bycatch, as starving animals may also be bycaught, while healthy animals with full stomachs may die from different causes. The conclusion of a necropsy should thus include a degree of uncertainty varying from "Certain bycatch" to "Possible bycatch" or "Unknown" and any additional characteristic that can aid in bycatch diagnosis will be of great value for improving the bycatch classification of dead stranded cetaceans.

Given that bycatch must result from nets set or towed in a particular part of the water column, specific food remains found in the stomachs, rather than their quantity, may provide further clues on the likelihood of bycatch. For instance, dolphins that are bycaught in mid-water trawls or seines are likely to have pelagic, rather than demersal prey in their stomachs (Perrin *et al*. 1973; Couperus 1997; Marçalo *et al*. 2015). Conversely, stomachs of dolphins bycaught in bottom trawls have a higher proportion of demersal prey (Scheinin *et al*. 2014). Following this reasoning, harbour porpoises bycaught in bottom-set gill nets are expected to have a relatively high proportion of demersal prey in their stomachs. Therefore we hypothesize that specific identification of prey remains in harbour porpoise stomachs may further improve the identification of bycatch. To test this hypothesis, we examined the stomach contents of porpoises that were known

(certain) or very likely (highly probable) bycatches, and used these to improve the classification of progressively less certain cases of bycatch. The study was conducted in a region where bottom-set gill nets are the type of fishery most commonly held responsible for porpoise bycatches (Reijnders *et al*. 2009; Camphuysen & Siemensma 2011).

Material and Methods

Necropsies

Dead, stranded harbour porpoises were collected by members of the Dutch strandings network and transported to Department of Pathobiology, Faculty of Veterinary Medicine, Utrecht University, for post-mortem study. In addition, bycaught porpoises were received directly from fishers. The porpoise carcasses were examined using a standard necropsy, following protocols of Kuiken *et al*. 1991). The probability of bycatch was assessed based on five criteria: 1) direct information of the fisher; 2) evidence of contact with fishing gear or fishers: superficial or penetrating line marks, digesta in oesophagus, bruises, amputations, skull fractures; 3) exclusion of other causes of death; 4) normal health status: sufficient to good nutritional body condition, stomach contents present; 5) hypoxia (signs of drowning): oedematous lungs, persistent froth in airways, following (Kuiken 1994). Based on the available evidence, each porpoise was placed in one of the following four categories: Certain bycatches were those that were handed in directly by fishers (criterion 1) and that lacked observations suggesting that the animal was already dead when it drifted into the net (e.g. extent of autolysis). Highly probable bycatches lacked this direct information from fishers, but met criteria 2 and 3 and had no characteristics that conflicted with criteria 4 and 5. Probable bycatches were those that met either criterion 2 or 3, and had no characteristics that conflicted with criteria 4 and 5. Possible bycatches were those that had some characteristics consistent with bycatch, but that were difficult to assess in full, due to advanced decomposition hampering mainly assessment of criteria 3 (exclusion of other causes of death). Finally, animals for which other causes of death were determined (grey seal, *Halichoerus grypus*, predation, Emaciation or Infectious disease), or for which no criteria either consistent or at odds with bycatch or any other cause of death could be determined (Unknown), were taken as negative control groups.

Stomach contents

Stomach contents of dead harbour porpoises stranded in The Netherlands (SE North Sea) were examined, following the procedures outlined in Leopold *et al*. (2015b). A total of 696 stomachs (Table S1) were examined in this study, 114 of which did not contain any prey remains (16%) and 27 that contained only remains of invertebrates (4%). These latter two categories were excluded from further analysis. Clearly, if no prey remains were found, no information on the last meal was available, while many of the invertebrates (annelids, crustaceans, molluscs, echinoderms) were probably secondary prey (Pierce & Boyle 1991) and all, but particularly annelids and squid have hard parts that survive much longer in the stomach of a predator than fish hard parts (Harris *et al*. 2015), and thus were probably often not indicative of the last meal before death. Prey species were assigned to several groups: demersal and pelagic fish prey, and various invertebrates (Table S2). We classified those fish species that were assumed to spend the majority of their time near the sea floor as "demersal prey", and all others as "pelagic prey". The pelagic prey species mostly consisted of forage fish (Engelhard *et al*. 2013), such as Clupeidae and Osmeridae and their pelagic predators Atlantic mackerel, Atlantic horse mackerel and European seabass (Table S2). Although sandeels are known to forage also high in the water column (Greenstreet *et al*. 2006), they spend a substantial amount of time in or near the bottom within reach of bottom-set gill nets, and hence were classified as "demersal". The three parties involved in collecting dead porpoises (Dutch strandings network), the necropsies (Utrecht University) and the study of stomach contents (IMARES Wageningen UR) are mandated by the Dutch Ministry of Economic Affairs to perform these actions.

The contribution of a specific prey taxon in the diet of a group of consumers was expressed as the frequency of occurrence (percentage of stomachs containing that prey (%O), or as the percentage of that prey in terms of total prey numbers (%N) or prey mass (%M), across all stomachs containing prey remains (Pierce & Boyle 1991).

Analysis

The mean (and uncertainty) of the percentage demersal prey in the diet for each bycatch category (i.e. Certain bycatch, Highly probable bycatch, Probable bycatch, Possible bycatch and Unknown) was estimated by including bycatch category as a discrete factor variable within a Generalized Linear Model (GLM).The percentages demersal prey in the diet (in occurrence %O, numbers %N and grams %M), were converted to fractions and treated as a quasi-binomial response variable. More

specifically, the number of stomachs, number of prey or summed prey mass in grams of all prey items treated as the number of 'trials', and the number of stomachs, number of prey or summed prey mass of demersal prey items as 'successes'. This specification of the response takes into account the accuracy of the estimated percentages: those percentages of demersal prey based on large stomach contents, which are more accurate, implicitly receive a higher weighting. The quasi-binomial distribution allows for over or under-dispersion. For the presentations of the results (i.e. figures and tables), these estimated fractions were converted back to percentages.

The discrete bycatch categories (excluding the Unknowns) were also converted to numerical classes 1 to 4, for respectively, Certain, Highly probable, Probable and Possible bycatch, and used as a continuous variable in a GLM to explain the observed variations in the percentage demersal prey in the diet as linear function (on logit scale) of bycatch certainty (i.e. numerical class 1-4). Since our aim was to detect if bycatch certainty increases or decreases with the percentage of demersal prey in their diet, only a linear function was explored. Significant negative slope coefficients indicate that the percentage demersal prey declines as a linear function of the decrease in certainty with which dead porpoise can be classified as bycatch.

Finally we used data on diet to improve the classification of those porpoises classified as Probable bycatch or Possible bycatch. For this analysis, two groups were generated: group 1: containing the Certain and Highly probable bycatches, and group 2: containing Grey seal victims, animals that had died from Emaciation, Infectious disease or from Unknown reasons. Next the probability of a porpoise belonging to either group was modelled as a function of the proportion of demersal prey (by mass) in the diet, where the response variable for group 1 was defined as '1' and the response variable of group 2 was defined as '0'.

Results

Numbers of available porpoises and prey species found

The number of available porpoise carcasses for this study increased with decreasing certainty of bycatch (Table 1). The average prey mass (in non-empty stomachs) was on average higher in the bycatch categories, than in the Emaciation, Infectious disease or Unknown carcasses. This could be due to the fact that a full stomach was also used as a bycatch criterion. Interestingly, there is an increase of reconstructed prey mass with decreasing certainty for bycatch. This suggests that prey mass alone is a poor criterion for defining bycatch probability.

Stomach filling was lowest in the group of animals with Unknown cause of death, that lacked any characteristics pro or contra bycatch or any other cause of death. Variation in reconstructed prey masses per stomach was large in all groups, with masses from 0.17 to 14 673 gram, across all categories.

Table 1. *Average reconstructed fish prey masses in grams for porpoise stomachs containing fish remains, per cause of death category, with standard deviations and numbers of stomachs (n). The numbers of empty stomachs and stomachs with only remains of invertebrates are also given.*

Cause of death	Prey mass in Non-empty stomachs	Empty stomachs	Stomachs with invertebrates only
Certain bycatch	661±706 (n=7)	0	0
Highly probable bycatch	704±1542 (n=17)	1	0
Probable bycatch	875±1357 (n=38)	9	0
Possible bycatch	1426±2538 (n=65)	3	1
Grey seal predation	992±1264 (n=112)	5	1
Emaciation	645±1304 (n=64)	18	5
Infectious disease	722±1311 (n=74)	24	8
Unknown	363±704 (n=178)	54	12

In total, remains of at least 71 different prey species were found: 44 fish species, five species of squid, six annelids, ten crustaceans, one gastropod, four bivalves and one echinoderm (Table S2). Among the fish prey species, 29 were considered demersal and 15 were considered pelagic species. Overall, fish constituted the bulk of the reconstructed prey mass, with 134136 individuals identified, that had a summed estimated fresh mass of 494.351 kg. The beaks of 1423 squids were found (summed mass 14.743 kg) and remains of 771 other invertebrates (1.271 kg). Thus, fish comprised 98% of prey numbers and 97% of prey mass. Of the fish prey, 126624 were demersal fish (94% of all fishes), with a summed reconstructed mass of 382.217 kg (77% of total reconstructed fish mass). Therefore, the vast majority of porpoise prey in this study were demersal fishes.

Incidence of prey types in various bycatch categories

Demersal prey occurred in most of the stomachs considered and were the dominant prey across all bycatch categories, both in terms of numbers and of mass. Considering all available stomachs with fish remains across the various bycatch categories (n=127), no change in relative importance of demersal prey was found with decreasing certainty of bycatch when this was expressed as %O (Slope estimate β=-0.1535, SE=0.4418, t-value=-0.348, p=<0.7288), but a decreasing non-significant trend was found for %N (Slope estimate β=-0.6985, estimate β=-0.6565, SE=0.2438, t-value=-2.693, p=0.008) (Figure 1).

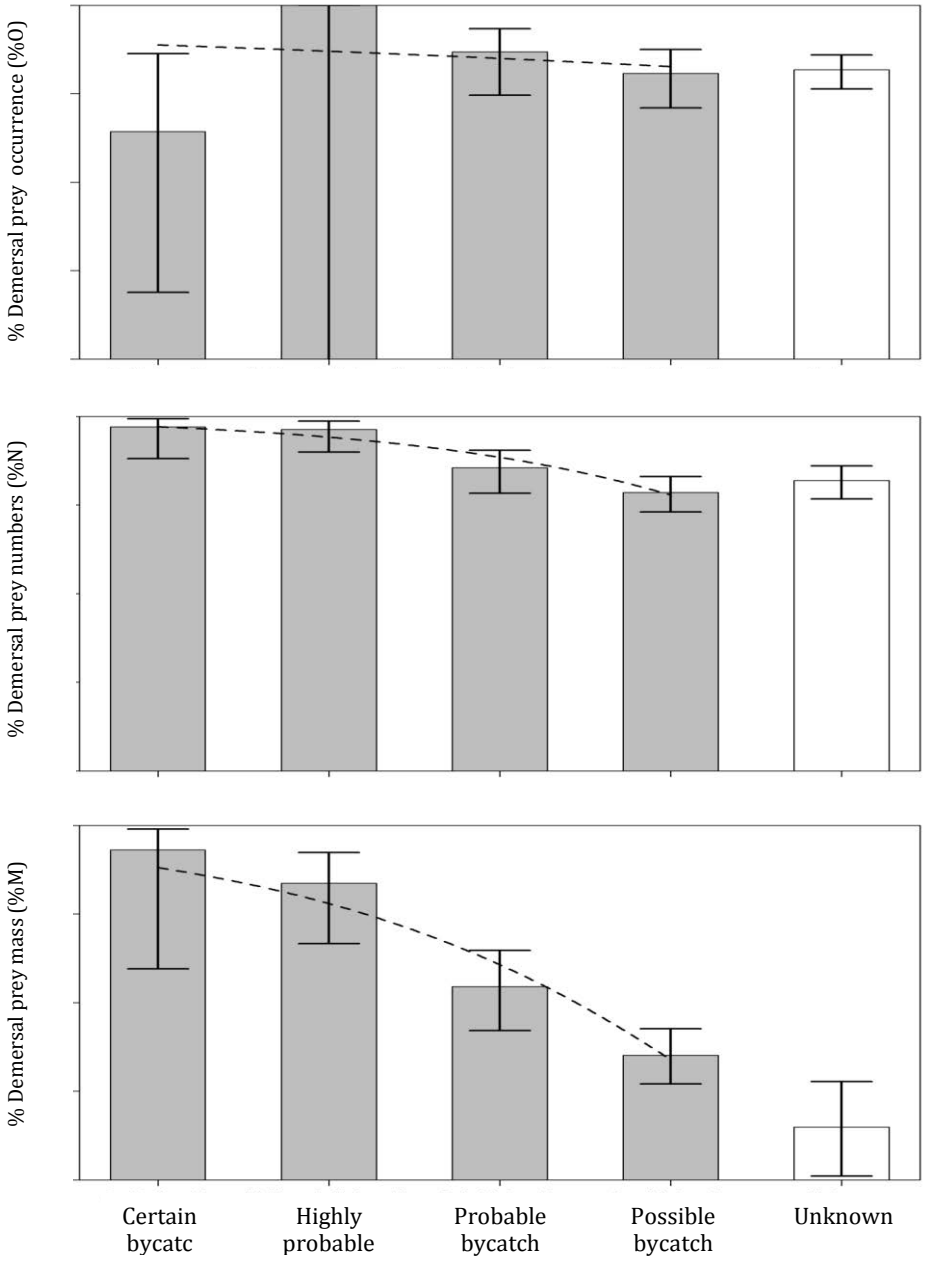

Figure 1. *Relative importance of demersal fish prey (against pelagic fish prey) for respectively Certain, Highly probable, Probable and Possible cases of bycatch (grey bars). Top: %O, Centre: %N, Bottom: %M (see text). For numbers of cases per bycatch category: see Table 1. Animals in the Unknown category (which is likely to include some bycatches) are depicted as a white bar for comparison, but these are excluded from the slope analyses.*

The observed % Demersal M-values were used to update the likelihood of bycatch for individual porpoises in the Probable and Possible bycatch categories. Using all (groups 1 & 2) stomachs with fish remains (n=555; see Table 1), we found that the probability that a porpoise belongs to group 1 (Certain or Highly probable bycatch) increases as a function of the demersal prey mass percentage (Figure 2: slope estimate β=0.010542, SE=0.003234, t-value= 3.260, p-value =0.00120). From this result, we considered that if the estimated probability exceeded 0.5+SE (the prediction standard error), the bycatch category should be upgraded: a Possible bycatch should be changed to a Probable bycatch, and a Probable bycatch to a Highly probable bycatch. Cases with an estimated probability below 0.5-SE should be downgraded: from Probable bycatch to Possible bycatch and from Possible bycatch to Unknown. Cases with estimated probabilities between these two values should remain in their respective categories.

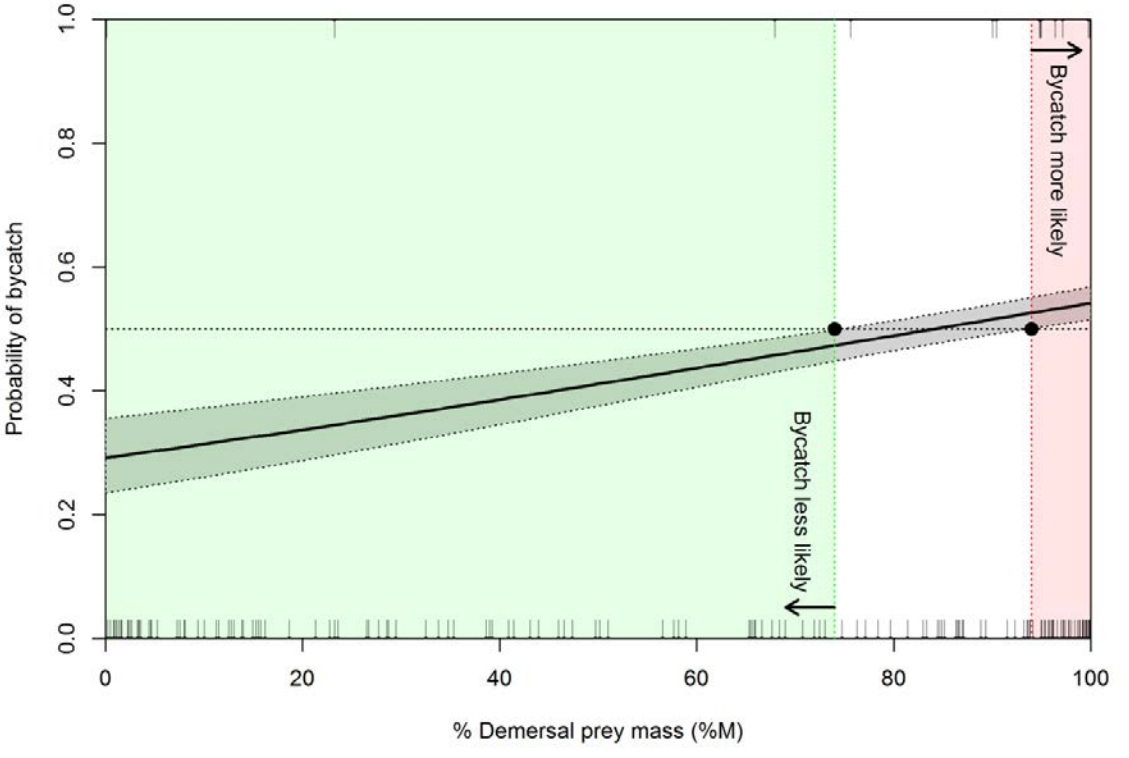

Figure 2. *Bycatch probability (line with shaded predicted SE) as a function of the proportion M reconstructed demersal fish prey in the individual stomachs (markers on X-axis: top=group1; bottom=group2). For this figure, the proportions have been converted to %M along the X-axis.*

If the predicted bycatch probability exceeds 0.5+SE, the level of the bycatch category was updated (from Possible -> Probable and from Probable -> Highly probable bycatch). The 0.5+SE threshold was exceeded when the %M demersal prey in the diet exceeded 94% (the pink area to the right of the right black dot). Similarly, if the threshold was below 74% (0.5-SE, the green area to the left of the left black dot), the level of the bycatch category was downscaled (from Probable -> Possible and from Possible -> Unknown).

Among the animals that were bycaught with certainty, demersal prey were the dominant prey in all but one stomach (Figs. 2 and 3). This outlier had the remains of 30 partly digested, ca. 12 cm long lesser pipefish in its stomach, and no other prey. Two Highly probable bycatches had % Demersal M values below the lower threshold value of 74%. Both animals had a mix of goby, sandeel and sprat otoliths in their stomach. This made it impossible to judge which of these two, demersal gobies and sandeels, or pelagic sprats, had constituted the last meals. Three further Highly probable bycatches had % Demersal M values between the thresholds of 74% and 94%. All other Certain and Highly probable bycatches had % Demersal M values >94%, irrespective of the summed prey mass (Figure 3).

Porpoises of the other bycatch categories more often had % Demersal M values below the lower threshold value of 74% (Figure 3). Animals from the lower bycatch categories that had relatively large reconstructed prey masses in their stomach mostly had a % Demersal M value above the upper threshold of 94%. Exceptions were three Possible bycatches, which had eaten rather large quantities of Atlantic mackerel, Atlantic horse mackerel or smelt. Several animals that were diagnosed to have died from Infectious disease or Emaciation also had high reconstructed prey masses in their stomachs. Most of these also had a % Demersal M value above the upper threshold of 94%. Large reconstructed prey masses were also found among the Grey seal victims and the animals with Unknown cause of death, but in these categories, relatively many such animals had relatively low % Demersal M values.

Given that, generally, high reconstructed prey masses tended to come with high % Demersal M values, and that both measures, if high, may be considered indicative of bycatch, we subsequently deleted cases with reconstructed prey masses below 100 g, 500 g and 1 kg from the analysis (Figure 4), and considered individual variation within each bycatch class. Removal of cases with low prey masses quickly reduced variation among both % Demersal N and M values for the Certain and Highly probable bycatches, but variation remained high for the Probable and Possible bycatches and for the Unknowns.

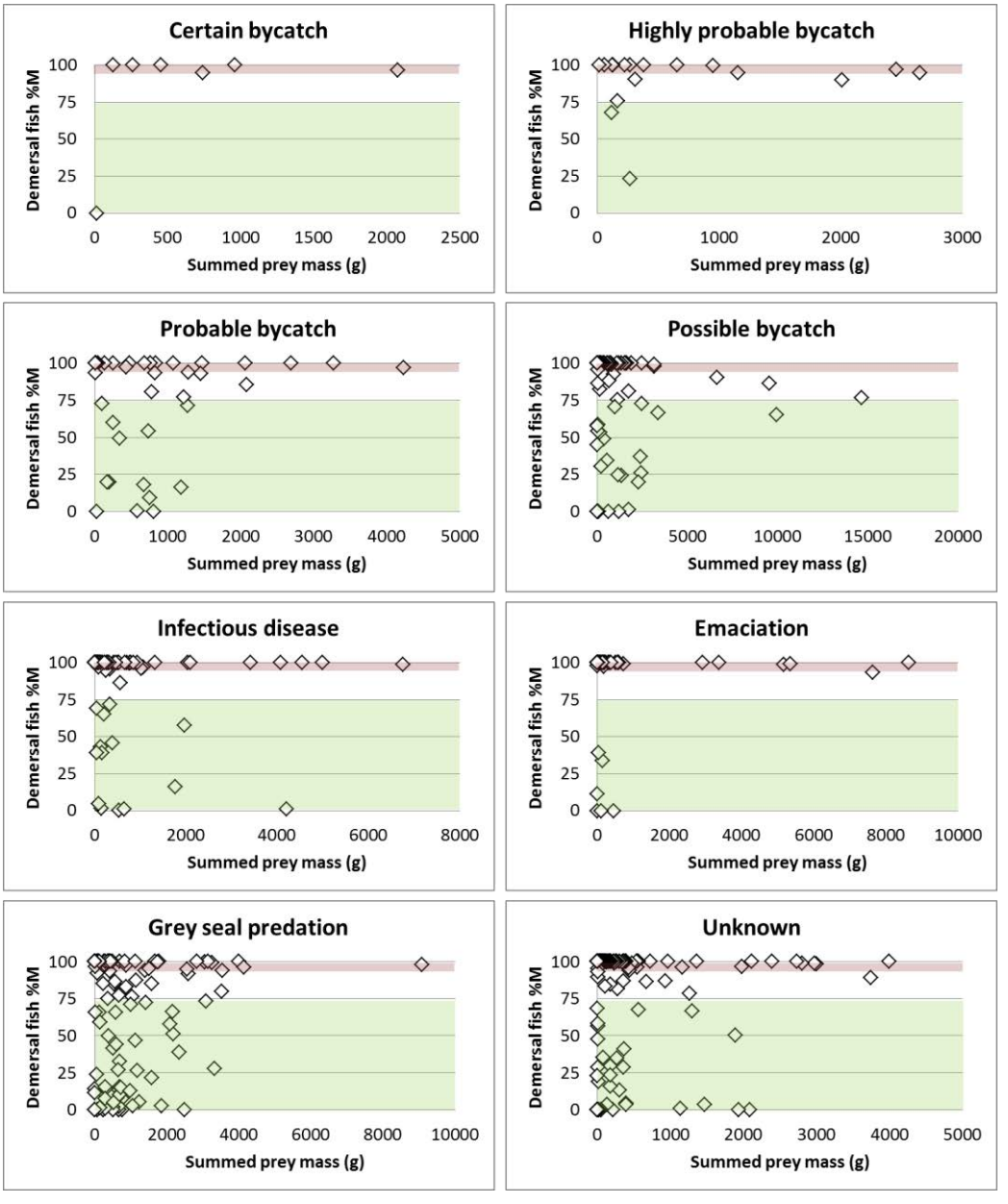

Figure 3. *The percentage of demersal prey (prey numbers) as a function of the summed prey mass of demersal and pelagic prey, for all porpoises, for different causes of death. Note different scaling along X-axes. Coloured areas as in Figure 2.*

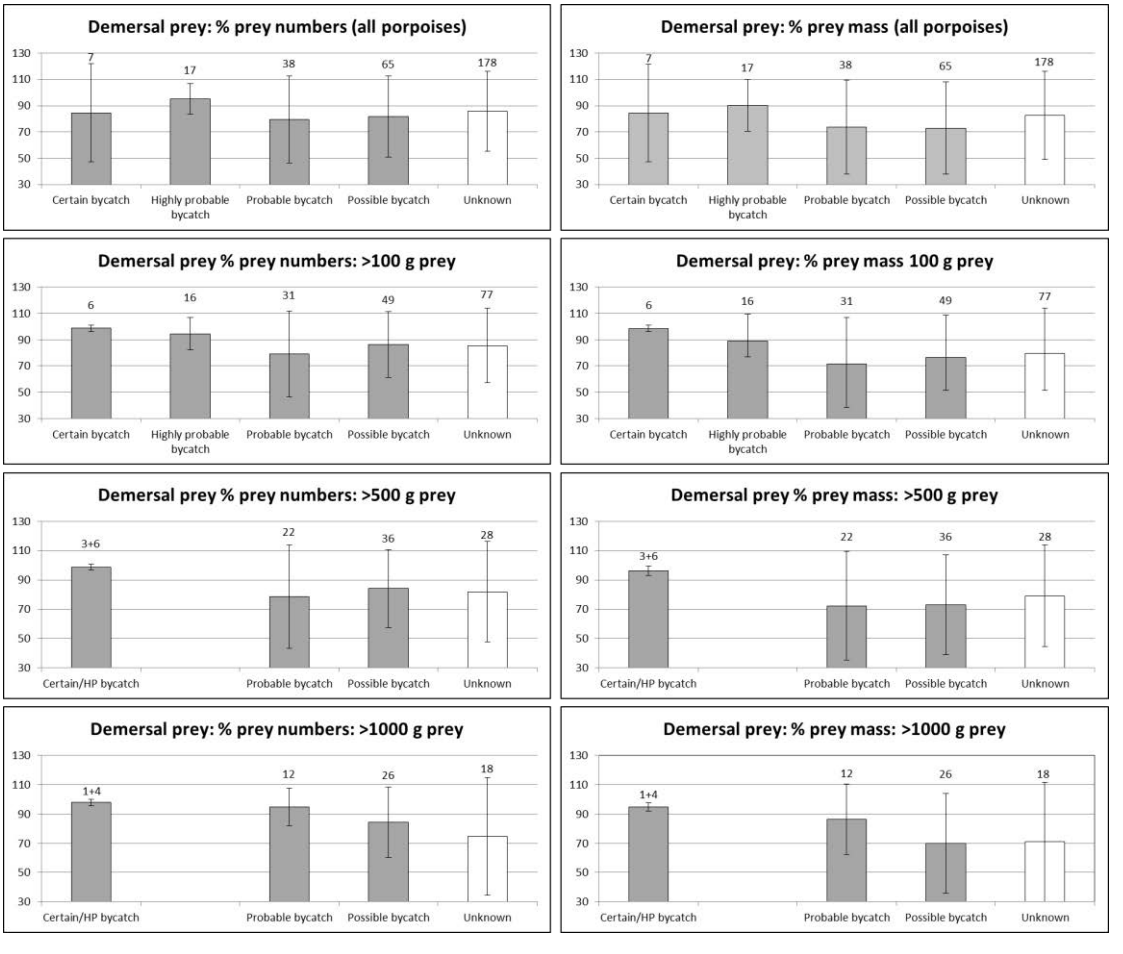

Figure 4. *Average (±SD) Demersal %N (left) and %M values (right) for the different bycatch classes, with, from top to bottom, all cases included, and cases with less than 100 gram, 500 gram and 1 kg summed prey mass removed. In the lower two rows, Certain and Highly probable bycatches were combined; numbers of cases indicated above error bars. Animals with Unknown cause of death depicted as white bars, for comparison.*

This suggests that summed prey mass by itself is little indicative for the probability of bycatch when bycatch is less certain, while the composition of the diet provides a better clue. This is also reflected in Table 1, showing a lower total prey mass for the Certain bycatches, compared to the Possible or Probable bycatches.

Amending Probable and Possible bycatch cases

Certain and Highly probable bycatches tended to have higher proportions of demersal prey in their stomachs, compared to animals that had died from other causes (Figure 2). This relationship was used to re-assess animals in the Probable and Possible bycatch categories and either upgrade or downgrade their bycatch likelihood status. For example, in several cases for which bycatch was suspected but less certain (i.e. Probable and Possible bycatch) high proportions of pelagic prey were present in their stomachs, suggesting that these are less likely to be bycatches. In summary, cases with % Demersal M values <74% were downgraded one category, while cases with Demersal M values >94% were upgraded one category, and cases with values between these two thresholds remained unchanged. Following these criteria, among the 38 cases hitherto considered Probable bycatches, 19 were upgraded to Highly probable bycatches, 6 remained unchanged and 13 were downgraded to Possible bycatches. Among the 65 original Possible bycatches, 32 were upgraded to Probable bycatches, 9 remained unchanged and 24 were downgraded to Unknown. The status of cases with Unknown cause of death were not amended, even though some bycatches are likely to occur in this category. However, as animals with other causes of death can also have % Demersal M values >94%, the Possible bycatches among the Unknowns cannot be identified on the basis of diet. Although the amount of demersal prey provides information on the likelihood that bycatch is the cause of death, it provides no certainty, since high % demersal M values were also found in carcasses of other causes of death. Therefore, none of the Unknowns were upgraded to Possible bycatches.

In conclusion, a more accurate likelihood of bycatch could be assigned in retrospect to many of the less certain bycatch cases, by including this extra diet information with the assessment of bycatch probability. Initial and amended assessments, by category, are given in Table 2.

Table 2. *Proposed changes in relative numbers of cases in the different bycatch classes, by using specific diet information.*

Bycatch category	Initial number of cases	Number of cases after using diet information
Certain bycatch	7	7
Highly probable bycatch	17	36
Probable bycatch	38	38
Possible bycatch	65	22
Unknown	178	202
total	**305**	**305**

Discussion

After several centuries of whaling, fishery bycatch has become a main cause of death in many cetaceans (Moore 2014). Assessing exactly the number of bycatch victims post-mortem is challenging, since indisputable characteristics are not always present. Currently, the lack of evidence of other causes of death and a full stomach (which provides evidence for an acute death), are used as criteria to define Possible and Probable bycatches. This study, however, shows that not a full stomach, but a stomach with prey species that are indicative of gear held responsible for the bycatch can be used to further improve pathological assessments.

Among the harbour porpoises from the SE North Sea studied here, demersal fish comprised the staple food. The main fisheries in the region are various types of bottom trawling and bottom-set gill nets (Camphuysen & Siemensma 2011; STECF 2014), so in all likelihood porpoise bycatches occur mainly near the sea floor. The fact that Certain and Highly probable bycatches were characterised by high proportions of demersal prey in their stomachs was therefore to be expected. Porpoises feeding on fishes that swim higher in the water column (pelagic prey) run less risk of being bycaught. Porpoises can take large numbers of both demersal and pelagic prey, and while a full stomach in a stranded porpoise provides evidence of an acute death, other causes than bycatch apparently contribute to the number of dead porpoises in the SE North Sea, like predatory grey seals (Jauniaux *et al*. 2014; Leopold *et al*. 2015a,b). Clearly, in areas without grey seal predation, or with different fisheries, e.g., drift netting, seining or long-lining, this situation may be quite different. It is thus important that assessors of bycatch know the local fishing practices and other circumstances that might contribute to porpoise deaths.

For our assessments, we are fairly confident that prey fish, classed in this study as demersal (Table S2), are mostly found near the sea floor. In contrast, prey classed

as pelagic, can be caught mid-water, and some species, such are mackerel, are probably rarely caught near the sea floor. Others, however, such as clupeids (Sprong *et al*. 1990), and smelts (Piersma *et al*. 1988) show a daily vertical migration, and can be very abundant near the sea floor during the day. The presence of clupeids of smelt in the stomach of a stranded porpoises does not, therefore, exclude the possibility of bycatch and our assessment of the probability of bycatch is therefore conservative. Indeed, diets dominated by sprat were found in a study based on bycaught porpoises in bottom-set turbot nets in the Black Sea (Tonay *et al*. 2007). On the other hand, a diet consisting largely of clupeids was considered indicative of mid-water, or even near-surface feeding in harbour porpoises killed by predatory grey seals, while a demersal prey spectrum in seal victims supposedly reflected seal attacks near the sea floor (Leopold *et al*. 2015b). This study presents another example that uses specific dietary information for inferring where, in the water column, harbour porpoises died, while foraging. These findings also present a warning, that diet studies based on bycaught porpoises in bottom-set gill nets (e.g., Recchia & Read 1989; Víkingsson *et al*. 2003), may provide a bias towards demersal prey.

For our assessment, we assumed that Certain and Highly probable bycatches were representative for all other bycatch categories. The Certain bycatches were brought in by Dutch fishermen, and they may represent a specific type of fishery. However, fishermen from other nations also operate in Dutch waters. If they operate differently, their bycatches may have different characteristics (e.g. show different types or frequencies of line marks, amputations, skull fractures or hypoxia). They may operate in different regions, and porpoise diet could be different. Still, we had to assume that the Highly probable bycatches, which were based on evidence from necropsies should apply to all types of fishery, and showed similar diets across fisheries, dominated by demersal prey.

We also had to assume, that causes of death were correctly assigned. In reality, some carcasses bore signs of more than one possible cause of death. For example, an Infectious disease may lead to Emaciation, and Emaciation may lead to increased risk of contracting an Infectious disease. Also some carcasses classified as fishery bycatch, could have been grey seal victims, and vice versa (see: Leopold *et al*. 2015a). Confusing cases of Infectious disease with Emaciation was not a problem for our analysis, as both were assigned to the same group (2). Confusing grey seal victims with bycatches would be a problem if this involved a carcass classed as a Highly probable bycatch. This would seem unlikely as considering a case as a Highly probable bycatch requires that other causes of death could be excluded, which is unlikely in the event of grey seal predation.

The core of this analysis (Figure 2) relies on having at least some Certain or Highly probable bycatches, whose characteristics can be used to consider other, less apparent cases. This study uses diet as an additional classification characteristic, but a similar quantitative assessment could be based on other signs. For example, if all Certain bycatches (i.e. those brought in by fishermen) also reveal signs of line marks or hypoxia, fresh porpoises with no such signs are unlikely bycaught individuals. However, if any fishery related characteristic is only apparent in a fraction of the Certain bycatches, many fresh porpoises without such signs may indeed be by-caught individuals, and the number of bycaught individuals might be underestimated. The number of available Certain bycatches was relatively small in this study, underlining the importance of obtaining more such carcasses for the study of bycatch in this small cetacean.

Acknowledgements

We thank Marije Siemensma (Marine Science & Communication), Bram Couperus and Meike Scheidat (IMARES) and Dutch fishermen for their efforts to land bycaught porpoises and make them available for pathological research. We thank the volunteers retrieving porpoise carcasses from Dutch beaches and the veterinary pathologists, technicians and the many volunteers that helped with the necropsies at the faculty of Veterinary Medicine of Utrecht University and with analysing stomach contents at IMARES.

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Supporting information has been requested by PLoS One: Tables S1 & S2, and can be found on line, after publication of this paper.

Table S1. Base data for all porpoises examined in this study: bycatch category (not corrected for stomach contents), an unique ID tag, and stomach contents in terms of numbers and summed masses of demersal and pelagic prey.

Table S2. All different prey species found in the porpoise stomachs examined, each assigned to be either pelagic (fish) species, demersal (fish) species, or various taxa of invertebrates (left column). The figures in the right columns are the number of porpoises of each bycatch class containing that specific prey species.


SG 2

Follow the fish: Do Harbour Porpoises (*Phocoena phocoena*) respond to better water quality up rivers?

Mardik F. Leopold, Liliane Solé, Lonneke L. IJsseldijk, Elze Dijkman, Thierry Jauniaux & Jan Haelters

5

Abstract

As rivers are being cleaned, life is returning to their estuaries and higher parts. Diadromous fish species are on the increase again in many major rivers discharging into the North Sea. Harbour porpoises (*Phocoena phocoena*), being predators of fish, have been noted to return to North Sea estuaries and rivers as well. Their mere presence in these rivers, however, is no proof that these small cetaceans actually exploit the returning fish. Diet studies of the porpoises found up-river can shed a light on their prey choice and ecological role in the system. Here we show that a major part of the diet of porpoises found in the river Western Scheldt (2007-2014) comprises diadromous fish, particularly juvenile European smelt (*Osmerus eperlanus*). Smelt contributed 46% to porpoise diet (% prey mass) in the Western Scheldt, against 14% in the river mouth and 3% in the North Sea at either side of the river mouth. Even though porpoise numbers are increasing in the river, not all is well, however. Animals found dead on the river banks were generally in a poor nutritional condition and had an elevated probability of being found dead with an empty stomach. Animals swimming very far upstream sometimes braved major water works such as sluices, which might have hindered their return to the sea. Relatively many animals were reported dead later, but to date, too few have been collected for stomach content analysis to make a valid comparison between diets in the lower and higher parts of this river system possible.

Keywords: diet, *Osmerus eperlanus*, river restoration, diadromous fish, habitat quality

Follow the fish: do harbour porpoises (*Phocoena phocoena*) respond to better water quality up rivers?

Mardik F. Leopold, Liliane Solé, Lonneke L. IJsseldijk, Elze Dijkman, Thierry Jauniaux & Jan Haelters

Introduction

Around the North Atlantic, the combination of habitat degradation, pollution and overfishing has stripped major rivers of their diadromous fish populations (de Groot 2002; Lotze 2005; Limburg & Waldman 2009). Conversely, river restoration programmes have resulted in the cautious come-back of several of the impacted fish species. Along the eastern North Sea and Channel seaboard, some species, such as European smelt *Osmerus eperlanus* (Linnaeus, 1758) (further: smelt), shads *Alosa sp.* and several salmonids are slowly returning into several rivers (Raat 2001; Maes *et al.* 2007, 2008; Buysse *et al.* 2008; Rochard *et al.* 2009; Breine & Van Thuyne 2014a,b, 2015). In their wake, predators like the harbour porpoise *Phocoena phocoena* may be expected to follow. Indeed, numbers of sighted porpoises have greatly increased in several estuaries along the eastern North Sea in recent years: in Germany in the rivers Ems, Jade, Weser and Elbe (Wenger & Koschinski 2012; GRD e.V. 2013) and in The Netherlands in the Marsdiep, (western Wadden Sea: Boonstra *et al.* 2013; IJsseldijk *et al.* 2015), the Eastern Scheldt (Zanderink & Osinga 2010; Jansen *et al.* 2013), and the river Scheldt, all the way into its upper parts, in Belgium. Here, Haelters (2013) reported a remarkable influx of harbour porpoises in spring 2013, coinciding with peak catches of smelts (Breine & Van Thuyne 2014a) and speculated that the two phenomena may be linked. If this would be the case, the porpoises should prey on this returning anadromous fish species. Alternatively, the recent incursions into these estuaries may be merely linked to an increased presence of harbour porpoises in the southern North Sea at large (Haelters & Camphuysen 2009; Wenger & Koschinski 2012; Hammond *et al.* 2013). However, in either case the ecological role of porpoises in the estuaries remains to be established. Jansen *et al.* (2013) have shown that porpoises confined to the semi-closed Eastern Scheldt foraged locally, on a more estuarine prey spectrum than their conspecifics in the adjacent North Sea, but porpoise diets in other estuaries and rivers remain unknown. In this study, we report the local increase of harbour porpoises in the

river Scheldt and we determine their diet from stomach content analysis, with particular emphasis on the importance of estuarine prey.

Methods

Sightings

Opportunistic sightings data of harbour porpoises in the Scheldt have been used to examine the increased presence of the species in this river. Sightings in the Dutch part of the river, were uploaded by the general public at www.waarneming.nl (2005-2014). Similar data from further upstream the river, in Belgium, were reported to www.waarnemingen.be, to www.zeezoogdieren.org or to the Royal Belgian Institute of Natural Sciences (RBINS), http://www.mumm.ac.be/FR/Management/Nature/search_strandings.php. The river is less wide in Belgium, and therefore harbour porpoises in the Scheldt and its tributaries here have a higher probability of being observed, often by multiple observers along their banks. Multiple reporting made it complicated to assess the exact number of harbour porpoises present in any stretch of the river, especially during 2013 in Belgium, when ≥25 animals swam up river (Haelters 2013). We have analysed the data available at the various platforms, without attempting to identify duplicates and simply plotted all sightings at the reported locations. As a proxy for harbour porpoise density in coastal North Sea waters, from where animals swimming up the river must originate from, we used yearly average numbers seen per hour of observation by Dutch seawatchers, taken from www.trektellen.org.

Collection of stranded animals

Carcasses of harbour porpoises found dead in The Netherlands have been collected since 2006, when logistically possible, within a nation-wide research program which aims to determine their cause of death, body condition and diet (Leopold & Camphuysen 2006). Carcasses were labelled (stranding location, date) and initially stored locally (-20˚C), before transport to IMARES-Texel (2006) or the Department of Pathobiology, Faculty of Veterinary Medicine, Utrecht University (2007-2014), where an necropsy was performed on each carcass. Harbour porpoises stranded in Belgium were reported to the Royal Belgian Institute of Natural Sciences (RBINS), where it was decided at an ad hoc basis if carcasses were to be collected or not. In Belgium, most animals found far inland were badly decomposed, or were reported in locations difficult to reach, leading to the collection of only a small number of carcasses, and only two of these have

been necropsied. Carcasses were labelled and initially stored at the RBINS (Ostend), at -20˚C. These necropsies were performed at the University of Liège.

Necropsies in The Netherlands and Belgium followed the same standardised protocol (Kuiken & Gacia Hartmann, 1991; Jauniaux *et al.* 2002a). Necropsy parameters used for the current analysis were: age, as adult or juvenile, judged from body length with a cut-off point at 130 cm (cf. Lockyer 2003) supplemented by observations on the gonads to determine sexual maturity when possible; gender (male/female); the decomposition code (DCC) of the carcass at necropsy, scored on a 5-point scale from 1 (live stranding) to 5 (mere bones or 'mummified'); and probable cause of death. The most frequent causes of death were drowning by entanglement in fishing gear (by-catch), infectious diseases (such as acute pneumonia, or severe parasitosis), seal attacks and emaciation (Jauniaux *et al.* 2002b; Leopold *et al.* 2015a). For this study we excluded animals that had died from seal attack or by-catch, as these were not encountered among the stranded animals in the Western Scheldt (see under Results). The nutritional body condition (NCC; see also Chapter 3, ES-1) for the Dutch animals was scored on a 6-point scale, from 1 (very fat and muscular) to 6 (emaciated), and for the Belgian animals as good, medium or poor nutritional status, judged from the general appearance of the animal and blubber thickness. Other information used was stranding location and stranding date.

Diet analysis

The main objective of the study was to compare the stomach contents of animals that stranded in the Western Scheldt with those of animals from seaward reference areas: the river mouth, i.e. the North Sea coastline from the Belgian/Dutch border to the western tip of the former island Walcheren; the 'Voordelta' north of the river mouth, from Walcheren to the Maasvlakte off Rotterdam; and the Belgian North Sea coastline south of the river mouth (Figure 1). Animals in the reference areas were selected for comparison, if they 1) had stranded in the same months as the Western Scheldt animals, and 2) had similar nutritional and decomposition conditions, and 3) had similar causes of death. Stomach contents were studied following methods outlined in Leopold *et al.* (2015b). In brief: prey hard parts were collected from stomach contents, identified to the lowest possible taxon, measured, their size corrected for wear and paired if possible. The minimum number of individual prey was determined and prey size and mass for each prey were determined. Prey species were assigned to an estuarine prey group (anchovy *Engraulis encrasicolus* (Linnaeus, 1758), smelt, sand smelt *Atherina presbyter* Cuvier, 1829, Nilsson's pipefish

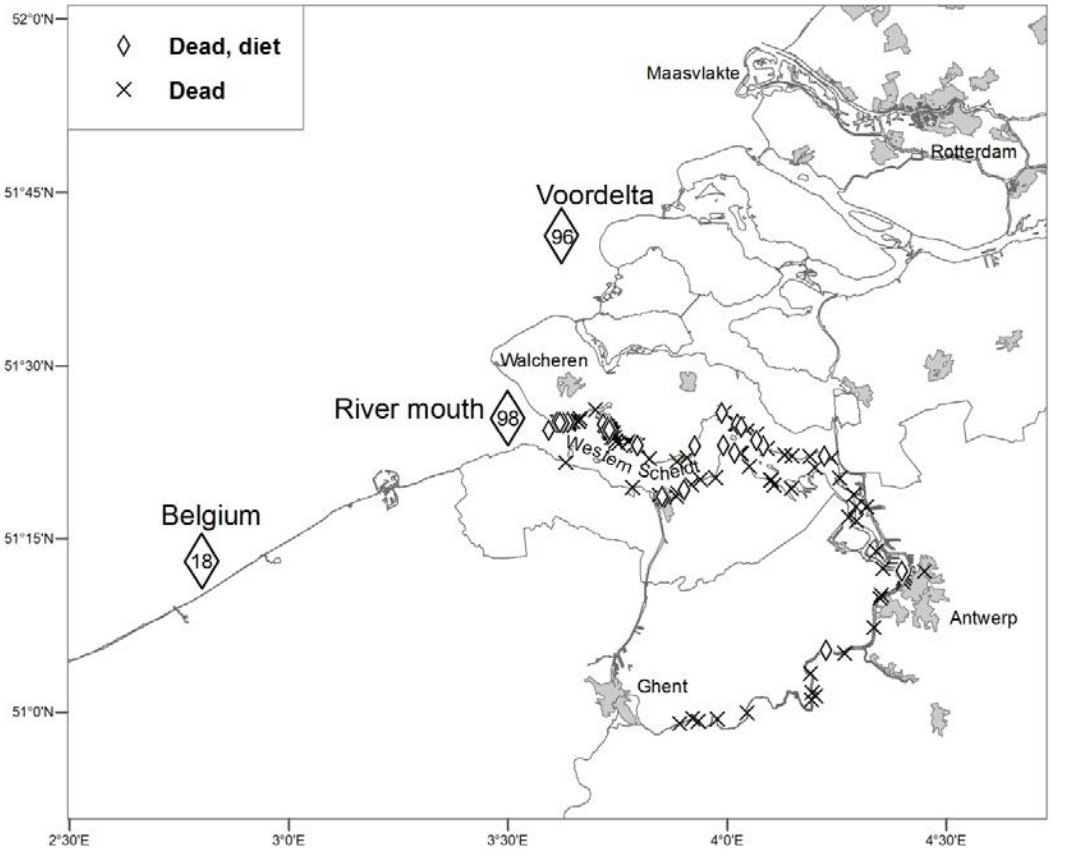

Figure 1. *Locations where carcasses of harbour porpoises were found. Specimens that were used for stomach content analysis are individually marked by small diamonds along the river Scheldt (n=23 in The Netherlands and n=2 in Belgium), and grouped in the reference areas (large diamonds). Outside the Western Scheldt, three reference areas were used: the river mouth: Dutch/Belgian border to the western tip of Walcheren (n=98), the Voordelta: from the tip of Walcheren to the Maasvlakte off Rotterdam (n=96), and the North Sea coastline of Belgium (n=18).*

Syngnathus rostellatus Nilsson, 1855 and European perch *Perca fluviatilis* Linnaeus, 1758), and a North Sea prey group (all other prey species found, see ES-1 for the full list of species found). Summed estimated prey masses per prey group and per harbour porpoise were used to compare diets between the animals found in the Western Scheldt, and in the three reference areas. Some prey remains may have stemmed from secondary prey, e.g. some of the invertebrates and some small fishes, but as this cannot be determined with certainty and because the relative

contribution of these small prey is nearly negligible, no distinction was made between supposedly primary and secondary prey.

Results

Increased occurrence in the Scheldt

Opportunistic sightings data show a sharp increase from 2011 in porpoise occurrence in the Western Scheldt (the Dutch part of the river; Figure 2).

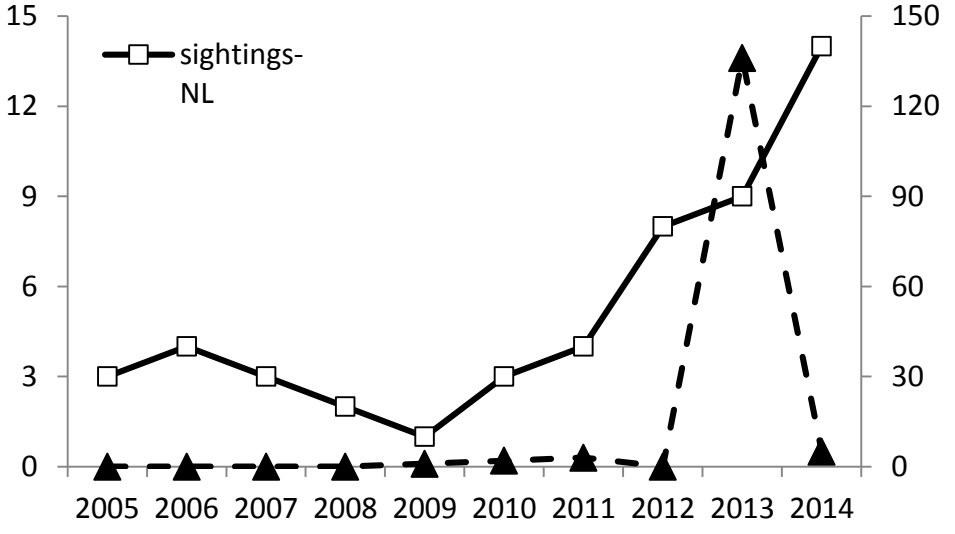

Figure 2. *Reported sightings of live harbour porpoises in the Dutch (open squares) and Belgian (filled triangles) parts of the river, 2005-2014. Note different Y-axis for Belgian sighting at the right. Average group size was 1.6 in The Netherlands and 2.0 in Belgium. Individual porpoises may have been seen and reported several times.*

Further upstream, in Belgium, numbers were lower in most years, but a remarkable number of sightings was reported in spring 2013, peaking on 11 April when at least 25 individuals were supposedly present (Haelters 2013). Many of these animals passed sluices, reaching port areas and stretches of the river system beyond tidal influence (Figure 3). This spring-influx in 2013 may have been a one-time event, with reported numbers of animals ten times those of 2009-2014 combined, while being confined to a few months only, in contrast to other years in which live porpoises were seen year round (Figure 4A). A total of 19 animals, probably the majority of those which took part in the 2013 influx, were reported dead about one month after their peak occurrence was seen (Figure 4B).

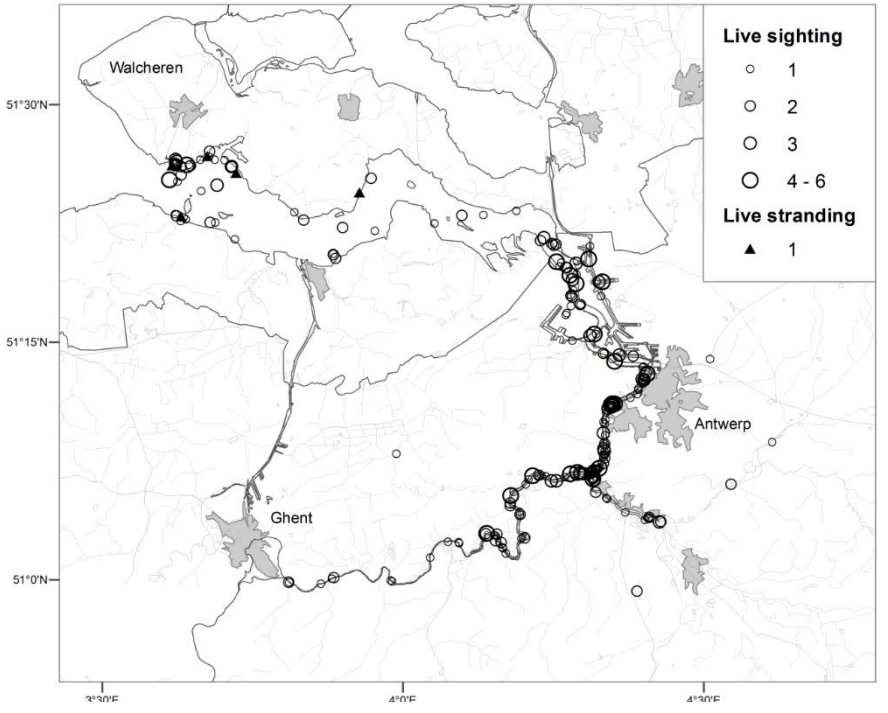

Figure 3. *Locations (circles) where harbour porpoises were seen alive in the river Scheldt or live-stranded (triangles), 2005-2014.*

Only two of these could be collected and necropsied; the others were found very decomposed, or observed in locations difficult to access and were not collected.

A slight spring peak in live sightings is also apparent in the Dutch data, but elevated numbers were also reported in December/January and in September (Figure 5).

Animals collected for stomach content analysis

All carcasses from the Dutch part of the river Scheldt that were necropsied were collected between 2007 and 2014 (Figure 6) and between March and October (Figure 7A). Five juvenile porpoises collected in the Western Scheldt (The Netherlands) and the two collected in the Belgian part of the river (both adults, in March 2011 and April 2013) had empty stomachs. The other 18 porpoises had prey remains in their stomachs. Four of these were adults and 14 were juveniles;

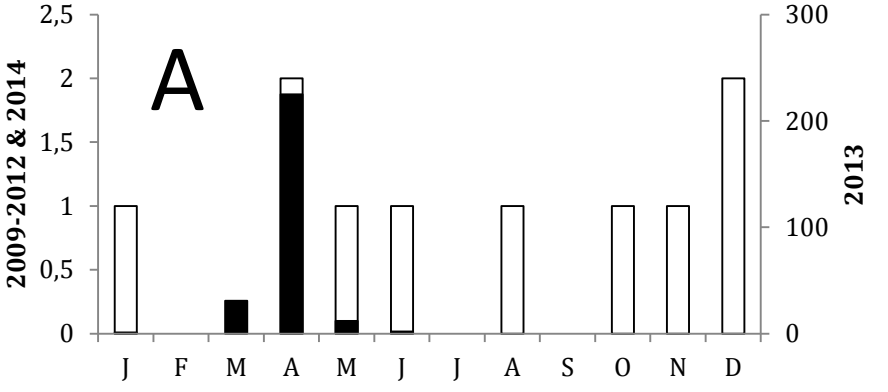

Figure 4A. *Monthly distribution of harbour porpoise sightings in the upper, Belgian parts of the river, summed for 2009-2014 (excluding 2013) as white bars (back), scaled along the left Y-axis, compared to those for 2013 as black bars (front), scaled along the right Y-axis. Note difference in scale.*

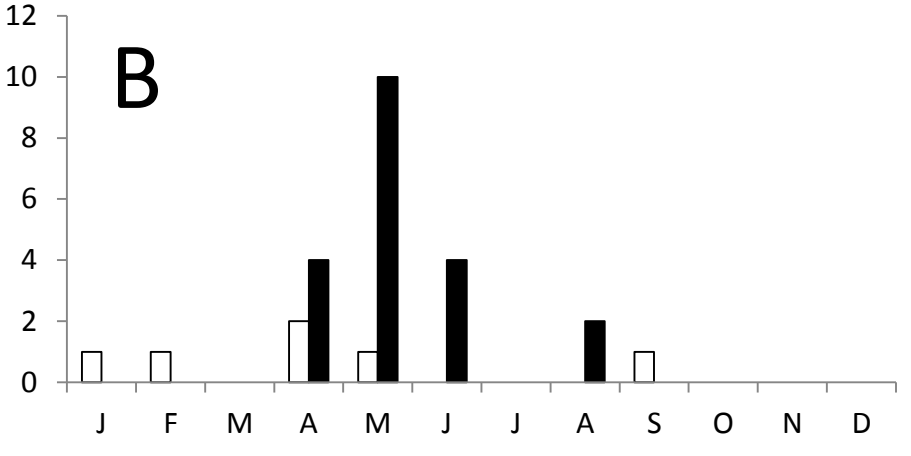

Figure 4B. *Monthly distribution of harbour porpoise strandings in the upper, Belgian parts of the river, summed for 2009-2014 (excluding 2013) as white bars, compared to those for 2013 as black bars. Same scaling for both groups.*

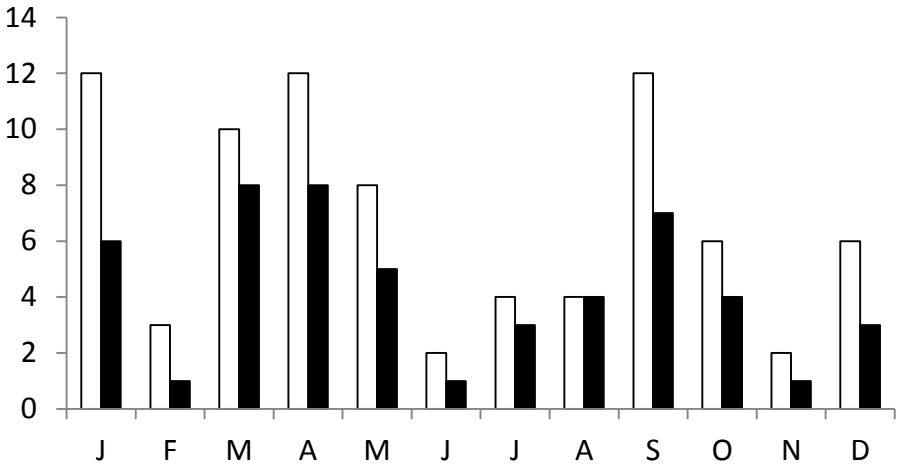

Figure 5. *Monthly distribution of harbour porpoise live sightings (white bars: animals; black bars: sightings) in the Dutch part of the river, summed for 2005-2014.*

six of 17 sexed animals with prey remains in their stomachs were males and eleven were females (versus four females and three males with empty stomachs). The two animals found in Belgium were both adult females, one was found dead emaciated, the other stranded alive (DCC 1) but died shortly afterwards, from an infectious disease. The decomposition codes (DCC) of the other porpoises used for stomach content analysis ranged from 2 to 5 (the full spectrum for animals stranded dead; average DCC with SD = 3.38±1.05, *n*=25). Most were emaciated: NCC ranged from 3 to 6. Probable causes of death included infectious disease (7), emaciation (2) and unknown (16). Importantly, no animals had been found that were clearly victims of grey seal attacks or fishery bycatch, both of which are rather common in the Voordelta (Leopold *et al.* 2015a). In addition, an 86 cm long porpoise was found dying (DCC 1) in the Western Scheldt in August 2013. This animal was a neonate that had not yet eaten solid foods, and was excluded from further analyses.

For comparison with the animals found in the river, we selected 212 animals that had stranded outside the Western Scheldt: 98 from the southern and northern coastlines of the river mouth, 96 from further north, from the adjacent Voordelta, and 18 from the Belgian North Sea coastline, further south (Figure 1). Animals from the years 2007-2014 were selected, that had been found between March and October, had a NCC 3-6 (or were in moderate to poor body condition as scored in Belgium) and had died from disease (27), emaciation (21) or unknown reasons (164).

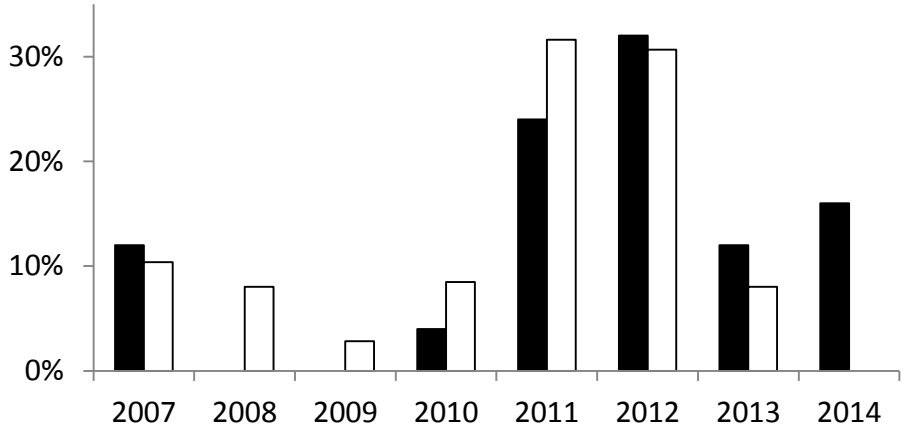

Figure 6. *Frequency distribution of harbour porpoises used for stomach contents analysis (with and without prey remains combined) over the years 2007-2014, compared for the river Scheldt (black bars) and the three reference areas combined (white bars). Monthly numbers are expressed as % of total numbers (25 porpoises from the river Scheldt and 212 from the reference areas).*

This selection yielded temporal distributions that were very similar between animals from the river Scheldt and from the reference areas (combined), both in terms of years (Figure 6) and months (Figures 7A,B).

Among the reference animals with prey remains in their stomachs, 17% (*n*=167 aged animals) were adults, which is rather similar to this percentage for the Western Scheldt (22%, *n*=18), given the low sample size of the latter. However, more males were present among the reference animals: 56% (*n*=165 sexed with prey remains in their stomach), versus 35% (*n*=17) among the animals found in the Western Scheldt with prey. Average DCC among the animals with prey remains in their stomachs from the reference areas (3.6±0.81; *n*=168) and in the river Scheldt (3.4±1.00; *n*=18) were not statistically different (T-test, T=0.82, df=184, p>0.1), but NCC was significantly lower among reference animals with prey remains (4.3±0.94; *n*=95), as compared to animals found in the Western Scheldt (The Netherlands, 5.2±0.75; *n*=11): (T=-3.66, df=104, p<0.001), indicating that animals found in the Western Scheldt had a poorer body condition. The proportion of empty stomachs 7/25 (28%) for the river Scheldt was also slightly higher than in the combined reference areas: 44/212 (21%).

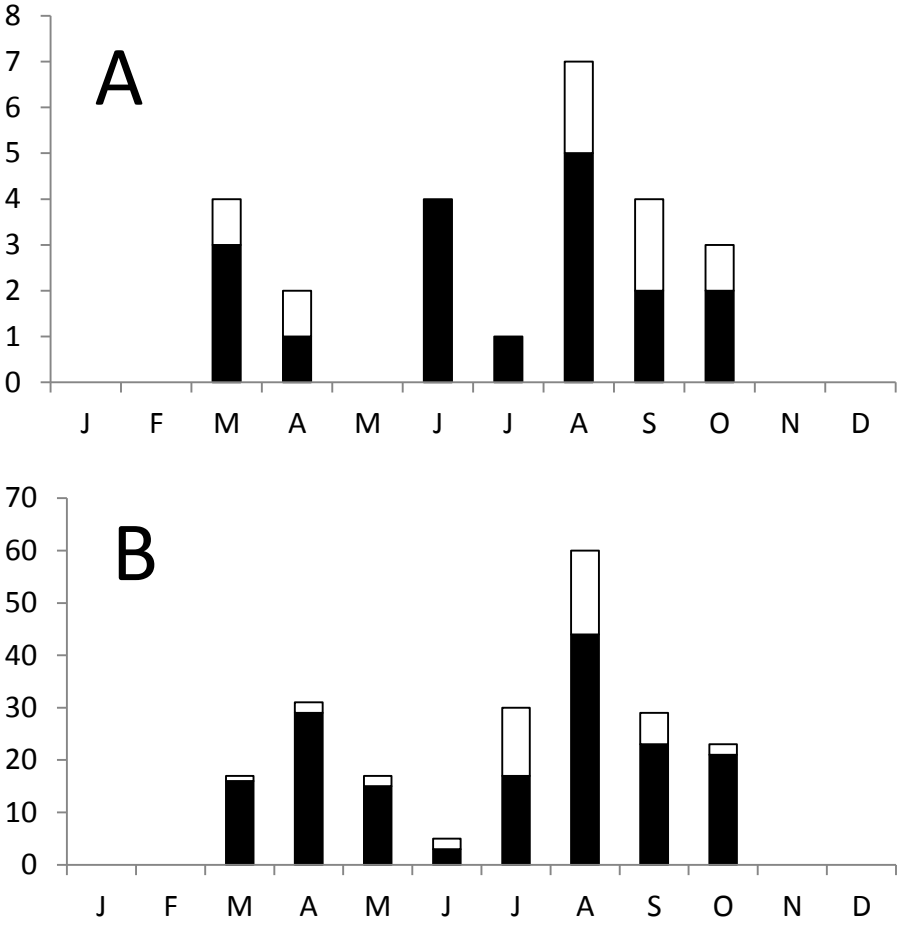

Figure 7A (top). *Monthly numbers porpoises from the river Scheldt used for stomach content analysis (n=18 animals with prey remains in their stomachs (black), and n=7 without (white)).*

Figure 7B (bottom). *The monthly numbers of reference animals (n=168 with prey (black) and n=44 without (white)).*

Evidence of porpoise births in the river Scheldt

Two of the animals collected for necropsy provided evidence that harbour porpoises might have given birth in the river Scheldt. A freshly dead, lactating female was found in Belgium, near Bornem, on 6 March 2011, circa 100 km up-river. The time of year for this case, however, probably signifies either a very long mother-calf bond, or dysfunctional mammary glands, rather than recent calving. The neonate found in August 2013 stranded near Hoofdplaat, 4.75 km into the Western Scheldt, half an hour after low tide. This timing suggests that this animal originated from the river, rather than having been carried in by a rising tide.

Stomach content analysis

Prey mass

The average reconstructed prey masses for animals with prey remains in their stomachs was 362 g for the Western Scheldt, and 505 g for the reference animals (all subareas combined, Table 1). Due to large standard deviations around these means, average reconstructed prey mass did not differ significantly between the Western Scheldt animals and animals from any of the reference areas, or animals in all reference areas combined.

Table 1. *Average and SD of reconstructed prey mass, per stomach containing prey remains, per sub-area. The value found for the Western Scheldt was tested (T-test) against those of each reference area, and against the value for all reference areas combined.*

	Average (g)	**SD**	***n***	**T**	**df**	***p***
Western Scheldt	**362**	**485**	**18**			
River Mouth	350	1080	73	0.070	89	>0.1
Belgian Coast	960	1411	16	-1.613	32	>0.1
Voordelta	557	1376	79	-1.013	95	>0.1
Total Reference Areas	**505**	**1264**	**168**	**-0.952**	**184**	**>0.1**

Prey species

Smelt comprised 99.2% of all estuarine prey mass, leaving the mass contributions of the 45 Nilsson's pipefishes, the four sand smelts, and the single European perch and anchovy across all studied stomachs (ES-1) insignificant. Among the 18 stomachs from the Western Scheldt with prey remains, nine contained remains of

Table 2. *Numbers of stomachs with prey, and with smelt, per sub-area.*

	Stomachs with prey	Stomachs with smelt	% Stomachs with smelt
Western Scheldt	**18**	**9**	**50**
River Mouth	73	5	6.85
Belgian Coast	16	2	12.50
Voordelta	79	8	10.13
Total Reference Areas	**168**	**15**	**8.93**

smelt (50%). Among the porpoises in the combined reference areas, this proportion was only 9% (Table 2).

The contribution of smelt to the total diet of individual porpoises in terms of reconstructed prey mass varied between 3.5% and 100% in the nine animals from the Western Scheldt that had eaten this prey species, and the average contribution of smelt to the diet (in terms of prey mass) was 46.11% for all 18 Western Scheldt animals combined (Table 3). Among the animals in the various reference areas that had remains of smelt in their stomachs, the contribution to the diet varied from 0.02% to 100% (n=15). In animals found in the river mouth, the total contribution of smelt was less than a third of this value for the animals found in the river itself, while in the two reference areas at either side of the river mouth, only circa 3% of the reconstructed prey mass comprised smelt (Table 3).

Table 3. *Summed total reconstructed prey masses (in g), across all animals per sub-area, and the proportions of smelt among these.*

	Summed total prey mass (g)	Summed mass smelt (g)	% smelt
Western Scheldt	**6510**	**3002**	**46.11**
River Mouth	25 518	3660	14.34
Belgian Coast	15365	393	2.56
Voordelta	43 943	1418	3.22
Total REF	**84 862**	**5471**	**6.45**

Table 4. *Minimum, maximum and average (±SD) distance (km) to the river mouth, of stranding locations of harbour porpoises with and without smelt remains in their stomachs.*

	n	**min**	**max**	**avg**	**sd**
with smelt	9	9.1	49.8	29.5	12.5
without smelt	9	9.1	58.6	23.3	17.8

The probability that smelt was found in a particular stomach from an animal from the Western Scheldt appeared independent from the distance to the river mouth, taken at 03˚33'E. The average distance of nine animals with and nine without smelt remains in their stomachs was not statistically different (Table 4: T=0.855, df=16, p>0.1).

Smelt size differed between the stomachs from animals found in the river (n=153 fishes) and in the river mouth and North Sea (n=203). The majority of smelts found in stomachs from animals that stranded in the river were around 8 cm reconstructed total length, with subsequent peaks in the distribution of fish lengths around 10-11 cm and around 14 cm. In the reference areas, reconstructed fish lengths peaked around 12 cm (Figure 8A). Masses of smelts found in river stomachs tended to be lower than in North Sea stomachs (Figure 8B). Most smelts taken were juveniles, but adult fishes, over 20 cm long, were also incidentally found (ES-2).

Discussion

Measures to diminish pollution levels and other ecological restoration measures such as restructuring of river banks have resulted in an ameliorated habitat quality, with some fish species returning to the river Scheldt. Among these are smelts; and monitoring along the upper, Belgian parts of the river system has shown that these are making a spectacular come-back. Smelt catches have greatly increased since 2010 in the river Scheldt and this species has become the most abundant fish species here (Breine & Van Thuyne 2014a,b, 2015). Concurrent with the return of the smelts in the river Scheldt, harbour porpoises have also started to return, but a dietary link between predator and prey was hitherto lacking. The return of the harbour porpoise in the Western Scheldt gained momentum in recent years, with a steady increase of the species in the river. Fike catches of smelt at Zandvliet (Belgium, near the Dutch border) also show an increase that commenced in 2009 (Breine & Van Thuyne 2015).

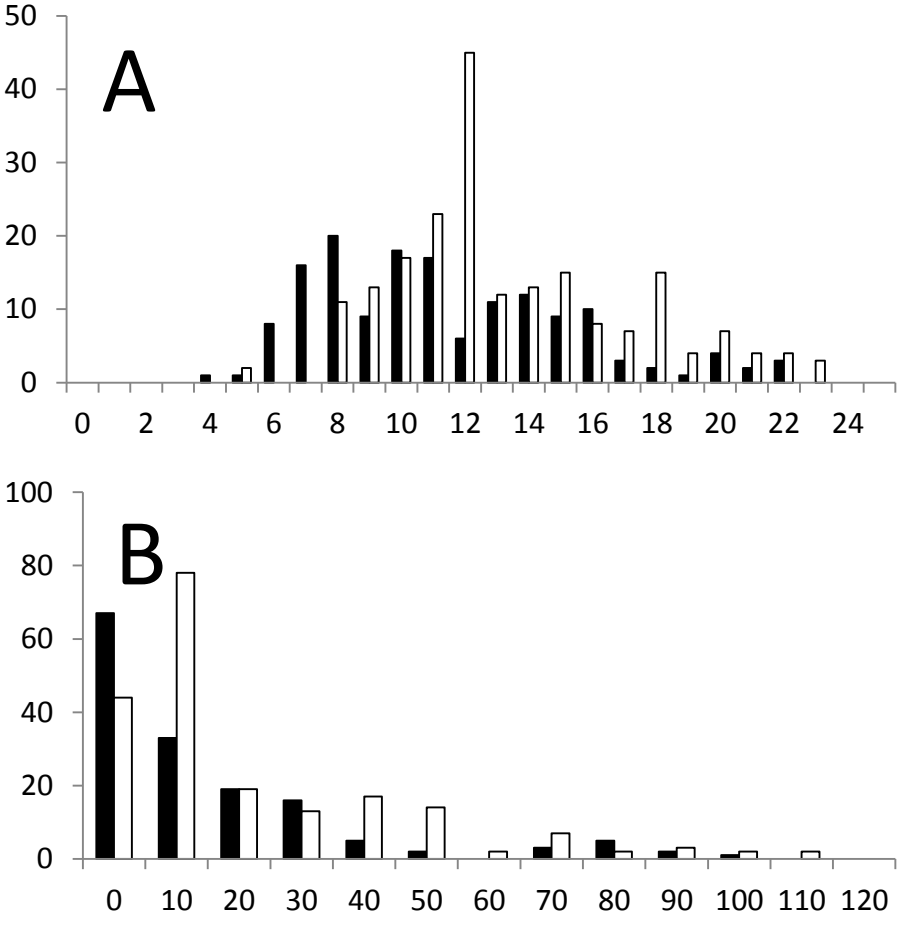

Figure 8A (top). *Length-frequency distribution of smelts (reconstructed total fish lengths in cm) from porpoises found in the river Scheldt (n=153 fishes; black bars) and from porpoises found in the reference areas combined (white bars: n=129, 11, and 63 for river mouth, Belgium and Voordelta, respectively).*

Figure 8B (bottom). *Mass-frequency distribution of the same smelts (g).*

Further upstream in the Belgian part of the river system, smelts were also abundantly caught, but harbour porpoises remained rare until a sudden influx in spring 2013. Whether or ot this mass influx was a one-time phenomenon, future observations will show.

The year 2013 was an anomaly, both in the Dutch and the Belgium part of the river. It might be expected that the numbers of porpoises entering the river are related to the density in the North Sea. The best, long-term trend data for porpoise presence in Dutch coastal waters are provided by seawatchers. Seawatching is primarily aimed at recording the passage of birds, but harbour porpoises are also recorded. The numbers seen per hour of observation show a long-term increase that closely matches yearly numbers of strandings in The Netherlands (Camphuysen & Siemensma 2011). Here, we use the yearly average number of sightings of harbour porpoises per hour of observation, across all seawatching sites in The Netherlands (data: www.trektellen.org). During the current study period, 2005-2014, the increasing trend, as reported by Camphuysen & Siemensma (2011) is near-significant (n=10, R^2=0.3894, 0.05<p<0.1; Figure 9A). The number of animals reported from the Western Scheldt over these years appears to follow the numbers reported by the seawatchers (Figure 9B: n=10, R^2=0.359, 0.05<p<0.1) but the numbers seen in 2013 were much lower than expected, while unprecedented numbers were seen in Belgium. Without this 2013-anomaly, the relationship between numbers reported by seawatchers in the North Sea, and by the general public in the Western Scheldt show a good correlation: n=9, R^2=0.6303, p<0.02), indicating that numbers at sea drive the numbers seen in the river. On the other hand, the increase in porpoise sightings in the Western Scheldt may also be correlated with smelt abundance, but good data from the Western Scheldt are lacking. In Belgian fike catches, numbers of smelts increased from 2009-2014 (Breine & Van Thuyne 2015), as did numbers of porpoises in the Dutch part of the river. Therefore, increasing numbers of porpoises were available for entering the Western Scheldt during the study years, and once in the river, they would have encountered increasing smelt densities, possibly providing better local feeding conditions.

The dietary study was performed on porpoises that were found dead, and had died from infectious disease, emaciation or unknown causes. Stranded animals are probably not representative for the general health status and diet of the animals in the study area. Many of the studied animals were not healthy. This could be explained by the fact that no animals had obviously died a violent, acute death, e.g. due to predation, collisions with ship propellers or fisheries bycatch.

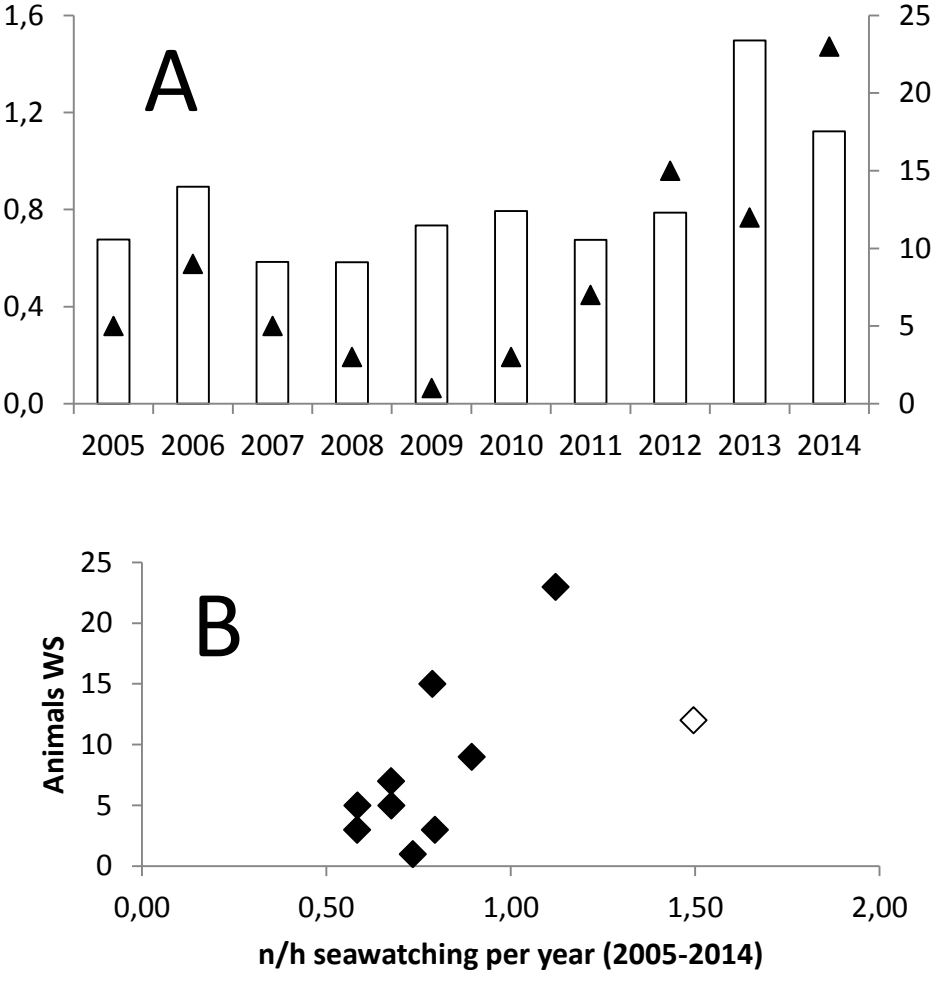

Figure 9A (top). *Numbers of harbour porpoises recorded per hour of observation per year, by Dutch seawatchers (www.trektellen.org, accessed 22 March 2015; all seawatching stations combined): bars and left Y-axis, and numbers of porpoises reported from the Western Scheldt per year, 2005-2014: filled triangles, right Y-axis.*

Figure 9B (bottom). *The relationship between the yearly seawatching average, as a proxy for porpoise density in North Sea nearshore waters, and numbers of porpoises reported per year in the Western Scheldt (2005-2014). Open symbol represents the year 2013.*

The peak in strandings along the Belgian, upper parts of the river, one month after the peak in live sightings in 2013 and the presumably high proportion of casualties here, suggests that most porpoises had wasted away here. The rather high NCC scores in general also indicated that most animals had probably died from infectious disease or from emaciation, although many such animals were also found along the shores of the river mouth and further away from the river, along Dutch and Belgian North Sea coastlines. From the animals stranded in the reference areas, we selected study subjects that were similarly emaciated or sick as the study animals in the Western Scheldt, to enable comparison of diets between these groups.

Relative to conspecifics from stretches of North Sea coastline at either side of the river mouth, harbour porpoises found up-river had similar reconstructed prey masses in their stomachs. However, porpoises found in the river tended to be in poorer body condition and had a slightly higher percentage of empty stomachs. The main difference between the two groups was that much more smelt had been consumed by animals in the Western Scheldt. Smelt was the most important single prey species in the Western Scheldt in terms of biomass (46.11% smelt), but was only of marginal importance in the adjacent North Sea (ca. 3%). Animals stranded along the river mouth had an elevated proportion of reconstructed smelt biomass in their diet (14.34%), as compared to animals found further away from the river. As the river is subject to tidal movements, river water will mostly flow into the North Sea during the ebb tide. With the riverine water mass, fishes may also move from the river into the river mouth, and so might carcasses of porpoises that died in the Western Scheldt. From the various reference areas, the river mouth should thus be most similar to the Western Scheldt as we found in the stomach contents from the various locations.

This study cannot resolve the question whether harbour porpoises in the Western Scheldt had purposefully swam up-river to feed on smelt. However, once in the river system, they clearly had been feeding on this anadromous fish, which was an important contribution of their diet here. Diets further upstream, e.g. in the Belgian part of the river, remain unknown as yet, but monitoring of fish has shown that smelts are common here (Breine & Van Thuyne 2014a,b, 2015). Very far upstream, other fishes, such as fresh water cyprinids, will become more dominant among the fish fauna. It would therefore be very interesting to study more stomach contents of animals found dead in the Belgian parts of the river system, and in other, similar river systems in the southern North Sea, to investigate if such fish species are, and in which quantities, taken by porpoises venturing far upstream.

Acknowledgements

We thank Jaap van der Hiele (Aseal) and the EHBZ team for retrieving the dead porpoises in and around the Western Scheldt, and the colleagues of the RBINS, the Research Institute for Nature and Forest (INBO) and The Agency for Nature and Forests (ANB) for notifying strandings and sightings in Belgium, and for their assistance in collecting dead porpoises. We acknowledge the help of the public for notifying sightings and strandings, and we thank the webmasters of www.waarneming.nl, www.waarnemingen.be, www.zeezoogdieren.org and www.trektellen.nl for their efforts in collecting data, and their consent to analyze the uploaded data on strandings and sightings. Waarnemingen.be is the website for information on nature managed by Natuurpunt and Stichting Natuurinformatie. This work was supported by the Dutch Ministry of Economic Affairs under Grant BO-11-018.02-004, and by the Belgian National Lottery under its programme on Sustainable Management. Peter Reijnders read and improved an earlier version of this paper.

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Supporting information has been requested by Marine Biology Research: Tables ES-1 & ES-2, and can be found on line, after publication of this paper.

Table ES-1. Prey list for the porpoises considered in this study, per sub-region. Total numbers of prey (and summed prey masses) were: Western Scheldt n=1119 (6510 gram); River Mouth n=11 062 (25 526 gram); Belgium n=5094 (15 364 gram); Voordelta n=11 582 (43 980 gram).

Table ES-2. Length and mass frequency distributions for smelts, per sub-region. Total fish lengths (cm) and prey mass (gram) were back-calculated from otolith sizes.

Detection of grey seal *Halichoerus grypus* DNA in attack wounds on stranded harbour porpoises *Phocoena phocoena*

Judith D.L. van Bleijswijk, Lineke Begeman, Harry J. Witte, Lonneke L. IJsseldijk, Sophie M.J.M. Brasseur, Andrea Gröne & Mardik F. Leopold

6

Abstract

DNA was analysed from external wounds on 3 dead harbour porpoises *Phocoena phocoena* that were stranded in the Netherlands. Puncture wounds as well as the edges of large open wounds were sampled with sterile cotton swabs. With specific primers that target the mtDNA control region of grey seal *Halichoerus grypus*, a 196 bp DNA fragment was amplified from 4 puncture wounds. Sequencing of the fragments confirmed the presence of grey seal DNA in the puncture wounds. DNA sequences differed between the cases, implying that 3 individual grey seals were involved. As 8 control swabs from intact skin and the transport bag as well as 6 swabs from open wounds on the same harbour porpoises were all negative, contamination with environmental DNA is considered unlikely. The results provide a link between strandings of mutilated harbour porpoises and recent observations of grey seals attacking harbour porpoises. Ours is the first study to use forensic techniques to identify DNA in bite marks from carcasses recovered from the marine environment. This approach can be extended to identify other marine aggressors, including cases involving persons mutilated at sea.

Keywords: mtDNA, Diagnostic PCR, North Sea, Inter-species interaction, Predation, Mutilation, Forensic analysis

Detection of grey seal *Halichoerus grypus* DNA in attack wounds on stranded harbour porpoises *Phocoena phocoena*

6

Judith D.L. van Bleijswijk, Lineke Begeman, Harry J. Witte, Lonneke L. IJsseldijk, Sophie M.J.M. Brasseur, Andrea Gröne & Mardik F. Leopold

Introduction

Increasing numbers of harbour porpoises *Phocoena phocoena* have stranded around the North Sea (Peltier *et al.* 2013). A particular feature of many porpoises stranded in the south-eastern North Sea is that they show severe, sharp-edged mutilations. Several causes for these mutilations have been suggested, such as ship strikes or fisheries by-catch (Camphuysen & Siemensma 2011). Recently, predatory or scavenging grey seals *Halichoerus grypus* have been held responsible based on morphological analyses of lesions on two porpoise carcasses (Haelters *et al.* 2012) and three field observations of grey seals feeding on harbour porpoises (Bouveroux *et al.* 2014). Leopold *et al.* (2015a) reported that most of the mutilated porpoises were juveniles in good nutritional condition (thick blubber layer) with full stomachs. Evidence that grey seals are truly responsible for killing the harbour porpoises that subsequently become stranded can only be gained from a combination of detailed pathology from a large number of carcasses (Leopold *et al.* 2015b) and from DNA studies. The latter can, in theory, take two approaches. Porpoise DNA in the alimentary tract of grey seals would prove that these seals have consumed (parts of) porpoises, whereas demonstrating grey seal DNA in the wounds of the mutilated porpoises would prove that the wounds were inflicted by grey seals.

In terrestrial settings, forensic DNA analyses has been successfully used to identify the species as well as the sex and individual identity of the predator (Williams *et al.* 2003; Blejwas *et al.* 2006). However, in situations in which a body has been submerged in water, this technique has been much less successful. In fact, we could only find one case where the DNA of a perpetrator was found in bite marks on a human body found in fresh water (Sweet & Shutler 1999), and we were unable to find a single case from the marine environment. Here, we report forensic DNA analyses of wounds on three stranded harbour porpoises. We developed a diagnostic PCR that provides a product when mitochondrial DNA of

grey seal is present. The test was designed in such a way that DNA of the victim (*Phocoena phocoena*) and of other potential predators (*Phoca vitulina, Orcinus orca*) or scavengers (*Canis lupus familiaris, Vulpes vulpes*) would give negative results. Forensic science protocols (Alaeddini *et al.* 2010) were followed in order to prevent contamination with grey seal DNA.

Material and Methods

Primer design

For the development of a diagnostic PCR for grey seal, sequences of the mtDNA control region of grey seal, the sympatric harbour seal *Phoca vitulina*, the harbour porpoise, and various other marine mammals and terrestrial scavengers were obtained from GenBank. For grey seal, we selected the data of Graves *et al*. (2009) re-edited by Fietz *et al.* (2013). In addition, new data were generated using the primer pair ThrL16272 and DLH16750 (Stanley *et al*. 1996) on DNA extracts of eight grey seals (GenBank accession numbers KM053398–KM053405) and 97 faecal samples of grey seals from various locations in the Dutch North Sea and Wadden Sea (GenBank accession numbers KM066992–KM067088). A multiple sequence alignment was created (Figure. S1 in the Supplement at www. int-res. com/ articles/ suppl/ m513 p277_ supp.pdf), and lists of species-specific primers were generated by the automated probe design option in 'ARB' (Ludwig *et al*. 2004).

Candidate primer sequences were checked for false matches with non-target species in GenBank using the Basic Local Alignment Search Tool (BLAST). Efficiency and specificity for grey seal was further confirmed for the primer pair HG_F1 (5'-CTT CGT GCA TTG CAT GCT-3') and HG_R1 (5'-CAT GGT GAC TAA GGC TCT-3') in PCRs on DNA extracts of tissues and faeces from grey and harbour seals.

Forensic test

Forensic DNA analyses were done on three stranded harbour porpoises found at three different locations along the Dutch coastline (in August, October and December 2013) and showing potential bite marks. On the beach, the carcasses were wrapped in clean plastic bags by transporters who wore clean clothes. Within 6 hours after discovery, the carcasses were investigated at Utrecht University in a laboratory that had not contained seal specimens for at least 10 days prior. Presumed attack wounds, puncture lesions and edges of large open

***Table 1**. Swab samples analysed in this study. Stranded* Phocoena phocoena *are indicated by a Pp number. +, ± or – indicates clear, unclear or no bands in the forensic test, respectively. s indicates that a sequence was retrieved successfully.*

Swab code	Type	Swab sample source	Test result
S1	Control	Human saliva	–
S2	Control	Human saliva	–
S3	Pp1	Puncture lesion	+s
S4	Pp1	Corner of open lesion	±
S5	Control	Control swab processed with wound swabs Pp1 & Pp2	–
S6	Control	Control swab processed with wound swabs Pp1 & Pp2	–
S7	Pp2	Edge of open lesion	–
S8	Pp2	Puncture lesion	+s
S9	Pp2	Puncture lesion	+s
S10	Control	Control swab processed with wound swabs Pp1 & Pp2	–
S11	Control	Control swab processed with wound swabs Pp3	–
S12	Control	Control swab processed with wound swabs Pp3	–
S13	Control	Body bag	–
S14	Pp3	Left flank skin	–
S15	Pp3	Corner of open lesion	–
S16	Pp3	Corner of open lesion	–
S17	Pp3	Corner of open lesion	–
S18	Pp3	Puncture lesion	+s

lesions were sampled with dry sterile cotton swabs (Table 1). Additional swabs were wiped over the intact skin of one of the porpoises and over the inside of the plastic bag used for transport. Negative control swabs were simply released from their packaging, in close proximity to the porpoise being autopsied, and processed along with the swabs taken from wounds. Swabs were individually packed, stored frozen at −20°C and transported to the Royal Netherlands Institute for Sea Research on the island of Texel for DNA analyses.

DNA was extracted and purified from the swabs using the QIAamp Investigator kit (Qiagen) following the protocol for surface and buccal swabs. Carrier RNA was not added because the total amount of DNA (from harbour porpoise, bacteria and predator combined) in test samples exceeded 2 ng μl^{-1}. All extractions were done at overpressure in a certified ISO-6 clean lab, well separated from the PCR lab (via a sluice and situated on a different floor), where no other marine samples had been located or processed for at least three weeks prior. DNA from each swab was

eluted from silica columns in 40 µl of buffer, quantified with a fluorometer and run on 1% agarose gels to verify the quality of the extract.

Fragments of 196 bp of the mitochondrial control region were amplified from 2 µl of DNA extract in a 50 µl PCR using the newly designed primers specific for grey seal (HG_F1 and HG_R1). The reaction mix contained 1× buffer, dNTPs, forward and reverse primers, BSA and Biotherm Polymerase. In a first PCR step, we ran 40 cycles of 20 s at 94°C, 20 s at 50°C and 30 s at 72°C. Subsequently, in a second PCR under similar conditions, 1 µl of product of the first PCR was re-amplified with 15 additional cycles. All samples from stranded porpoises were analysed in 4-fold. Negative PCR controls were run, but positive controls, i.e. DNA extracts of grey seal DNA, were not included, to prevent cross contamination. All PCR products were loaded on 2% agarose gels along with a size marker (SmartLadder or SmartLadder SF) and stained with SybrGold. The presence of bands was scored visually. DNA was extracted from the bands (Qiagen Gel extraction kit) and concentrated (Qiagen Minelute kit). PCR products were sequenced with forward and reverse primers by BaseClear (Leiden). Consensus sequences were BLAST searched and compared in a multiple alignment as specified in the primer design section above. New sequences obtained from puncture lesions on stranded harbour porpoises were submitted to GenBank (under accession numbers KJ863396–9).

Results

Two out of the nine harbour porpoise wound swabs (S8 and S9 from porpoise Pp2, Table 1) showed amplification products with grey seal-specific primers after the first PCR (40 cycles), and two more swabs (S3 from Pp1 and S18 from Pp3) after the second PCR (15 additional cycles). PCR replicates (4-fold) always showed consistent results (triplicates are shown in Figure S2 in the Supplement). The positive results were obtained from puncture lesions with underlying haemorrhages on three different harbour porpoises. Swabs from edges and corners of open lesions did not provide PCR products, nor did swabs from intact skin or negative control swabs, making contamination with environmental DNA highly unlikely. Sequencing the PCR products obtained from the puncture wounds delivered good quality reads from both the forward and the reverse primer (chromatograms in Figure S3 in the Supplement).

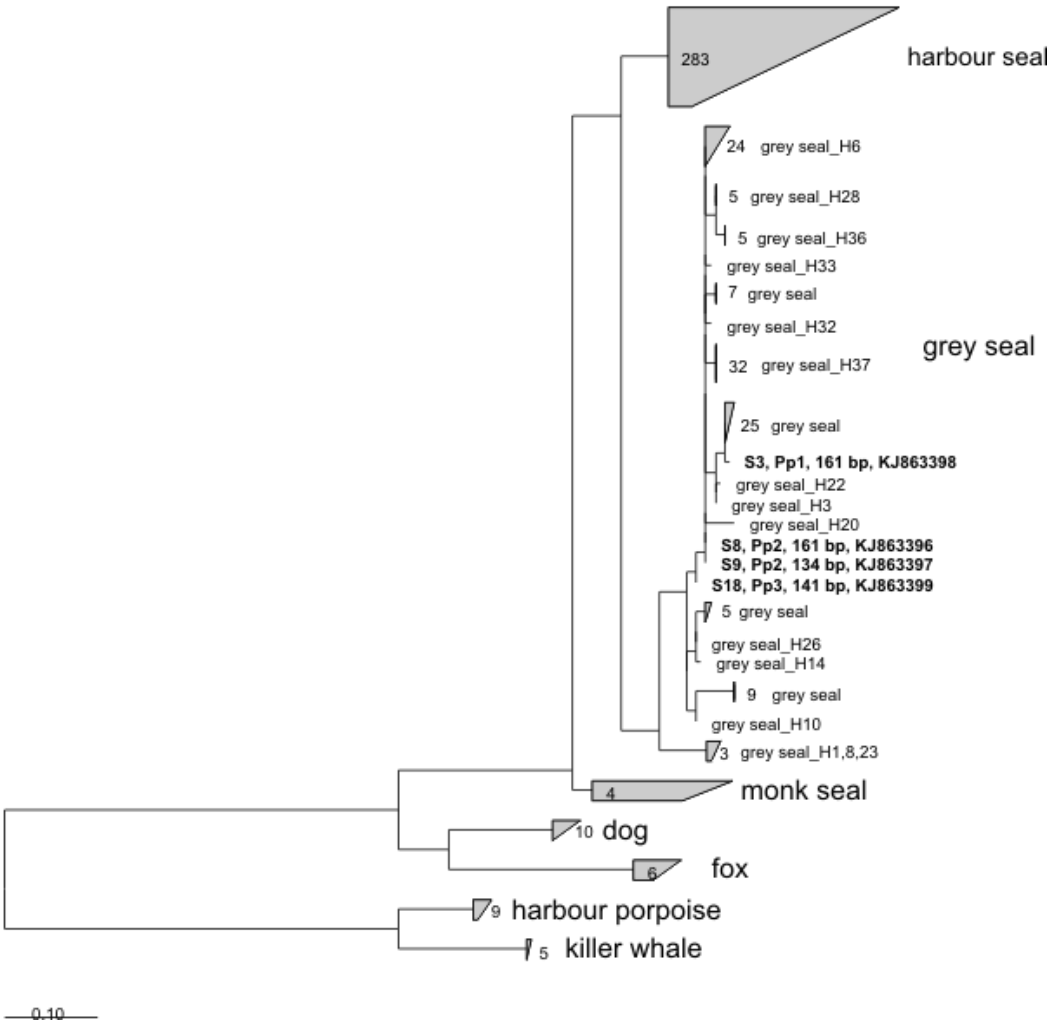

Figure 1. *Distance tree (ARB neighbour joining) of 354 positions of the mtDNA control region. mtDNA from bite marks on stranded* Phocoena phocoena *was added to this tree via ARB parsimony and is indicated in bold with swab number (S),* Phocoena phocoena *number (Pp) and GenBank accession number. Scale bar indicates relative amount of substitutions. Numbers associated with groups indicate the number of sequences in that group. Hg: grey seal* Halichoerus grypus*. Haplotype numbers (e.g. _H6) are according to Fietz* et al. *(2013). Note that the sequences obtained from 2 different bite marks on porpoise Pp2 are similar but differ from the sequences obtained from porpoises Pp1 (2 bases) and Pp3 (1 base).*

Consensus sequences with primer sequences trimmed off (no ambiguities, 134–161 bp) matched sequences of the control region of grey seals (Figure 1). The grey seal sequences differed among the three cases, implying that three different grey seal individuals had attacked the harbour porpoises.

Discussion

We assume that the grey seal DNA detected in wounds on three stranded mutilated harbour porpoises came from saliva remaining after a grey seal bite (Haelters *et al.* 2012; Leopold *et al.* 2015b). Of the nine wounds that were swabbed in total, only four were positive. These were all relatively small and deep punctures that may have been pressed closed quickly after the bite. Salivary DNA of the perpetrator is more likely to be preserved in such wounds than in larger, more open lesions due to the heavier bleeding and the open structure of the latter, which allows rinsing by sea water. Indeed, the other wounds that were swabbed were more severe and open in structure, and all came up negative. These results, together with the negative results for one intact skin swab and eight blanks, enable us to exclude environmental DNA (DNA freely floating in sea water) or contamination as the source of the positive results.

For future cases of stranded, mutilated harbour porpoises, we recommend swabbing puncture lesions, to objectively score inter-species interactions. Additional histological observation of haemorrhages in tissues underlying these puncture lesions can provide evidence for either attacks on live animals (haemorrhage present) or scavenging (haemorrhage absent). With these techniques combined, it is possible to discriminate between human-induced mutilation and inter-species aggression.

Our study is the first successful application of a forensic DNA technique in the marine environment and could be extended to identify other marine aggressors (Bolt *et al.* 2009; Estes *et al.* 2009), including cases involving persons mutilated at sea (Sweet & Shutler 1999).

Acknowledgements

We thank Stefan Schouten for valuable advice, Nicole Bale for improving the grammar of the manuscript, Hans Malschaert for ARB and Linux support, Okka Jansen and Jasper de Goey for lab work on primer testing and sequence preparation and Jaap van der Hiele,

Arnold Gronert and Kees Kooimans for collecting and transporting the carcasses. The Dutch Ministry of Economic Affairs financed the forensic tests, under project HD3456.

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Supplement

Sequence information used for primer design (Figure S1), gels showing results of grey seal PCR (Figure S2) and chromatograms of reads from swabs of harbour porpoise wounds (Figure S3).

***Figure S1**. Multiple alignment of newly designed primer regions (HG_F1 and HG_R1) given for 6 mammal species, both marine and terrestrial. Consensuses built from a certain number of sequences are shown: e.g.* Phoca vitulina *cons283 means that the consensus was built from 283 sequences. Blue colour indicates a match with the primer sequence; red indicates a mismatch.*

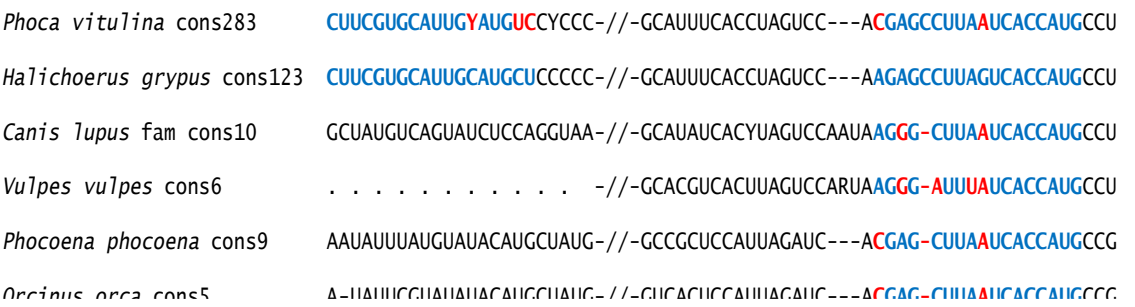
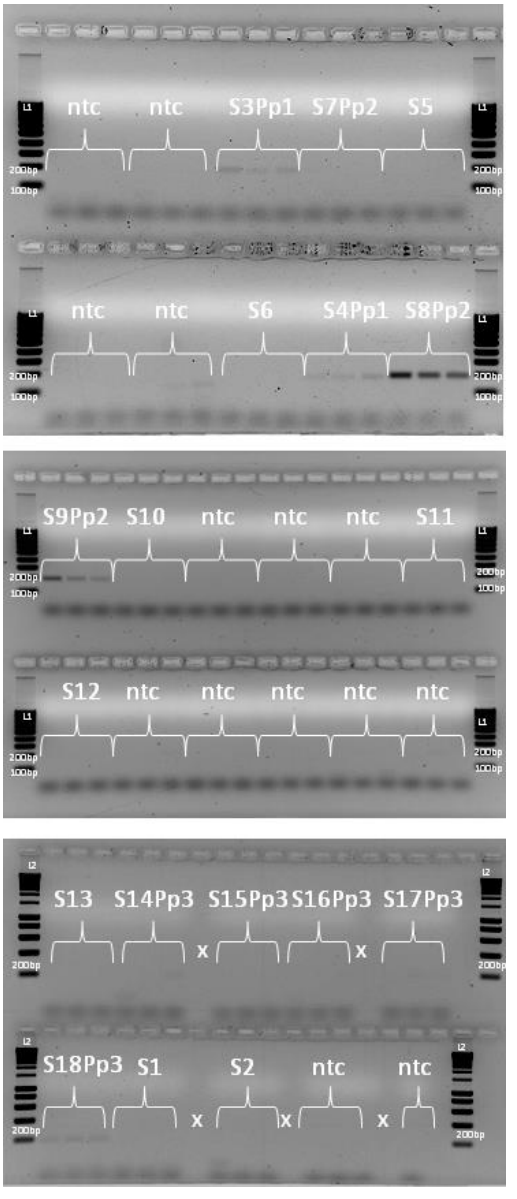


Figure S2. *Agarose gel showing results of grey seal* Halichoerus grypus *specific PCR applied to DNA extracts of swabs taken from several wounds on stranded harbour porpoises Phocoena phocoena. S: swab number, Pp:* P. phocoena *number, ntc: non template control. SmartLadder SF (L1) and Smartladder (L2) were loaded as size reference markers.*



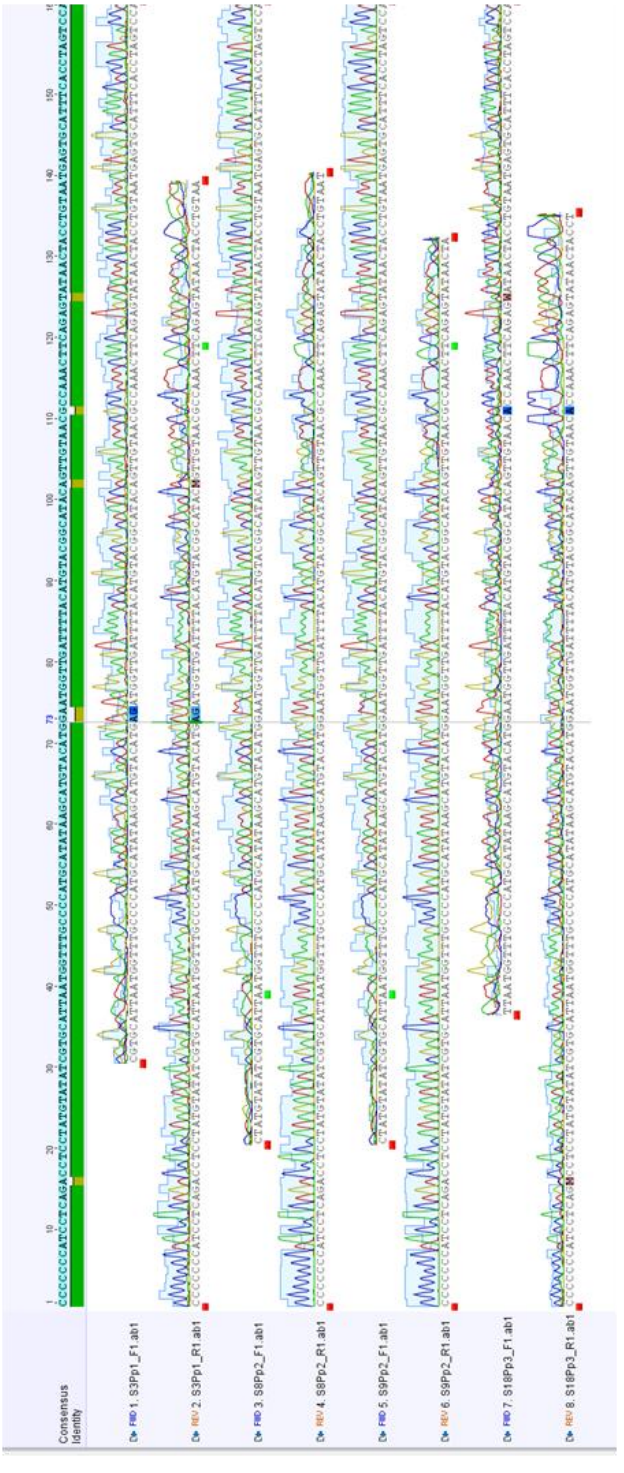
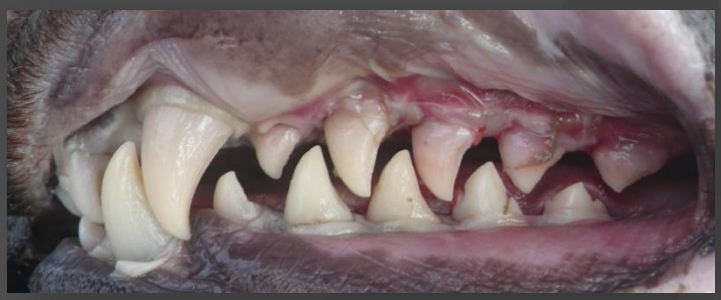


Figure S3. *Chromatograms of sequences of forensic DNA obtained from swabs of 4 puncture wounds on 3 different stranded harbour porpoises* Phocoena phocoena. *Blue boxes mark the differences between the sequences retrieved from porpoise Pp2 and the others. Red boxes indicate start and end of sequences. Green boxes indicate manual corrections. Pink boxes indicate ambiguities.*

Exposing the grey seal as a major predator of harbour porpoises

Mardik F. Leopold, Lineke Begeman, Judith D.L. van Bleijswijk, Lonneke L. IJsseldijk, Harry J. Witte & Andrea Gröne



7

Abstract

Harbour porpoises (*Phocoena phocoena*) stranding in large numbers around the southern North Sea with fatal, sharp-edged mutilations have spurred controversy among scientists, the fishing industry and conservationists, whose views about the likely cause differ. The recent detection of grey seal (*Halichoerus grypus*) DNA in bite marks on three mutilated harbour porpoises, as well as direct observations of grey seal attacks on porpoises, have identified this seal species as a probable cause. Bite mark characteristics were assessed in a retrospective analysis of photographs of dead harbour porpoises that stranded between 2003 and 2013 (n=1081) on the Dutch coastline. There were 271 animals that were sufficiently fresh to allow macroscopic assessment of grey seal-associated wounds with certainty. In 25% of these, bite and claw marks were identified that were consistent with the marks found on animals that had tested positive for grey seal DNA. Affected animals were mostly healthy juveniles that had a thick blubber layer and had recently fed. We conclude that the majority of the mutilated harbour porpoises were victims of grey seal attacks and that predation by this species is one of the main causes of death in harbour porpoises in The Netherlands. We provide a decision tree that will help in the identification of future cases of grey seal predation on porpoises.

Keywords: marine mammals, mutilation, predation, DNA, bite mark, decision tree

Exposing the grey seal as a major predator of harbour porpoises

7

Mardik F. Leopold, Lineke Begeman, Judith D.L. van Bleijswijk,
Lonneke L. IJsseldijk, Harry J. Witte & Andrea Gröne

Introduction

Marine mammals strand occasionally with large, fatal wounds. Suggested causes include ducted propellers (Thompson *et al*. 2010), fishermen confronted with by-catch (Kuiken & Baker 1991), and predators or scavengers (Long & Jones 1996; Haelters *et al*. 2012; Bouveroux *et al*. 2014). Over the past decade, hundreds of severely mutilated harbour porpoises (*Phocoena phocoena*) have been found along the southeastern North Sea coastline (Leopold *et al*. 2015), the cause of the wounding being unknown. This has resulted in controversy among scientists, the fishing industry and conservationists as to whether such mutilations were anthropogenic in origin or naturally inflicted by predators.

Research on predated livestock and protected wildlife species has demonstrated that the presence of salivary DNA of predators in bite wounds can be used to specifically identify the predator species (Williams *et al*. 2003; Williams *et al*. 2004; Imazato *et al*. 2012). Acute haemorrhages in the bite wounds and other lesions found during necropsy aid evaluation of the cause of death, and help distinguish between predation of a live animal and post-mortem scavenging. DNA degradation and/or the flushing out of predator saliva occurs quickly in bodies submerged in water (Sweet & Shutler 1999), and therefore, in mutilated marine mammals, the predator's DNA is most likely to be demonstrated in victims that are found fresh after having died rapidly from the wounds. As there is frequently a long interval between death and necropsy of stranded marine mammals, diagnosis of a predator attack by DNA is difficult. Despite this, grey seal (*Halichoerus grypus*) DNA has recently been demonstrated within bite wounds on mutilated harbour porpoises (van Bleijswijk *et al*. 2014).

The aims of this study were to evaluate the characteristics and incidence of grey seal-associated wounds found on harbour porpoises stranded along the Dutch coastline, determine criteria to establish if these were made ante- or post-mortem, and develop a decision tree to help investigators undertaking autopsies of small cetaceans to identify interactions with grey seals accurately. We show that a

substantial proportion of harbour porpoises that stranded on the Dutch coast were mutilated by grey seals. We also conclude that most cases involved active killing and that only a small proportion can be attributed to post-mortem scavenging. This makes predation by grey seals one of the main causes of death in harbour porpoises currently stranding in The Netherlands.

Material and Methods

Porpoises used for characterization of grey seal-associated wounds

Grey seal DNA was demonstrated in various bite marks on three mutilated harbour porpoises (van Bleijswijk *et al*. 2014). These wounds showed macroscopic and microscopic acute haemorrhages, indicating that these lesions had been inflicted during life, just prior to death. Figure 1 shows the lesions that were present on these animals and Table 1 shows which lesions were swabbed and which lesions were positive for grey seal DNA. All three animals were in good nutritional condition and had fed shortly prior to death, as shown by the presence of partly digested prey in their stomachs. The mutilations were considered fatal and exsanguination was the most likely cause of death.

Table 1. *Wounds presence and number of swabs tested in three mutilated harbour porpoises (Pp 1–3); numbers give swabs taken/swabs that tested positive for grey seal DNA; ns = lesion present but not swabbed; abs = lesion absent.*

wound	Pp1	Pp2	Pp3
blubber defect (edge)	ns	1/ 0	2/0
tailstock punctures	1/ 1	2/ 2	abs
head punctures	1/ 0	ns	1/1
flipper punctures	ns	ns	ns
parallel scratches	ns	ns	abs

The incidence of grey seal bite marks

The incidence of grey seal attacks on harbour porpoises was determined with a retrospective study of 1081 harbour porpoises that stranded on the Dutch coastline and were autopsied between 2003 and 2013. Porpoises were collected on the basis of available local logistics, irrespective of the preservation of the carcass. All carcasses had been photographed, paying special attention to any skin and blubber lesions. We used these photographs to assess the presence or absence of lesions associated with grey seal interactions. When the preservation state of the carcass, the absence of body part, or the quality of the pictures made assessment impossible, cases were scored as ‘unknown’.

Distinguishing ante-mortem grey seal-associated wounds from post-mortem scavenging

For each suspected grey seal mutilation case, the necropsy report was reviewed. Criteria used to denote an attack rather than post-mortem scavenging by a grey seal were: no definitive other cause of death (e.g. infectious disease or emaciation), presence of macroscopic or microscopic acute haemorrhages associated with the presumed bite marks, a good nutritive condition (see below) and evidence that the porpoise had fed shortly prior to death (i.e. prey remains in the stomach).

Nutritional condition code

For each porpoise, the nutritional condition code (NCC) was scored on a scale from 1 (very fat and muscular) to 6 (emaciated) (Kuiken & Baker 1991, and see Chapter 3, ES-1). The relationship between NCC and the probability of the presence of grey seal-associated interaction was analysed by generalized linear modelling (including a binomial error distribution and logit link) in which we used the ordered categorical variable NCC as a continuous variable. To test whether NCC could be used as a continuous variable, we first fitted a generalized additive model (GAM) to see if there was a nonlinear pattern between the probability of predation and the NCC status. A nonlinear pattern would suggest that the different levels of NCC have different lengths (e.g. from NCC1 to NCC2 is not the same as the distance between NCC 2 and 3). The GAM showed that the relationship was strictly linear (electronic supplementary material, Figure S1), confirming that NCC can be used as a continuous variable, and 95% confidence limits were determined using a simulation (Gelman & Hill 2007). Porpoises have a thicker blubber layer in winter (Lockyer *et al*. 2003), and this seasonal effect is likely to be reflected in the NCC. As probable grey seal victims were more commonly found in winter (Figure S2), we restricted this analysis to those porpoises found stranded from December up to and including March, to remove this seasonal effect.

Results

Three harbour porpoises (Figure 1a,c,e) were examined. Wounds that contained grey seal DNA were small, repetitive incisions present on the head (Figure 1b) or bilaterally on the tailstock (Figure 1d,f). In addition, presumed grey seal bite marks were present on the flippers (Figure 1g) and presumed grey seal nail rake marks (Haelters *et al.* 2012) were present as five parallel scratches on the bodies of the DNA-positive porpoises (Figure 1h). Large, presumably fatal defects in the

epidermis (which extended through the full thickness of the blubber, with substantial parts of blubber missing) were present in all three cases in which grey seal DNA was detected. These defects mostly showed straight edges and angles, and grey seal DNA could not be demonstrated in these lesions (Table 1). Given the DNA evidence from the smaller lesions present, five different types of skin wounds could be associated with grey seal interactions:

(1) The main mutilation: this comprised a skin and full thickness blubber defect. We set a minimum threshold of a 5 x 10 cm area of missing skin and blubber as representative of a grey seal bite wound and ignored smaller defects as these were interpreted as peck wounds made by birds.
(2) Head marks: one or multiple series of at least three repetitive, parallel puncture wounds anywhere on the head separated by a consistent distance of 0.5–2.0 cm (Figure 1b).
(3) Tailstock marks: repetitive puncture wounds on the tailstock, present bilaterally, and running approximately dorsoventrally in two or more parallel lines (Figure 1d,f).
(4) Flipper marks: a series of three or more repetitive incisions present on one or both of the flippers (Figure 1g).
(5) Scratches: a series of three to five parallel running scratches anywhere on the body (Figure 1h).

The presence or absence of lesions likely to be seal-related was determined in 721/1081 porpoises (Figure 2); the remainder were too decomposed or not photographed in sufficient detail. Major blubber defects (main mutilation) were present in 444/721 (62%) porpoises. In 202 (46%) of these 444 cases, the presence or absence of marks on the tailstock, head, flippers or body could also be reliably assessed. In 120/202 (59%), head marks and/or tailstock marks were visible, and in 37 of the 120 porpoises both were present. In harbour porpoises that had no major blubber defects, head or tailstock marks occurred significantly less frequently (38/306, 12%; Fisher's exact test, $p<0.001$). Flipper marks and/or scratches were found in 60% (95/158) of the porpoises that had head and/or tailstock marks (Figure 2), whereas these occurred significantly less frequently in animals that had no head or tailstock marks (11/327, 3%; Fisher's exact test, $p< 0.001$). The significant concurrent incidence of a major blubber defect with one or more of the four types of marks prompts us to conclude that 120 animals were highly likely to have been victims of grey seal attacks ('probably yes' in Figure 2).

Figure 1. *Macroscopic photographs of the harbour porpoises with grey seal DNA-positive wounds. (a) Pp 3, left side shows absence of large pieces of skin, blubber and musculature. (b) Pp 3, right side of the maxilla showing repetitive puncture lesions on the head ('head mark'). (c) Pp 1, absence of large amounts of skin and blubber in the mandible and throat area, leaving the fractured mandibular bone bare. (d) Pp 1, two lines of parallel running puncture lesions on the tailstock, the lesions were bilateral symmetrical (not visible in picture) ('tailstock mark'). (e) Pp 2, large skin and blubber defects on the body wall leaving ribs and musculature bare. (f) Pp 2, repetitive bite marks on the tailstock similar to Pp 1, figure 1d. (g) Pp 2, flipper with repetitive punctures on the dorsal surface that were mirrored on the palmar surface (not visible in picture) ('flipper punctures'). (h) Pp 2, five parallel running scratches on the left lateral body wall ('scratches').*

Sixteen porpoises with a major blubber defect (2%) had no visible head or tailstock marks, yet did have flipper marks or scratches (n=14), or both (n=2). We consider these possible victims of grey seal attacks ('maybe' in Figure 2: 2%). In 242 of the 444 (55%) porpoises with blubber defects, puncture wounds could not be reliably assessed and therefore the cause of the mutilations in these cases remains unknown. A final category of porpoises that had evidence of a seal encounter were those that lacked a blubber defect but did show marks on the head, tailstock, flippers or body. These animals may have been grabbed or bitten by a seal but probably escaped an immediate fatal seal attack (46/721, 6%: 'possible escape' in Figure 2). In conclusion, based on the proposed assessment criteria, 25% (182/721)of the evaluated porpoises, the 'probably yes', 'maybe' and' possible escape' categories (Figure 2), had wounds attributable to a grey seal.

Gender and age distribution for the animals in the categories 'probably yes' and 'probably not' are shown in Table 2. No significant difference was found for gender between the two groups ($\chi2=0.05$, df=1, p=0.824). Juveniles were significantly more likely to be victims of grey seal attacks than adults ($\chi2=8.0331$, df=1, p=0.005).

***Table 2**. Distribution over age and gender of the probable seal victims ('probably yes' category) and for 'probably not' category. For 110 out of 120 and 537 out of 539 cases, respectively, gender and age could still be assessed.*

	Male	**Female**		**Male**	**Female**
Probably yes			**Probably not**		
Adult	7	9	Adult	51	79
Juvenile	53	37	Juvenile	208	126
Neonate	1	1	Neonate	45	28

The distinction between attack wounds and scavenging defects was considered for the porpoises in the 'probably yes' category (Figure 2). The cause of death could not be determined in 20 of the 120 available cases due to advanced decomposition or organ loss associated with the mutilation. In 90 of the remaining 100 animals, no definitive cause of death other than the presumed grey seal attack could be found. Four of the remaining 10 animals were emaciated and six may have died due to (an infectious) disease. Macroscopic haemorrhages were noted in 26 of the 90 animals for which no other cause of death could be determined. Eight of these were confirmed by histology.

The stomach contents were studied in 113 of the 120 porpoises in the 'probably yes' category. In 84 (74%) of these, prey remains were found in the stomach, whereas 29 (26%) had empty stomachs. Based on a detailed study of the stomach contents of grey seal victims, it was inferred that the nature of the wounding

reflected their last meal (Leopold *et al*. 2015): porpoises with the main mutilation on the side of their body had eaten mainly demersal fish, whereas porpoises that had been mutilated in the throat region had eaten mainly pelagic, schooling fish.

The NCC could be reliably scored in 97/120 of the identified probable grey seal victims and in 271/539 harbour porpoises that did not show any signs of grey seal interaction (the 'probably yes' and 'probably not' categories, respectively: see Figure 2). Animals in the 'probably yes' category had significantly lower NCC's than animals in the 'probably not' category ($p<0.001$) and were thus nutritionally in a better condition.

These findings all indicate that the majority of 120 animals in the 'probably yes' category had been killed by grey seal predation and not scavenged post-mortem.

Discussion

The estimated frequency of harbour porpoise–grey seal encounters (25% of 721) includes the possible cases of grey seal attacks ('maybe' in Figure 2: 2%) and animals that probably escaped an attack (6%). These findings suggest that grey seal attacks were the cause of death in at least 17% of the stranded animals. This is probably a conservative estimate as mutilated carcasses with an opened abdominal or thoracic cavity are likely to sink rapidly and decay, therefore going unrecorded. Moreover, animals that initially escaped an attack may have died later from the wounds inflicted. If dead stranded and autopsied harbour porpoises are representative of porpoise deaths in the region, then grey seal attacks (more than 17%) together with fisheries bycatch (approx. 20%), infectious disease (approx. 18%) and emaciation (approx. 14%) are the most important causes of death for harbour porpoises in the southeastern North Sea (Utrecht University 2009–2013, unpublished harbour porpoise necropsy results).

If grey seals benefit nutritionally from this inter-species interaction, then according to optimal foraging theory, they would preferentially target the most energy-rich parts of easily caught large prey (Benoît *et al.* 2011). Porpoise blubber fits this description of optimal diet better than most prey tissue. The porpoise population may suffer in ways other than loss of individuals as most of the mutilated animals were healthy and fat prior to the attack, suggesting that grey seals primarily target juvenile harbour porpoises that are in prime condition and so probably reduce recruitment to breeding age. For this reason, predation by grey seals may have significant cumulative effects on porpoise ecology as, under predation pressure, they may avoid profitable feeding grounds or adjust their diving behaviour in the

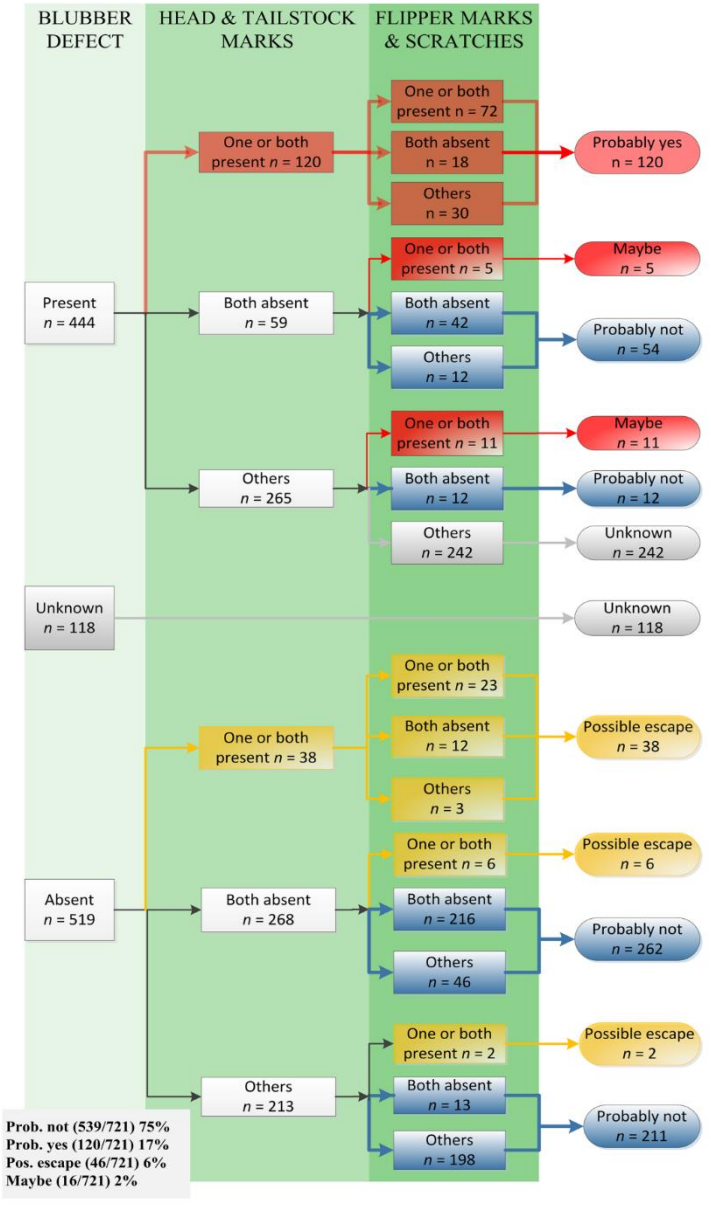


Figure 2. *Decision tree showing number of cases that had presence, absence or 'unknown' for blubber defects, head and tailstock marks, and flipper marks and scratches, respectively. Others = absence of one characteristic, with the other characteristic 'unknown'. 'Probably yes' = probable grey seal victim. 'Maybe' = possible grey seal victim. 'Unknown' = not possible to determine if grey seal victim. 'Possible escape' = victim that probably escaped from a grey seal attack. 'Probably not' = not a grey seal victim.*

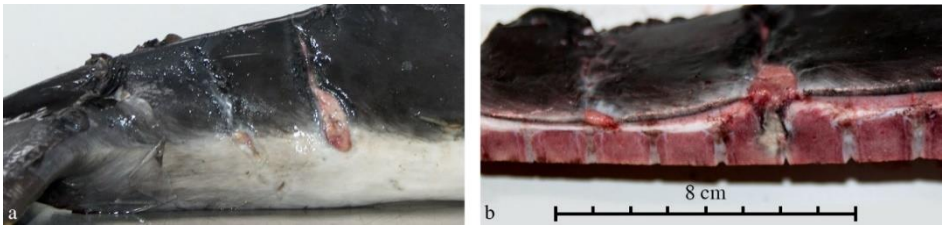


Figure 3. *Example of a 'possible escape' case. Macroscopic photograph of an inflamed 'tailstock mark': (a) lateral view showing a skin wound similar in shape, location and size to 'tailstock mark' as shown in figure 1d,f, which shows partial healing; (b) cut section through the tailstock showing the same skin wound and inflammation extending into underlying tissue, new bone formation of the vertebrae and inflammation in the intervertebral disc.*

presence of predators (Heithaus & Dill 2002; Baird *et al*. 2008). There is also increasing evidence that animals faced with a significant predation pressure may respond by losing weight to allow them to move faster, thereby increasing the probability of escaping attack (Piersma *et al*. 2003; MacLeod *et al*. 2007; Heithaus *et al*. 2009; van den Hout *et al*. 2010). Similar to the well-reported lethal aggression shown by bottlenose dolphins (*Tursiops truncatus*) (MacLeod *et al*. 2007), porpoises faced with the likelihood of seal predation may respond by becoming leaner and faster swimmers. However, weight loss makes a porpoise more prone to emaciation, another major cause of death for this species, and porpoise health may be impaired in a wider sense. As the smallest cetacean, the large surface-area-to-volume ratio means that porpoises lose relatively large amounts of body heat to their environment, forcing them to maintain high feeding rates. Both losing feeding time due to increased vigilance for predators and living leaner may pose a serious challenge for a harbour porpoise faced with a predation risk–starvation trade-off (MacLeod *et al*. 2007).

Grey seal attacks on harbour porpoises are not always fatal, as shown by the animals in the 'possible escape' category (Figure 2). Over 50% of the bite marks on these animals showed clear inflammation or healing, indicating that these animals had escaped an attack (25/46; Figure 3). Such escapes would allow animals to learn to avoid grey seals, but at the costs mentioned above.

Another well-reported and frequent cause of sudden death in harbour porpoises is drowning due to fisheries bycatch. In these cases, post-mortem findings include all the characteristics of sudden death seen in grey seal attack victims except the bite wounds and associated haemorrhages. Without haemorrhages in the bite wounds, we cannot exclude the possibility that grey seals feed on porpoise carcasses bycaught in gill nets as they are known scavengers of fish entangled in such nets

(Benoît *et al*. 2011; Moore 2003; Chouinard *et al*. 2005). However, relatively few (n=5, or 4%) of the 'probable yes' animals showed net marks on their skin, suggesting that if this phenomenon occurs, it happens infrequently. Still, it is tempting to speculate that harbour porpoises entangled in such nets may have triggered grey seals to turn from scavenging to attacking live animals. The first grey seal victim was found in 2003 (Leopold *et al*. 2015), but without accurate information from earlier years it is not possible to determine when this behaviour first occurred. Increasing numbers of mutilated animals have been found from 2003 to 2013, but this trend parallels the increasing trend in the number of harbour porpoises stranded (Leopold *et al*. 2015). Certain prerequisites must be present for this behaviour to develop. These include sympatry of predator and prey, and possibly a high incidence of fisheries bycatch of the prey in static fishing nets to induce this behaviour.

Finally, many of the mutilated porpoises were found on Dutch shores used frequently by human bathers and surfers, and there would appear to be no *a priori* reason why humans may not be at risk from grey seal attacks.

Acknowledgements

We would like to thank all the people who reported, photographed and collected dead porpoises, in particular Arnold Gronert, Kees Kooimans and Jaap van der Hiele, who provided the majority of the porpoises. We thank all veterinary pathologists, technicians, our histology laboratory and volunteers who helped with more than 1000 autopsies over the years. Finally, we thank Mark Dagleish, Rachel Thomas, Stefan Schouten and Thijs Kuiken for guidance with setting up this paper, Erik Meesters for help with the statistics and Joop Fama for formatting the images.

Funding statement

This work was financed by the Dutch Ministry of Economic Affairs under the BO programme (BO-11-011.04-044, HD3500) and 140000353.

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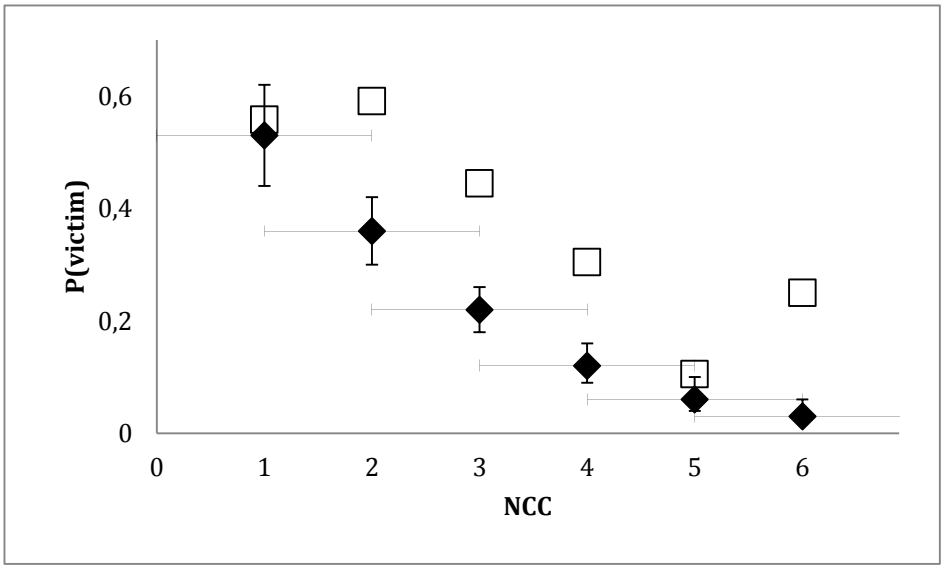
Data Supplement

Three file were supplied with the publication of this paper in Proc. R. Soc. B 282: 20142429. This material is available at http://dx.doi.org/10.1098/rspb.2014.2429 or via http://rspb.royalsocietypublishing.org.

Figure S1. - *The mean probabilities for a carcass to be a probable seal victim, with 95% confidence limits for the whole dataset (Jan-Dec: filled diamonds) and for the key period (Dec-Mar: open squares).*



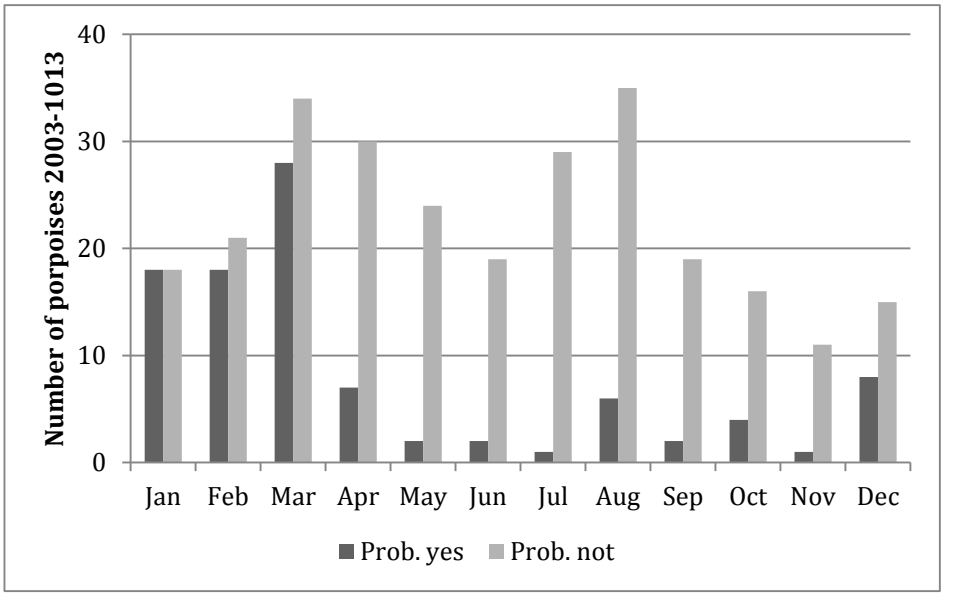


***Figure S2**. Numbers of harbour porpoises in the categories 'Probably yes' and 'Probably not', per month, 2003-2013 for which the nutritional condition code could be determined.*

Basedata S3. - The data used for this study. For each porpoise studied, identified by a unique Idcode, month and year of stranding are given, followed by its NCC, the presence/absence (or unknown) of, respectively, blubber defect, tailstock marks, head marks, flipper marks, and scratches; evidence of, respectively, recent feeding ("stomach contents") and haemorrhages, whether or not the carcass had been kept frozen prior to necropsy; porpoise gender and age category (Neonate, Juvenile or Adult) and our final conclusion regarding grey seal interaction.

(not reproduced here). Data are open access and may be downloaded from:

http://rspb.royalsocietypublishing.org/content/royprsb/suppl/2014/11/21/rspb.2014.2429.DC1/rspb20142429supp3.xls

Porpoises: From predators to prey

Mardik F. Leopold, Lineke Begeman, Eileen Heße, Jaap van der Hiele, Sjoukje Hiemstra, Guido Keijl, Erik H. Meesters, Lara Mielke, Dorien Verheyen & Andrea Gröne

8

Abstract

Along the Dutch shores hundreds of harbour porpoises *Phocoena phocoena* are stranded each year. A recurrent phenomenon in the Netherlands is a surge of strandings in late winter and early spring of severely mutilated porpoises, that are mostly in good nutritional body condition (thick blubber layer). These mutilated porpoises have parts of the skin and blubber, and sometimes of the muscle tissue missing. By reviewing photographs of stranded animals taken at the stranding sites as well as necropsy results we found 273 mutilated animals from 2005 to 2012. Mutilations could be classified into several categories, but wounds had been mostly inflicted to the sides of these animals, in a zigzag fashion, or to the throat/cheek region. The stomach contents of 31 zigzags, 12 throats/cheeks and 31 control animals that were not mutilated, from the same age and blubber thickness categories were compared; all these animals had stranded between December and April, 2006–2012. The diet of individuals with zigzag lesions to their sides consisted for a large part of gobies, while animals that had wounds at the throat/cheek had been feeding predominately on clupeids. In comparison, animals without mutilations had a more varied diet, including gobies and clupeids, but also a large proportion of sandeels and gadoids. The finding that the type of mutilation corresponds to a certain diet suggests that porpoises that were feeding on different prey, or in different micro-habitats, were hit in different ways. Animals feeding at the sea floor (on gobies) apparently run a risk of being hit from the side, while animals supposedly feeding higher in the water column (on schooling clupeids), were predominantly hit from below, in the throat region. The wider variation in the diets of non-mutilated porpoises is suggestive of them using a larger variety of micro-habitats.

Keywords: *Phocoena phocoena,* strandings, mutilation, diet, grey seal, *Halichoerus grypus*

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8

Introduction

Harbour porpoises *Phocoena phocoena*, being relatively large piscivores, are considered apex predators in the southern North Sea, where bigger marine predators such as large sharks or killer whales *Orcinus orca* are largely absent. Still, dozens of severely mutilated porpoises wash ashore yearly in the Netherlands. These animals have sharp, smoothly curved or erratic zigzag cuts over their bodies and have parts of the skin and blubber missing (Leopold *et al.* 2015). Earlier, such mutilations have been tentatively attributed to fishermen confronted with bycatches (Camphuysen and Oosterbaan 2009; Haelters & Camphuysen 2009; Leopold & Camphuysen 2006), ship propeller strikes (Camphuysen & Siemensma 2011, cf. Thompson *et al.* 2010), sand dredgers (Oudenaarden 2012a,b) or scavenging grey seals *Halichoerus grypus* (Camphuysen & Siemensma 2011). North Sea dolphins, particularly bottlenose *Tursiops truncatus* and white-beaked dolphins *Lagenorhynchus albirostris* have also been considered, as these may harm and even kill porpoises, but could be excluded as actors in this respect. Dolphins are rather rare in the SE North Sea and the lesions inflicted by these attacks are well described and quite different from those found in the mutilated porpoises in the Netherlands (Barnett *et al.* 2009; Haelters & Everaarts 2011; Patterson *et al.* 1998; Ross & Wilson 1996).

In the SE North Sea, grey seals were first implicated as predators of porpoises in Belgium and France (Haelters *et al.* 2012; Bouveroux *et al.* 2014). Two decades ago grey seals were seen incidentally to catch and partly consume harbour porpoises in the Isle of Man and in Northumberland, UK (Vodden 1995). Van Bleijswijk *et al.* (2014) identified grey seal DNA in bite marks on harbour porpoise carcasses stranded in the Netherlands, linking the scarce observations of actual attacks to the large numbers of mutilated porpoises currently washing up dead in the SE North Sea.

Grey seals re-colonised Dutch waters around 1980 and the subsequent population development showed exponential growth (Reijnders *et al.* 1995; Brasseur *et al.* 2010). In 2012, 835 grey seals were counted in the Delta area in the southwest of

the country (Strucker *et al.* 2013) and 3059 in the Dutch part of the Wadden Sea (Common Wadden Sea Secretariat 2012). Grey seals range widely from their haul-out sites (Aarts *et al.* 2008; Brasseur *et al.* 2010; Russell *et al.* 2013) and occur anywhere off the Dutch and Belgian coastlines. Numbers of porpoises have also increased markedly in the southern North Sea in recent decades (Haelters & Camphuysen 2009; Camphuysen 2011; Camphuysen & Siemensma 2011; Scheidat *et al.* 2012; Hammond *et al.* 2013) and therefore, interactions between seals and porpoises have potentially become much more frequent here in recent years.

Hundreds of stranded harbour porpoises are reported per year in The Netherlands and 25% of these bear the tell-tale marks of grey seal attacks (Leopold *et al.* 2015). Mutilations take different forms and several types are distinguishable, which may provide clues as to how these porpoises were attacked. In addition, the stomach contents of the mutilated animals may yield information on where the victims were feeding, when attacked. In this paper, we consider the different types of wounds inflicted, in concert with the stomach contents of mutilated porpoises to gain insight in the circumstances under which these porpoises were attacked.

Material and Methods

Examining photographic evidence and necropsy data

The Dutch coastline (523 km, including the Wadden Sea and Western Scheldt) is mostly readily accessible to the public and most stranded cetaceans are probably reported, to www.walvisstrandingen.nl. Meta-information was collected for each animal, including date, location, and fate (animal collected or discarded). Since 2006, stranded porpoises were routinely collected for necropsy along several stretches of coastline, and incidentally elsewhere. Necropsies took place on Texel in 2006 (Leopold & Camphuysen 2006) and from 2007 at the Faculty of Veterinary Pathology Medicine, Department of Pathobiology of Utrecht University (Gröne *et al.* 2012). From animals that went through necropsy, body length, sex and blubber thickness, taken dorsally, laterally and ventrally, just anterior of the dorsal fin were also recorded. These animals were all photographed, with special attention for external lesions.

In addition to the photographs taken during necropsies, all photographs of animals that were stranded between 2003 and 2012 that were made by the general public and uploaded to www.walvisstrandingen.nl were examined. For this study, we reviewed photographs of 1974 stranded porpoises, including 857 that went through necropsy. For each animal, we established, if possible, external damage.

The state of decomposition at recovery (DCC: decomposition code) was established for all animals examined, on a 5-point scale: 1 = live stranding; 2 = fresh; 3 = visibly starting to decompose; 4 = rotten; 5 = remains (mere bones or "mummified"). Animals that were too rotten (mostly DCC 4 and 5 but also many DCC 3 animals) and animals for which only poor-quality photographs (taken at the strandings site) were available, were not analysed. Three observers assessed the photographs independently, categorising lesions as possibly inflicted by seals or probably caused by other agents such as ship propellers, knives or axes, and trauma inflicted by scavengers (birds, dogs, foxes, etc.). Data were entered into a database, discussed and amended afterwards if different observers had classed damaged porpoises differently.

Considering the traumas now known to have been inflicted by seals (van Bleijswijk *et al.* 2014; Leopold *et al.* 2015), we distinguished five types of wounds within the "major blubber defects" category:

a. Zigzag patterns: animals with multiple traumas inflicted mainly to the sides of the bodies, under various angles, with parts of the skin and blubber apparently torn off; some of these parts missing or hanging loose from the body (see Appendix A for photographs of these and other lesions);
b. Head–tails: animals with the head and tail sections largely intact, but with most of the soft parts in between lost;
c. Throat/cheek: animals with a large part of the skin and blubber missing from the side of the head, usually under the eye, extending to or from the throat area;
d. Circular body: animals with large cuts behind the head, at or near the widest part of the body and often with large sheets of the skin and blubber missing;
e. Body parts: loose pieces of the skin and blubber, loose dorsal fins, pectoral fins, flukes, or tailstocks, with or without loose pieces of the skin and blubber attached.

Lesions, considered not related to seals, were not used in the analysis. These included defects with very smooth edges that were supposedly inflicted with a cutting force, rather than a tearing force: animals cut straight in two, animals with amputated dorsal fins, pectoral fins or tail flukes and cuts and stabs to the body apparently inflicted with knives (see: Haelters & Camphuysen 2009). Small (< 5 × 10 cm diameter), often multiple lesions with irregular edges, with more superficial penetration were supposedly inflicted by scavenging birds, and not considered.

All photographs taken from the same animal were examined in concert. For each animal photographed, we noted if it showed a major blubber defect and if so, which type.

Selecting animals for stomach content analysis

Stomach content analysis was performed on three groups of porpoises: zigzag animals, animals with mutilations to the throat or cheek and animals that were not mutilated. Intact, non-empty stomachs were available for 36 zigzag animals. As most of these were juveniles (31 animals <130 cm total length, cf. Lockyer 2003 for North Sea porpoises) that were found between December and April 2006–2012, this group was selected to reduce heterogeneity. Average blubber thickness of these 31 animals was 20.5 ± 5.3 mm and most was fresh or starting to decompose (DCC <3). For comparison, we used animals that had been mutilated at the throat or cheek (n = 12) and animals that were not mutilated (n = 31). Selection criteria for these were: juvenile, found between December and April 2006–2012, blubber layer >15 mm, DCC <3, intact, and non-empty stomach. For animals with "circular body lesions" or animals reduced to "head–tails" or to mere body parts, only 2, respectively 0 stomachs were available, so these groups were left out of the analyses.

Stomach content analysis

During necropsy, porpoise stomachs were removed and carefully cut open for a brief inspection for pathology. Stomachs were then bagged and stored frozen for later study. All food remains found in the fore stomach, the fundic stomach, and the pyloric stomach (Smith 1972) and in the oesophagus were included in the analyses.

Relatively undigested prey were identified to the species level and measured directly. Most samples contained partly digested prey. These were collected in a large beaker. Prey hard parts were isolated by letting a gentle water flow make the beaker overflow, removing most of the soft particles. Care was taken to retain hard, but light parts that were useful for identification, such as squid beaks and shrimp claws. When a more or less clean sample of prey hard parts remained at the bottom of the beaker, this was sorted under a dissecting microscope. Alternatively, samples that contained large amounts of partly digested prey were packed in a 300-µm mesh bag, which in turn was put into a 120-µm mesh bag. The sealed package was then washed at 70 °C in a washing machine with standard washing powder. This procedure effectively removed soft material, while prey hard parts were retained

within the inner bag. The 120-µm mesh bag served to protect the bones and otoliths in the inner bag from damage and provided an extra safety measure against loss of material that should have been retained in the inner bag. After washing, the samples were not spun dry in the washing machine, to prevent damage to the hard prey remains.

Prey remains used were: fish sagittal otoliths, bones, eye lenses and scales, cephalopod beaks, crustacean, and gadoid–parasite exoskeleton parts. First and foremost, otoliths were used to identify fish species, and to estimate fish length and weight, following Leopold *et al.* (2001). We used Clarke (1986), Härkönen (1986) and Leopold *et al.* (2001), as well as our reference collection of otoliths and fish bones for species identification. Prey remains were photographed with a Zeiss camera stereoscope (Stereo Discovery.V8 Achromat S, 0.63 × FWD 115 mm) and measurements were taken using Axiovision software (AxioVs 40 v.4.7 & 4.8). The minimum number of individual prey (MNI) was estimated for each prey species per porpoise. Otoliths were ordered in accordance with species, size and side (left/right). Pairs were made of otoliths that were visually assumed to originate from the same fish. The remaining single (left or right) otoliths were considered to represent one fish each. The upper and lower squid beaks and eye lenses were treated in a similar manner.

Other fish remains, such as vertebrae and premaxillae (see Watt *et al.* 1997), were also used to identify fish species and estimate their size and MNI. These other fish remains were used to complete and verify the findings by matching these to the paired otoliths. Remains of the parasitic copepod *Lernaeocera branchialis* were taken as proof of whiting *Merlangius merlangus* presence (Kabata 1992; van Damme & Hamerlynck 1999). In the absence of otoliths, fish eye lenses > 2 mm cross-section present in the same sample were considered to stem from whiting if *Lernaeocera* remains were present, and if no remains of other large fish were present. The regression: whiting length (in cm) = 8.4427 (fish eye lens length, in mm; Leopold *et al.*, unpublished) was used to estimate fish length in such cases. Likewise, the presence of another parasitic copepod, the eye-maggot *Lernaeenicus sprattae* was taken as proof for the presence of sprat *Sprattus sprattus*, allowing in a few cases, worn clupeid vertebrae or otoliths to be used for identifying sprat as prey (Schram 1991; Groenewold *et al.* 1996).

If stomachs contained very large (hundreds or thousands) numbers of goby *Pomatoschistus* sp. or sandeel *Ammodytes* sp. otoliths, these were sorted in 5–8 batches of similar size and wear (see below) which were counted. MNI per batch was taken as half the number of otoliths in that batch and per batch, the smallest

and largest otoliths were measured. The sizes of the largest and smallest fish per batch were estimated from these, after correction for wear (see below) and the sizes of all other fish within that batch were estimated by linear intrapolation.

Even though sagittal otoliths are the parts of a fish that are most resistant to digestion, they do wear down in the acidic, grinding environment of a predator's stomach. Most retrieved otoliths are thus smaller than the original size and a correction is needed for an unbiased estimate of fish size (Tollit *et al.* 2004). All retrieved otoliths were examined for signs of wear and the amount of wear in each otolith was assessed as:

Wear class 0:	no wear noticeable; otolith in pristine condition;
Wear class 1:	slight wear, otolith shape still largely intact, but some wear at margins;
Wear class 2:	moderate wear, otolith rounded but shape and otolith sulcus still well visible;
Wear class 3:	severe wear; otolith badly worn, shape and size severely affected, sulcus barely visible.
Wear class 4:	otolith worn down to such an extent that size is no longer related to original size.

Correction factors specific to each wear class 1–3 were obtained from a separate project on the diet of piscivorous predators (harbour porpoise, great cormorant *Phalacrocorax carbo* and Atlantic puffin *Fratercula arctica*) consuming considerable quantities of sand gobies *Pomatoschistus minutus*, whitings, smelts *Osmerus eperlanus*, herrings *Clupea harengus* and lesser sandeels *Ammodytes marinus*. This was accomplished by selecting predator stomachs that contained large numbers of otoliths of one of these fish species that were all of the same age group and that contained sufficient numbers of otoliths of all wear classes 0–3. Wear was assessed and length and width were measured for each individual otolith. Median sizes were calculated for each wear class and grade-specific correction factors were calculated by comparing median sizes of the various wear classes 1–3 to medium sizes of wear class 0 otoliths. For both length and width, and for all species except whiting, grade-specific correction factors were close to 1.05, 1.1 and 1.2 for wear classes 1–3, respectively and these values were used for all fish species, except whiting. Correction factors for whiting were determined as 1.06, 1.14 and 1.24 for wear classes 1–3, respectively. Lengths and widths of otoliths of wear classes 1–3 were corrected accordingly, before fish length and fish mass were calculated. Otoliths of wear class 4 were given the average size of all other otoliths, in the same sample or across samples for the same month of stranding, after

correction for wear. Average size was only assigned to wear class 4 otoliths if their number was relatively small. If such numbers were larger (particularly in gobies) they got a randomly estimated size assigned to them from the other, less worn otoliths in the sample, thus preventing large peaks in numbers of otoliths of average size. In order to reduce heterogeneity (inter-observer differences), wear class was always assessed by the senior author.

Fish length was calculated from regression equations (Leopold *et al.* 2001), using lengths and width of both otoliths of presumed pairs, or length and width of single otoliths, or just length or just width of otoliths with damage preventing taking the other measurement. To obtain a single estimate for original total fish length, the average of all 4 (maximum) otolith measures was used. The total fish length value was then used to calculate the fresh wet weight of the fish (Leopold *et al.* 2001).

The number of cephalopods was defined as the more numerous number of the upper or lower beaks, or pairs of eyes. Because squid beaks are less sensitive to digestion (Sekiguchi & Best 1997; Tollit *et al.* 1997; Phillips & Harvey 2009), no corrections for wear were made of these remains. The length and weight of cephalopods were calculated from relationships between lower beak size according to Clarke (1986) or from our reference collection. When upper beaks were more numerous, squid size was estimated from the less numerous lower beaks. For the remaining upper beaks the average size of the other individuals within the same stomach was given.

When shrimp claws were found, the equation proposed by Doornbos (1984) was applied to estimate weight. For shrimp remains that could not be translated to shrimp size, such as shrimp eyes, a standard weight of 1.0 g was used, the average for all shrimps for which the size could be estimated.

Statistical analysis

For each porpoise stomach, the number of prey (MNI per species) was estimated, and from length–weight relationships (Leopold *et al.* 2001), total prey mass (Appendix B). Prey numbers and biomass data were fourth root transformed and the Bray–Curtis dissimilarity was calculated of each matrix. The resulting distance matrix was analysed using Principal Coordinate Analysis. Differences between groups were assessed using Permanova (Anderson 2001; McArdle & Anderson 2001). Analyses were performed using R (R core team 2012) and the package vegan (Oksanen *et al.* 2012).

Results

Examining photographic evidence for seal inflicted trauma

Between 2000 and 2012 a total of 4724 harbour porpoise strandings were recorded in The Netherlands. From 2006 to 2012, 857 animals were necropsied and these were all photographed. For 2005–2012, photographs were available for another 1117 animals on www.walvisstrandingen.nl. Over time, the proportion of animals that was photographed increased (Figure 1). Five types of major blubber defects have been identified from photographs (Figure 2). Increasing numbers of mutilated animals were identified over the years, but this trend parallels the trend in general numbers stranded (Figure 1). We found 273 porpoises with major blubber defects among reported porpoise strandings from 2005 to 2012, and a single earlier case in 2003. The largest proportions of mutilated animals were found in 2010 (21.4%) and 2012 (20.5%); the overall percentage of identified mutilated animals was 14.4%, or even 17% when only animals were considered that were necropsied (Leopold *et al.* 2015).

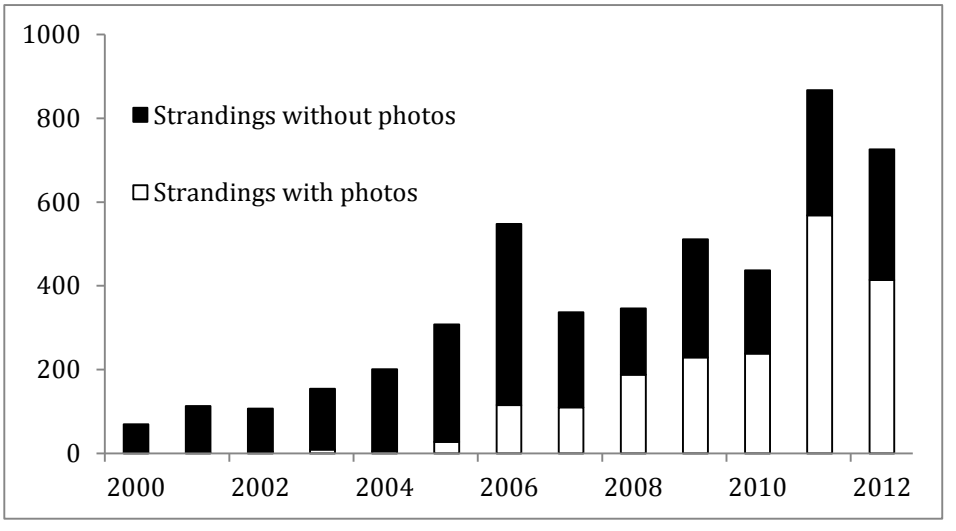

Figure 1*. Total numbers of reported strandings (n=4724, 2000–2012) and the relative proportions of porpoises that were photographed (white), either on the necropsy table or at the stranding site, or both.*

Animals used for stomach content analysis

A record number of animals with major blubber defects were stranded in 2012, including many with zigzag or throat/cheek lesions (Figure 2). Zigzag lesions were most common and across all years, animals with these lesions were predominantly found in winter (December to April: Table 1).

The animals with zigzag lesions found from 2006 to 2012 and from December–April were predominantly juveniles (<130 cm, 91.5%) that were in a good nutritional body condition (average blubber thickness mostly >15 mm: Appendix B). Thirty-one zigzag juveniles were available from this period. For comparison, we selected all intact porpoises from our diet database, that were <130 cm long, had stranded between December and April 2006–2012, had >15 mm of blubber and a non-empty stomach (also 31 animals) and animalswith throat/cheek lesions under the same criteria (12 animals).

***Table 1**. Numbers of identified porpoises with zigzag lesions per year and per month, 2003–2012. Order of months is centred around late winter. Grey highlight indicates the period selected for stomach content analyses.*

Month	2003	2004	2005	2006	2007	2008	2009	2010	2011	2012	Totals
10					1	1			1		**3**
11								1			**1**
12						3				1	**4**
1			1	1			3	3	2	1	**11**
2					2		4	3	6	5	**20**
3	1			2	4	3	2	3	3	15	**33**
4				1	2						**3**
5				1							**1**
6											**0**
7					1	1			1		**3**
8								2			**2**
9								1		1	**2**
Totals	**1**	**0**	**1**	**5**	**10**	**8**	**9**	**13**	**13**	**23**	**83**

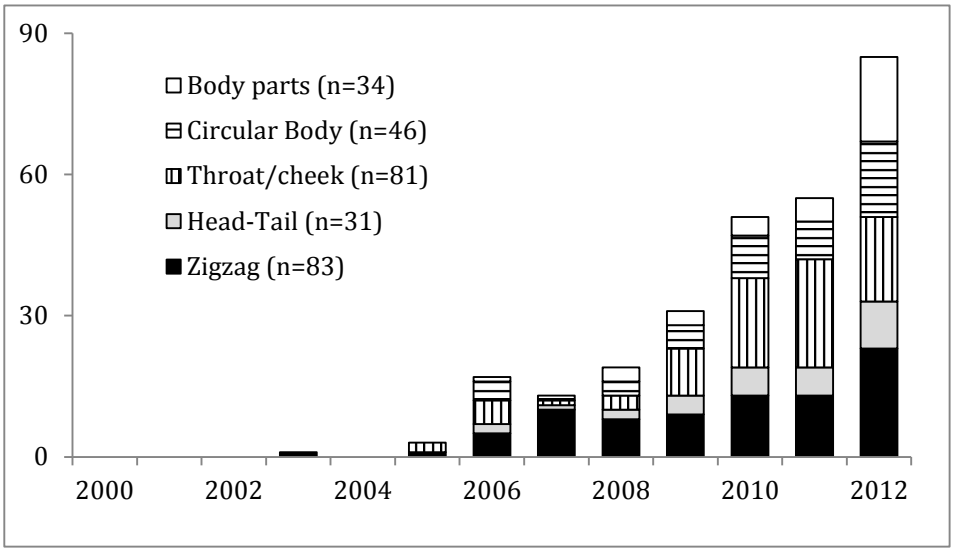

***Figure 2**. Different types of major blubber defects among stranded porpoises in The Netherlands.*

Diet

The majority of zigzag animals available for the diet study were found in 2012 (Table 2). Therefore, we first compared the diet of 2012 zigzags (n = 12 stomachs, incidentally all found in March) with the diet of zigzags in earlier winters (n = 19). Both diets were dominated by gobies (Figure 3) and in subsequent analyses all years were pooled.

Compared to the zigzag animals, the diet of the animals hit at the throat/cheek (n=12) comprised a much larger proportion of clupeids (herring and sprat) and gadoids (mostly whiting) and a much smaller proportion of gobies (Figure 4). We note, however, that the contribution of gadoids to the diet of porpoises wounded at the throat or cheek was mainly due to the stomach contents of one animal. The group of animals that were not mutilated (n= 31) had the most varied diet, with rather equal proportions of gobies, clupeids, gadoids and sandeels, but also less full stomachs.

***Table 2**. Pairwise comparisons of diets of three groups of stranded porpoises: animals with zigzag lesions, with lesions to throat or cheek, and animals that were not mutilated. Permanova tests are used to test for differences.*

Groups	T (prey biomass)	P (perm)	perms	T (prey numbers)	P (perm)	perms
Zigzag-Throat	2.4661	0.001	997	2.8114	0.001	999
Zigzag-Not mutilated	2.1211	0.002	999	2.3625	0.002	997
Throat-Not mutilated	1.1914	0.249	999	1.1786	0.235	999

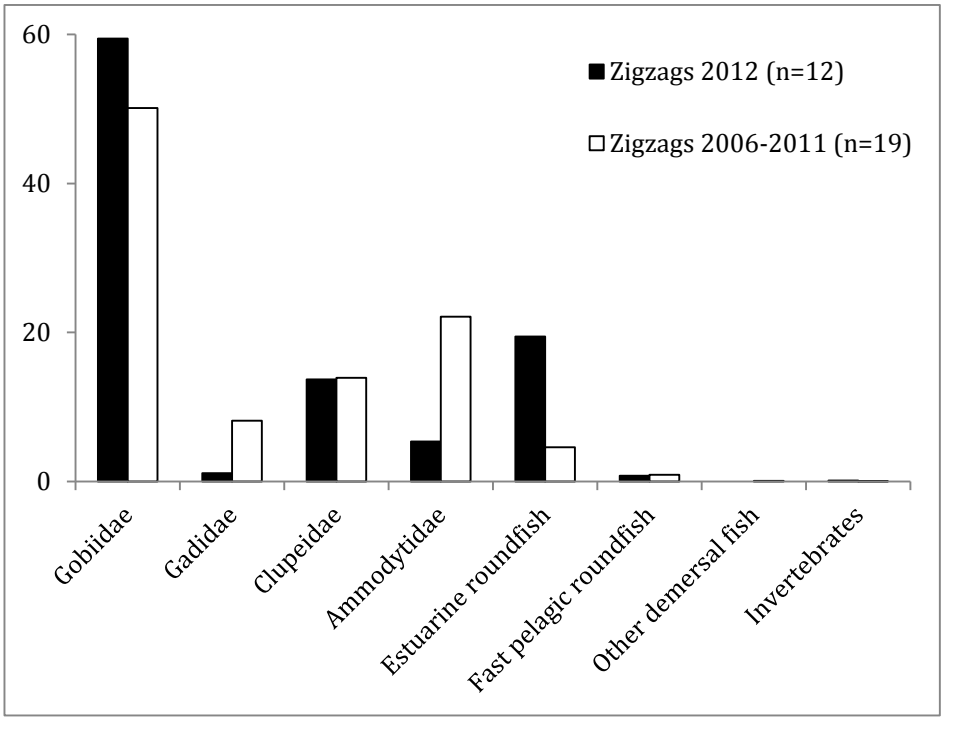

***Figure 3**. Comparison of diets (summed preymasses) of porpoises found with zigzag lesions in 2012 and 2006–2011. Average reconstructed prey mass per stomach was 1010±1176 g (2012), and 1507±1066 g (2006–2011), respectively. Prey species included in each prey group are listed in Appendix B.*

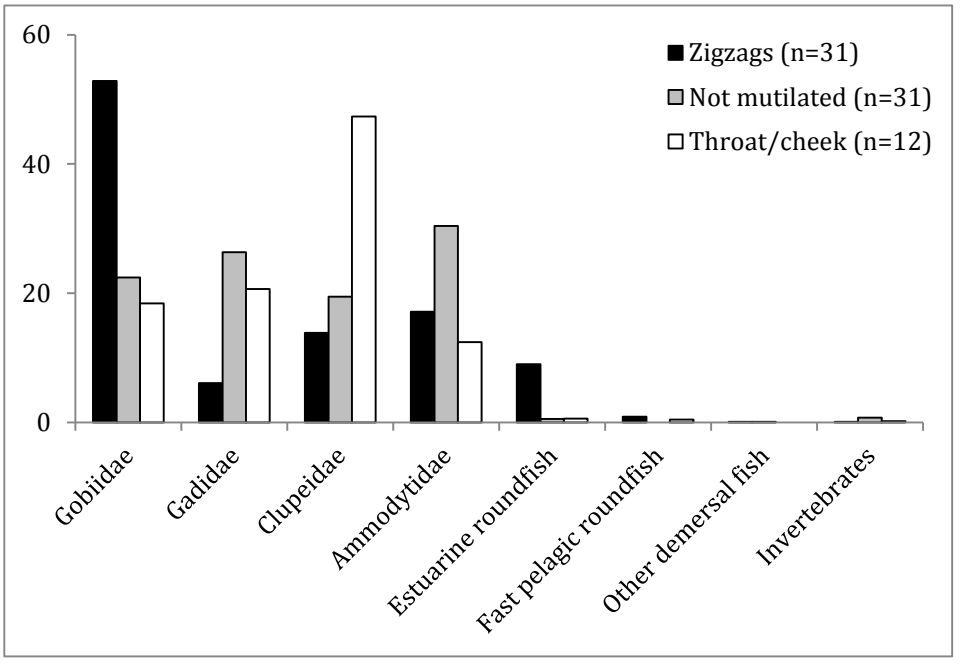

***Figure 4**. Comparison of diets (summed preymasses) of porpoises found with zigzag lesions, lesions to the throat or cheek and animals that were not mutilated (all years combined in each category). Average reconstructed prey mass per stomach was 1315±1117 g (zigzags), 1072±809 g (throat/cheek), and 598±680 g (not mutilated) respectively.*

Comparing the three groups in concert, a Principal Coordinates Analysis (PCO) explains 73% of the variance in prey numbers within the total group of animals considered, and 64% of the variance of prey biomass (Figure 5a,b). The differences between the prey spectra of zigzag and throat/cheek animals and between zigzag and non-mutilated animals were highly significant (Table 2); the difference between non-mutilated animals and throat/cheek animals was not significant.

Discussion

In this study we investigated if the combination of specific wounds and stomach contents of mutilated porpoises would provide clues as to how and where these animals were attacked. This approach could only be successful if porpoises were feeding when being attacked, or at least, were still swimming in the same micro-habitat where they had been feeding last. The relatively full stomachs of mutilated individual suggest that this condition may have been met. Our study demonstrates that: 1) most affected animals were apparently in good nutritional body condition,

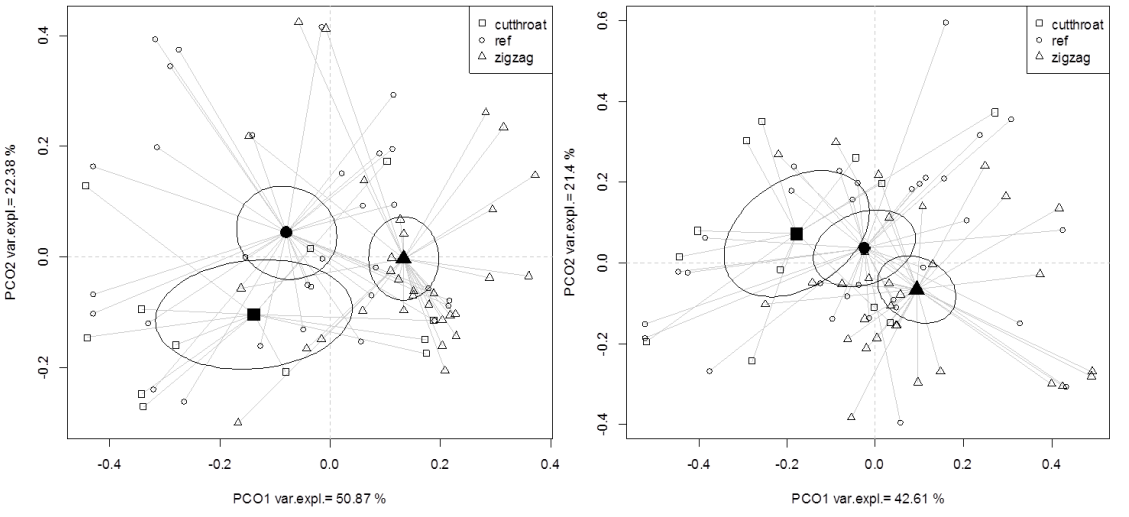

Figure 5a *(left). PCO plot of the diet composition (prey numbers) of all porpoises analysed.*

Figure 5b *(right). PCO plot of the diet composition (prey mass) of all porpoises analysed. Midpoints of groups in bold, 95% confidence ellipses are given around these centroids.*

and had comparatively full stomachs (Figure 4), and 2) that their diet differed with the type of lesions inflicted. Porpoises with zigzag wounds had been feeding mostly on gobies, i.e., close to the sea floor. Animals with wounds to the throat or cheek had been feeding predominantly on clupeids, i.e., higher in the water column. The combination of attack wounds and attack-specific diets shows that porpoises are never safe from seal attacks and may be hit both at the sea floor and higher in the water column. The relationship between the specific attack wounds and diet cannot be explained by grey seals scavenging on already dead porpoises as a link with porpoise diet would have been lost. The difference in diets of porpoises with zigzag wounds and porpoises wounded in the cheek/throat region strongly indicates that the porpoises were attacked alive, while feeding. Their good nutritional body condition and filled stomachs would also indicate a sudden death. All these findings are consistent with predation during feeding, or shortly after feeding.

Non-mutilated animals, that is porpoises without major blubber defects, had the most varied diet. Net marks, i.e., thin linear impressions, either on the skin or on the lips, presumably from bottom-set gillnets (see: Haelters & Camphuysen 2009), were found on eight of the 31 non-mutilated animals examined (Appendix B), indicating drowning as the cause of death. Incidentally, net marks were also found on one of the zigzags, and animal that also had a tailstock bite mark (UT047), from

the teeth of a grey seal (see: Leopold *et al.* 2015; van Bleijswijk *et al.* 2014). This combination of lesions may indicate an attack on a porpoise stuck alive in a net, or a grey seal scavenging on a porpoise corpse, after this animal had drowned. Grey seals are known to take fish from set nets (Moore 2003; Stenson *et al.* 2013) and it would seem a small step to start feeding from entangled porpoises.

Our findings indicate that the occurrence of mutilated harbour porpoises is much more common in the Netherlands than reported in bordering countries, and is seemingly rising, in concert with an increase in strandings. Major blubber defects were found on 17% of all stranded porpoises that were sufficiently fresh to be necropsied (Leopold *et al.* 2015). In some years this incidence was >20%, indicating that grey seal attacks are an important cause of death.

Both harbour porpoises and grey seals have greatly increased in numbers in Dutch nearshore waters in recent decades. The seals may have found porpoises to be a new food resource, carrying a large blubber store with a high energy density. Our results provide further arguments in favour of the hypothesis that grey seals cause these mutilations, now found on dozens of stranded porpoises per year. Alternative hypotheses, that porpoises were first by-caught in e.g. bottom set-nets (cf. Camphuysen & Oosterbaan 2009) and mutilated later, either by fishermen or by scavenging seals, or that they were hit by ducted ship propellers (cf. Thompson *et al.* 2010), were not supported. This difference between anthropogenic causes of death and predation has important implications for policy making and mitigation measures for the protection of this vulnerable small cetacean, since predation, in contrast to man-induced mortality, is a natural phenomenon.

Acknowledgements

Every porpoise used in this study has its own history, from the moment it was found on the beach until it arrived at the necropsy table. Collecting dead porpoises, particularly mutilated ones, is by no means an easy or pleasant task. Animals have been collected by many individuals, always on a voluntary basis. This work cannot be appreciated too much and we would like to thank all people who reported, photographed and collected dead porpoises. This research was funded by the Dutch Ministry of Economic Affairs (140000353).

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Appendix A. Examples of different lesions

Figure A1. *Zigzag (Jaap van der Hiele).*

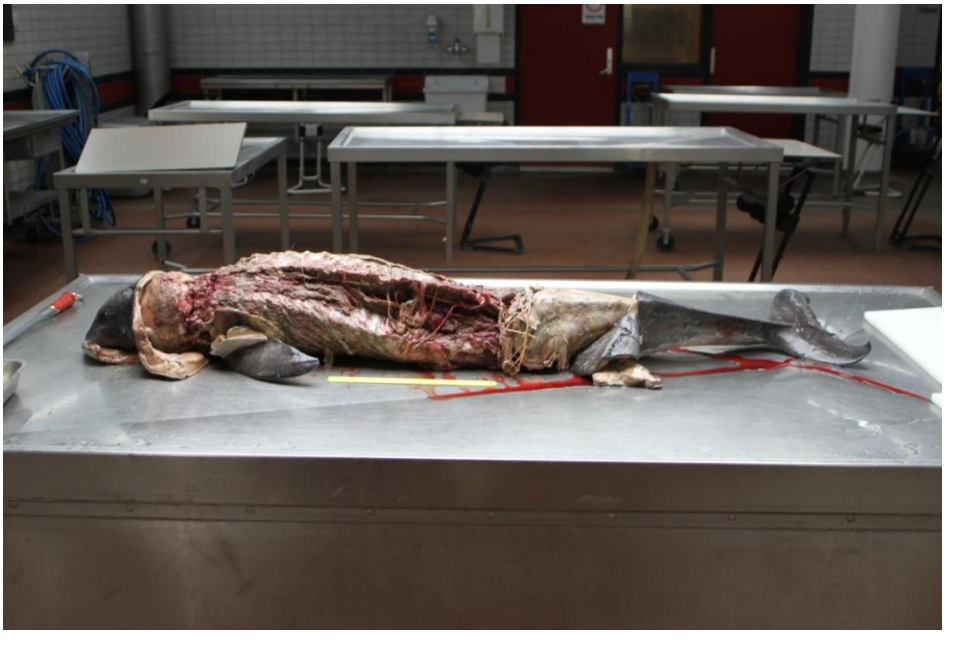

Figure A2. *Head-tail (Hans Verdaat, IMARES).*

Figure A3. *Throat/cheek (Utrecht University).*

Figure A4. *Circular body (Naturalis: www.walvisstrandingen.nl).*

Figure A5. *Body parts (Arnold Gronert).*

Figure A6. *Claw marks (Utrecht University).*

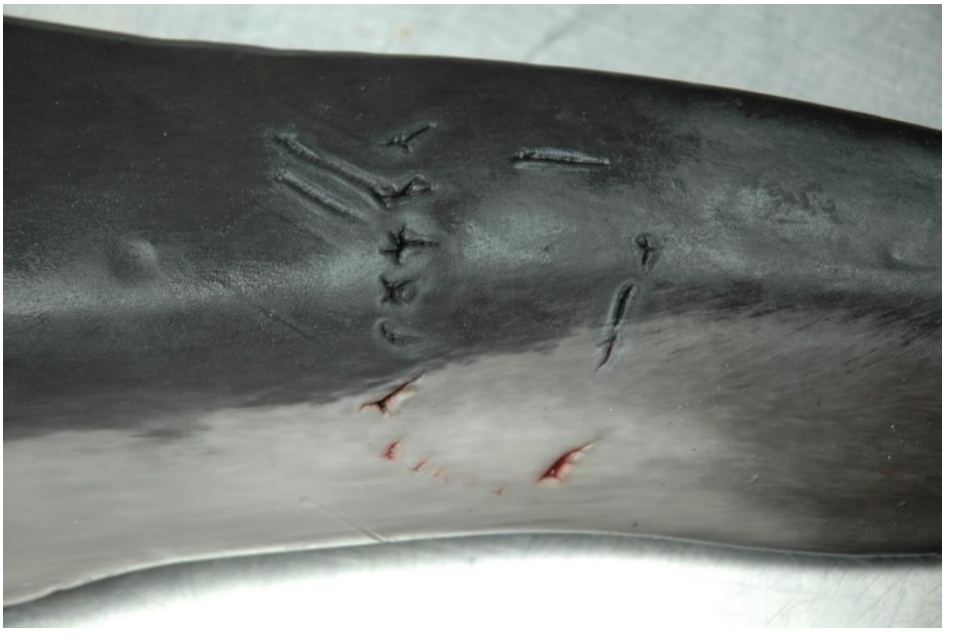

Figure A7. *Tailstock bite mark (Kees Camphuysen, NIOZ).*

Figure A8. *Cut in half (Utrecht University).*

Figure A9. *Anthropogenic: knife cuts (Jaap van der Hiele).*

Appendix B. Basic data for all porpoises included in the diet-part of this study, by mutilation category: zigzags, throat/cheeks and controls (overleaf).

ID: porpoise identifier; Marks: Minor marks, separate from major blubber defect, None (only when photographs showing all sides were available), Tailstock (bite) mark, Claw marks, Net marks, or unknown; Month and Year refer to stranding date, Lat and Long to stranding location; TBL is the total body length (cm), Sex: male (M), female (F), and unknown (?); Blubber is the average blubber thickness (see Material and methods section); Final columns give summed prey masses and prey numbers (in parentheses) for, respectively: gobies (common, Lozano's, sand, painted and transparent gobies), gadoids (bib, poor cod, whiting), clupeids (herring, sprat), Ammod. (Ammodytidae: greater, lesser and small sandeels), estuarine (estuarine roundfish: European perch, golden grey mullet, sand smelt, smelt, Nilsson's pipefish), fast pelgs (fast pelagic fish: Atlantic mackerel, Atlantic horse mackerel, European seabass), Demersal (other demersal fish: five-bearded rockling, viviparous blenny, flatfishes) and invert (invertebrates: brown shrimp, squids).

Cat.	ID	Marks	Month	Year	Lat	Long	TBL	Sex	Blubber	Gobies	Gadoids	Clupeids	Ammod.	Estuarine	Fast pelgs	Demersal	Invert
Zigzag	TX044	None	3	2006	51.8267	3.8438	119	F	17.7	1400.38 (1552)	0	69.61 (10)	30.48 (6)	61.54 (10)	196.67 (1)	0	0
Zigzag	TX024	?	4	2006	53.1781	4.8131	108	F	?	1244.35 (1979)	0	1.54 (1)	434.33 (298)	0	0	0	0.70 (1)
Zigzag	UT035	Claws	2	2007	52.9044	4.6915	130	F	24.3	95.77 (48)	0	606.80 (38)	431.92 (61)	0	0	0	0
Zigzag	UT008	?	3	2007	51.8516	3.9254	117	?	22	3312.06 (2578)	0	200.73 (13)	4.20 (3)	18.31 (1)	0	0	3.00 (3)
Zigzag	UT037	Tailstock	3	2007	51.8516	3.9254	~ 112	?	17.7	448.02 (1391)	0	0	2.63 (2)	0	0	0	4.88 (1)
Zigzag	UT038	Tailstock	4	2007	51.8267	3.8438	~ 88	?	16	1277.90 (1392)	37.40 (1)	0	0	59.17 (1)	22.85 (1)	0	1.00 (1)
Zigzag	UT047	Tailstock & net marks	3	2007	51.8516	3.9254	~ 100	M	11	428.93 (675)	0	0	0	0	0	0	0
Zigzag	UT129	None	3	2008	51.4571	3.5094	103	F	30	164.85 (225)	784.37 (9)	164.03 (3)	12.14 (3)	0	0	0	6.00 (6)
Zigzag	UT130	Tailstock & claws	3	2008	51.8516	3.9254	113	M	22	164.99 (336)	0	9.21 (2)	42.22 (9)	0	0	0	0
Zigzag	UT131	Claws	3	2008	51.8516	3.9254	111	?	?	1790.52 (2163)	587.25 (6)	74.16 (7)	4.95 (2)	146.58 (7)	0	0	0
Zigzag	UT195	Tailstock & claws	12	2008	53.0796	4.7081	116	M	22.7	1.88 (3)	0	264.48 (20)	41.07 (2)	0	0	0	0
Zigzag	UT204	Tailstock & claws	2	2009	53.1781	4.8131	121	M	22	0	91.77 (1)	674.98 (56)	2721.90 (219)	42.65 (1)	0	0	0
Zigzag	UT207	Tailstock & claws	2	2009	51.8516	3.9254	102	M	19.3	15.02 (22)	0	4.24 (1)	0	652.10 (12)	0	0	0
Zigzag	UT208	Tailstock & claws	2	2009	51.4571	3.5094	114	F	25	0.33 (1)	736.18 (10)	243.28 (31)	292.03 (45)	112.66 (2)	16.24 (3)	0	1.84 (1)
Zigzag	UT227	?	3	2009	53.1781	4.8131	105	M	15	150.40 (230)	0	196.64 (25)	39.11 (7)	0	0	0	1.00 (1)
Zigzag	UT280	Claws	1	2010	52.3216	4.4578	92	F	13.7	429.11 (282)	0	195.62 (16)	0	223.69 (42)	27.35 (4)	28.84 (3)	0
Zigzag	UT386	?	3	2010	51.8516	3.9254	~ 100	?	?	999.87 (2285)	0	149.33 (8)	6.77 (1)	0	0	0	0
Zigzag	UT675	?	2	2011	52.859	4.6806	118	?	?	1082.92 (1226)	0	1127.66 (96)	0	0	0	0	0
Zigzag	UT674	Tailstock & claws	3	2011	53.0434	4.685	110	M	23	767.87 (395)	102.73 (1)	0	2272.25 (484)	0.75 (1)	0	0	0
Zigzag	UT670	Claws	3	2012	53.4734	5.6038	104	M	23.7	1769.13 (2866)	0	753.60 (76)	485.65 (72)	79.04 (34)	0	0	9.98 (10)
Zigzag	UT693	None	3	2012	53.4287	5.8273	~ 110	F	13	3070.79 (5369)	42.55 (1)	0	111.66 (17)	27.92 (20)	0	0	0
Zigzag	UT697	T.stock? & claws	3	2012	51.8267	3.8438	104	M	?	6.01 (10)	0	0	0	0	0	0	0
Zigzag	UT701	Claws	3	2012	51.8516	3.9254	109	M	30	87.00 (158)	0	27.03 (3)	0	18.87 (1)	0	0	0
Zigzag	UT702	Claws	3	2012	51.8267	3.8438	107	F	20	1224.27 (1691)	94.25 (1)	211.78 (23)	35.62 (4)	21.12 (3)	3.39 (2)	0	0
Zigzag	UT704	Tailstock & claws	3	2012	51.8567	4.0029	96	M	15	224.86 (383)	0	349.52 (27)	0	117.33 (11)	0	0	0
Zigzag	UT711	Tailstock	3	2012	51.8267	3.8438	112.5	F	30	257.29 (397)	0	0	0	0	0	0	0
Zigzag	UT713	Tailstock	3	2012	51.8516	3.9254	101	F	21	45.95 (63)	0	120.24 (5)	15.94 (2)	478.08 (41)	0	0	4.51 (7)
Zigzag	UT714	Tailstock	3	2012	51.8516	3.9254	~ 99	M	22.7	45.93 (54)	0	96.07 (5)	0	1616.19 (89)	93.54 (10)	0	1.43 (4)
Zigzag	UT716	Tailstock	3	2012	51.8516	3.9254	123	F	16	465.94 (1014)	0	84.81 (8)	2.43 (1)	0	0	0	0
Zigzag	UT718	?	3	2012	51.8267	3.8438	~ 126	F	?	3.92 (5)	0	17.10 (1)	0	0	0	0	0
Zigzag	UT715	?	3	2012	51.8516	3.9254	~ 112	F	19.7	3.25 (5)	0	0	0	0	0	0	0
Throat/Cheek	UT197	Tailstock & claws	2	2009	53.1781	4.8131	103	F	19.7	0	0	232.06 (55)	0	0	0	0	0
Throat/Cheek	UT182	Tailstock & claws	12	2008	51.5067	3.4115	111	M	21.7	0	60.31 (1)	619.53 (19)	8.53 (2)	0	0	0	1.00 (1)
Throat/Cheek	TX046	Tailstock & claws	3	2006	51.6858	3.8107	101	M	28.3	4.64 (8)	711.71 (8)	96.35 (3)	15.41 (2)	0	53.82 (4)	0	0.70 (1)
Throat/Cheek	UT198	Claws	1	2009	52.9867	4.6978	128	M	20.7	0	1207.07 (5)	885.89 (47)	0	0	0	0	1.00 (1)
Throat/Cheek	UT203	Tailstock & claws	2	2009	52.7421	4.6247	107	M	22.7	7.46 (11)	0	738.06 (125)	1422.32 (227)	0	0	0	0
Throat/Cheek	UT219	Tailstock & claws	2	2009	53.0796	4.7081	104	M	26.7	0	0	1.58 (1)	0	0	0	0	0
Throat/Cheek	UT221	Tailstock	3	2009	52.9748	4.7624	127	M	23	0	0	1029.24 (35)	24.28 (4)	0	0	0	1.00 (1)
Throat/Cheek	UT235	None	2	2009	52.3216	4.4578	103	M	21.3	27.10 (35)	321.65 (1)	0	12.23 (2)	0	0	0	11.73 (6)
Throat/Cheek	UT450	Claws	3	2011	52.1505	4.2969	116	M	21.7	496.31 (454)	352.43 (5)	1466.36 (179)	37.48 (10)	41.45 (5)	0	0	10.50 (11)
Throat/Cheek	UT452	None	3	2011	52.3768	4.4921	~ 97	M	15.3	478.97 (769)	0	72.98 (8)	9.04 (2)	3.72 (2)	0	0	0
Throat/Cheek	UT205	Tailstock & claws	2	2009	52.7421	4.6247	111	M	23.3	0	0	715.09 (76)	58.25 (4)	0	0	0	0
Throat/Cheek	UT454	Tailstock & claws?	3	2011	52.3768	4.421	107	M	20	1351.70 (736)	0	235.87 (20)	9.37 (2)	28.40 (5)	0	0	0
Control	TX025	None	3	2006	53.0434	4.685	99	F	16	3.70 (7)	0	2.71 (1)	0	0	0	0	0
Control	TX030	None	4	2006	53.1309	4.7543	~ 110	F	27.3	25.46 (13)	0	155.78 (10)	13.68 (3)	1.63 (1)	0	0	0
Control	TX033	None	3	2006	53.1035	4.9269	107	M	28	0	0	0	0	0	0	0	1.96 (1)
Control	TX057	Net marks	4	2006	52.7009	4.6138	102	M	18.3	35.02 (36)	0	38.17 (4)	45.82 (10)	0	0	0	1.96 (1)
Control	UT111	Net marks	3	2008	51.4571	3.5094	114	F	24	9.04 (11)	194.84 (5)	0	21.82 (7)	0	0	0	0
Control	UT114	None	3	2008	52.1505	4.2969	105	M	25	711.24 (713)	105.98 (3)	2.59 (1)	131.94 (58)	0	0	0	2.96 (2)
Control	UT120	None	2	2008	53.0434	4.685	89	M	16	106.56 (255)	0	76.28 (5)	386.02 (27)	0	0	0	0
Control	UT209	None	12	2008	52.9867	4.6978	106	M	20	7.48 (11)	0	0	0	0	0	0	0
Control	UT210	Tailstock & claws	2	2009	52.3768	4.4921	112	M	22.3	22.83 (16)	177.41 (3)	336.00 (59)	187.95 (53)	0	0	13.99 (1)	1.00 (1)
Control	UT211	Tailstock & claws	2	2009	52.7009	4.6138	119	F	25.7	0	0	200.16 (12)	1811.45 (207)	0	0	0	0
Control	UT212	Tailstock & Claws	2	2009	52.7421	4.6247	128	M	23.7	0	0	0	9.68 (1)	0	0	0	0
Control	UT213	Tailstock & claws	2	2009	52.8094	4.6539	107	M	26.3	0	0	283.38 (53)	0	0	0	0	0
Control	UT214	Claws?	2	2009	52.7421	4.6247	101	M	22.7	0	0	811.20 (27)	0	0	0	0	0
Control	UT216	Tailstock & claws	2	2009	52.859	4.6806	117	M	20	0	0	276.15 (7)	21.53 (3)	0	0	0	0
Control	UT220	Tailstock & claws	3	2009	52.8094	4.6539	98	M	22.7	0.83 (3)	135.33 (3)	280.06 (52)	806.79 (58)	0	0	0	0
Control	UT223	None	3	2009	52.7421	4.6247	92.5	M	20.3	39.04 (46)	0	273.57 (29)	27.66 (2)	0	0	0	1.00 (1)
Control	UT224	Tailstock & claws	2	2009	53.0796	4.7081	103.5	M	21	233.80 (156)	102.11 (1)	298.51 (34)	1442.21 (178)	0	0	0	1.00 (1)
Control	UT228	Net marks	12	2008	53.1781	4.8131	129.5	F	18.7	150.77 (104)	2238.15 (17)	70.27 (5)	0	0	0	0	2.46 (3)
Control	UT229	Net marks	3	2009	51.5067	3.4115	109	M	26.3	71.52 (45)	1399.54 (11)	0	2.56 (1)	0	0	0	5.00 (5)
Control	UT231	None	4	2009	52.9044	4.6915	121	F	24	0.57 (1)	41.27 (1)	172.00 (2)	126.56 (5)	0	0	0	0
Control	UT232	None	2	2009	52.1505	4.2969	102.5	F	18	0	0	0	0	0	0	0	7.78 (9)
Control	UT243	Claws?	12	2008	53.1309	4.7543	124.5	F	23.7	0	461.49 (8)	0	0	0	0	0	0
Control	UT393	None	1	2011	52.1505	4.2969	120	M	20.3	0	0	10.34 (1)	0	0	0	0	0
Control	UT395	None	3	2010	53.4734	5.6038	122	M	25	34.89 (41)	0	0	206.71 (41)	0	0	0	111.17 (48)
Control	UT421	Net marks	3	2011	53.4838	5.918	117	F	20.3	407.76 (494)	0	34.46 (3)	293.37 (47)	4.29 (2)	0	0	0
Control	UT422	None	3	2011	52.0794	4.1988	108	M	15	9.75 (9)	23.35 (1)	0	0	14.88 (8)	0	0	0
Control	UT435	Net marks	4	2011	52.9044	4.6915	105	M	16.3	1080.66 (1169)	5.04 (2)	34.80 (4)	10.62 (2)	24.29 (5)	0	0	0
Control	UT453	Net marks	3	2011	52.3216	4.4578	125.5	F	24.3	0	0	141.19 (44)	34.76 (11)	0	0	0	0
Control	UT668	Net marks	3	2011	52.7421	4.6247	110.5	M	18.7	1167.43 (1185)	0	59.93 (4)	40.30 (3)	15.39 (4)	0	0	0
Control	UT669	Tailstock?	3	2011	52.1088	4.2103	105.5	M	20	36.67 (75)	0	53.37 (12)	19.13 (1)	27.53 (15)	0	0	0
Control	UT682	Claws?	3	2011	53.4838	5.918	109.5	F	23.7	6.79 (10)	0	0	2.38 (1)	11.27 (3)	0	0	0

Synthesis

Are porpoises opportunistic foragers?

9

9 Are porpoises opportunistic foragers? What are the constraints for feeding of this supposed top-predator?

Synthesis

The principal goal of the research presented in this thesis was to gain a better understanding of the feeding ecology of harbour porpoises in Dutch waters. At the start of this work, I realised that no two porpoises are likely to be equal. Because of this I was not satisfied with the classic approach of the many diet studies already conducted, that describe a "population average" of porpoises diet, or for that matter, of any other animal, in a particular situation. Important questions were: which factors are at play in pushing diets of individual porpoises away from this population average? How much is diet governed by the prey on offer (which is largely unknown in sufficient detail) or are for instance ontogenetic developments important in the prey choice of porpoises? Do males differ from females in their prey choice, and if so, are such differences age-related? How is sampling affected by the particular year(s) in which animals can be sampled? What is the seasonal variation in prey taken? Are there differences between individual porpoises, in requirements for specific foods or in their skills to obtain these prey? Are there, in other words, more or less fixed patterns, or "rules" governing the prey choice of individuals, given the prey on offer?

The mere fact that such questions are not being addressed in most diet studies may indicate that these questions may be easily asked but not easily answered. Three main obstacles stand in the way of those trying to address such questions. The first is that for each animal studied, basic information needs to be available on the parameters that are to be considered (age or length, gender, health status, time and cause of death, location were the last meal was ingested). Some of these (such as gender, location, length) can be solved by simple book keeping, others are more difficult or even impossible to pinpoint directly. Close cooperation with veterinary pathologists ensured that the best possible information on health status and cause

of death was obtained, but this in itself was a process with a learning curve. Learning curves are unavoidable in research, but also unwanted as these all too often result in finding a "year effect" later. We were therefore very careful, from the onset of this project, to document all findings, so that preliminary conclusions could later be changed if necessary, after new information, or new insights had become available. This became evident after we realised that some 200 mutilated carcasses, that had initially been labelled as highly probable cases of fisheries bycatch, were in fact victims of grey seal predation (Chapter 7), and, of particular relevance to the diet study, that several types of attack could be inferred from the wounds present, and documented in sufficient detail for post-hoc evaluation. Subsequently, from the specific types of attack wounds inflicted on these porpoises, we could identify two ways of foraging: close to the bottom or higher in the water column, as shown by clearly different stomach contents (Chapter 8). This –unexpected- new insight also made us explore the possibility that stomach contents could help identify cases of bycatch better (Chapter 4). The co-operation between biologists and pathologists thus payed off, both ways.

The second obstacle is the matter of sample size. If one lets go the idea that all porpoises are equal and splits the available sample of analysed stomach contents in e.g. those of males and females, sample size for each becomes circa half that of the original sample size. Going further, looking also at season further divides sample sizes in each remaining cell, by 12 if the data are broken up by month. Adding year, porpoise age (or size as a proxy), cause of death, health status makes one quickly reaching the point where sample sizes for particular combinations become too small for statistical testing: reality stands in the way of ambition here. One answer to this problem is that one tries to sample as many animals as possible. Adding extra years is one possibility, even if year itself is one of the dividing factors: it is only one such factor and in any one year we can increase sample size for all other factors. Another way forward is to use stratified sampling: sample those animals preferably, that represent categories for which still relatively little information is available. In the course of this study it was realised that the majority of the sampled animals were juveniles, and therefore more effort was put into collecting, and analysing large adults. Likewise, when we realised that few animals were available from the fringes of the study area: the Eastern and Western Scheldt, the Wadden Sea and the River Eems, we specifically tried to have more dead animals collected from these places. Estuarine waters may be ecologically quite different from the open North Sea, also for harbour porpoises and hence the foods taken there may be quite different. This approach has been successful for the Western Scheldt (Chapter 5), while sample size for the Eastern Scheldt and Wadden Sea still fall short of allowing separate analyses and discussing the situation in these waters in much detail. The

situation for the river Eems is the worst: to date only two harbour porpoises could be collected for research here, so more work is needed and every effort will be made to increase sample size here.

The third problem that needed tackling was the complexity of a diet study that takes many co-variables into consideration. In all likelihood, several factors are at play simultaneously, pulling or pushing into the same, opposite or unrelated directions. This also touches upon the problem of biased (stratified) sampling. Care should be taken not to sample only in one particular region in one particular year. Interactions between the several factors acting upon porpoise prey choice are best identified if roughly equal sample sizes are available for all relevant cells. However, by using multi-variate analysing techniques, this prerequisite could be slightly relaxed and effects of e.g. year, month, and porpoise length could be assessed while taking the other co-variables into account.

The main findings of this study

The first main finding is, that gobies (in fact, mostly sand gobies *Pomatoschistus minutus*), gadoids (mostly whiting *Merlangius merlangus*), clupeids (herring *Clupea harengus* and sprat *Sprattus sprattus*) and sandeels (both *Ammodytes marinus* and *A. tobianus*, as well as *Hyperoplus lanceolatus*) were the "big four" of porpoise diet. Considering that these four stand out, over nine years, 12 months, the full length of the Dutch coast line, the two genders and all ages and health classes of porpoises, this may seem quite a restricted diet, but probably contains everything a porpoise needs during its life time: easy-to-catch small prey for first-time foragers on solid food; protein for growth and energy for thermoregulation and movement and large prey for those with a big appetite. On the side, porpoises take a wide variety of other prey that may simply be taken opportunistically, for no better reason than that these are available, or because these contain some critical component for general sustenance.

The second main finding is that there is a clear ontogenetic development in prey choice. Porpoises start taking solid foods while still accompanied by their mother, and probably while still nursing. The first prey are small: mostly gobies. These are bottom-dwelling fishes of only a few cm long, and were found to be on average one gram in body mass. Young porpoises quickly become very efficient foragers on gobies. We have seen many stomachs containing hundreds, and 30 containing the remains of over one thousand gobies (the record-holder had remains of 5,369 gobies in its stomach). Although this is impressive when seen from a foraging effort

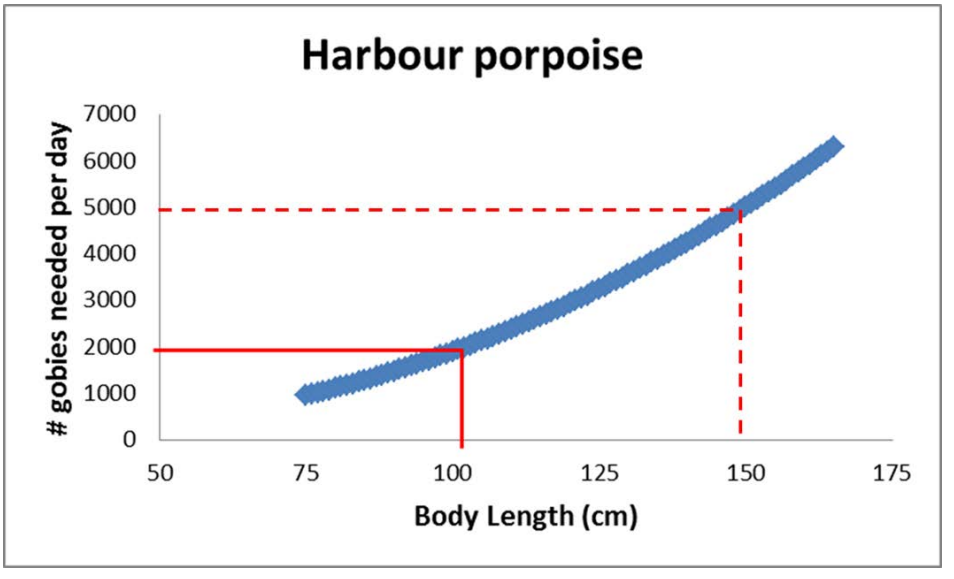

Figure 1. *The number of 1 gram gobies needed per day for porpoises of various lengths to remain energetically neutral. Body mass is estimated using the equation that relates body mass to TL for animals in very good nutritional condition (NCC 1: Chapter 3, Table 8) and the outcome is divided by 10 to get the number of gobies needed, daily.*

point of view, this number of gobies could satisfy the caloric needs of a full-grown harbour porpoise for just one day. I have summarised in Chapter 3 the available information on the daily rates of food needed for a porpoise to maintain a neutral energy balance. This is roughly estimated to be 10% of its own body mass. Even for a small porpoise, it would seem to require a lot of effort to reach that amount, if feeding solely on 1 gram gobies (Figure 1).

A young porpoise of 1 meter long would require 2000 gobies daily. This must be a feasible number, given the numbers of otoliths found in some of the examined stomachs. Note, however, that a 24h day has 1440 minutes, and that unlike rorqual whales, porpoises are no filter feeders. A porpoises must find, catch and ingest each prey in turn and a rate of 2000 gobies per day equals nearly 1.4 such sequences per minute, if feeding would be continuous. Clearly, a porpoise cannot be feeding continuously at the sea floor as it also has to engage in other activities, so the actual rate of ingestion must be quite a bit higher than this. If a porpoise growth to a length of 150 cm, the required rate goes up to 5000 gobies per day, or 3.5 per minute at continuous feeding. There must be a limit to the foraging performance of porpoises, and from this it follows that larger porpoises simply must switch to larger, and/or energy-richer prey, to keep the daily intake at the required level. It seems feasible

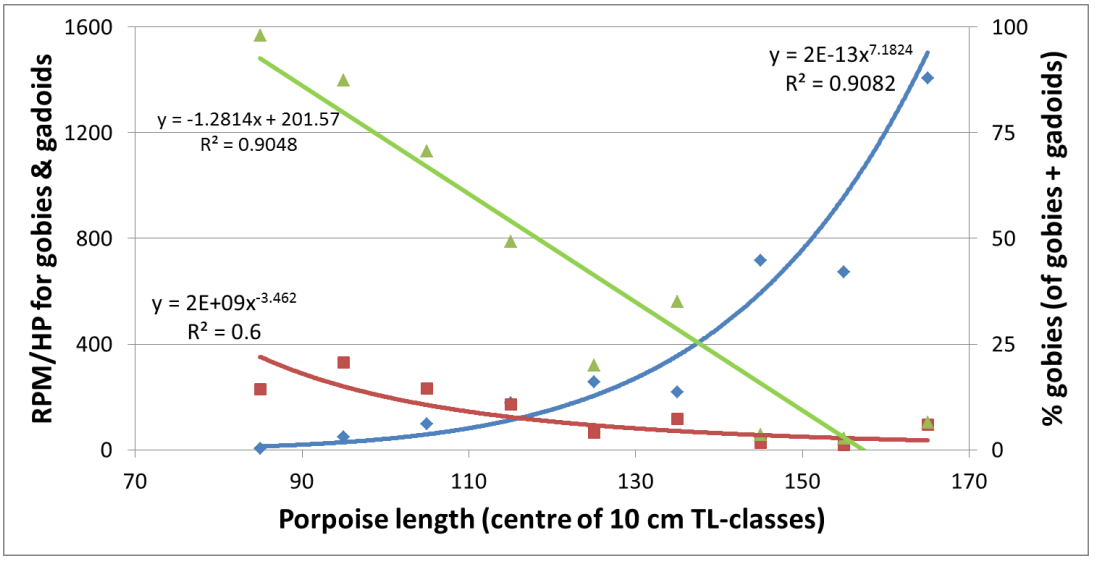

***Figure 2**. Absolute amounts of reconstructed prey mass per harbour porpoise (non-empty stomachs only) for gobies (red) and gadoids (blue), with the percentage of gadoid mass in green (right Y-axis).*

that young porpoises must learn to catch larger, faster prey and that they need to master the necessary skills in time to counter their own increase in energy demands.

This is exactly what we find (Figure 2). Considering only gobies, numerically the most important prey, and gadoids, most important in terms of prey mass, we see that the absolute amount of reconstructed mass of gobies found in porpoise stomachs decreases slowly but steadily (and significantly) with porpoise length. At the same time the amount of gadoid prey mass increases, and does so more steeply, indicating that the total prey intake of porpoises increases with their own body mass (Chapter 2).

The third major finding is that of seasonal variation. In summer, reconstructed prey masses are significantly lower than at other times of year. Also in summer, porpoise blubber thickness is at its lowest, across all length classes (Figure 3), with the smaller animals with the thickest blubber, losing most, in summer.

At first glimpse both may simply be adaptations to needing less energy for thermoregulation in summer. However porpoises are ill-adapted to live in warmer waters (Gaskin 1982) and may quickly reach the upper limit of their thermo-neutral zone, particularly when exercising (feeding). Like the larger whales, known to be

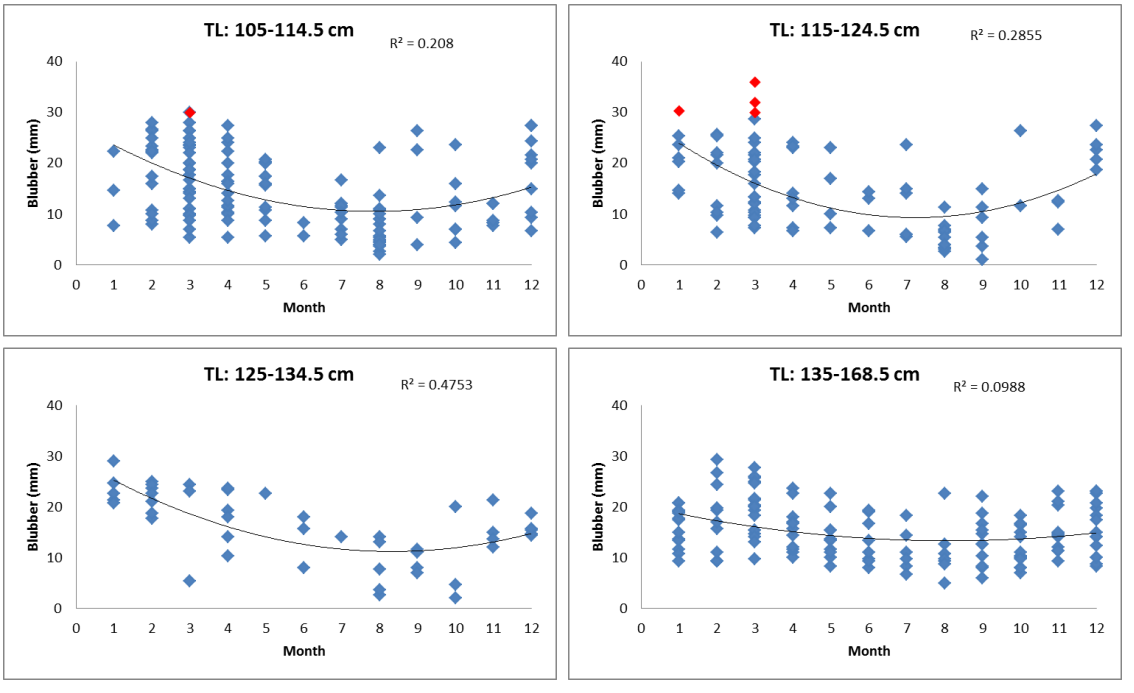

***Figure 3**. Blubber thickness (see Chapter 3) for individual porpoises in this study, grouped by body length and by month. Few reach 3 cm blubber thickness (red dots) and only so in winter (January-March).*

subject to overheating when exercising, harbour porpoises may also have trouble to keep feeding efficiently in summer, and lose blubber as a result (Rosen *et al*. 2007). The fact that particularly young animals that died in summer, do so in a very poor nutritional state (Chapter 2, Figure 3), points in this direction.

The fourth major result is the finding that, once a porpoise loses body mass, it may get into a downward spiral of reduced insulation, higher costs for thermoregulation and possibly poorer diving performance due to a less positive buoyancy (Rosen *et al*. 2007). Should this lead to a very poor nutritional condition (NCC 5 or 6) the animal may lose the capacity to catch the most profitable, high-energy prey (Chapter 3, Figure 5), that it would seem to need especially when reaching such a condition, and the process may become irreversible, leading to its death.

Chapters 4 and 8 focus on bottom-feeding versus mid-water feeding. The prey to be caught in these two different micro-habitats are different and this is reflected in the stomach contents of animals that died suddenly, and violently. Chapter 4 shows that animals that probably died by drowning in bottom-set gillnets mostly had been

feeding near the sea floor. Therefore, the specific stomach contents of animals presumed drowned, may help to identify victims of such bycatch. Along similar lines, we could show in Chapter 8, using porpoise stomach contents, that grey seals, as newly discovered predators of porpoises, catch their prey both near the sea floor and higher in the water column and with this finding we could better interpret the various attack wounds left on their victim's bodies, which helped convincing the many sceptics of the hypothesis that grey seals are not just cuddly animals, but fierce predators.

Chapters 6 and 7 provide the definite proof, both by DNA evidence and by careful pathological study, that grey seals are indeed predators of harbour porpoises and that (Chapter 7) large numbers of porpoises are involved: 25% of the carcasses bore bite and claw marks attributable to grey seals. The hard evidence laid out in these two chapters helped convince most, if not all who first thought it impossible that a "fish eating seal" would take on another marine mammal, as prey, although apparently, some work still needs to be done across the Atlantic, where vast numbers of mutilated seals have been found, that have been attributed to shark predation (Haelters *et al*. 2015).

Future work

As stated earlier: reality has stood in the way of ambition, during this study. Although we got more than we bargained for, i.e. the studies on seal predation, some other leads have not yet been followed in full. The evidence for dietary differences between male and female porpoises could be made a lot stronger, if more adult animals can be included in this study. Suggestions in the scientific literature (Jansen *et al*. 2013), that harbour porpoises in the Eastern Scheldt are trapped there and suffer a relatively high mortality rate have not yet been fully verified due to small sample sizes for this former estuary. Suggestions that the supposed high mortality would be related to poor nutritional condition, which in turn might be caused by a lean diet in the Eastern Scheldt, can thus also not yet be checked thoroughly. Although the current state of our knowledge shows that other factors (porpoise size, health status and season) are more important in shaping porpoise food intake (both quantitatively and qualitatively) than regional or gender-related factors, these could still be important, either locally, or for animals of a certain age.

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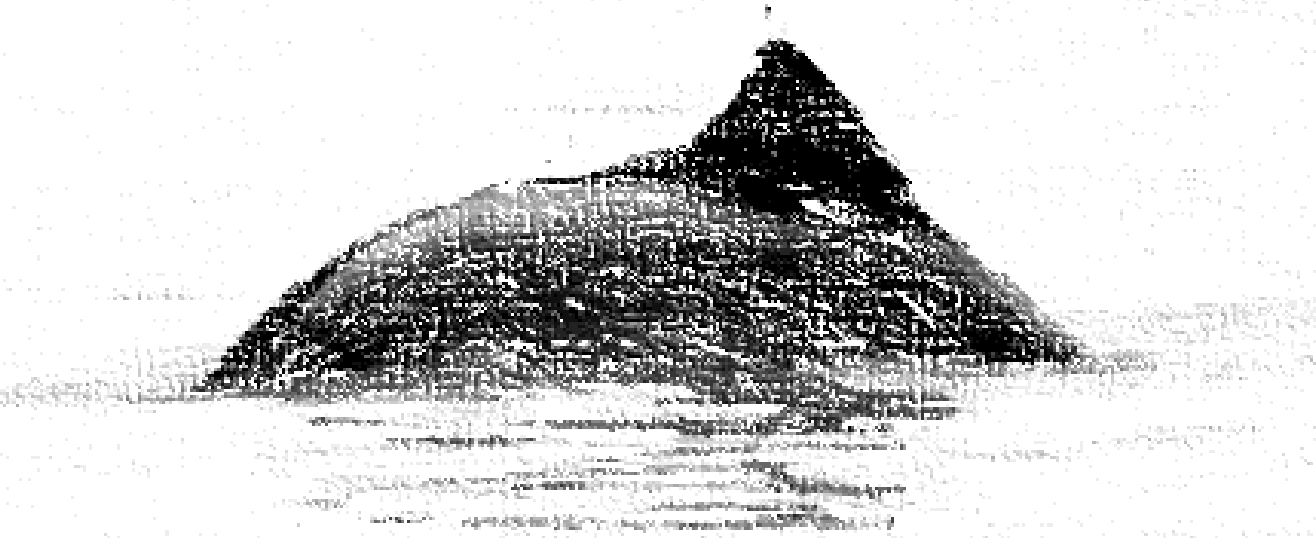

Addendum

10

10 Addendum

Curriculum vitae

Maarten Frederik (Mardik) Leopold werd geboren op 1 november 1957 in Haarlem. Na het doorlopen van het VWO aan het Coornhert Gymnasium in Gouda, ging hij biologie studeren in Utrecht. Tijdens de doctoraalfase van die studie, mocht hij van zijn Universitair hoofddocent Dr Aldo Voûte, de "betonnen" Utrechtse Uithof verruilen voor Texel, waar hij onderzoek ging doen aan het NIOZ, onder leiding van Kees Swennen, eerst aan de voedselecologie van scholeksters, en vervolgens aan het voorkomen van zeevogels op het Friese Front, in de Noordzee. Na het afstuderen volgde een veel groter onderzoek aan de verspreiding van zeevogels op de gehele Noordzee. Bij dit onderzoek werd ook Kees Camphuysen betrokken, vanwege zijn ongeëvenaarde kennis van de Nederlandse zeevogels. De beide "Kezen" hadden ook al ervaring met het doen van dieetonderzoek aan visetende zeevogels, aan de hand van in de maag achterblijvende gehoorsteentjes van vissen, de otolieten. In de marge van het onderzoek vonden drie gebeurtenissen plaats die later bepalend bleken voor de verdere loopbaan: de stranding van een "exotische" zeehond, een zadelrob, op Texel, die nog voedselresten in maag, darm en feces bleek te hebben; een olie-incident waarvan talloze alken en zeekoeten slachtoffer werden op Texel, en aanvankelijk schaarse, maar allengs talrijkere waarnemingen van bruinvissen tijdens het tellen van zeevogels op de Noordzee. Dit laatste maakte Mardik tot een van de weinige "bruinvisdeskundigen" in Nederland. Ondertussen was ook een onderzoek gestart naar prooiresten in de magen en darmen van roodkeelduikers, wintergasten onder de Nederlandse zeevogels, die in slechts kleine aantallen op onze kust aanspoelen en waarvan vrijwel niets bekend was van hun dieet. Deze combinatie van factoren leidde tot het besef dat er legio kansen waren voor dieetonderzoek aan zeevogels en zeezoogdieren. Alleen een goede referentiecollectie van otolieten van de vissen uit onze regio ontbrak nog. Dit gemis werd opgevuld door het zelf aanleggen van een dergelijke collectie, op basis van >10.000 vissen, en het vastleggen en toegankelijk maken van die collectie op CD-ROM. Met dit gereedschap, en steeds betere microscopen en groeiende kennis van de otolieten van de vissen in onze regio, werd een waaier van dieetstudies opgezet:

Kees & Kees waren lichtende voorbeelden als het ging om het grijpen van kansen voor (dieet)onderzoek en het publiceren van de bevindingen. Langzaam maar zeker werd een overgang gemaakt van werken bij het NIOZ naar (voorgangers van) IMARES. Daarbij bleven de banden met Kees Camphuysen en andere onderzoekers van het NIOZ altijd hecht en kon het dieetwerk worden voortgezet en uitgebreid. Een groot onderzoeksproject aan bruinvissen dat startte in 2006 maakte grote aantallen van deze zeezoogdieren beschikbaar voor onderzoek: een enorme kans om het dieet van deze moeilijk toegankelijke dieren nader te onderzoeken. Aanvankelijk vond dit onderzoek plaats op Texel, opnieuw in een IMARES/NIOZ combinatie; vanaf 2007 verschoof het pathologie-deel van het werk naar de faculteit Diergeneeskunde aan de Universiteit van Utrecht, onder leiding van professor Andrea Gröne. In de snijzaal in de Uithof (!) werd intensief samengewerkt met achtereenvolgens (en overlappend in de tijd) Lidewij Wiersma, Lineke Begeman en Lonneke IJsseldijk en dit samenspel leidde tot een grote opbouw van kennis van de bruinvis en tot een reeks van publicaties, uitmondend, onder andere, in dit proefschrift, onder auspiciën van professor Peter Reijnders. De focus op de bruinvis maakte dat andere dieetonderzoeken tijdelijk op een lager pitjes moesten, maar het dieetonderzoek eindigt niet met het voltooien van dit proefschrift: er staat nog een reeks studies aan zowel bruinvissen als andere zeezoogdieren en (zee)vogels op stapel.

Summary

The harbour porpoise is one of the smallest cetaceans worldwide, and the most numerous cetacean in the North Sea. With its small size and general diet (mostly small fish), it shares major ecological characteristics with large fishes, but also is clearly different from these, as it is warm-blooded. This, together with its large body surface to volume ratio, makes that a porpoise needs relatively large amounts of food. Prey with a high energy density should thus be preferred, if these are readily available. Across its range, harbour porpoises have been found to take many such prey species: small schooling fish with a high lipid content. However, leaner prey species are also taken, indicating that porpoises are not obligatory food specialists of energy-rich prey. Specific needs, as well as foraging skills, probably differ between individual porpoises and this thesis first focusses on how these differences influence the diet. Porpoise age and body condition, as well as time of year were found to be important factors (chapter 2), within the southeastern North Sea where this study was conducted. The relationship between porpoise body condition (its nutritional state) and its diet is further explored in chapter 3; the relationship between location, both in a geographical sense and vertically, in the water column, is examined in chapters 4, 5, and 8. Chapters 4 and 8 are cross-overs to two other main subjects in Dutch harbour porpoise studies, the correct identification of fisheries bycatch in stranded porpoises and finding the perpetrator responsible for the hundreds of severely mutilated porpoises that have washed up on Dutch shores during the past decade, respectively. In chapter 4 we show that stomach content analysis can help identify bycatch victims. Chapters 6-8 give an in-depth analysis of the mutilations, revealing predatory grey seals as the perpetrators in a "who-dunnit". Harbour porpoises were found to face a complex of constraints, complicating their already intensive foraging. They are not the top predators they may have been supposed to be, but were found to be prey themselves as well.

Chapter 1 introduces the subject of this study, the harbour porpoises as a small, warm-blooded piscivorous predator, with a big appetite. It also introduces the central question of this thesis: what do harbour porpoises eat, and for what reason, and which constraints do they face while foraging?

Chapter 2 explores the various factors that influence the diet of individual porpoises:

Diet studies of marine mammals typically summarise prey composition across all individuals studied. Variation in individual diets is usually ignored, but may be more than just "noise" around an optimal foraging strategy that should be the same across the entire population. Instead, different individuals may have both different

needs, and different skills to fulfil their requirements and diets may differ structurally between different groups of individuals within a population. Here we show that diets of harbour porpoises differ with age and nutritional condition of individuals, as well as seasonally. Even though all porpoises should probably strive to feed, at least partially, on energy-rich prey, such as clupeids or sandeels, the diet of juveniles is dominated by small, lean, gobies, and that of adults by larger, but also lean gadoids. Prey with a relatively high energy density was found in only a third of the porpoises with non-empty stomachs, and in about one quarter of all porpoises. In a multivariate assessment of prey composition against factors such as porpoise size, season and porpoise body condition, we found the highest proportion of empty stomachs, the lowest reconstructed prey masses in non-empty stomachs, and the lowest proportion of energy-rich prey in summer. We also found lower reconstructed prey masses in porpoises in poorer condition. Our results show that individual differences matter, in that porpoise diet develops with porpoise size (as a proxy for age) and that this development may be affected by the change of the seasons, and by individual mishap, leading to starvation.

Chapter 3 focuses on the interplay between porpoise body condition and diet, in a region where relatively many lean prey are included in the diet:

The distribution of harbour porpoises in the North Sea has shifted southwards in recent years. Apparently, many animals left areas previously rich in sandeels and moved to a region where much leaner gobies and gadoids are important prey. This shift in range, and presumably in diet, does not seem to have affected the body condition of all porpoises in the South. Body condition varies in stranded specimen found in The Netherlands, from very good to very poor. Emaciation is a common cause of death in this species, indicating that periods of decreased quantity or quality of prey can be detrimental to the species. The question thus arises whether emaciated harbour porpoises could not find sufficient food or whether their food was of insufficient quality. Stomachs of emaciated animals are not necessarily empty but, in fact, often contained food remains. In this study we examine these remains and compare the prey composition of well-nourished porpoises to that of progressively leaner specimens, collected between 2006 and 2014. We hypothesize that porpoises might starve by eating relatively too much prey with a low fat content that has a low energy density. Such food may be referred to as junk food: prey that is too lean for maintaining a good body condition. Results show that there is a significant difference in prey composition between animals in a good body condition and animals in a poor body condition, that starving animals have fewer prey remains in their stomachs, and that these prey, on average, are of lower quality. Healthy harbour porpoises take a mixture of fatty fish and leaner prey: the "big four" in dietary terms are clupeids and sandeels with a relatively high fat

content, and gadoids and gobies, which are leaner prey. Our findings show that there is a negative correlation between the loss of body mass and the ingestion of fatty fish. This indicates that the emaciation is likely due to a lack of energy-rich prey, and that harbour porpoises need these prey in their diet to prevent starvation.

Chapter 4 looks at the diets of porpoises supposedly bycaught in bottom-set gill nets, aiding in the correct identification of bycatch victims:

Fisheries bycatch, particularly in bottom-set nets, is an important cause of death in harbour porpoises. Identifying bycatch from post-mortem studies on stranded porpoise carcasses is often difficult and relies on a combination of features. One characteristic considered consistent with bycatch is a full stomach, as this signals an acute death. Here we show that when porpoises are mostly bycaught in bottom-set nets, prey species composition, rather than the quantity of prey remains in their stomachs is the most informative characteristic to identify bycatch. Certain and highly probable bycatches (i.e., those porpoise carcasses brought in by fishers or with net marks and other evidence of bycatch) had a high proportion of demersal fish prey in their stomachs, usually >94% by mass of all fishes identified. Less certain cases, so-called probable and possible bycatches, included progressively more animals with lower percentages of demersal fish prey mass. The certain and highly probable bycatches also tended to have higher proportions of demersal prey in their stomachs, compared to animals that had died from other causes of death (e.g., emaciation, infectious disease, grey seal predation or unknown causes). This relationship was used to improve the classification of those porpoises classified as probable or possible bycatch. Prey species composition may thus be used as an additional bycatch criterion during post-mortem studies of stranded cetaceans, if the type of fishery responsible for the bycatches is known.

Chapter 5 deals with the diet of porpoises returning into the river Scheldt, in the wake of river clean-up and returning diadromous fishes:

As rivers are being cleaned, life is returning to their estuaries and higher parts. Diadromous fish species are on the increase again in many major rivers discharging into the North Sea. Harbour porpoises, being predators of fish, have been noted to return to North Sea estuaries and rivers as well. Their mere presence in these rivers, however, is no proof that these small cetaceans actually exploit the returning fish. Diet studies of the porpoises found up-river can shed a light on their prey choice and ecological role in the system. Here we show that a major part of the diet of porpoises found in the river Western Scheldt (2007-2014) comprises diadromous fish, particularly juvenile European smelt (*Osmerus eperlanus*). Smelt contributed 46% to porpoise diet (% prey mass) in the Western Scheldt, against

14% in the river mouth and 3% in the North Sea at either side of the river mouth. Even though porpoise numbers are increasing in the river, not all is well, however. Animals found dead on the river banks were generally in a poor nutritional condition and had an elevated probability of being found dead with an empty stomach. Animals swimming very far upstream sometimes braved major water works such as sluices, which might have hindered their return to the sea. Relatively many animals were reported dead later, but to date, too few have been collected for stomach content analysis to make a valid comparison between diets in the lower and higher parts of this river system possible.

Chapter 6 provides the hard (DNA) evidence that grey seals are predators of porpoises:

DNA was analysed from external wounds on 3 dead harbour porpoises that were stranded in the Netherlands. Puncture wounds as well as the edges of large open wounds were sampled with sterile cotton swabs. With specific primers that target the mtDNA control region of grey seal, a 196 bp DNA fragment was amplified from 4 puncture wounds. Sequencing of the fragments confirmed the presence of grey seal DNA in the puncture wounds. DNA sequences differed between the cases, implying that 3 individual grey seals were involved. As 8 control swabs from intact skin and the transport bag as well as 6 swabs from open wounds on the same harbour porpoises were all negative, contamination with environmental DNA is considered unlikely. The results provide a link between strandings of mutilated harbour porpoises and recent observations of grey seals attacking harbour porpoises. Ours is the first study to use forensic techniques to identify DNA in bite marks from carcasses recovered from the marine environment. This approach can be extended to identify other marine aggressors, including cases involving persons mutilated at sea.

Chapter 7 gives the pathological evidence and incidence of grey seal predation of harbour porpoises:

Harbour porpoises stranding in large numbers around the southern North Sea with fatal, sharp-edged mutilations have spurred controversy among scientists, the fishing industry and conservationists, whose views about the likely cause differ. The recent detection of grey seal DNA in bite marks on three mutilated harbour porpoises, as well as direct observations of grey seal attacks on porpoises, have identified this seal species as a probable cause. Bite mark characteristics were assessed in a retrospective analysis of photographs of dead harbour porpoises that stranded between 2003 and 2013 (n=1081) on the Dutch coastline. There were 271 animals that were sufficiently fresh to allow macroscopic assessment of grey seal-

associated wounds with certainty. In 25% of these, bite and claw marks were identified that were consistent with the marks found on animals that had tested positive for grey seal DNA. Affected animals were mostly healthy juveniles that had a thick blubber layer and had recently fed. We conclude that the majority of the mutilated harbour porpoises were victims of grey seal attacks and that predation by this species is one of the main causes of death in harbour porpoises in The Netherlands. We provide a decision tree that will help in the identification of future cases of grey seal predation on porpoises.

Chapter 8 looks into the stomach contents of grey seal victims, as compared to porpoises that died from other causes and provides evidence that porpoise prey is related to the wounds inflicted on them, showing that grey seals may attack porpoises anywhere in the water column:

Along the Dutch shores hundreds of harbour porpoises are stranded each year. A recurrent phenomenon in the Netherlands is a surge of strandings in late winter and early spring of severely mutilated porpoises, that are mostly in good nutritional body condition (thick blubber layer). These mutilated porpoises have parts of the skin and blubber, and sometimes of the muscle tissue missing. By reviewing photographs of stranded animals taken at the stranding sites as well as necropsy results we found 273 mutilated animals from 2005 to 2012. Mutilations could be classified into several categories, but wounds had been mostly inflicted to the sides of these animals, in a zigzag fashion, or to the throat/cheek region. The stomach contents of 31 zigzags, 12 throats/cheeks and 31 control animals that were not mutilated, from the same age and blubber thickness categories were compared; all these animals had stranded between December and April, 2006–2012. The diet of individuals with zigzag lesions to their sides consisted for a large part of gobies, while animals that had wounds at the throat/cheek had been feeding predominately on clupeids. In comparison, animals without mutilations had a more varied diet, including gobies and clupeids, but also a large proportion of sandeels and gadoids. The finding that the type of mutilation corresponds to a certain diet suggests that porpoises that were feeding on different prey, or in different micro-habitats, were hit in different ways. Animals feeding at the sea floor (on gobies) apparently run a risk of being hit from the side, while animals supposedly feeding higher in the water column (on schooling clupeids), were predominantly hit from below, in the throat region. The wider variation in the diets of non-mutilated porpoises is suggestive of them using a larger variety of micro-habitats.

In the final chapter (9) the individual component of porpoise diet is underlined. Diet is shaped, within the possibilities of local prey assemblages, by the individual qualities of the predator, such as its age (and presumably, foraging skills) and nutritional body condition. The importance of such co-variance can only be appreciated if porpoises are seen individually, through amalgamation of data linked to date and place of stranding, and detailed data from pathological studies. With the sample size available, several factors were found to shape porpoise diet; more such factors may become known with future larger sample sizes. The main findings of this study were: 1) that there is a "big four" in porpoise diet, consisting of a mix of relatively lean gobies and gadoids, and more fatty fish: clupeids and sandeels, while other prey, such as smelts are only locally important; 2) that there is an ontogenetic shift from a predominance of gobies in the diet of juvenile porpoises towards eating more gadoids as porpoises get older (larger); 3) that diet, but also the nutritional condition of porpoises varies with the seasons, with the summer appearing to be a particularly difficult time for porpoises in Dutch coastal waters; 4) that porpoises which lack fatty fish in their diet are often in poor nutritional condition; 5) that the stomach contents, more specifically the proportion of demersal fish in the diet, may help identify victims of bycatch in bottom-set gill nets; and that, 6) likewise, the type of foods found in the stomachs grey seal victims can be linked to the wounds found on the carcasses and, presumably the foraging micro-habitat where the attack took place; and that 8) grey seals are important predators of porpoises, further complicating the feeding ecology of the latter.

Nederlandse samenvatting

De bruinvis is een kleine, onopvallende walvisachtige die talrijk voorkomt in de (Nederlandse) Noordzee. De soort kan gezien worden als een evolutionair geminiaturiseerde walvis, die qua omvang de grenzen heeft bereikt van hoe klein een walvisachtige kan worden. Het kleine formaat, samen met warm-bloedigheid en leven in relatief koud zeewater brengt een aantal specifieke problemen met zich mee. Om warm te blijven en zijn lichaamsfuncties te onderhouden, moet een bruinvis veel eten. Geschat wordt dat een bruinvis dagelijks 10% van zijn eigen lichaamsgewicht aan voedsel moet eten. Dit gaat samen met een hoog metabolisme, dat theoretisch gevoed zou moeten worden door prooien met een hoog energiegehalte. Over zijn gehele range van voorkomen, van Japan en beide zijden van Amerika, tot in de gematigde zeeën van Europa, is bij verschillende dieetstudies gevonden dat vette, scholende rondvissen, inderdaad een belangrijke dieetcomponent vormen. Echter, ook een keur van andere, vaak minder vette prooien vormen deel van het bruinvisdieet.

In het Nederlandse deel van de Noordzee waren bruinvissen decennia lang schaars, maar in de afgelopen 25 jaar kwam hierin verandering. Klaarblijkelijk verruilden veel dieren delen van de Noordzee waar (vette) haring en zandspiering vermoedelijk belangrijke prooien zijn, voor de zuidelijke Noordzee. Eerdere studies hebben laten zien dat in onze streken juist relatief magere vissen, zoals grondels, kabeljauwachtigen en platvissen belangrijke prooien zijn, iets dat niet strookt met de gedachte dat bruinvissen alleen kunnen gedijen op een dieet van hoog-energetische prooi. In dit proefschrift wordt het dieet van de bruinvis in Nederland onder de loep genomen, aan de hand van de inhoud van de magen van vele honderden bruinvissen, die dood op onze kust aanspoelden, tussen 2005 en 2014. In die magen zijn vaak nog resten aanwezig van de laatst gegeten prooien, zoals gehoorsteentjes (otolieten) en botten van vissen, kaken van inktvissen en wormen en delen van de uitwendige skeletten van andere ongewervelde dieren. Deze kunnen worden herleid tot zowel de soort als de grootte van de gegeten prooien. Voor dit onderzoek werden 829 magen onderzocht, waarvan er 158 “leeg” bleken te zijn (bevatten geen herkenbare prooiresten). In de 671 niet-lege magen werden resten aangetroffen van in totaal ruim 140.000 prooien. Per maag varieerde dit aantal van 1 tot vele duizenden. Met behulp van een –eveneens grote- referentiecollectie van otolieten en andere harde prooidelen en bijbehorende statistische verbanden (regressies) tussen de grootte van deze onderdelen en de lengte en het gewicht van de bijbehorende vis of ander prooidier, werd ook het gewicht van de gegeten prooien gereconstrueerd, althans van die prooien waarvan zich nog resten in de magen van de dode bruinvissen bevonden. Het dieet van “de bruinvis” kon vervolgens worden beschreven in termen van het relatieve aantal

bruinvissen dat resten van een bepaalde prooi in de maag had, en van het relatieve gezamenlijk aantal en gewicht van de verschillende gereconstrueerde prooien.

“De bruinvis” bestaat echter niet. De populatie bestaat uit allemaal verschillende individuen en ieder individu maakt eigen afwegingen, of wordt door zijn omgeving gestuurd, bij de keuze van de te eten prooien. De centrale vraag bij dit dieetonderzoek was daarom, welke factoren het dieet beïnvloeden, of anders gezegd, bij een zeker individu wegtrekken van het gemiddelde dieet. Om dit te kunnen onderzoeken moet de status van iedere bruinvis worden onderzocht, in samenhang met de maaginhoud. Deels gebeurde dit door het goed bijhouden van de strandingsgegevens (plaats en datum van stranden); dit gebeurt centraal via www.walvisstrandingen.nl, maar ook werd een eigen database opgebouwd. Ook werd de conditie van iedere bruinvis nauwkeurig onderzocht, door gespecialiseerde veterinair pathologen van de faculteit Diergeneeskunde aan de Universiteit van Utrecht. Deze kennis was niet aanwezig in Utrecht (of op Texel waar het dieetonderzoek werd uitgevoerd) en moest dus worden opgebouwd. Dit gebeurde door in de eerste jaren van het onderzoek specialisten uit het buitenland (vooral uit België, het Verenigd Koninkrijk en Duitsland) uit te nodigen bij grootschalige bruinvis-snijsessies, zodat hier het vak kon worden geleerd. Ook bij latere gelegenheden (massa-secties) werden buitenlandse specialisten uitgenodigd, niet alleen om dergelijke klussen te kunnen klaren, maar ook om van elkaar te blijven leren. Het onderzoek naar doodsoorzaken was de hoofdzaak voor de pathologen, maar ook een goede boekhouding van biologisch relevante zaken als lengte, gewicht, geslacht, lichaamsconditie, versheid (of juist niet!) van het kadaver waren voor het dieetonderzoek belangrijk. Gaandeweg het onderzoek bleek ook de doodsoorzaak en factor van belang bij het dieetonderzoek en er is veel werk gemaakt van het correct vaststellen van de doodsoorzaak. Hierbij doken twee problemen op, die een prominente plaats kregen in het dieetonderzoek: het vaststellen van bijvangst in warnetten die op de zeebodem worden geplaatst, en de duiding van veelvuldig voorkomende, opvallende wonden op aangespoelde bruinviskadavers.

In hoofdstuk 1 wordt de bruinvis geïntroduceerd als een kleine, warmbloedige predator van (vooral) vissen, met een grote voedselbehoefte. Ook wordt de centrale vraag van dit dieetonderzoek geformuleerd: welke factoren zijn (mede) bepalend voor wat een individuele bruinvis eet?

In hoofdstuk 2 wordt deze vraag uitgewerkt. Er is gekeken naar een reeks van factoren: de grootte (leeftijd) van de bruinvis, zijn geslacht (man of vrouw), zijn lichamelijke conditie, de mate van versheid van het kadaver, en plaats en datum van stranden. Van deze reeks bleken leeftijd, conditie en tijd van het jaar de meest

bepalende factoren. Jonge bruinvissen eten vooral grondels. Dit zijn kleine (gemiddeld circa 1 gram) en magere visjes, waarvan er dus heel veel gegeten moeten worden om voldoende calorieën binnen te krijgen. Blijkbaar zijn grondels zeer talrijk en makkelijk te vangen. Een jonge bruinvis heeft enkele duizenden grondels per dag nodig, als hij zou moeten leven op alleen deze prooien: dergelijke aantallen werden ook daadwerkelijk aangetroffen in enkele magen. Berekend werd (zie hoofdstuk 9, Figuur 1) dat een jonge bruinvis rond de 2000 grondels per etmaal zou moeten eten. Dit is ruim meer dan 1 grondel per minuut, zelfs al zou een bruinvis dag en nacht niets anders doen dan foerageren. Wordt een bruinvis ouder, en neemt zijn lichaamsgewicht toe, dan zijn nog veel meer grondels nodig: reden om over te stappen naar een dieet met veel meer kabeljauwachtigen, meest wijtingen van rond de 100 gram per stuk. Ook neemt de relatieve hoeveelheid vette vis (haringachtigen, zandspieringen en snelle roofvissen zoals makreel) toe met het ouder worden. Echter, sommige bruinvissen slagen er niet in om (voldoende) vette vis te eten en vallen af. Of omgekeerd: bruinvissen die om de een of andere manier vermageren, slagen er niet in om nog voldoende vette vis te eten en kunnen zo in een vicieuze cirkel belanden, waardoor ze uiteindelijk in sterk vermagerde toestand komen, en vaak nog met resten van vooral magere prooien in hun maag, sterven. Dit fenomeen wordt in detail uitgewerkt in hoofdstuk 3: bij vermagerende bruinvissen verdwijnt eerst haring en sprot uit het dieet, en vervolgens ook zandspiering, waardoor een dieet overblijft van nog vooral wijting en grondels. Dat de tijd van het jaar ook medebepalend is voor wat een bruinvis eet, hangt vermoedelijk samen met het aanbod in zee, of met door jaar heen verschillen in vetgehalte van bepaalde vissen, waardoor ze meer of minder aantrekkelijk worden als prooi. Dit aspect (het prooiaanbod in kwantitatieve en kwalitatieve zin) is echter niet onderzocht.

In hoofdstuk 4 wordt nader ingezoomd op bijvangst als doodsoorzaak. Bijvangst is, wereldwijd en ook in Nederland een belangrijke doodsoorzaak, maar deze is aan gestrande kadavers niet makkelijk vast te stellen. In feite kan de diagnose "bijvangst" alleen met zekerheid worden gesteld, als de visser zelf de bruinvis ter beschikking stelt voor de wetenschap, iets dat door steeds betere samenwerking tussen vissers en onderzoekers gelukkig steeds vaker gebeurt. In de meeste gevallen moet de diagnose van doodsoorzaak echter worden gesteld aan de hand van een aangespoeld kadaver. Kenmerken van verstrikking (indrukken van het net) kunnen echter vervagen, zeker als een kadaver enige tijd heeft rondgedreven voordat het aanspoelde. In tegenstelling tot landzoogdieren (inclusief de mens) die verdrinken, krijgen bruinvissen die verdrinken geen zeewater in de longen. Wel is er vaak schuim in de longen aanwezig door geknapte longblaasjes, maar dit kan ook een gevolg zijn van een longontsteking. Zelfs afdrukken van een net kunnen zijn ontstaan op een bruinvis die dood in een net verstrikte: er zijn dus geen

ondubbelzinnige kenmerken van verdrinking en de patholoog moet het doen met een optelsom van verschillende kenmerken. Twee daarvan zijn een volle maag en een goede lichamelijke conditie, omdat deze wijzen op een plotseling intredende dood. De diagnose "bijvangst" krijgt dus altijd een aantekening, met de mate van zekerheid van de diagnose, op basis van het aantal nog zichtbare kenmerken die kunnen wijzen op bijvangst, en een "volle" maag is er daar een van. Tijdens het onderzoek kwamen er echter diverse magen op tafel, van vermoedelijke bijvangstslachtoffers, die vol zaten met vissen die zich niet of nauwelijks aan de zeebodem ophouden. Aangezien verdrinking in staand want, het meest waarschijnlijke verantwoordelijke vistuig, aan de bodem moet plaatsvinden, wierp dit de vraag op of niet zozeer de hoeveelheid maaginhoud, als wel de aard van die maaginhoud bekeken zou moeten worden. De maaginhouden van "bijvangst bruinvissen" met een verschillende mate van zekerheid ten aanzien van de diagnose van deze doodsoorzaak, werden vergeleken. Zekere en hoogst waarschijnlijke bijvangsten waren inderdaad gekenmerkt door een zeer hoog percentage (>94% van het totale gereconstrueerde gewicht aan prooien) bodemvis en dit percentage daalde met iedere stap van minder zekerheid van de diagnose. In de reeks: zekere, hoogst waarschijnlijke, vermoedelijke, en mogelijke bijvangst kunnen we op grond van de specifieke maaginhoud de diagnose scherper stellen voor de minder zekere gevallen. Met een statistisch model laten we zien dat vermoedelijke en mogelijke bijvangsten een categorie omhoog moeten in deze reeks, als het massapercentage aan bodemvis in de maag >94% is, terwijl gevallen in die categorieën met <74% een categorie dienen te zakken (vermoedelijke bijvangst wordt dan mogelijke bijvangst, en mogelijk bijvangst wordt dan: "onbekend").

In hoofdstuk 5 wordt een regionaal aspect van het dieet onderzocht. In het algemeen verschilde het dieet niet sterk tussen bruinvissen die in verschillende delen van Nederland aanspoelden (deels, vermoedelijk doordat nog niet van ieder deelgebied, over het hele jaar heen, voldoende bruinvissen konden worden onderzocht). Eén regio liet echter een duidelijk afwijkend beeld zien: de Westerschelde. Deze rivier raakte in de tweede helft van de vorige eeuw sterk vervuild en de bruinvis en de meeste trekvissen verdwenen hier. Door een reeks milieumaatregelen verbeterde de kwaliteit van de rivier sterk, en keerden trekvissen, vooral spieringen, weer terug, zo laten onder meer fuikvangsten in het Belgische deel van de rivier zien. Op ons verzoek zijn zoveel mogelijk kadavers geborgen die in de Westerschelde werden aangetroffen, zodat een vergelijking gemaakt kon worden van het dieet in de rivier zelf, in de monding en in de Voordelta, waarin de Westerschelde uitmondt. Uit de Voordelta waren voldoende bruinvissen onderzocht, en zo ook langs de noordelijke oever van de monding (Vlissingen-westpunt van Walcheren). Langs het kustgedeelte Breskens-Belgische

grens waren echter vrijwel geen bruinvissen geborgen voor onderzoek, maar hier boden bruinvissen die iets verderop, in België strandden, uitkomst. Uit magen die al in België waren onderzocht, waren de harde prooiresten bewaard, zodat deze op Texel opnieuw konden worden onderzocht (om er zeker van te zijn dat er geen verschil in onderzoeksmethode zou zijn), waardoor ook uit het zuiden voldoende materiaal voorhanden was. In de magen van dieren uit de rivier zelf werd een zeer hoog aandeel (46%, prooimassa) aan trekvis, vooral spiering gevonden. In de monding van de rivier (punt Walcheren tot in België) was dit 14% en in de Voordelta 3% (vergelijkbaar met de rest van Nederland). Bruinvissen die de rivier opzwemmen eten dus relatief veel trekvissen, die ook onderdeel uitmaken van het rivier-ecosysteem. Toch lijken de bruinvissen nog aanzienlijke problemen te hebben op de Westerschelde. Gemiddeld waren de onderzochte dieren erg mager. Dieren die heel ver de rivier optrokken, tot diep in België, kwamen vermoedelijk allemaal om het leven. Om zover de rivier op te kunnen zwemmen moesten sluizen worden gepasseerd en was de weg terug wellicht niet meer te vinden: overleven op alleen riviervis is wellicht nog te lastig voor deze dieren.

In hoofdstukken 6-8 wordt de grijze zeehond ontmaskerd als een belangrijke predator van bruinvissen. Eerder was al door Belgische collega's, op grond van enkele in België aangespoelde, verminkte dode bruinvissen aannemelijk gemaakt dat grijze zeehonden hier achter zaten. Deze suggestie wekte echter nogal wat weerstand en hard bewijs ontbrak. Gezien de honderden gevallen die we in Nederland hadden gezien, was dit voor onze situatie een belangrijk punt. DNA-bewijs zou de zaak kunnen beslechten, maar de kansen om dit bewijs te kunnen leveren werden alom laag ingeschat. Er waren in principe twee mogelijkheden: of we zouden bruinvis-DNA moeten vinden in de maag, darm, of feces van een grijze zeehond, of we zouden grijze zeehond-DNA moeten kunnen aantonen in de veronderstelde bijtwonden op de bruinviskadavers. Omdat we niet wisten welke individuele zeehond te bemonsteren (en hoe dit te doen), werd voor de tweede mogelijkheid gekozen. Drie verse, zwaar verwonde bruinvissen, afkomstig uit verschillende delen van het land (ZW Nederland, Zuid-Holland, en Noord-Holland) werden verzameld, door drie verschillende teams, waarbij de kadavers al op de strandingslocatie goed werden verpakt om contaminatie te voorkomen, en meteen naar Utrecht gebracht voor sectie. Hier werden, voorafgaand aan de sectie, *swabs* genomen, van de diverse wonden (wattenstaafjes in de wond gedrukt of erlangs gehaald), plus een groot aantal blanco monsters. Deze zijn onderzocht in het DNA *clean-lab* van het NIOZ op Texel, waar men ervaring heeft met de detectie en determinatie van zeehond-DNA. In Utrecht werden de verwondingen uitvoerig beschreven en gefotografeerd en werd nagegaan of er ook bloedingen aanwezig waren in het weefsel direct rond de wonden. Dit zou namelijk wijzen op een nog aanwezige bloeddruk op het moment van verwonden. De wonden op deze drie

bruinvissen werden vervolgens vergeleken met wonden, gevonden op alle eerder onderzochte verminkte bruinvissen. Hiertoe werden alle pathologische rapporten opnieuw nagezien, evenals alle foto's gemaakt tijdens de eerdere secties. In diepe, smalle "bijt"wonden op alle drie de *geswabde* bruinvissen werd DNA van grijze zeehonden aangetoond. Andere soorten potentiële bijtende zoogdieren (gewone zeehond, hond, wolf, vos, orka) konden worden uitgesloten. Bloedingen onder de bijtwonden in deze en een aantal eerder onderzochte kadavers lieten zien dat hier sprake was geweest van predatie en niet van aas eten. De retrospectieve analyse van alle eerder onderzochte dode bruinvissen bracht aan het licht dat 25% van de dieren die in Nederland in verse staat aanspoelen (zodat de verwondingen goed onderzocht kunnen worden) bijt- en klauwsporen dragen die op grond van de wonden op de *geswabde* bruinvissen, toe te schrijven zijn aan een aanval door een grijze zeehond. In een nader onderzoek van de foto's van verminkte dieren kwam naar voren dat er twee hoofdtypen waren in de verwondingen: sommige dieren leken aan de zijkant van het lichaam gepakt, waarna repen huid en spek volgens een soort zig-zag patroon van het lijf waren gerukt, terwijl andere dieren juist bij de keel waren gepakt. Maagonderzoek liet vervolgens zien dat de "zig-zag" dieren vooral bodemvis hadden gegeten, terwijl de dieren die van onderen, bij de keel waren gegrepen veel meer haring- en sprotresten in de maag hadden. We interpreteren dit als: bruinvissen die aan de bodem foerageren worden aangevallen door een zeehond die hen vanaf de zijkant, over de bodem benadert, terwijl bruinvissen die hoger in de waterkolom op scholen haring en sprot jagen, van onderen worden aangevallen. Anders gezegd: een bruinvis is nergens veilig.

Predatie op bruinvissen door zeehonden is vermoedelijk een relatief nieuw verschijnsel. Er zijn enkele oudere beschrijvingen gevonden van incidentele aanvallen, maar dit heeft zich voor zover bekend nooit eerder tot een meer algemeen voorkomende foeageerstrategie van zeehonden ontwikkeld. Secties op bruinvissen in het verleden, bijvoorbeeld bij Naturalis in Leiden, hebben nooit het beeld opgeleverd van de verminkingen zoals we ze nu hebben gezien. Een vervolgvraag is hoe bruinvissen om zullen gaan met deze nieuwe predatiedruk. Enkele dieren waren klaarblijkelijk ontsnapt aan een aanval: ze hadden geheelde of helende wonden. Er is dus enige ruimte om te leren van een aanval. Bruinvissen die te maken hebben met aanvallen van dolfijnen reageren hierop door met minder vet (blubber) door het leven te gaan. Verondersteld wordt, dat dit een aanpassing is om wendbaarder te worden, wat de kans op ontsnappen aan de aanvaller zou moeten verkleinen. Echter, een bruinvis heeft maar relatief kleine vetreserves en een dunnere blubberlaag verhoogt de kans op verhongering, als het foerageersucces eens een paar dagen lager is dan gewoonlijk. Predatie is dus een extra complicerende factor, bij het al complexe probleem waarmee een bruinvis te maken heeft bij het vinden van voldoende van het juiste voedsel om te kunnen overleven.

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Dankwoord / Acknowledgements

Stel, je loopt ergens in Nederland langs het strand en je vindt het kadaver van een dode bruinvis. Loop je dan door, of meld je de vondst? Vele honderden mensen deden dat laatste, de afgelopen jaren en vaak namen ze ook een of meer foto's om de determinatie te laten bevestigen. Zonder deze (eerste) meldingen geen onderzoek: ik wil iedereen die zijn vondst heeft gemeld, al dan niet direct bij walvisstrandingen.nl, of via politie, dierenambulance, de EHBZ (Eerste Hulp Bij Zeezoogdieren), de Zeehondencrèche in Pieterburen, Ecomare op Texel, SOS dolfijn in Harderwijk of bij Arnold Gronert, "onze man in Petten", de plaatselijke reddingsbrigade of strandpaviljoenhouder, Staatsbosbeheer, Rijkswaterstaat, de Noordwester op Vlieland of het Natuurcentrum op Ameland, of direct bij mij, van harte bedanken voor de gedane moeite. Nóg meer moeite hebben zij zich getroost die de dode bruinvissen van het strand hebben gehaald, en soms zelfs hebben bezorgd in Utrecht (in een enkel geval zelfs met eigen auto!). Ik heb zo goed mogelijk geadministreerd wie de bruinvissen hebben gevonden en opgehaald voor ons onderzoek, maar bij enkele kadavers zijn de labels verloren gegaan of onleesbaar geworden, dus ik moet me excuseren voor iedere naam die hier ontbreekt: A Dijkman, Albert Dijkstra, Alieke Talsma, Andre Dijkman, Anneke Kleinstra, Anton Duijnhouwer, Arjen Dijkstra, Arjen Schaap, Arnold Gronert, Arnout de Vries, Arthur Oosterbaan, B Henstra, Barbara van der Molen, Bemanningen van Phoca, Krukel, Harder en Navicula, Bert Teeuwen, Bram Couperus, Brandweer Spijkenisse, dhr Broek, Carl Zuhorn, Cees van Hoven, Christine Koersen, D Visser, DB Vlaardingen, Dave de Koning, Dierenambulances: Den Haag, Den Helder, Kennemerland, Oestgeest, Vlaardingen, Dierenbescherming Vlaardingen, Dirk Bruin, Dirk Visser, Dolf Vogelzang, de Douane, E vd Berg, fam van der Berg, Enno..., Evelien van Wijk, Eveline Dekker, Ewout Adriaans, Frank Lek, Gemeentewerken Ameland, Gerard Ridderhof, H Brinkman, H. Wijnberg, HMS Den Helder, Hans Verdaat, Henk Brugge, Hessel Wiegman, Hielke Bruining, J Bloem, J Bruin, J Butter, J Verschoor, J vd Hoorn, J. Kienstra, J. F. de Jong, Jan Harthoorn, Jan vd Star, Jaap van der Hiele, Jeroen Hoekendijk, Johan Krol, John Daalder, de KLPD (meldkamer), de KNRM, Karin van Veen, Kees Camphuysen, Kees Kooimans, Kees Soepboer, Fam. Kelder, Kim de Haan, fam. Kruk, L vd Vaart, Leen van Duijn, Leo de Mooij, Lianne Blaauw, Linda van Asselt, Lou van Fraaijenhove, M Geerse, M Masen, MG Boon, Maarten Brugge, Maarten Jan Boon, Marc Plomp, Mariëtte Smit, Mart Gul, Martin de Jong, Dhr. Muijze, Nynke Osinga, P van Tuinen, PP Ternaard, Paul de Jong, Pierre Bonnet, Piet Metz, Politie Den Haag, den Helder, Haaglanden, Rotterdam, Reddingsbrigades van Bloemendaal, Egmond aan Zee, Petten, Sint Maartenszee, Vlissingen, Westerschouwen, Remke Mulder, Rene Jager, Rens Roos, Rens van der Zwaag, Rinus Nieuwstad, Rinus Noort, Rob van Bemmelen, Roos Kamsma, RP

Meerman, kapitein M.S. Esterella (rederij vrolijk), Ruud Koelewijn, S Waasdorp, Salko de Wolf, Saskia Verbr..., Simon Mostert, Simon de Vries, Stichting Strandexploitatie Den Helder, Strandwacht Julianadorp, Strandwacht SNN, Peter Gooijer, Simon Mostert, T. Kolk, Tanya Campton, Tim van Nus, Teo & Mary Schoos, Theo de Wijs, de VPGO (Vereniging Paardrijden Gehandicapten Oost), Wagemaker, Waterschap Zeeland, Willem Stel, Willem de Rover, Carl Zuhorn, en enkele anoniem gebleven "toeristen" en strandpaviljoenhouders.

Slechts drie van deze mensen, te weten Arnold Gronert, Kees Kooimans en Jaap van der Hiele zijn onderscheiden: met de Duboispenning (Naturalis). Deze onderscheiding wordt uitgereikt aan personen die zich belangeloos inzetten voor de wetenschap. De onderscheidingen voor deze drie mannen is zonder meer terecht, maar volgens de voorwaarden voor toekenning verdienen eigenlijk alle hierboven genoemden een onderscheiding!

Een klein aantal, maar o zo belangrijke bruinvissen is direct aangeleverd voor onderzoek door hier niet nader te nomen vissers. Ik wil hen van harte bedanken voor hun hulp, inzet en samenwerking, in de persoon van hun "voorman" Rems Cramer. Ten aanzien van het organiseren van de komst van deze bijzondere bruinvissen gaat mijn dank ook uit naar Marije Siemensma, Bram Couperus en Meike Scheidat.

De secties op de eerste serie bruinvissen werd gedaan op de kade van de NIOZ haven op Texel, met goedkeuring van de toenmalige directeur, Herman Ridderinkhof, en de havenmeester Ewout Adriaans. Het werk werd uitgevoerd in een grote tent van Tentorent op Texel (je moet maar durven) en werd ingericht met behulp van een serie oude labtafels die "nog ergens in de loods stonden" (NIOZ) en voorzien van verlichting en stromend door Siem Groot (NIOZ); tussendoor werd ook nog even een vriescontainer geregeld. De catering ter plaatse werd verzorgd door de vaste NIOZ cateraar, Ruud Boom. Ik kan de uitmuntende samenwerking met het NIOZ niet genoeg bejubelen... en dan zou ik nog bijna de langlopende samenwerking vergeten met de "vissenmannen" van het NIOZ, Hans Witte en Sieme Gilles, die mij voorzagen van ontelbare vissen (voor de gehoorsteentjes) en hielpen bij lastige determinatiekwesties. Evenzo kon ik altijd terecht bij Marc Lavaleije, als er "onmogelijke" stukjes krab of schelpdier gedetermineerd moesten worden.

Kees Camphuysen was onmisbaar: samen hebben we het onderzoek opgezet, een database structuur ontwikkeld (die tot vandaag de dag wordt gebruikt), het eerste deel van het onderzoek uitgevoerd en beschreven. In de jaren die volgden bleef Kees aanvankelijk direct betrokken bij het bruinvisonderzoek, maar kon het

geleidelijk loslaten en aan mij over laten. Uiteraard voerden we vele discussies met bruinvissen en dieetonderzoek als onderwerp: ik beschouw Kees als mijn bruinvisgeweten (en heel veel meer dan dat) en ik ben trots en gelukkig dat je een van mijn paranimfen wilt zijn bij de promotieceremonie!

Porpoise pathology is an art that we needed to master. At the onset of this study, in our tent at Texel, several veterinary pathologists and veterinarians came over, without pay, to help out with the first 60 necropsies. I am very much indebted to Thierry Jauniaux, who came over in his veterinary van from Liege, Belgium, with his students: Hélène Beguerie, Joseph Schnitzler, and Olivier Drouget, to take the lead: merci beaucoup! We were extremely fortunate this first year, as we were also helped out by Susanne Prahl and Kristina Lehnert (Forschungs- und Technologiezentrum Westküste-FTZ, Germany), Pierre-Yves Daoust, of the Department of Pathology and Microbiology, Atlantic Veterinary College, University of Prince Edward Island, Charlottetown, Canada; Maria Morell Ybarz, of Centre Tecnològic de Vilanova i la Geltrú Universitat Politècnica de Catalunya, Barcelona, Spain; Paulien Bunskoek (Dolfinarium Harderwijk). Additional assistence was provided by / Aanvullende assistentie werd verleend door: Marjan Addink, Coby en Dirk Kuiken, Edward Soldaat, Okka Jansen, Rob Witbaard, Kees Jan van Bergeijk, Arnold Gronert, Sophie Brasseur, Ester Dias, Cindy van Damme en Sharon Boekhout.

In 2007 we were convinced that it would be better to move the entire pathology operation to the much better equipped facilities of the Department of Pathobiology, Faculty of Veterinary Medicine, at Utrecht University, where we were kindly invited by professor Andrea Gröne and her staff. Lidewij Wiersma was appointed to the porpoise job and performed this task diligently: het was een voorrecht en een genoegen met je te mogen werken in de snijzaal, Lidewij! Al snel verscheen er een tweede ster in de snijzaal, Lineke Begeman, en een derde, al wisten we dat toen nog niet: Lonneke IJsseldijk. Lineke nam geleidelijk de taken over van Lidewij, en bracht het werk aan de bruinvis vele stappen verder, door een enorme inzet, een bijzonder serieuze taakopvatting (nooit eens kort door de bocht) en vooral een scherpe blik en een *open mind*. Met Lineke had ik de discussies die de biologie en de pathologie samen brachten, waardoor we samen heel veel verder kwamen in ons gezamenlijke vak. Vele groten stoppen helaas (voor de rest van de wereld) op hun piek: in jouw geval was dat meteen na onze mooiste publicatie, in de *Proceedings*. Ik zag je met lede ogen vertrekken uit Utrecht, want we hadden afzonderlijk nooit het niveau kunnen halen dat we samen hebben gehaald, en los daarvan, was onze samenwerking zeer inspirerend. Ik ben heel blij dat jij ook paranimf wilt zijn bij mijn promotie!

En nu is Lonneke hoofd-bruinviszaken in Utrecht. Je kwam binnen als een piepjonge vrijwilliger (net de HAVO klaar?), vervolgens wervelde je door de opleiding van Hall-Larenstein heen (ze zijn daar nog steeds niet helemaal van de schrik bekomen volgens mij: je was absoluut de meest bijzondere student die ooit van die opleiding kwam!) en je had één doel in het leven: werken aan walvisachtigen. Dat is je uiteraard gelukt: met zoveel *drive* kon dat ook niet missen. Als ik zie hoeveel we samen nog op stapel hebben staan, gaan we nog een mooie toekomst tegemoet: ik kijk er naar uit!

Ook in Utrecht werden we geholpen door vele pathologen en dierenartsen met verstand van bruinvissen en een grote groep "helpers": de opeenvolgende massa-secties waren altijd ware happenings. ***Many vets, veterinary pathologists, and others came to Utrecht to assist us with mass-necropsy sessions, always providing your services for free (much appreciated!) in happenings that were always inspiring teaching and learning sessions, for all of us. I am immensely grateful for all your input: this is science at its best! In the years from 2007, we were joined by:*** Marjan Addink, Erik Agrer, James Barnett, Danielle Beekman, Pieter Beer, Lineke Begeman, Chantal Bleumink, Marijke de Boer, Pierre Bonnet, Judith van den Brand, Elisa Bravo Rebolledo, Caroline Brorer, Natashja Buijs, Paulien Bunskoek, Judith van den Brand, Kees Camphuysen, Bram Couperus, Herman Cremers, Pieter Crucq, Nick Davison, Rob Deaville, Mariel ten Doeschate, Susanne van Duijne, Eva Dujardin, Niels van Elk, Tilen Genov, Jan Geertsema, Reza Gerretsen, Thomas Ghisbain, Frank de Goot, Alexia Grondin, Andrea Gröne, Arnold Gronert, Ilka Hasselmeier, Jasper Hetterschijt, Jannes Heusinkveld, Martine van den Heuvel, Jaap van de Hiele, Sjoukje Hiemstra, Bert Hoeve, Okka Jansen, Thierry Jauniaux, Nora de Jeu, Els de Jong, Juthatip Keawacharoen, Bekah Keesler, Guido Keijl, Sylvia Keijser, Marja Kik, Willem-Jan Kitslaar, Erwin Kompanje; Amrit Knoppers, Christine Koersen, Kees Kooimans, Polona Kotnjek, Katharina Kramer, Dirk and Coby Kuiken, Thijs Kuiken, Kristina Lehnert, Frank Lek, Manon Lock, Klaus Luke, Marjet van der Meijden, Aleksija Neimane, Ivanna Nijenhuis, Matthew Perkins, Susanne Prahl, Marianne Psaradellis, Barbara Rodenburg, Jolianne Rijks, Joseph Schnitzler, Henrike Seibel, Marije Siemensma, Valerie Stekke, Elodie Stolear, Wouter Jan Strietman, Maaike Tamis, Rachel Thomas, Dorien Verheyen, Adrie Vonk, Lilian de Vos, Ruby Wagensveld, Bas Wassink, Erik Weerts, Babeth van der Weide, Lidewij Wiersma, Tjitske Wiersma, Richard Witte, Lonneke IJsseldijk, Jooske IJzer, Maria Zijlstra.

De logistiek rond snijsessies, maar ook van ieder afzonderlijk transport van een dode bruinvis die naar Utrecht moest worden gebracht (soms op stel en sprong, omdat de sectie onmiddellijk, op het nog verse dier moest plaatsvinden) was een enorme uitdaging. Vanuit Texel/Den Helder werd dit vooral verzorgd door Piet

Wim van Leeuwen, André Meijboom, Hans Verdaat, Simon de Vries en Elisa Bravo Rebolledo. De “grote drie” van het bruinvissen verzamelen, Arnold Gronert, Kees Kooimans en Jaap van der Hiele, waren nooit te beroerd om bijzondere bruinvissen zelf naar Utrecht te brengen en als dat toch niet kon, waren daar de chauffeurs van de landbouwhuisdieren faculteit, Rik van Walderveen, Willy Elberse en Ewout Boom.

Speciale vermelding verdienen Johan Krol (Ameland), Jeroen Hoekema en Lonneke IJsseldijk, omdat zij zelfs bruinvissen in hun eigen auto vervoerden voor het onderzoek!

Dode bruinvissen komen zelden gelegen en zijn bij nacht en ontij van het strand gehaald. In het verlengde daarvan zijn ze ook op alle dagen (en nachten, vermoedelijk) van de week in Utrecht nader onderzocht. De inzet hierbij van Andrea, Lidewij, Lineke, Lonneke en Sjoukje was fenomenaal: klachten heb ik nooit gehoord, integendeel. De snijzaal werd professioneel, wat geenszins in tegenspraak is met: enthousiast en met veel plezier, gerund door Johan van Amerongen, Louis van den Boom, Natashja Buijs, Sjoukje Hiemstra en Ruby Wagensveld. Dames en heren: zeer bedankt voor jullie inzet!

Het uitzoekwerk aan de maaginhouden vond plaats op Texel. Omdat we dit echt goed wilden doen, was dit een zeer arbeidsintensieve klus, die alleen maar kon worden geklaard door de geweldige inzet van een hele reeks studenten (door mij wel liefkozend “maagmeisjes” genoemd, een eretitel, ook voor de heren in het gezelschap). In chronologische volgorde waren dit: Joyce van den Berg, Cindy van Damme (pre-2001: otolieten CD); Angela Folmer, Maarten Debruyne (2006), Annika Krauthof, Marjoleine Roos, Martijn Moorlag (2007), Amber Beerman, Ellen Nieuwenhuijsen (2008), Lennert Wolfs (2009), Robin Kop, Clasina Jansen (2010), Tessa van Heumen, Lisette Mekkes (2011), Dorien Verheyen (2012), Lara Mielke, Eileen Heße (2012 én 2013: recidivisten!), Tara Schelling, Lizzy van der Steeg (2013), Kelly Witteveen, Liliane Solé, Carolien Strating (2014). Guido Keijl was ook jaren lang, op de maandagen, bij ons op het lab te vinden voor otolieten-uitzoekwerk, en verzorgde daarnaast de database achter walvisstrandingen.nl; samen konden we nog een flink aantal “vage” bruinvissen duiden en zo de bruinvisboekhouding op orde brengen.

Het werk aan bruinvissen is gedaan in opdracht van het Ministerie van Economische Zaken, in het kader van “BO” (beleidsondersteunend) onderzoek aan het dieet van de bruinvis (IMARES), onderzoek naar de doodsoorzaken (Universiteit Utrecht), en aanvullend onderzoek aan DNA bij verminkte bruinvissen (IMARES/UU/NIOZ). Het is bijzonder dat het bruinvisonderzoek voortdurend kon

worden gefinancierd en dit is vooral te danken geweest aan de inzet van Folchert van Dijken, Annegien Helmens, Jan Huinink, Wilmar Remmelts en Jeroen Vis (allen EZ), Tia Hermans (Alterra) en Oscar Bos (IMARES). Henk den Boon (E-Connection) zorgde voor sponsoring *in natura* van Arnold Gronert, door hem jaren lang een 4WD pick-up truck ter beschikking te stellen om aangespoelde vogels en zeezoogdieren op het Noord-Hollandse strand te tellen en te bergen voor onderzoek. Evenzo sponsorde de Zeehondencrèche Pieterburen twee EHBZ auto's (in Noordwijk en Zeeland), recent werd dit overgenomen door de Stichting Dierenlot en ASeal. De Zeehondencrèche verzamelde ook zelf dode bruinvissen voor ons onderzoek of fungeerde als tussenstation voor opslag en vervoer van dode bruinvissen naar Utrecht: Nynke: dank voor je hulp hierbij! Ecomare op Texel haalde jaren lang, met eigen auto's bruinvissen van het Texelse strand en hetzelfde geldt voor de andere opvangcentra langs de Nederlandse kust en instellingen als Rijkswaterstaat en Staatsbosbeheer. Veel kosten van het werk op de stranden zijn door de mensen zelf gedragen. Hopelijk brengt in de toekomst de Stichting Zeezoogdierenhulp Nederland (i.o.) hierin verandering.

Wetenschappelijke discussies werden gevoerd met vele collega-onderzoekers, vooral met Jan Haelters en Kees Camphuysen (dieet, verspreiding, zeehond-bruinvis interacties, bijvangst problematiek) en meer diffuus, over allerlei biologische vragen, met de mannen en vrouwen van de "vogel-vleugel" op het NIOZ. Ik zal jullie missen! Op IMARES (Texel) werkte ik in een warm nest, met gewaardeerde collega's, waarvan sommigen ook gegrepen zijn door het maagonderzoeksvirus (Jan Andries van Franeker, Suze Kühn, Elisa Bravo Rebolledo, Sophie Brasseur, en in de begintijd van het onderzoek, Okka Jansen). Als er logistiek iets geregeld moest worden stond iedereen altijd klaar en (bijna) niemand klaagde ooit (echt) over de soms krachtige geuren die uit het lab de gang op dwarrelden. Geert Aarts en Erik Meesters waren onmisbaar bij de meer ingewikkelde statistische analyses, en zo ook Jaap van der Meer (NIOZ). De mooiere (GIS) kaarten in dit proefschrift zijn gemaakt door Elze Dijkman. Harry Witte en Judith van Bleijswijk deden fantastisch werk in het NIOZ DNA-clean lab: ik zal nooit het moment vergeten dat Judith door de gang kwam aangerend: "we hebben een bandje!" (= een positief geval van grijze zeehond DNA). Alleen dat al was een film waard, en die kwam er ook: dank ook aan Corlijn de Groot en haar VPRO/Kennis van Nu team voor een prachtig stukje werk.

I am greatly indebted to you, Thierry, for all you have done for us. If Jan Haelters is the "founding father" of seal-porpoise interactions, you are the archangel of porpoise pathology for me. We have come so far, and achieved so much, thanks to your help and teaching, from the onset of our work, back in 2006.

I will never become a true pathologist, but as much as I enjoyed discussing porpoise problems with Lineke, I have enjoyed the discussions with others in the international community of porpoise pathologists: Johanna Baily, Andrew Brownlow, Mark Dagleish, Nick Davison, Rob Deaville, Andrea Gröne, Ilka Hasselmeier, Thierry Jauniaux, Paul Jepson, Marja Kik, Thijs Kuiken, Kristina Lehnert, Barry McGovern, Susanne Prahl, Ursula Siebert, Lidewij Wiersma and Jooske IJzer. Yours is a fascinating world and I am grateful to you all, for inviting me into it. Fascinating discussions were also held on the subject of the porpoises mutilations, when the culprit was not yet identified with certainty, with Jan Haelters, Thibaut Bouveroux, Jaap van der Hiele, Abbo van Neer, Han Reijnhoudt, Eligius Everaarts and Jolanda Meerbeek.

De foto's die zijn gebruikt in dit proefschrift zijn gemaakt, en beschikbaar gesteld door; Geert Aarts (hoofdstuk 1), Arnold Gronert (omslag en hoofdstukken 3 en 7(inzet)); Albert Dijkstra (hoofdstuk 5), Piet Van den Hout (hoofdstuk 6), Martijn de Jonge (hoofdstuk 4), Wouter Jan Strietman (hoofdstukken 2, 7, 9 en 10). De opmaak van dit proefschrift werd verzorgd door Bas Engels.

Het schrijven van dit proefschrift kreeg pas echt vaart toen we hadden besloten vol te gaan voor publicatie van de zeehond-bruinvis interacties in een tijdschrift met een hoge impact factor. We kregen hierbij begeleiding van Stefan Schouten: dank daarvoor, Stefan! Peter Reijnders heeft er altijd in geloofd dat ik dit proefschrift ook echt zou schrijven, en, niet onbelangrijk, wist een rem te zetten op al te ongebreidelde ambities mijnerzijds. En zo is het gekomen: Peter, dank voor alle hulp, steun en focus!

En uiteraard, een warm "dank jullie wel" aan Katja, Kyra en Tom. Avond- en weekendwerk was (is) routine en daarvoor was altijd alle begrip en steun. Sterker nog: jullie gingen tijdens de laatste, "schrijf-zomer" zonder mij op vakantie, omdat dat *beter was voor mij*, zodat ik dan rustig thuis door kon stressen. Wat een weelde! ☺