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A multi-method approach reveals long- and short-term dietary differences in individual harbour porpoises *Phocoena phocoena* in the southern North Sea

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ABSTRACT: Understanding predator–prey relationships is essential for revealing the complex role of marine mammals in exerting top-down control within marine ecosystems and is crucial for developing effective conservation strategies. The harbour porpoise *Phocoena phocoena* is the most abundant cetacean species in the North Sea, and most studies on its diet are based on traditional hard part analysis in stomachs providing limited knowledge of its complex feeding ecology. Here, we combined stomach content analysis (SCA), metabarcoding and stable isotope analysis on the same 48 individuals, stranded between 2005 and 2021, to elucidate the diet of harbour porpoises in the southern North Sea. We aimed to increase prey species detection rates and to uncover temporal changes in the diet by comparing individual diets immediately prior to stranding with assimilated diets. By using SCA and metabarcoding complementarily, we were able to increase species detection by 49% on an individual sample level and uncovered a previously unknown prey species, hooknose *Agonus cataphractus*. Adult harbour porpoises primarily obtain energy from common sole *Solea solea* and sandeels, while juveniles rely mainly on whiting *Merlangius merlangus*, reflecting distinct energy sources aligned with biomass estimates. Direct method comparison revealed great temporal dietary differences in adult and juvenile porpoises. Near-shore species with a benthic carbon source contributed most to the short-term diet, whereas offshore species with a pelagic carbon source contributed most to the long-term diet. This framework can be extended to other ecosystems and predator species to elucidate the species-specific diets of animals where direct observations are not feasible.

KEY WORDS: Feeding ecology · Cetaceans · Metabarcoding · Stomach content analysis · Stable isotope analysis · Mixing models · North Sea

1. INTRODUCTION

Insights into predator–prey relationships can help to unravel the complex roles of marine mammals as top-down regulators in marine ecosystems (Baum & Worm 2009, Roman & Estes 2018). Additionally, ecological data on foraging are crucial for the conservation of threatened species and ecosystems, and provide important baseline parameters for sophisticated food web models (Püts 2021). The intricate nature of many ecosystems, such as the North Sea (Dickey-Collas et al. 2014), coupled with the elusive behaviour of most marine mammals, often leaves gaps in our understanding of complex predator–prey interactions within marine

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food webs. This lack of clarity can lead to biased and ineffective management decisions (Brodeur et al. 2017).

The North Sea stands out as one of the most heavily utilized, yet productive, shelf regions globally (Couce et al. 2020), facing numerous anthropogenic pressures such as shipping, pollution, fisheries and offshore development activities like wind and tidal energy (Burthe et al. 2014, Emeis et al. 2015). It is also undergoing climate-induced changes (Belkin 2009), including an increase in Lusitanian (warm-favouring) fish species due to rising sea surface temperatures (Jones et al. 2023) and a surge in jellyfish occurrences, which correlate with the North Atlantic Oscillation index (Attrill et al. 2007). The impact of these stressors on the availability of prey for marine mammals in the North Sea remains uncertain.

The harbour porpoise *Phocoena phocoena* is the smallest and most abundant cetacean species in the North Sea, with the current population exceeding 300 000 individuals (Gilles et al. 2023). Harbour porpoises in the North Sea have shown a southward shift in distribution, resulting in an increased abundance of porpoises in the southern North Sea (Hammond et al. 2013, Gilles et al. 2023). Harbour porpoises feed on a multitude of prey taxa, with fish being their primary food source (Leopold 2015). In the southern North Sea, their diet varies significantly with age; juveniles mainly consume gobies (*Pomatoschistus* spp.), while adults have a more varied diet including lean fish like whiting *Merlangius merlangus* and Atlantic cod *Gadus morhua*, as well as high-energy fish like Atlantic herring *Clupea harengus* and sandeels (*Ammodytes* spp.) (Leopold 2015). The families Gobiidae, Ammodytidae, Gadidae and Clupeidae are particularly important for the overall diet (in terms of biomass) of harbour porpoises in the southern North Sea (e.g. Gilles et al. 2008, Leopold et al. 2015).

However, existing studies, primarily based on traditional methods, provide limited insights into the complex feeding ecology of high-energy-demand predators like harbour porpoises (Spitz et al. 2012). Many questions remain, such as potential biases in the diet of stranded individuals towards coastal prey species (Jansen et al. 2013). Furthermore, most studies focus on stranded individuals from over a decade ago (Haelters et al. 2012, Leopold et al. 2015, Mahfouz et al. 2017) and are especially lacking for certain areas, like the German coastline of the North Sea (Benke et al. 1998, Gilles et al. 2008, Lick 1991).

Traditionally, the diet of marine animals has been studied using stomach content analysis (SCA), which examines hard parts like otoliths and squid beaks found in stomach samples, to determine the individual's last meal (Bowen & Iverson 2013). However, this method can be biased because soft-bodied prey and fragile otoliths are often overlooked (Pierce et al. 2004) and digestion times vary (Tollit et al. 2003); therefore, stomach contents may not accurately represent the full spectrum of an individual's diet. Nevertheless, to date, SCA remains the only method to accurately assess consumed biomass (Härkönen 1986, Leopold et al. 2015).

Recent advances in aquatic dietary studies include DNA metabarcoding, a non-invasive molecular approach which can reveal new aspects of food web relationships, offering a more sensitive and higher taxonomic resolution than traditional SCA (Boyi et al. 2022). DNA metabarcoding provides a powerful tool for ecological studies, enabling insights into the composition, structure and dynamics of biological communities, using environmental samples like soil, water and scats, in a variety of ecosystems where direct observations are limited or impossible (Bohmann et al. 2014, Ruppert et al. 2019). Biases of conventional SCA can be overcome, as metabarcoding is more sensitive and offers a higher taxonomic resolution (Massey et al. 2021). One major advantage of metabarcoding is that it can also target prey species without hard parts (e.g. jellyfish) (Jarman et al. 2013) or species with otoliths that are rapidly digested, e.g. sandeels (Grellier & Hammond 2006). Main drawbacks of metabarcoding are that sequence reads cannot currently be reliably translated into accurate biomass estimates (Boyi et al. 2022) and taxonomic resolution is highly dependent on the primers used (Baetscher et al. 2023).

Stable isotopes analysis (SIA) is another compelling tool to study the diet of marine mammals, mainly using nitrogen (^{15}N) and carbon (^{13}C) isotopes (Das et al. 2003, Damseaux et al. 2021, Ogilvy et al. 2022). SIA provides insights into an individual's trophic position and primary sources of carbon (coastal versus offshore, pelagic versus benthic) in the food web (Peterson & Fry 1987, Newsome et al. 2010). It is based on the understanding that the stable isotope composition of a predator is a weighted mixture of the assimilated isotopic composition of its food sources, modified by isotopic fractionation (Newsome et al. 2010), reflecting the consumer's diet over longer time periods (Dalerum & Angerbjörn 2005). The time period reflected depends on the analysed tissue; for example, half-life turnover rates for carbon and nitrogen isotopes in dolphin skin were estimated to be 24.16 ± 8.19 d and 47.63 ± 19 d, respectively (Giménez et al. 2016). Predators typically have higher $\delta^{15}N$ values than their prey, while $\delta^{13}C$ values remain relatively similar across trophic levels (McConnaughey & McRoy 1979, Post 2002). Bayesian mixing models can estimate the pro-

portional contributions of various prey sources to a consumer's diet (Moore & Semmens 2008). Inherent limitations (e.g. Layman & Post 2008) include a lack of high taxonomic resolution (Polito et al. 2011), the inappropriate use of fractionation factors as well as temporal and spatial variation of isotopic signatures of different prey sources (Shiffman et al. 2012).

SCA, metabarcoding and SIA each offer a unique perspective on the diet of marine organisms, capturing either a direct snapshot or a more assimilated view of their dietary intake (Inger & Bearhop 2008). Whereas SCA is unambiguously biased towards stranded and bycaught individuals (Sekiguchi et al. 1992, Das et al. 2003), metabarcoding and SIA can, in principle, also be applied to free-ranging animals by applying less invasive techniques like biopsy sampling and non-invasive scat collection (Johnson & Davoren 2021). Combining these methods can therefore compensate for biases introduced by either method and can also help to provide a comprehensive picture of the complete diet of harbour porpoises, rather than a glimpse of the dietary spectrum.

To date, there appears to be a lack of studies that have employed a combination of SCA, metabarcoding and SIA on the same marine mammal individuals to track changes in prey selection over time. This gap in research highlights an opportunity for significant advancements in our understanding of marine mammal diets and their dynamic nature.

Here we investigated the diet of harbour porpoises in the southern North Sea by integrating 3 methodologies: SCA, metabarcoding and SIA. This integrated approach is expected to identify a broader range of prey species, thereby enhancing our understanding of their dietary patterns. Additionally, the study explores temporal variations by comparing diets immediately prior to stranding with long-term, assimilated diets, providing insights into dietary shifts over time and offering a more dynamic understanding of the feeding ecology of harbour porpoises.

2. MATERIALS AND METHODS

2.1. Sample collection

As part of a long-term health monitoring programme, harbour porpoises stranded along the coastline of Schleswig-Holstein, Germany, have been necropsied since the 1990s to determine their health status and cause of death (Siebert et al. 2006). Organs, including muscle, intestine and stomach, are assessed pathologically, and then stored at −20°C until further analysis (Siebert et al. 2001, 2020). For this study, we selectively included only those stranded harbour porpoises (n = 48 individuals) for which it was feasible to apply all 3 dietary analysis methods: metabarcoding, SCA and SIA (Fig. 1). These 48 porpoises, which

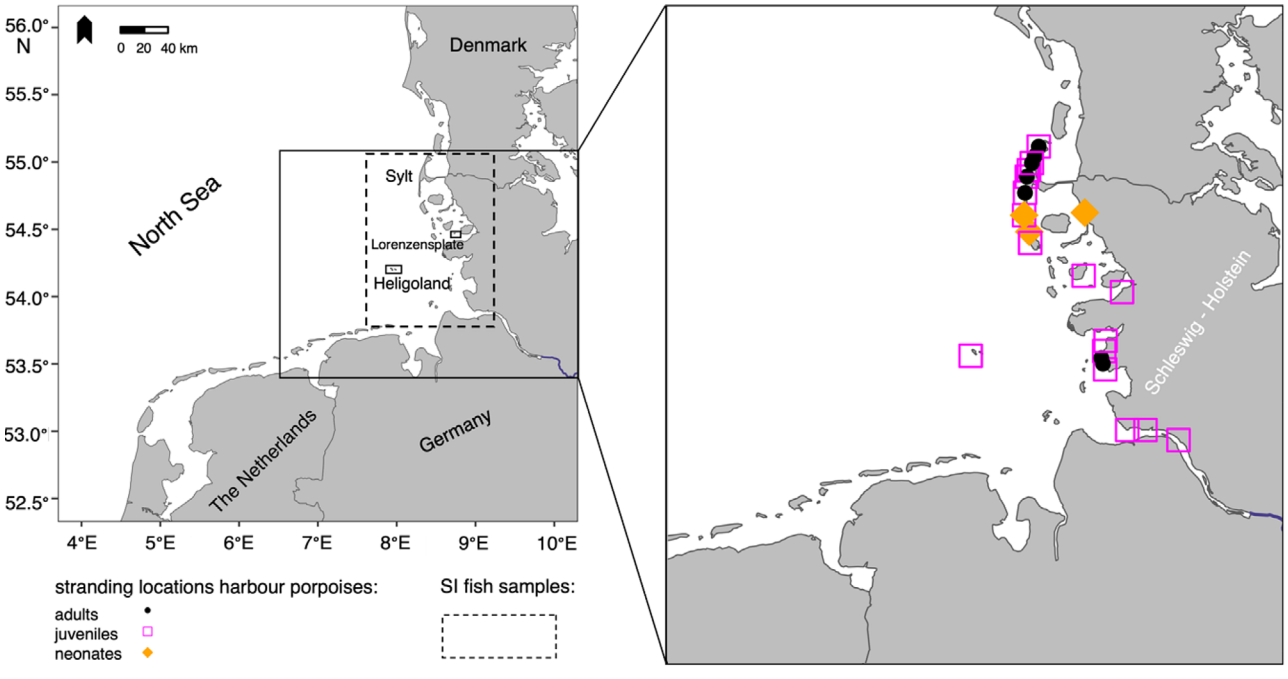

Fig. 1. Stranding locations of harbour porpoises included in this study and sampling area for prey fish stable isotope (SI) samples

stranded during the period 2005–2021, comprised 3 neonates, 24 juveniles and 21 adults. Classification into different age categories was based on body lengths or, where available, on the determined age by counting annual growth layers in lower incisors (Siebert et al. 2001) (metadata in Table S1 in the Supplement at www.int-res.com/articles/suppl/m755p115_supp.pdf).

2.2. SCA

SCA was performed on 48 harbour porpoise stomachs following the methodology outlined by Leopold et al. (2015). Stomachs were either rinsed and prepared for analysis during necropsies or stored at −20°C until further processing. Briefly, relatively undigested prey remains were identified to species level and measured directly. Subsequently, all remaining stomach contents were rinsed into a large glass beaker, where heavier prey items (e.g. otoliths and squid beaks) stayed at the base of the beaker, and lighter prey items (tissues, fluids) were gradually flushed out of the sample by means of overflow (Leopold et al. 2015). To ensure that nothing was lost during the overflow process (e.g. small vertebrae), the overflowing beaker was placed on a 500 µm metal mesh sieve. The remains were then sorted and identified under a stereo microscope. Otoliths were primarily used to identify fish species and to estimate their length and weight (based on available regressions in Leopold et al. 2001 and Härkönen 1986). To avoid overlooking any consumed prey with hard parts, other prey remains (e.g. vertebrae, scales, cephalopod beaks and crustacean remains) were also used for prey species identification. Hard parts were foremost identified according to Camphuysen & Henderson (2017), Härkönen (1986), Leopold et al. (2001) and an internal reference collection at the Institute of Terrestrial and Aquatic Wildlife of the University of Veterinary Medicine Hannover Foundation. Prey remains were photographed with an Olympus UC90 camera attached to an Olympus SZX 10 stereomicroscope and measurements were taken using Olympus cellSense software (version 3.2). Otolith wear was accounted and corrected for using correction factors described by Leopold (2015).

After back-calculating length and weight of all recorded prey, frequency of occurrence (FO), percentage frequency of occurrence (%FO), consumed biomass (g) and percentage consumed biomass (%g) were calculated per prey species and also per prey guild (Table S2). Additionally, to assess the overall significance of each prey guild, we employed the index of relative importance (IRI = %FO × [%N + %M], where %FO is number of stomachs where prey was found divided by the total number of stomachs, %N is the numerical percentage of each prey in relation to the total number of individual prey found in the stomachs, and %M is the percentage of total reconstructed prey weight). IRI serves as a concise and widely applied measure to summarise dietary composition (Hyslop 1980). Prey guilds were structured around the categorization of fish prey primarily according to ecological factors, while still maintaining taxonomic relevance (Leopold et al. 2015).

2.3. Metabarcoding

A universal 16S rRNA primer, targeting marine and freshwater fish species, was applied to stomach and/or intestinal samples of 48 individuals. Intestinal samples were utilized in instances where SCA had already been performed and the stomach was no longer available for DNA sampling. For one individual, we had 2 different samples: 1 stomach and 1 intestinal sample to check for differences between tissues, resulting in a total of 49 samples. A detailed description of the metabarcoding approach and fish primer design can be found in Boyi et al. (2022). In short, DNA was isolated from 200–250 mg of harbour porpoise stomach or intestinal content using the QIAamp Fast DNA Stool Mini Kit (Qiagen). Subsamples were taken from scat cores at 2 to 3 different places and subsequently homogenized. Negative controls (blanks without stomach or intestinal content) were included for each isolation procedure. DNA isolated from a vouchered fish species (common rudd *Scardinius erythrophthalmus*), using a QIAamp DNA Micro Kit (Qiagen), was included as a positive control and for validation. Afterwards, DNA was subsampled into 2 volumes of 50 µl to circumvent repeated freeze–thaw processes and to protect DNA integrity. All samples were stored at −20°C until further processing.

A 2-step enrichment PCR was employed to effectively amplify limited amounts of prey DNA. In the initial step, locus-specific primers were utilized to amplify the target region. Subsequently, in the second step, primers incorporating both the locus-specific sequence and a universal 5′ tail, as delineated in Illumina's Nextera library protocol, were employed. In this second step, the products of the first PCR served as templates, facilitating the enrichment of the target region. In the third step, unique indices (bar-

codes) and Illumina adapters were appended to all second-step PCR products, followed by further amplification of the products. The paired-end sequencing using an Illumina MiSeq sequencer with a MiSeq Reagent Kit V2 (500 cycles) (2 × 250 bp) (Illumina) was executed at Microsynth Next Generation Facilities, Switzerland.

After quality control, operational taxonomic units (OTUs) were assigned. OTU sequences underwent comparison with a manually compiled reference database utilizing NCBI accessions. A confidence threshold at the species level was then applied to further categorize OTUs. Species were found to be sufficiently classified if OTUs had a confidence level of >0.9.

2.4. SIA of carbon (C) and nitrogen (N)

2.4.1. Harbour porpoise samples

Of the 48 samples mentioned above, only 45 were available for carbon ($\delta^{13}C$) and nitrogen ($\delta^{15}N$) stable isotope composition analysis. First, muscle tissue (approximately 200 mg) from each porpoise, usually taken from the longissimus dorsi, was carefully weighed prior to placing it into an Eppendorf tube. Samples were stored at −80°C overnight before freeze-drying for 48 h. Post freeze-drying, the samples were ground into a fine, homogenized powder using a mortar. Homogenized samples were weighed in tin capsules using a microbalance (precision 0.01 mg). To optimise the combustion of the sample, approximately the same mass of tungsten (W) was added to each capsule.

Stable isotope measurements were conducted using an isotope ratio mass spectrometer (IsoPrime PrecisION) coupled with an N-C-S elemental analyser (Vario MICRO cube, Elementar) to enable automated analysis. Isotope ratios are expressed in δ notation (parts per thousand, ‰):

$$\delta X = \left(\frac{R_{\text{sample}} - R_{\text{standard}}}{R_{\text{standard}}}\right) \times 1000 \quad (1)$$

where X corresponds to ^{13}C or ^{15}N and R is the ratio of heavy to light isotopes of $^{13}C/^{12}C$ or $^{15}N/^{14}N$.

International standards used for correcting isotope ratios were Vienna Pee Dee belemnite (VPDB) for $\delta^{13}C$ and atmospheric air for $\delta^{15}N$. International Atomic Energy Agency (IAEA)-certified reference materials for sucrose (IAEA-C_6, $\delta^{13}C = -10.8 \pm 0.5$‰; mean ± SD), ammonium sulphate (IAEA-N_2, $\delta^{15}N = 20.4 \pm 0.12$‰) and silver sulphate (IAEA-S_2, $\delta^{34}S = 22.62 \pm 0.08$‰) were used as primary analytical standards. Sulphanilic acid (Sigma-Aldrich, $\delta^{13}C = -28.61 \pm 0.31$‰ and $\delta^{15}N = -0.38 \pm 0.22$‰) was used as a secondary analytical standard. European seabass *Dicentrarchus labrax* was used as standard control. Standard deviations on multiple batch repeat measurements of secondary and internal laboratory standards analysed interspersed with samples (1 repeat of each standard after 12 samples) ranged from 0.18 to 0.23‰ for $\delta^{13}C$ and from 0.16 to 0.24‰ for $\delta^{15}N$. For a detailed description of the method applied in this study, see Damseaux et al. (2021) or Pinzone (2021).

Raw results were analysed and fixed if the C:N ratio was between 3.1 and 3.8. Within this range, the lipid concentration was low enough to not influence the abundance of stable isotopes in the samples (Post et al. 2007, Skinner et al. 2016). Outside this range, samples were normalised for lipid content using the equation of McConnaughey & McRoy (1979), adapted by Post et al. (2007), for aquatic animals:

$$\delta^{13}C_{\text{normalised}} = \delta^{13}C_{\text{untreated}} - 3.32 + 0.99 \times (C{:}N) \quad (2)$$

2.4.2. Fish samples

Fish samples were collected in coastal waters of the German North Sea (approximately between 53.858–55.012° N and 7.893–8.879° E) for the FishNet project between 2019 and 2021 (Schückel et al. 2023) and covered all important prey species based on our own SCA database (Fig. 1). For the analysis of $\delta^{13}C$ and $\delta^{15}N$ of fish prey, a large piece of tissue (usually a piece of the tail) was removed from each fish, labelled and frozen at −20°C in a cryotube.

The measurements of stable isotope ratios of $\delta^{13}C$ and $\delta^{15}N$ were carried out at the Leibniz Institute for Zoo and Wildlife Research in Berlin. Here, most of the samples were first subjected to fat extraction using a Soxtherm (type SE406, C. Gerhardt). Each sample was placed in a glass fibre filter (VWR®, 70 mm, type 698), which was transferred in an extraction sleeve (glass fibre MN649: 33 × 80 mm) into an extraction cup (glass, macro, Ø × L: 54 × 130 mm) with a metal insert. The glass container was then slowly filled with 110 ml of a methanol–chloroform solution (1:2 ratio) and 2–3 boiling stones.

For the SIA of $\delta^{13}C$ and $\delta^{15}N$, 0.5 ± 0.1 mg of the samples were weighed into tin capsules (type IVA, 4 × 6 mm) and measured using an elemental analyser isotope ratio mass spectrometer with autosampler function (FlashEA 1112, Thermo Fisher Scientific). The international standards used were atmospheric

air for $\delta^{15}N$ and VPDB for $\delta^{13}C$. The analytical precision was <0.15‰ (1 SD) for the stable carbon isotope ratio and <0.15‰ for the nitrogen isotope ratio.

2.5. Statistical analysis

All data were analysed within R (version 4.2.3; R Core Team 2024).

2.5.1. SCA

Permutational multivariate ANOVA (PERMANOVA) was used to assess the influence of age and sex on harbour porpoise prey species composition. Additional variables that might affect individual prey selection, e.g. season or location, were ignored due to the rather low sample size.

2.5.2. SIA and MixSIAR modelling

After a preliminary visual inspection of the 45 samples analysed, 3 samples were categorized as outliers, either having increased $\delta^{15}N$ levels or being too depleted in $\delta^{13}C$ compared to the rest of the samples and were therefore excluded from further analysis. Also, neonates were excluded as they could have biased results due to the possibility of nursing. Subsequently the assumptions of the quantitative model, grounded in frequentist probability, were tested using a mixing polygon simulation to evaluate the ability of the model to accurately calculate source contributions in explaining consumer isotopic values (Erftemeijer & Robin Lewis 2006, Phillips et al. 2014, Wild et al. 2020) (Fig. S1). Individuals outside the mixing polygon had to be removed, as the model could most likely not account for those individuals. The remaining data set consisted of 34 individuals, which were included in the mixing model (Table S1) and used for the analysis of differences in $\delta^{13}C$ and $\delta^{15}N$ between adult and juvenile harbour porpoises, using non-parametric Kruskal-Wallis tests.

MixSIAR modelling was performed using the 'MixSIAR' R package (version 3.1.12, Stock et al. 2018). Kruskal-Wallis tests were used to assess differences in $\delta^{13}C$ and $\delta^{15}N$ values of harbour porpoise muscle samples with respect to age class and sex. The 'SIDER' ('Stable Isotope Discrimination Estimation using R') package (version 1.0.0.0; Healy et al. 2018) was used to calculate trophic enrichment factors (TEFs; difference between stable isotope ratios of a consumer and its food source) for harbour porpoises based on available data of taxonomically closely related species.

Predator and prey samples were corrected for the oceanic Suess effect (Keeling 1979, Gruber et al. 1999) to allow the comparison of $\delta^{13}C$ values from individuals sampled over a wide temporal scale. This was done by using the 'SuessR' package (version 0.1.4, Clark et al. 2021). Data were adjusted to the year 2020 (Table S1), which was the average year of collected fish prey stable isotope data. Note that $\delta^{13}C$ values corrected for the Suess effect were used if not indicated otherwise.

Briefly, MixSIAR, using Markov chain Monte Carlo (MCMC) simulations, was applied to model the probability of proportions of fish prey sources in the diet of adult and juvenile harbour porpoises. However, mixing models are unable to distinguish between sources with similar isotopic values (Phillips et al. 2014). Further, it is unlikely that mixing models with more than 7 sources present legitimate and interpretable source estimations (Stock et al. 2018).

Harbour porpoises have a broad prey spectrum; therefore, a reduced set of prey species or merging of those prey species, due to the possibility of overlapping isotopic values, was required (Sorensen et al. 2009). Thus, cluster analysis was performed prior to running the model. $\delta^{13}C$ and $\delta^{15}N$ stable isotope values were available for 34 prey species. To reduce the data set, only known prey species (based on SCA and metabarcoding) were kept for further analysis, resulting in 19 prey species. Those 19 species were indexed based on the subsequent information. The transition from coastal to offshore waters, identified through suspended particle composition, typically occurs between 20 and 75 km from the Elbe outlet (Desmit et al. 2024). Isoscape models for the North Sea reveal a gradient of decreasing $\delta^{13}C$ values along the German coastline, with values around −18 west and north of Heligoland extending towards the central North Sea (MacKenzie et al. 2014, St. John Glew et al. 2019). Using these models and considering suspended particle composition, prey species were classified as coastal or offshore. Moreover, prey species were categorized based on either being influenced by benthic or pelagic sources, as planktonic algae exhibit lower $\delta^{13}C$ values than benthic algae (de la Vega et al. 2016). Whiting and Atlantic cod were categorized into 2 length classes due to significant differences in their $\delta^{13}C$ values. Finally, 22 fish units were included in the cluster analysis, with sample sizes ranging between 3 and 125 (Table S3, Fig. S2). Subsequently, the mean isotope values for each unit were

Table 1. Mean, SD and sample size (n) of $\delta^{13}C$ and $\delta^{15}N$ for the 6 prey clusters included in MixSIAR modelling. Species included in each cluster are given under the cluster name. Clusters with benthic sources have enriched $\delta^{13}C$ of >−18 and clusters with pelagic sources have depleted $\delta^{13}C$ of <−18. Some species are divided into different clusters depending on their $\delta^{13}C$ values. See Table S3 for $\delta^{13}C$ and $\delta^{15}N$ values of individual species

Cluster	Mean $\delta^{13}C$	SD $\delta^{13}C$	Mean $\delta^{15}N$	SD $\delta^{15}N$	n
1 **Coastal fish | benthic $\delta^{13}C$ source** *Ammodytes marinus, Eutrigla gurnardus, Pomatoschistus pictus, P. minutus, Osmerus eperlanus*	−17.3	0.8	17.3	0.8	110
2 **Offshore fish | pelagic $\delta^{13}C$ source** *Ammodytes marinus, Callionymus lyra, Gadus morhua* (<10 cm), *Hyperoplus lanceolatus, Limanda limanda, Sardina pilchardus, Sprattus sprattus*	−19.0	0.6	15.5	1.3	64
3 **Flatfish | benthic $\delta^{13}C$ source** *Buglossidium luteum, Platichthys flesus, Pleuronectes platessa, Solea solea*	−16.4	1.2	16.5	1.0	172
4 **Bentho-pelagic fish | pelagic $\delta^{13}C$ source** *Clupea harengus, Trisopterus esmarkii*	−18.9	0.8	13.3	1.6	24
5 **Bentho-pelagic fish | benthic $\delta^{13}C$ source** *Gadus morhua* (>10 cm), *Merlangius merlangus* (>10 cm)	−16.0	0.6	18.1	0.8	62
6 **Bentho-pelagic/pelagic fish | pelagic $\delta^{13}C$ source** *Merlangius merlangus* (<10 cm), *Scomber scombrus*	−18.3	1.0	16.0	1.3	74

grouped using a hierarchical cluster analysis (k = 6; Ward's minimum variance method; Table 1). The mean and associated standard deviation of each source were determined by averaging the mean $\delta^{13}C$ and $\delta^{15}N$ values for each unit forming the cluster.

Currently, no TEFs are available specifically for harbour porpoises, and informative priors on porpoise diet to include are also largely lacking. To enhance the accuracy and reliability of dietary estimates, we ran models including different factors (age, sex or no factor) for the consumers, different priors for the sources (Fig. S3) and different TEFs for $\delta^{13}C$ and $\delta^{15}N$ (Table 2). In total, 24 different sets of models, with each set containing 3 distinct models, were run (n = 72) (Table S4).

A process × residual error model structure was used, and each mixing model was run with the following MCMC parameters: 3 chains, 100 000 iterations, first 50 000 iterations burn-in and sampling at intervals of 50 iterations (Stock & Semmens 2016). Model convergence was evaluated with the Gelman-Rubin diagnostic, which compares estimates of variance within and between Markov chains, where values <1.01 indicate convergence (Gelman et al. 2013). Model evaluation was based on (1) Gelman-Ruben diagnostics to assess model convergence, ensuring that MCMC sampling had reached a stable solution and that the results were reliable; (2) leave-one-out cross-validation (LOO) information criterion results to provide a measure of model fit and complexity, helping to identify the model that best balances predictive accuracy and parsimony; and (3) posterior density plots to evaluate how well the model accounted for the data, with particular attention to distributions that reflected realistic ecological interpretations without artifacts such as double peaks.

2.5.3. Direct model comparison

For comparison of methods, SCA and metabarcoding data sets were grouped into the prey clusters identified in the cluster analysis for SIA prey samples included in the mixing models. Metabarcoding and SCA data were arranged in a (class × sample) table including sequence read counts and consumed bio-

Table 2. Mean ± SD trophic enrichment factors (TEFs) for $\delta^{13}C$ and $\delta^{15}N$ used for MixSIAR models

Source TEFs	$\delta^{13}C$	$\delta^{15}N$
Borrell et al. (2012)	1.29 ± 0.56	2.73 ± 0.58
Caut et al. (2011)	1.26 ± 0	1.23 ± 0.1
Giménez et al. (2016)	1.01 ± 0.37	1.57 ± 0.52
Hobson et al. (1996)	1.3 ± 0.1	2.4 ± 0.3
Mèndez-Fernandez et al. (2012)	0.8 ± 0	1.4 ± 0
'SIDER' R package; Healy et al. (2018)	1.38 ± 1.95	3.42 ± 1.61

mass estimates, respectively (Tables S5 & S6). Biomass instead of counts was used for SCA data, as it provides the best measure of relative importance to the diet (Swan et al. 2020). Species that belonged to either of 2 clusters (lesser sandeel *Ammodytes marinus*, *Gadus morhua*, *Merlangius merlangus*) were grouped into the cluster most appropriate based on SCA results. A seventh cluster was added for prey species which could not be assigned to one of the 6 other classes because of the lack of stable isotope data. From these tables, the mean (±SD) proportion of each class contributing to the diet of harbour porpoises was calculated, separately for juvenile and adult predators.

3. RESULTS

3.1. SCA

Out of 48 stomachs, 3 stomachs (1 adult, 1 juvenile and 1 neonate) were completely empty, and an additional 3 stomachs only had remains in the oesophagus. As oesophagus samples were only available for some individuals, they were also excluded from further SCA.

Harbour porpoise age class had a significant effect on prey species composition (biomass on prey guild level; PERMANOVA, pseudo-$F_{1,35}$ = 1.78, p = 0.029). Adults consumed almost 4 times more flatfish in terms of biomass than juveniles (9352.94 and 2356.86 g, respectively), while juveniles consumed approximately 12 times as many gadoids compared to adults (11 584.03 and 953.97 g, respectively) (Table S7). Harbour porpoise sex had no significant effect on prey species composition (biomass on prey guild level; PERMANOVA, pseudo-$F_{1,35}$ = 1.10, p = 0.349).

Overall, the prey guilds sandeels (n = 18) and flatfish (n = 18) dominated the diet of harbour porpoises in terms of frequency of occurrence (FO), closely followed by gadoids (n = 17) and gobies (n = 15) (Fig. 2a). Regarding prey biomass (%W), gadoids and flatfish had the highest percentages, 38 and 35.13%, respectively (Fig. 2c). Sandeel biomass was less than half (17.51%) compared to that of gadoids or flatfish. Gobies only contributed 1.44% to the total biomass.

The IRI for all sampled individuals also indicated that flatfish, gadoids, sandeels and gobies were of primary importance; clupeids, demersal roundfish (e.g. dragonet *Callionymus lyra*) and estuarine roundfish

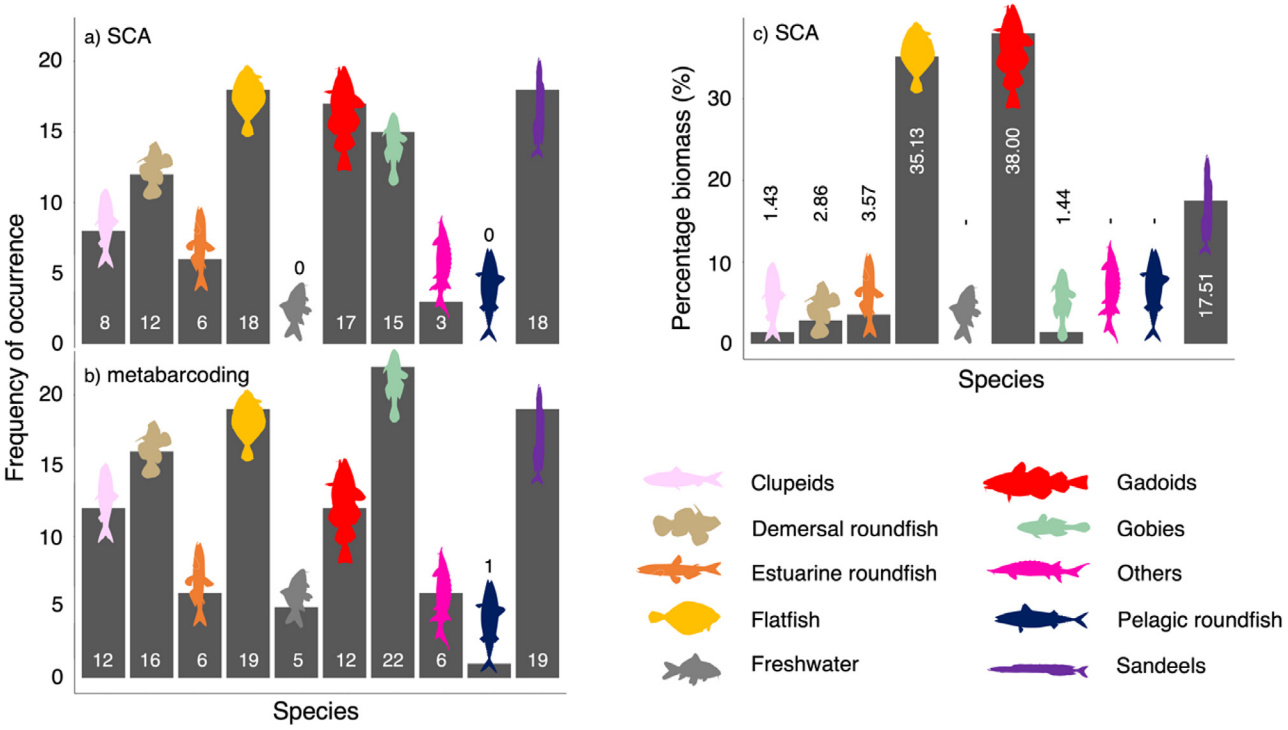

Fig. 2. Results for overall prey composition based on prey guilds for stomach content analysis (SCA) (n = 42 samples) and metabarcoding (n = 49). (a,b) Frequency of occurrence (FO); (c) percentage biomass (%W) obtained from SCA (not applicable for metabarcoding)

(European smelt *Osmerus eperlanus*) were of secondary importance; and invertebrates, other fish species (pipefish Syngnathidae spp., European sturgeon *Acipenser sturio* and lamprey *Lampetra* spp.) and squid were of negligible importance (Table S2).

3.2. Metabarcoding

Of the 49 processed stomach and intestine samples for metabarcoding, 1 sample did not reveal any fish DNA and was excluded from further analysis. A total of 1 469 826 merged sequence reads over 48 samples passed the quality filtering (Q > score 20) and were correctly indexed. Of those, 213 161 merged sequence reads (n = 29; 14.5%) were host DNA. The lack of DNA in the 2 negative controls (PCR blanks) confirmed the absence of contamination during PCR. From the 2 positive control samples (both *Scardinius erythrophthalmus*), a total of 69 642 merged sequence reads were obtained after quality filtering, indicating successful amplification of targeted fish DNA.

The sequence reads were classified into 27 unique OTUs (Table S8) belonging to marine, brackish and freshwater fish species. Of those, 74.07% (n = 20) were unambiguously identified to species level with 100% BLAST identity. Two OTUs, also identified to species level, Lozano's goby *Pomatoschistus lozanoi* and *Merlangius merlangus*, had confidence levels of 0.97 and 0.96, respectively. Five OTUs were identified as a 'complex', meaning they were closely related species either belonging to the same family (Ammodytidae: small sandeel *Ammodytes tobianus* and greater sandeel *Hyperoplus lanceolatus*; Pleuronectidae: European plaice *Pleuronectes platessa* and European flounder *Platichthys flesus*; dab *Limanda limanda* and long-rough dab *Hippoglossoides platessoides*) or genus (*Alosa* and *Lampetra*).

Overall prey composition in terms of FO showed prey species grouped into gobies (n = 22), flatfish (n = 19), sandeels (n = 19) and demersal roundfish (n = 16) to be the most important prey guilds (Fig. 2b). The results for the one individual from which both a stomach and intestinal sample were analysed showed a 100% overlap in the prey sequences detected.

3.3. SIA

C:N ratios of all harbour porpoise samples were between 3.3 and 3.8 and therefore did not require lipid corrections. The overall mean (±SD) $\delta^{13}C$ value was −17.6 ± 0.5‰, ranging from −18.4 to −16.5‰. Mean $\delta^{15}N$ was 17.6 ± 0.9‰, spanning a range of 15.2 to 19.4‰ (detailed in Table S1). There were no significant differences in $\delta^{13}C$ values between adult and juvenile harbour porpoises (Kruskal-Wallis $\chi^2 = 0.50$, df = 1, p = 0.48). However, adults had significantly higher $\delta^{15}N$ values than juveniles (Kruskal-Wallis $\chi^2 = 4.35$, df = 1, p = 0.04). No significant differences between sexes were found for $\delta^{13}C$ (Kruskal-Wallis $\chi^2 = 0.55$, df = 1, p = 0.46) and $\delta^{15}N$ (Kruskal-Wallis $\chi^2 = 0.20$, df = 1, p = 0.65).

3.4. MixSIAR

Isotope ratios of harbour porpoises and their potential prey predominantly assembled within the mixing polygon, which was expressed by the mean and associated error of each of the 6 prey clusters used for MixSIAR modelling (Fig. 3).

Overall, models including age as a factor and an either uninformative or semi-informative prior performed best (Table S4). The selected model

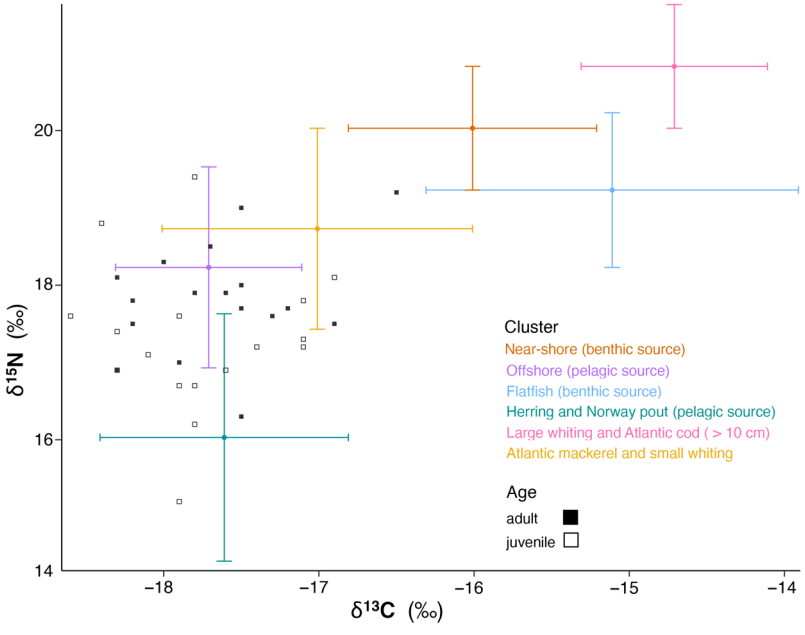

Fig. 3. Isotope mixing polygon showing putative prey and harbour porpoise $\delta^{13}C$ and $\delta^{15}N$ values. Isotope values of harbour porpoises are grouped by age (adults and juveniles). Sources are colour-coded according to the cluster analysis and have been corrected for trophic enrichment ($\delta^{13}C$: 1.29 ± 0.56; $\delta^{15}N$: 2.73 ± 0.58; Borrell et al. 2012). Standard deviations for each source are represented by error bars

(Model 62; Table S4) included TEFs based on Borrell et al. (2012), age as a factor and an uninformative prior (alpha = 1) (Fig. S3). MixSIAR model output revealed that Cluster 2 (Offshore fish | pelagic $\delta^{13}C$ source) contributed most to the long-term diet of adult harbour porpoises with a mean contribution of 46.8% (95% CI: 14.0, 73.3) followed by Cluster 4 (Bentho-pelagic fish | pelagic $\delta^{13}C$ source) and 6 (Bentho-pelagic/pelagic fish | pelagic $\delta^{13}C$ source), contributing 33.0% (95% CI: 14.3, 56.5) and 11.0% (95% CI: 4.0, 34.8), respectively. Cluster 4 contributed most to the long-term diet of juvenile harbour porpoises, with a mean contribution of 47.5% (95% CI: 17.7, 75.3). Cluster 2 contributed slightly less to the diet of juveniles with 41.4% (95% CI: 8.1, 75.9) followed by Cluster 6, contributing 6.2% (95% CI: 1.0, 24.4). Clusters 1 (Coastal fish | benthic $\delta^{13}C$ source), 3 (Flatfish | benthic $\delta^{13}C$ source), and 5 (Bentho-pelagic fish | benthic $\delta^{13}C$ source) each contributed less than 5% to the overall diet. Posterior distributions and correlation plots are provided in the supplementary materials (Figs. S4 & S5).

3.5. Direct comparison SCA versus metabarcoding

In total, 42 samples were available for the direct comparison of metabarcoding and SCA results (Fig. 4). Only fish prey was compared, as metabarcoding samples were solely analysed with a fish primer in this study. Metabarcoding only displayed a slightly higher species detection rate compared to SCA (26 versus 22 identified fish species, respectively). Of those, 11 fish species were only detected with SCA and 15 species were only detected via metabarcoding. Species overlap for both methods was 11 species. Overall, prey species detection was higher for metabarcoding than for SCA for the majority of the samples (n = 26; samples that had at least 1 fish species identified with both methods). On an individual sample level, we were able to increase prey species detection by 49% on average. Differences in FO for sandeels, flatfish and demersal roundfish were only marginal compared to SCA results, likely due to the larger metabarcoding sample size. Nevertheless, goby occurrence increased by 11.23%.

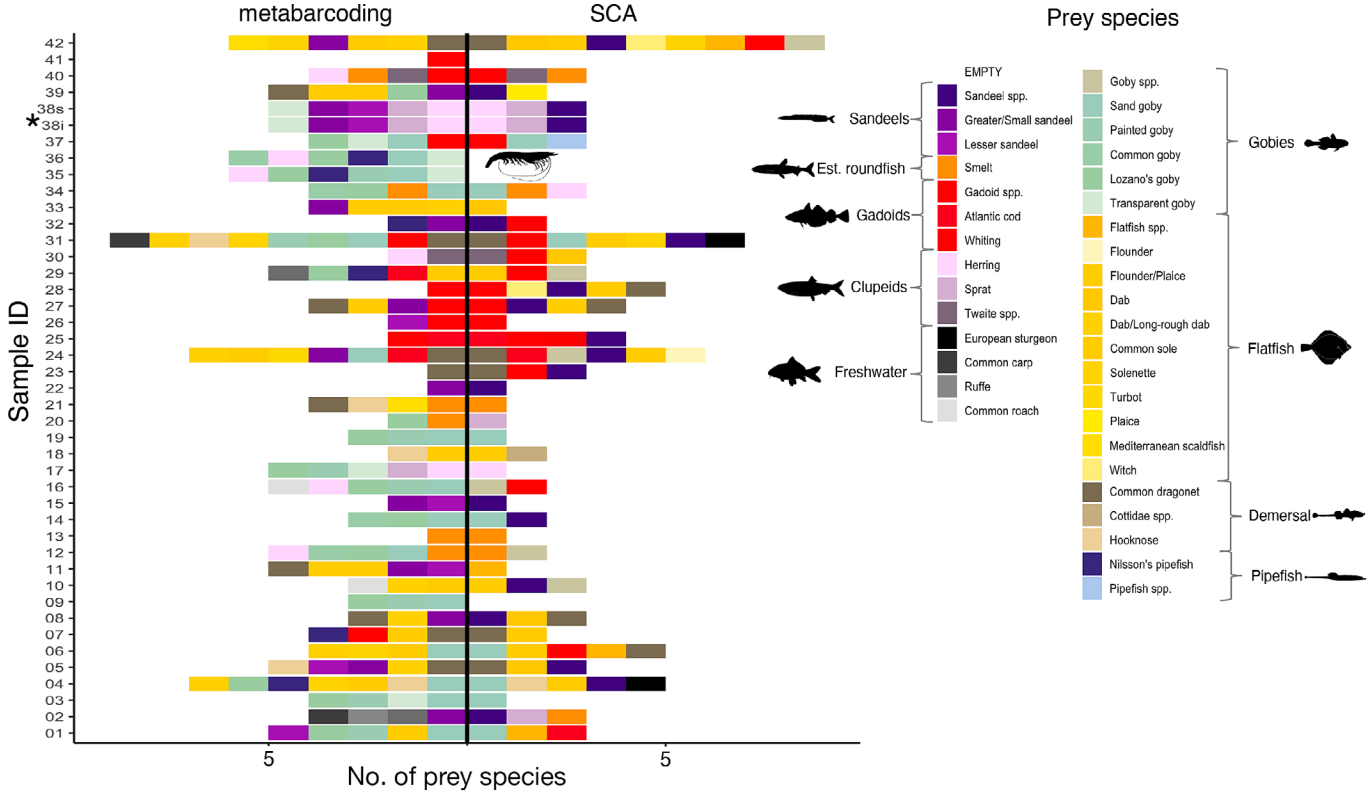

Fig. 4. Direct comparison of prey, at the species level, between metabarcoding samples on the left and hard parts (from stomach content analysis, SCA) on the right. Samples are plotted in the same order left and right of the vertical black line, starting from the inside out. Colour coding is based on functional groups and matches colours used in Fig. 2. Samples 38i and 38s, indicated by an *, were collected from the same individual with one sample from the intestine and one from the stomach, respectively. The shrimp symbol indicates that only shrimp remains were found during stomach content analysis

3.6. Direct comparison of all 3 methods

Direct method comparison was possible for 34 individuals (number of individuals that were included in the mixing model). The mean proportions of prey clusters to the diet of adult and juvenile harbour porpoises varied greatly for each method (Fig. 5). As mentioned above, Cluster 2 (Offshore fish | pelagic $\delta^{13}C$ source) had the highest contribution to the diet of adult harbour porpoises analysed with MixSIAR followed by Cluster 4 (Bentho-pelagic fish | pelagic $\delta^{13}C$ source) whereas this pattern was reversed in juvenile porpoises. The contribution of Cluster 2 decreased strongly for juveniles analysed via SCA and contributed equally as Cluster 1 (Coastal fish | benthic $\delta^{13}C$ source) to the diet of adults analysed via SCA. Cluster 1 also showed the highest contribution to the diet of juvenile porpoises via SCA. Adults analysed via metabarcoding also had highest mean proportions of Cluster 2, albeit less strong than MixSIAR adults. Cluster 4 only provided a minor contribution to the diet of adults and juveniles analysed via SCA and metabarcoding. Metabarcoding juveniles showed the highest contribution of Clusters 1 and 7 (Cluster 7; species not included in MixSIAR; hooknose *Agonus cataphractus*, Allis shad *Alosa alosa*, transparent goby *Aphia minuta*, Mediterranean scaldfish *Arnoglossus laterna*, common carp *Cyprinus carpio*, ruffe *Gymnocephalus cernua*, European brook lamprey *Lampetra planeri*, *P. lozanoi*, common goby *Pomatoschistus microps*, common roach *Rutilus rutilus*, turbot *Scophthalmus maximus* and Nilsson's pipefish *Syngnathus rostellatus*) to their diet. Cluster 6 (Bentho-pelagic/pelagic fish | pelagic $\delta^{13}C$ source) contributed <0.01% to the diet of any harbour porpoise analysed via SCA.

4. DISCUSSION

This research marks a pioneering effort in combining SCA, metabarcoding and SIA to study the diet of individual harbour porpoises. By integrating these methods, we have gained insights into both the short- and long-term dietary patterns of harbour porpoises, revealing previously unidentified prey species and increasing prey species detection rates, thereby filling current knowledge gaps in the feeding ecology of harbour porpoises in the southern North Sea. Multi-method approaches for dietary analyses of marine wildlife have been performed before on various taxa, including seals (Dehn et al. 2007), dolphins and porpoises (Giménez et al. 2017, Mahfouz et al. 2017,

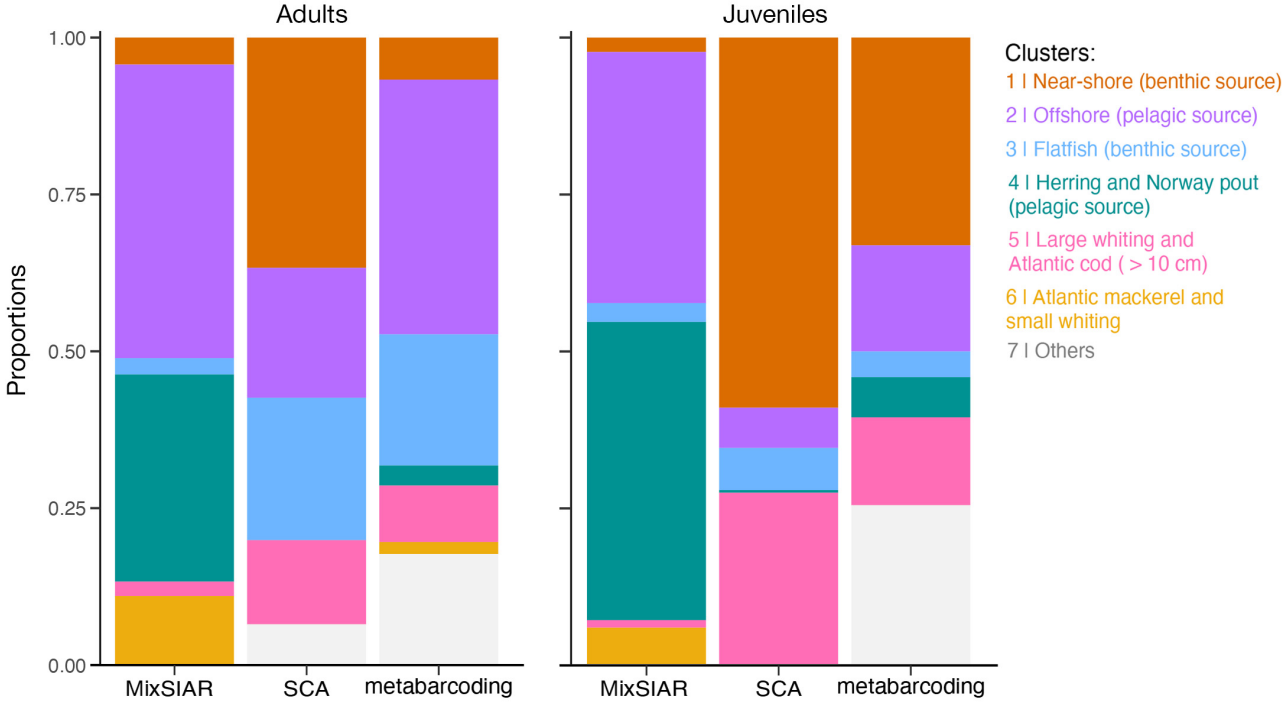

Fig. 5. Mean proportions of each prey cluster in the diet of adult and juvenile harbour porpoises. Cluster 7 includes prey species that could not be assigned to any of the 6 other clusters (*Agonus cataphractus, Alosa alosa, Aphia minuta, Arnoglossus laterna, Cyprinus carpio, Gymnocephalus cernua, Lampetra planeri, Pomatoschistus lozanoi, Pomatoschistus microps, Rutilus rutilus, Scophthalamus maximus* and *Syngnathus rostellatus*)

McCluskey et al. 2021), sea birds (Polito et al. 2011, Kuepfer et al. 2023), fish (Lin et al. 2023) and invertebrates (Cordone et al. 2022). However, only a few included metabarcoding and most compared SCA and SIA results from different individuals, locations and time spans. Understanding how environmental factors influence marine mammal diet is complex and difficult to capture with a single method, as important aspects of an individual's feeding ecology may be overlooked. The differentiation between long- and short-term diet, as determined by combining the 3 methodologies, is crucial for management decisions regarding the conservation of harbour porpoises, as well as ecological and fishery impact assessments.

4.1. Dietary composition

Profound differences were found in the diet of individual harbour porpoises as estimated by MixSIAR compared to SCA and metabarcoding, suggesting differences in their long- and short-term diet. Unlike the diet of strandings reflecting coastal, benthic species like gobies and flatfish, the long-term diet of adult and juvenile harbour porpoises in this study seems to be more offshore-oriented where they feed on pelagic, schooling species like sprat *Sprattus sprattus* or herring and bentho-pelagic sandeels. Although seasonal influences on the dietary composition of porpoises are known (e.g. Leopold 2015), due to the relatively small sample size, those were not considered here. Also, as some harbour porpoises seem to spend their time predominantly in the Wadden Sea (Scheidat et al. 2024), it cannot be excluded that an increased sample size would show different long-term diets. However, a similar pattern concerning long- versus short-term diet was also described in an earlier study on harbour porpoises stranded in the Netherlands (Jansen et al. 2013).

As the 3 methods were performed on the same individuals, temporal differences in the diet represent the true diet rather than methodological errors and highlight that single-method studies most likely do not uncover the full dietary spectrum of a species.

Most abundant prey guilds identified with SCA and metabarcoding were largely in line with previous studies conducting SCA in the southern North Sea, namely gobies, sandeels and gadoids (e.g. Benke et al. 1998, Gilles et al. 2008, Haelters et al. 2012) where sandeels, gobies, gadoids and clupeids were found to be the top 4 prey guilds concerning harbour porpoise diets in the Netherlands (Leopold 2015). In contrast, we found flatfish to be 1 of the top 4 prey guilds contributing to the short-term diet, likely replacing energy-rich clupeids (>5 kJ g^{-1}) whose overall importance in terms of IRI was very low (Table S2). Earlier SIA studies found that flatfish (Pleuronectidae spp.) and gadoids only covered a minor contribution to the long-term diet of harbour porpoises from the southern North Sea (Das et al. 2003) and highlighted the importance of clupeids (herring and European pilchard *Sardina pilchardus*) and gobies (Mahfouz et al. 2017), which is only partly in line with our findings (Fig. 3; Fig. S4). However, given that some diet studies in the same area were conducted more than 2 decades ago, temporal shifts in diet composition cannot be excluded and should be further assessed in future studies.

All methods showed an ontogenetic variation in diet, although with less strong variation revealed via SIA (Fig. 5). In terms of reconstructed biomass (SCA), flatfish, especially common sole *Solea solea*, and sandeel biomass were much higher in adults than in juveniles (Table S7), which could either be due to differences in foraging habitat use or suggesting that foraging on flatfish or sandeels burrowed in the seabed requires experience. Here, we found that goby biomass was generally low but higher in adults compared to juveniles (219.91 and 137.55 g, respectively), whereas earlier studies showed that gobies were predominately targeted by juveniles (e.g. Santos et al. 2004, Andreasen et al. 2017). Adult harbour porpoises gained most energy from common sole (5 kJ g^{-1}, Spitz et al. 2010) and sandeels (5.8 kJ g^{-1} for *Ammodytes tobianus*, Spitz et al. 2010), whereas juveniles gained by far most energy from whiting (3.9 kJ g^{-1}, Spitz et al. 2010) (Fig. S6), advocating that adults and juveniles meet their main energy demands via different sources which are in line with biomass estimates. However, that does not always seem to be the case, and other studies showed that energy availability of certain prey species can be more important than biomass availability (Lockyer et al. 2003, Spitz et al. 2018).

Both metabarcoding (n = 5) and SCA (n = 1) uncovered a previously unrecorded prey species of harbour porpoises in our study area, namely the demersal, commercially unimportant hooknose *Agonus cataphractus*. Hooknose has been described as minor part of the diet of harbour seals *Phoca vitulina* in the Baltic Sea (Sørlie et al. 2020) and in the Wash and Moray First estuaries in the UK (Tollit & Thompson 1996, Hall et al. 1998) but has recently also been found in grey seals *Halichoerus grypus* from the German part of the North Sea (Boyi et al. 2022). Incongruously, monitoring of *A. cataphractus*, at least in the Wadden Sea, shows a decreasing trend since around 2012 in

many regions, resulting in densities dropping to their lowest levels recorded throughout the entire time series (Tulp et al. 2022).

Although the IRI provides an estimation of the relative importance of prey and proves useful when comparing results with other studies, this index does not include the abundance of available prey species. Hence, the low IRI of sturgeon and lamprey may partly be due to their relatively low occurrence in our study area compared to e.g. sandeels or flatfish. To determine if prey choices are truly a preference or rather abundance-related would require detailed abundance data on prey species, which are, unfortunately, lacking for many prey species, especially non-commercial ones.

4.2. Powerful combination

SCA relies on the opportunistic acquisition of stomachs (McCluskey et al. 2021). Dietary information obtained from stranded individuals likely returns biased results, as injured or sick individuals may need to modify their prey choices prior to death (Sekiguchi et al. 1992, Das et al. 2003). Including SIA may not only increase species detection rate but also unravel the diet of healthy animals (health status that is not compromised by acute illness or injuries). Neonates and juveniles are prone to acute starvation and/or emaciation due to their high metabolic requirements (Lockyer & Kinze 2003, IJsseldijk et al. 2022). If the energy consumed by an adult does not meet its energy demands, individuals could metabolize stored energy reserves in blubber and muscle, leading to a decrease in body condition and possible starvation (Rojano-Doñate et al. 2018). Therefore, an assimilated diet over 3 mo is assumed to represent the diet of healthy animals.

SIA showed that both adults and juveniles mainly rely on energy-rich fish species, including clupeids and sandeels (Cluster 2; Offshore fish | pelagic $\delta^{13}C$ source and Cluster 4; Bentho-pelagic fish | pelagic $\delta^{13}C$ source) as major contributors to their diet, whereas SCA proposed that prey guilds consumed prior to death differ significantly by age class, with juveniles especially relying on low-energy gadoid species (Table S7). Further, even though Clusters 2 and 4 contributed similarly to both the long-term diet of juvenile and adult harbour porpoises, they differed significantly in their mean $\delta^{15}N$ values, suggesting they mainly feed on the same prey species but on different sizes or in different proportions (Tucker et al. 2007).

By using SCA and metabarcoding complementarily, we were able to increase overall prey species detection by about 10%, and to 49% on average at the individual sample level. This highlights the variability of goby species consumed and the addition of freshwater fish species to porpoise diets, which has previously gone unnoticed (Fig. 4). Further, the direct comparison of metabarcoding and SCA suggests that, for the majority of the samples, results of both methods overlap, meaning that metabarcoding includes the past 1 or 2 meals prior to death otherwise highlighted by SCA. Also, metabarcoding excels in estimating diet on a fine-scale taxonomic resolution and in detecting species when prey remains were not visible in stomachs identified as empty for SCA (Fig. 4).

Juvenile porpoises analysed via metabarcoding showed the highest contribution of Cluster 1 (Coastal fish | benthic $\delta^{13}C$ source) and 7 (Others) to their diet. Given the importance of Cluster 7 for some animals analysed via SCA and metabarcoding, those prey species included in Cluster 7 should be included in future SIA studies on harbour porpoise diets. Furthermore, we suggest using modelled proportions of SCA and metabarcoding for a more accurate comparison in the future. Unfortunately, it is not possible to apply the structure of the Bayesian mixing model applied here to SCA and metabarcoding data due to the complexity and different dimensions of these approaches. One way to overcome this might be to apply bootstrapping techniques to SCA and metabarcoding data (e.g. Planque et al. 2021).

Although we have highlighted that a combination of multiple techniques provides a more comprehensive dietary spectrum of a predator, some research questions may be sufficiently answered with only 1 or a combination of 2 methods (Giménez et al. 2017). SCA may be more useful in ecosystem models where consumed biomass is necessary (Püts 2021). SIA is useful in assessing trophic niche overlap of co-occurring top predators (Planque et al. 2021), and metabarcoding can enhance the fine-scale taxonomic resolution of consumed diets (Massey et al. 2021). This combined approach proves particularly beneficial when one method faces constraints such as empty stomachs, absence of species-specific dietary data or overlapping carbon isotope values among potential food sources (McClain-Counts et al. 2017).

4.3. Method caveats

Generally, results may be biased by the relatively small sample size, which is due to the availability of

individuals that were necropsied and had samples for all 3 methods available. Individuals used in this study should be seen as means to validate the complementary method approach.

A study based on pinniped scats used frequency of occurrence methods and Monte Carlo simulations to analytically determine the consequence of sample size on the dietary analysis (Trites & Joy 2005). In our study, porpoises had an average of 3 species per stomach, suggesting that a minimum sample size of 118 is needed, depending on the prey distribution (linear, exponential, uniform), to detect differences between 2 populations while confirming statistical differences with 80% power. At best, the same sample size would be available for metabarcoding and SIA as well.

Another aspect likely causing biased results is the assimilation efficiency of different prey types in SIA (Giménez et al. 2017) and fast metabolic rates influencing metabarcoding and SCA results (Gaskin 1978); both cause different sensitivities to overall prey detection. In this study, unequal assimilation of different prey taxa may explain the lack of species deemed to be important based on the other 2 methods, e.g. gobies (Taylor et al. 2017).

Since fish is the most important diet component, we chose a universal fish primer. However, we recommend that future studies include multiple primers that can elucidate the full range of different prey groups (e.g. crustaceans and squid, Table S2).

Within this study framework, providing the best possible input for the mixing models proved to be the most challenging task. While considering the uncertainty in isotopic variability of predators and different prey sources, Bayesian mixing models use isotope values and fractionation factors to estimate the assimilated diet of the consumer (Stock et al. 2018). Yet, implementing them and creating desirable model outputs can be challenging, as SIA cannot distinguish between food sources with similar isotopic profiles (Whitaker et al. 2019). The clusters used in the model determine the groups which can be used for the method comparison as they are fixed in terms of their distinctive isotopic signatures. Here, unlike in other studies, it was not possible to describe sources purely based on their $\delta^{13}C$ values and hence approximate distance from shore (Ogilvy et al. 2022), neither in terms of biological nor taxonomical relevance (Leopold et al. 2015). This is especially problematic for Cluster 6 (Bentho-pelagic/pelagic fish | pelagic $\delta^{13}C$ source). From an energetic as well as from a management point of view, it would be important to know if porpoises associated with that cluster fed on small whiting with a low energy content or on bigger, energy-rich Atlantic mackerel. In the future, more data on prey stable isotopes, reducing variation, are desirable to enhance model performance.

The use of appropriate TEFs is crucial to reduce uncertainty in mixing models and hence biased results (Bond & Diamond 2011). Trophic enrichment is influenced by a number of environmental (e.g. temperature) and physiological (e.g. age and metabolic rate) factors and varies per species, making species-specific TEFs necessary (Vanderklift & Ponsard 2003). However, obtaining such data is nearly impossible due to logistical and ethical constraints associated with conducting experimental studies on cetaceans. Here, models with 6 different TEFs (Table 2), based on previous studies and 'SIDER', were run. TEFs established for muscle tissue from fin whales *Balaenoptera physalus* (Borrell et al. 2012) performed best. TEFs provided by Borrell et al. (2012) were determined to yield the most reliable results, with fewer outliers and biologically plausible posterior estimates. However, we acknowledge that the TEFs provided by Giménez et al. (2016) and Hobson et al. (1996) produced similar results when visualized within the isotopic mixing polygon. Despite this, their scaled posterior distributions appeared less realistic, often displaying either bimodal distributions, overly broad peaks, or a single dominant peak near 100% with all other values close to 0%. Such outputs are not representative of the ecological reality as we understand it, even when prior knowledge was incorporated into the model. Yet, species-specific TEFs for harbour porpoises would be desirable given their extremely high metabolic rates (Rojano-Doñate et al. 2018). Such specific TEFs would likely change the contribution of different prey clusters found here.

Mixing models allow previous knowledge of prey species (priors) to be included (Stock et al. 2018). However, including priors can cause a substantial reduction in model performance (Swan et al. 2020) and requires explicit consideration of how much weight the prior should have in any analysis (Stock et al. 2018). As the temporal coverage of SCA is much shorter than that of SIA, we attempted 4 differently weighted priors based on previous knowledge obtained from SCA and another study performing SIA on harbour porpoises from the southern North Sea (Jansen et al. 2013). Our study suggests that priors based on SCA from the same individuals caused a substantial reduction in model performance (Table S4). One reason could be that these priors were informed by short-term diets, which differ substantially from assimilated diets, leading to overly informative priors that are not well-supported by the data.

We argue that an increased sample size could further enhance the posterior model and account for species-specific characteristics as mentioned above. Moreover, an increased sample size of prey species included in the cluster analysis will likely decrease the variance of individual prey species, returning more well-defined clusters.

4.4. Conclusions

By combining SCA, metabarcoding and SIA, this study revealed substantial differences between the short- and long-term diet of individual harbour porpoises in the southern North Sea. Whether these differences are related to changes in environmental, anthropogenic or health conditions remains unknown at this stage, and such factors should be included in future studies. Although every technique used for dietary analysis has its own set of drawbacks, our research indicates that a multi-method approach is necessary to accurately reflect the complexity of porpoise diets and can address the shortcomings of one method by leveraging the advantages of another. Understanding underlying mechanisms that cause individual changes in diets over time and the chances in adaptability when preferred prey is unavailable are necessary to adapt conservation measures in fast-changing marine environments. For future studies, it would be useful to include fatty acid analyses and updated bomb calorific measurements of prey species as well, given that those methods can provide valuable information about the quality and nutritional value of different food sources (Pethybridge et al. 2018), which is essential for understanding the importance of certain key prey species in the diet of harbour porpoises.

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