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Original Article

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Limited resource use overlaps among small pelagic fish species in the central Baltic Sea

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Abstract

Small pelagic fish have a central position in pelagic food webs, linking plankton production to higher trophic levels. They often favour crustacean mesozooplankton and are thought to compete for the same resource, an assumption that relies on microscopy diet identification that neglects digested and soft-bodied prey. Here, we aimed to systematically identify the entire resource use and overlap among the dominant small pelagic fish in the central Baltic Sea at high taxonomy resolution. The diet composition of two clupeid species, herring (*Clupea harengus*) and sprat (*Sprattus sprattus*), and the three-spined stickleback (*Gasterosteus aculeatus*) was assessed in May and October using DNA metabarcoding, stable isotopes, and microscopy. All three methods gave consistent results. The clupeids shared a similar diet in May when prey diversity was low, composed mainly of the copepods *Pseudocalanus* and *Acartia*, whereas three-spined stickleback favoured different copepod species and the rotifer *Synchaeta*, which was confirmed by a different isotopic value as compared to the two clupeids. In October, all forage fish preyed on diverse zooplankton species, mainly composed of the copepods *Acartia, Eurytemora*, and *Temora*, while *Pseudocalanus* was only important for herring. The observed resource use partitioning between sprat and herring was confirmed by the stable isotope values from October, suggesting that different prey species were targeted during the summer period. Our study highlights that resource use overlaps among small pelagic fish were limited and varied with prey availability. This suggests that shifts in zooplankton dynamics, rather than competition for resources, have the potential to drive small pelagic fish population fluctuations.

Keywords: forage fish; zooplankton; diet composition; DNA metabarcoding; stable isotopes

Introduction

Small pelagic fish, also called forage fish, provide multiple ecosystem services. They support fisheries, piscivorous fish, seabirds, and marine mammals, and because of their ability to feed directly on planktonic organisms, they exert direct control on lower and higher trophic levels (Nissar et al. 2022). Planktivorous fish represent the lowest trophic level with the capacity to move against currents, enabling them to actively migrate to feeding and spawning grounds (Bakun 2006). Their recruitment success is highly dependent on environmental conditions, which, if unfavourable, can hinder their population fitness (Cushing 1990, Beaugrand et al. 2003). Altogether, this makes small pelagic fish populations prone to high fluctuations with consequences for zooplankton and predatory fish, marine mammals, and seabirds (Cury et al. 2000). Forage fish often share a similar diet, consisting mainly of crustacean zooplankton prey (Raab et al. 2012, Bachiller et al. 2021). However, zooplankton species composition is diverse, including not only diverse copepod and cladoceran species but also soft-bodied organisms. Moreover, prey availability and diversity vary throughout the seasons (Fanjul et al. 2018), and in turn, fish diet composition may also change with prey availability (Casini et al. 2004). Consequently, it is important to assess prey availability and the entire composition of fish diet at high taxonomy resolution over the seasons to identify resource use overlap that may lead to competition among small pelagic fish.

Fish populations are strongly driven by environmental parameters, particularly temperature, and by the spatial and temporal co-occurrence with their plankton prey (Beaugrand et al. 2003, Mackenzie et al. 2007, Ferreira et al. 2023), as fish recruitment depends on the synchronization of reproduction timing with periods of high prey availability, both in terms of quantity and quality (Cushing 1990). Copepods are often dominating the prey composition of small pelagic fish, but in coastal areas, other taxa, including cladocerans, rotifers, and benthic organisms, can substantially contribute to the biomass of available prey (Jan et al. 2024, Maathuis et al. 2024). Moreover, in seasonal systems, zooplankton are fuelled by a succession of phytoplankton pulses (Behrenfeld and Boss 2014, Hjerne et al. 2019), and their availability for upper trophic levels varies throughout the year (Eloire et al. 2010). These dynamics can affect small pelagic fish diet composition, which may have different favoured prey depending on the fish size (Barnes et al. 2010), selectivity, and prey availability (Casini et al. 2004). Density-dependent processes and resource use overlap can lead to competition among forage fish (Möllmann et al. 2005, Casini et al. 2006, Pedraza-Garcia and Cubillos 2008), eventually driving the system into a new regime characterized by alternative dominant species and trophic

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interactions (Tomczak et al. 2022). Such fluctuations are expected to be exacerbated due to climate change, notably by shifting the phenology of fish and their plankton prey (Moyano et al. 2022), and by altering fish prey species and size composition, potentially leading to a mismatch between energy demand and resource availability (Richardson 2008, Thackeray et al. 2016, Asch et al. 2019, Hedberg et al. 2024). Hence, changes in prey species composition can have unexpected consequences on the entire trophic network if species interactomes are not well understood.

Currently, most of the knowledge on fish trophic interactions relies on identification of undigested parts, such as exoskeletons, bones, and otoliths, by microscopy (Baker et al. 2014). This results in observations at broad taxonomy resolution neglecting digested and soft-bodied prey (Hyslop 1980). Traditional methods can be complemented with DNA metabarcoding and stable isotope analyses to obtain further information on species interactions (Hardy et al. 2010, Miya 2022). DNA metabarcoding, similarly to microscope observations, identifies a snapshot of the fish diet but at a much higher taxonomic resolution and is not biased towards hard-bodied prey (de Sousa et al. 2019). Bulk stable isotopes allow to estimate seasonal or annual trophic niches based on the ratio of heavy to light carbon and nitrogen isotopes in fish tissues and provide an integrated measure of resource use over time (Boecklen et al. 2011). Despite the increasing accessibility of such molecular techniques (van Dijk et al. 2014) and their promising application for management purposes (Jacquemot et al. 2024, Kelly et al. 2024, Urban et al. 2024), they are still underused for investigating trophic interactions, and especially the link between plankton and fish.

Two clupeid species, the Atlantic herring (*Clupea harengus*; herein referred as herring) and the European sprat (*Sprattus sprattus*; herein referred as sprat) that are both filter and particulate feeders (Blaxter and Hunter 1982), and the three-spined stickleback (*Gasterosteus aculeatus*) that uses a particulate-feeding strategy (Tugendhat 1960), are coexisting in the pelagic Baltic Sea. Over the last decades, clupeids have been the dominant forage fish species in terms of biomass, but sprat and herring stocks have recently declined while three-spined stickleback has increased (Olsson et al. 2019, Olin et al. 2022, ICES 2024). The latter contributes now to more than a tenth of the total small pelagic fish biomass in the pelagic Baltic Sea (Olsson et al. 2019). Based on the recent fish dynamics and their shared diet preferences, previous studies suggested that competition for resources has impacted small pelagic fish populations (Jakubavičiūtė et al. 2017b, Olsson et al. 2019, Donadi et al. 2024). In the central Baltic Sea, sprat and herring generally favour mesozooplankton prey, such as the copepods *Pseudocalanus*, *Temora*, and *Acartia*, yet species exhibit different diet composition across seasons and locations (Möllmann et al. 2004). Moreover, sprat is reported to be strictly planktivorous, whereas herring commonly consumes nektobenthos (Casini et al. 2004), linking benthic and pelagic productions (Kiljunen et al. 2020). While *Pseudocalanus* are mainly consumed in spring in the pelagic (Bernreuther et al. 2018), the copepod *Eurytemora* is a key prey species for both clupeids in summer in coastal areas (Ojaveer et al. 2018). Jakubavičiūtė et al. (2017b) reported seasonal patterns from coastal locations where three-spined stickleback and clupeids shared similar resources, especially in autumn when all species consume cladoceran prey. However, only few studies have explored the diet of the three-spined stickleback, and most of them are restricted to the coastal areas (Jakubavičiūtė et al. 2017a, Jakubavičiūtė et al. 2017b) or gulfs of the Baltic Sea (Peltonen et al. 2004, Lankov et al. 2010), albeit three-spined stickleback spend most of their life cycle in the pelagic Baltic Sea, where they share the same feeding grounds as herring and sprat (Olin et al. 2022). Consequently, resource use overlap among the three dominating fish remains to be investigated at a larger spatial scale in the pelagic Baltic Sea.

Building on a study conducted in the southern Baltic Sea showing that three-spined stickleback and clupeids used different resources in spring (Novotny et al. 2022), we aimed to better understand their resource use before and after the summer zooplankton peak, when prey diversity and availability differ. We hypothesized that clupeids and three-spined sticklebacks vary in diet composition and that their resource use overlap decreases with increasing prey diversity. We examined small pelagic fish trophic interactions in May and October using DNA metabarcoding, stable isotopes, and microscopy, and further estimated resource use overlap and relative prey selectivity.

Methods

Sampling

Sprat, herring, and three-spined stickleback were collected during the Sprat Acoustic Survey (SPRAS) in May 2022 and 2023 and during the Baltic International Acoustic Survey (BIAS) in October 2022 in the Gotland Basin (ICES subdivisions 27 and 28.2; Fig. 1a) using a pelagic fishing trawl. Sampling protocol was approved by Göteborgs djurförsöksetiska nämnd and was conducted according to the ethical permit Dnr 5.8.18-09 303/2024. Overall, sprat dominated the catches in May, whereas three-spined stickleback represented more than half of the sampled individuals in October (Fig. 1b–d, Table S1). For diet analysis, the three fish species were randomly subsampled from all trawling events, defined as instances where the pelagic trawl was lowered and retrieved, and total length was measured to the nearest 0.5 cm. Analysed three-spined stickleback ranged from 4 to 8 cm, sprat from 8 to 13.5 cm, and herring from 14 to 19.5 cm in total length (Fig. S1). During the SPRAS, fish guts were dissected on board, and during the BIAS, whole fish were frozen at −20°C on board, and dissection was performed in the laboratory. In addition, zooplankton samples were collected in vertical tows using a 90 μm-WP2 net during the SPRAS, with a sampling depth of 80–0 m in 2022 and 60–0 m in 2023. Fish gut and zooplankton samples were stored in 95% ethanol at −20°C until further analyses.

DNA metabarcoding

Gut content of three individuals per species and per trawling event was removed and homogenized. Half of the content was mixed with 360 μl ALT lysis buffer for DNA extraction, and the other half was preserved in 95% ethanol for microscope counts. A subsample of 1 ml of homogenized plankton samples was concentrated to remove the ethanol and mixed with 360 μl ALT. DNA was then extracted using QIAmp DNA Micro Kit (Qiagen) with an additional glass bead beating step.

A first PCR reaction was performed on a 400 bp long fragment of the V4 region of the 18S *rRNA* gene (18S) targeting all eukaryotic prey using the primers 528F and 706R (Elwood et al. 1985) and on a 313 bp long fragment of the

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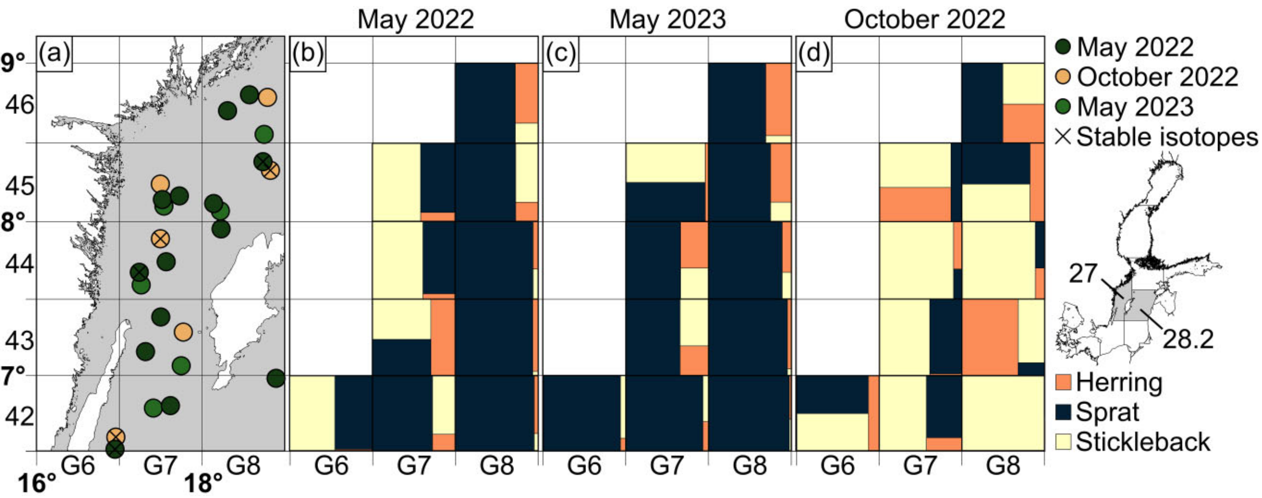

Figure 1. Map of the study area and average relative proportions of forage fish in each trawl per ICES statistical rectangle. (a) Sampling locations in the ICES subdivisions 27 and 28.2 (grey shaded area) in the Baltic Sea. Filled points denote the May and October surveys; cross symbols show the locations from where stable isotope analyses were performed. (b–d) Average proportion of caught forage fish abundance in each ICES statistical rectangle during the surveys in May 2022 and 2023 and October 2022.

mitochondrial cytochrome c oxidase I (COI) targeting metazoans prey using mlCOIintF and dgHCO2198 (Leray et al. 2013) as described in Novotny et al. (2022). Indexes and Illumina adapters were then added in a second PCR reaction as described in Zemora-Terol et al. (2020). Pair-end Illumina sequencing was performed on MiSeq v3-600 (2 × 300 bp reads) with addition of 10% PhiX, and demultiplexing was performed using bcl2fastq (v. 2.20.0.422) from the CASAVA software suite at the National Genomics Infrastructure, Stockholm, Sweden.

Processing of the raw fastq data was facilitated using the nf-core/ampliseq pipeline (Ewels et al. 2020, Straub et al. 2020). Different versions of the pipeline were used to process the SPRAS 2022 (v. 2.8.0) and the SPRAS 2023 and BIAS 2022 (v. 2.9.0) sequencing data. Briefly, primers were trimmed with Cutadapt (Martin 2011), and DADA2 (Callahan et al. 2016) was used to infer amplicon sequence variants (ASV) in R (R Core Team 2024). Taxonomy assignment was then performed against the PR^2 (v. 5.0.0; Guillou et al. 2012) database for 18S, and the COI amplicons were assigned against a custom reference database combining the MARine Eukaryote Species (MARES) database (Arranz et al. 2020) and a 636 bp sequence of *Synchaeta baltica* retrieved in GenBank (accession: MK905848; Wilke et al. 2020).

Microscope counts and stable isotopes

Microscope counts and stables isotopes were performed for the samples collected in 2022. A fraction of the gut content was identified under a stereomicroscope at the highest taxonomic resolution possible. For the WP2 samples, zooplankton abundance was estimated by counting a minimum of 1 ml of sample and at least 100 individuals from the most abundant genera. For stable isotopes analyses, stomach tissue from nine (eight for herring collected during SPRAS) individuals per fish species and surveys in 2022 were rinsed with Milli-Q water and oven-dried at 60°C for 24–48 h until completely dried and ground to a fine powder using a mortar and a pestle before being encapsulated in tin capsules. Stable carbon and nitrogen isotopic compositions were determined using a Thermo Delta V continuous flow isotope ratio mass spectrometer coupled to a Thermo FlashEA1112 elemental analyser at Center for Physical Sciences and Technology, Vilnius, Lithuania. Stable carbon and nitrogen isotopic compositions were calibrated relative to the VPDB and AIR scales using USGS24, IAEA-CH3, IAEA-N-1, IAEA-N-2, and IAEA-600. Precision (u(Rw)) was determined to be ± 0.12‰ for δ^{13}C and ± 0.18‰ δ^{15}N on the basis of repeated measurements of calibration standards and sample replicates. The total analytical uncertainty was estimated to be ± 0.16‰ for δ^{13}C and ± 0.20‰ for δ^{15}N.

Data analysis

Data were analysed and visualized in R (v. 4.3.3; R Core Team 2024) using the tidyverse core packages (v. 2.0.0; Wickham et al. 2019) if not specified otherwise. Metadata, ASV, and taxonomy tables were processed using the phyloseq R package (v. 1.46.0; McMurdie and Holmes 2013). ASVs assigned to Teleostei (18S) or to the host fish genus (COI) were removed, and a standardized subsampling of 10 000 reads was performed to limit the bias caused by uneven sequencing depth across samples. For each barcode, dominant prey ASVs, defined as occupying at least 0.1% of the read counts in at least 50% of the samples for each forage fish collected during each survey, were kept for further analyses. Samples with less than 1000 reads after these filtering steps were discarded. Rarefaction curves showed that all samples reached a plateau after these processing steps (Fig. S2). Phyloseq-class objects were then converted to data frames to be analysed as relative read abundance at the taxon level (Table S2). Non-metric multidimensional scaling (NMDS) was performed based on Bray–Curtis distances using the function metaMDS with the default parameters from vegan (v. 2.6.4; Oksanen et al. 2022). To test whether the diet differed among fish species, permANOVAs followed by pairwise comparison using pairwiseAdonis (v. 0.4.1; Arbizu 2017) were performed for each survey and barcodes. Differences in variance among groups were tested using the function betadisper from vegan (Oksanen et al. 2022). Resource use overlap among all samples was calculated based on the relative read abundance with the Schoener's *D* index (Schoener 1968) using spaa (v. 0.2.2; Zhang 2016).

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We used a modified Chesson index (α; Chesson 1978) to estimate the relative selection for each prey taxa (i) among fish species that takes into account the relative prey read abundance in the fish gut (R_i) in relation to a pooled diet composition (R_{pool}) (Equation 1). This pooled diet composition contained all consumed prey taxa and represented the average diet composition of all fish species at a given time and location. For this analysis, protists were excluded as they are likely occurring in the fish gut due to secondary predation, as well as unspecified copepods, crustaceans, insects, arthropods, and unassigned reads. Relative selectivity was compared with absolute selectivity calculated using the Chesson index, which compares R_i against the relative read abundance in the environment instead of R_{pool}. The neutral selection threshold (α_0) was calculated for each month and barcode as $\alpha_0 = \frac{1}{m}$, where m represents the total number of prey taxa. To estimate the significance of the divergence from neutral selection, we calculated the 95% confidence interval (CI) for α_i of each prey taxon using 1000 bootstrap iterations. Values of $\alpha_i \pm \text{CI} > \alpha_i$ indicate that relative selectivity for a prey taxon is higher than would be expected under random feeding, termed positive selection, $\alpha_i \pm \text{CI} < \alpha_0$ indicate negative selection, and $\alpha_i - \text{CI} \leq \alpha_0 \leq \alpha_i + \text{CI}$ indicate neutral selection. Fish prey relative selectivity was visualized as a network with the link being proportional to the average α_i using igraph (v. 2.0.3; Csardi and Nepusz 2006).

$$\alpha_i = \frac{\frac{R_i}{R_{\text{pool},i}}}{\sum_i^m \frac{R_i}{R_{\text{pool},i}}}. \tag{1}$$

Relative 18S and COI read abundances and relative microscope counts for total copepods, cladocerans, and rotifers were compared using Spearman's rank correlations. *P*-values were then adjusted using the false discovery rate method to account for multiple testing.

Difference in δ^{13}C and δ^{15}N values among forage fish species were assessed for each season separately, due to the potential change in baseline values using one-way ANOVAs followed by Tukey's honestly significant difference (HSD) tests with the stats R package (R Core Team 2024).

Results

DNA metabarcoding

Metabarcoding produced 12.08 and 15.73 million reads for the 18S and COI, respectively. After quality filtering and subsampling, 218 out of 245 samples for the 18S and 228 out of 244 samples for the COI were retained for further analyses (Table S3).

In May, the zooplankton community in the water column was dominated by copepods (18S: 56.1%; COI: 28.6%), especially *Temora* and *Pseudocalanus*, and rotifers (18S: 30.1%; COI: 69.0%), but their relative abundances differed between the two barcodes (Fig. 2). Both 18S and COI comprised benthic organisms albeit at low relative abundance (18S: 1.57%; COI: 1.33%), including the annelids *Marenzelleria* and *Bylgides* and the mollusc *Macoma*, suggesting meroplanktonic life stages of these organisms in the water column. The 18S barcode also identified protist reads, including dinoflagellates and ciliates that were on average five times more abundant in 2023 than in 2022, and gelatinous plankton, such as ctenophores that represented on average up to 1.60% of the reads. During both May surveys, the COI had a higher taxonomic resolution for non-copepod species and identified rotifers to *Synchaeta* and cladocerans mainly to *Evadne* and to *Pleopis* in 2023.

DNA metabarcoding showed the dominance of copepod reads in the diet composition for all three fish species in May and October (Fig. 2), but the taxonomic composition of their diet differed across all surveys among fish species with both COI and 18S barcodes (permANOVA: $R^2 \geq 0.29$, $P \leq .001$). In May, sprat and herring shared a similar diet composition, different from that of three-spined stickleback in both years (Table 1). The copepods *Acartia* and *Pseudocalanus* contributed to more than half of herring and sprat diet in May, independently of the barcode used (Fig. 2). Gelatinous plankton and benthic organisms, identified with 18S, represented together on average up to 12.7% of the clupeid diet reads and were at least twice as abundant in 2023 as in 2022. The third most dominant copepod associated with sprat and herring was *Temora*, contributing to more than a tenth of the 18S reads but to less than 4% of the COI reads. In contrast, the dominant copepods associated with three-spined stickleback were *Temora*, *Eurytemora*, and *Acartia*, whereas *Centropages* and *Pseudocalanus* together contributed to a lower proportion of three-spined stickleback diet in May (Fig. 2). Rotifers contributed up to a quarter of the 18S reads and half of the COI reads in three-spined stickleback guts, while they represented less than 1% and 7% of the 18S and COI reads associated with clupeids, respectively. The remaining prey associated with three-spined stickleback comprised cladocerans and few benthic organisms that together represented less than 2% of the reads.

In October, the diet composition differed among all fish species (pairwise permANOVA: $R^2 \geq 0.19$, $P \leq .004$; Table 1; Fig. 2). The copepods *Temora*, *Eurytemora*, *Acartia*, and *Centropages* dominated in the diet of all fish species independently of the barcode used, but their relative contribution varied across species. These four copepod genera contributed to more than 93.4% of the reads associated with sprat and three-spined stickleback and up to 61.8% of reads in herring diet. The remaining prey taxa for herring were the copepod *Pseudocalanus* (18S: 20.4%; COI: 28.7%) and ctenophores (18S: 20.4%), and to a lesser extent the cnidarian *Aurelia* and cladocerans that each represented up to 1.5% of the reads. Despite sharing similar prey taxa, they varied in their relative abundance between three-spined stickleback and sprat. For three-spined stickleback, 18S identified twice as many *Temora* reads associated with three-spined stickleback (43.9%) as with sprat (23.4%), about the same *Eurytemora* contribution (sprat: 32.8%; three-spined stickleback; 40.1%), and a 5-fold difference in *Acartia* relative abundance, which represented more than a third of sprat gut content and only 6.6% of the reads associated with three-spined stickleback. COI showed a clear preference of *Eurytemora* (59.1%) over *Temora* (24.3%) and *Acartia* (11.0%) for three-spined stickleback, while sprat favoured *Acartia* (40.4%) and *Eurytemora* (36.4%) at similar levels, followed by *Temora*, which contributed to about a fifth of the read counts of their diet.

The ordination of the fish diet composition visualizes resource use partitioning among the fish species (Fig. 3a–f). In May, sprat and herring diets overlapped but differed for three-spined stickleback, while diet overlap among all three fish species was low in October, although three-spined stickleback diet composition was more similar to that of clupeids than it was in May. The dissimilarity between three-spined stickle-

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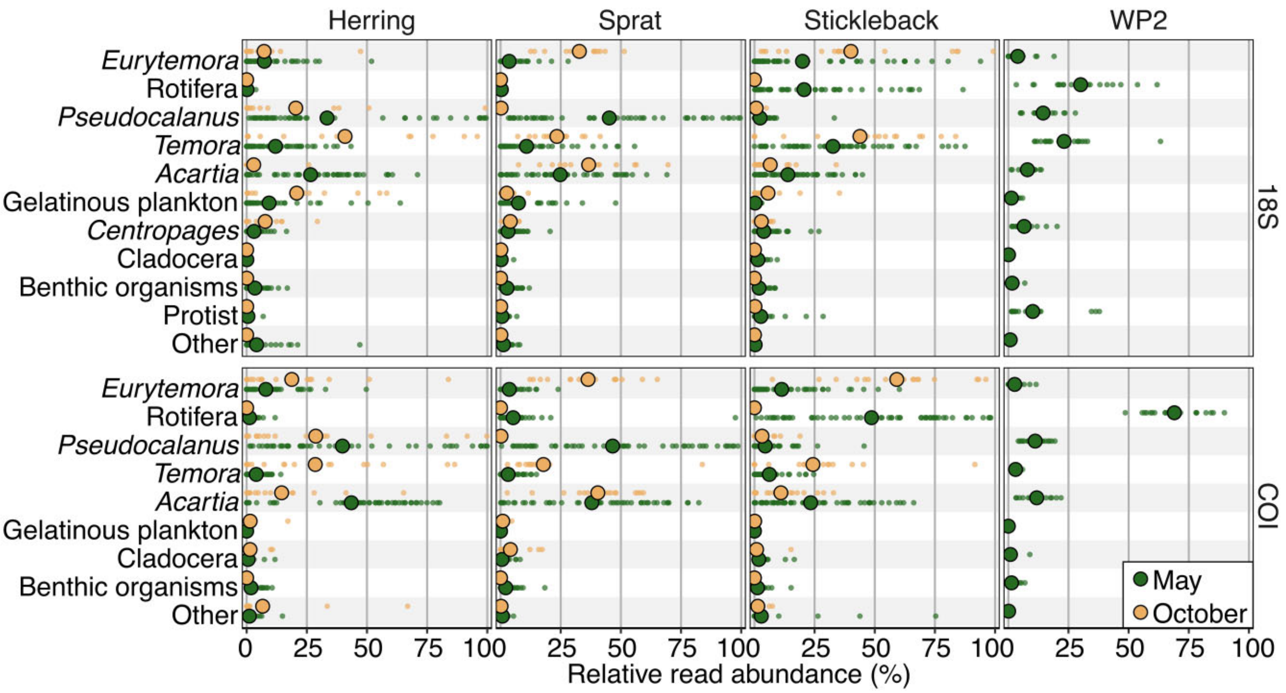

Figure 2. Relative 18S (upper panels) and COI (lower panels) read abundances of herring, sprat, and three-spined stickleback diet composition and prey availability in the water column (WP2) in May (averaged for 2022 and 2023) and October 2022. Each point represents a unique biological sample, and the larger points represent the average read contribution for each prey group. Prey taxa are ordered from the overall highest average diet contribution to the lowest.

Table 1. Pairwise PermANOVA output of diet composition between herring, sprat, and three-spined stickleback for the May 2022 and 2023 and October 2022 surveys.

Survey	Species	Herring	Sprat	Stickleback
May 2022	Herring	–	0.02	0.40∗
	Sprat	0.03	–	0.40∗
	Stickleback	0.27∗	0.36∗	–
May 2023	Herring	–	0.01	0.34∗
	Sprat	0.02	–	0.35∗
	Stickleback	0.29∗	0.37∗	–
October 2022	Herring	–	0.23∗ !	0.22∗ !
	Sprat	0.39∗ !	–	0.28∗
	Stickleback	0.19∗	0.26∗	–

Upper diagonal shows R^2 values based on COI, and lower diagonal is based on 18S relative read abundance. Significance is indicated with the asterics (∗when $P < .05$). Differences in variance among fish species are shown with !, indicating that outcomes need to be interpreted with caution, as variance also drives the statistical output.

back and clupeid diet composition in May is supported by low Schoener's D indices for both barcodes (median Schoener's $D \leq 0.31$), but were higher between sprat and herring (median Schoener's $D \geq 0.47$) and comparable to their intraspecific diet overlap (median Schoener's $D \geq 0.46$; Fig. 3g, h). In October, sprat and three-spined stickleback had the highest median interspecific Schoener's D (18S: 0.48; COI: 0.57), indicating some resource overlap, which was, however, lower than their respective intraspecific diet overlap (Fig. 3i). The high variance of herring diet composition compared to the other fish (Table 1) was reflected by the lowest intraspecific (median Schoener's $D \leq 0.35$) and interspecific diet overlap index (Fig. 3c, f, i).

In May, both relative and absolute selectivity indices identified similar patterns and showed different feeding preferences between three-spined stickleback and clupeids (Fig. 4a, b; Fig. S3). Three-spined stickleback had on average a higher relative selectivity than the neutral selection based on 18S ($\alpha_0 = 0.09$) for rotifers ($\alpha \pm \text{CI} \geq 0.16$), cladocerans ($\alpha \pm \text{CI} \geq 0.12$), and the copepods *Temora* ($\alpha \pm \text{CI} \geq 0.11$) and *Eurytemora* ($\alpha \pm \text{CI} \geq 0.11$). Selectivity indices based on COI ($\alpha_0 = 0.1$) were in line with 18S but showed a neutral relative selection for *Eurytemora* ($\alpha \pm \text{CI} = [0.1, 0.14]$), while revealing relative positive selections for *Synchaeta* ($\alpha \pm \text{CI} \geq 0.2$) and *Evadne* ($\alpha \pm \text{CI} \geq 0.14$). However, absolute selectivity revealed that cladocerans and *Eurytemora* were positively selected by three-spined stickleback with both barcodes. Sprat and herring had on average a positive relative selectivity for the copepods *Pseudocalanus* ($\alpha \pm \text{CI} \geq 0.15$) and *Acartia* ($\alpha \pm \text{CI} \geq 0.1$). In addition, 18S identified clupeid positive relative selectivity for ctenophores ($\alpha \pm \text{CI} \geq 0.14$) that was consistent with the absolute selectivity index. COI showed differences in benthic or meroplanktonic relative prey selectivity, three-spined stickleback selected for *Marenzelleria* ($\alpha \pm \text{CI} \geq 0.12$), while clupeids showed positive selection for *Bylgides* ($\alpha \pm \text{CI} \geq 0.11$). However, no absolute prey selectivity was observed for benthic or meroplanktonic prey. In October, less taxa were selected by all fish species, increasing the neutral selection threshold to $\alpha_0 = 0.14$ and $\alpha_0 = 0.13$ for COI and 18S, respectively (Fig. 4c, d; Fig. S3). Sprat showed positive selection for the copepod *Acartia* ($\alpha \pm \text{CI} \geq 0.15$) and the cladocerans *Bosmina* (18S; $\alpha \pm \text{CI} \geq 0.2$) and *Pleopis* (COI; $\alpha \pm \text{CI} \geq 0.15$), and negative selection for *Pseudocalanus* ($\alpha \pm \text{CI} \leq 0.06$). Herring had a positive selection for *Pseudocalanus* ($\alpha \pm \text{CI} \geq 0.15$), and three-spined stickleback had a positive selection for the copepods

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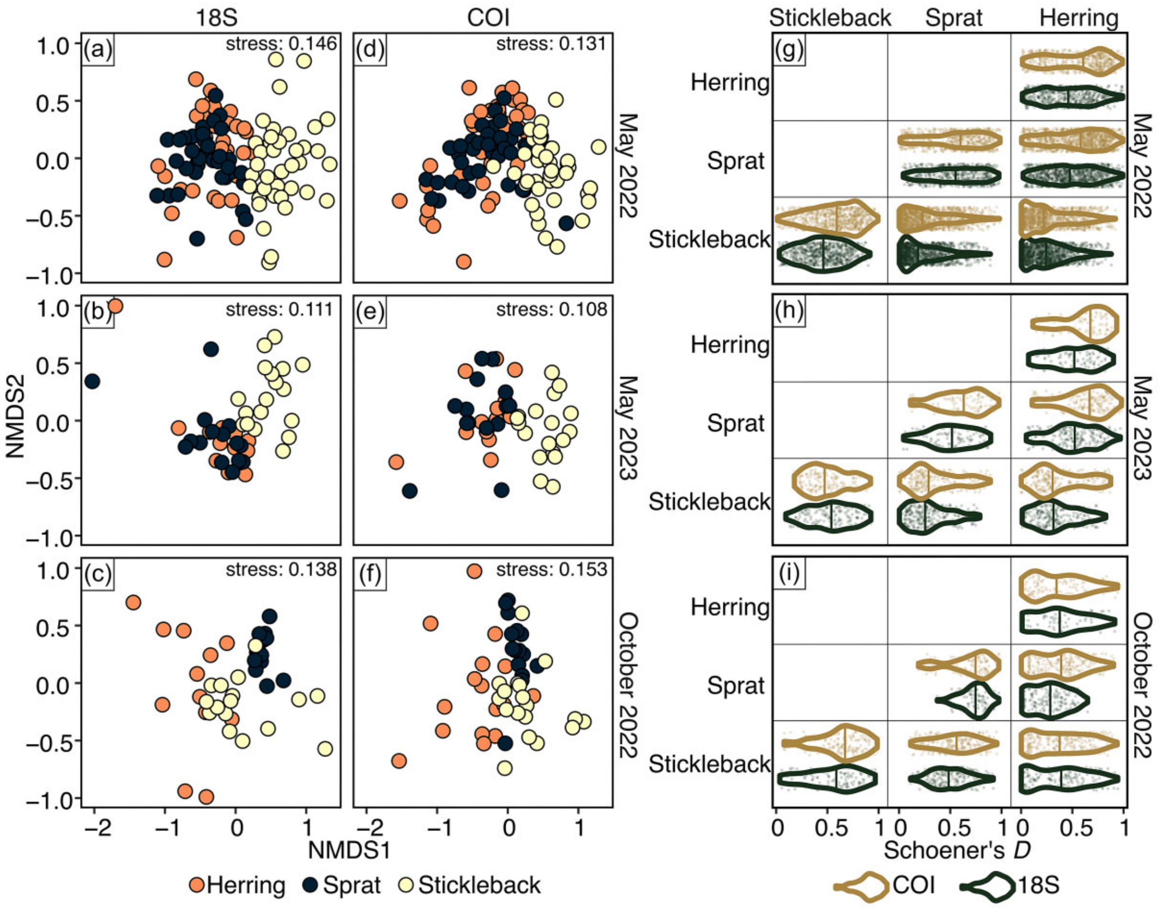

Figure 3. Sprat, herring, and three-spined stickleback diet composition ordination and resource use overlap. (a–f) NMDS plot of diet composition by species for 18S (a–c) and COI (d–f) in May 2022 (a, d) and 2023 (b, e), and October 2022 (c, f). (g–i) Diet composition overlap among and across each fish species is represented by the Schoener's *D* index in May 2022 (g) and 2023 (h) and October 2022 (i) for the 18S and COI barcodes. Dots represent sample combinations, the violin represents data distribution, and the vertical line the median. The diagonal represents the intraspecific diet overlap. A Schoener's *D* index of 0 denotes no overlap, and 1 denotes full overlap.

Eurytemora ($\alpha \pm \mathrm{CI} \geq 0.19$) and *Temora* ($\alpha \pm \mathrm{CI} \geq 0.15$), and negative for cladocerans ($\alpha \pm \mathrm{CI} \leq 0.11$).

Microscope counts

Microscope counts confirmed the dominance of copepods in herring and sprat diet. In contrast, three-spined stickleback diet was dominated by rotifer eggs, while mature *Synchaeta* were identified in the water column in May (Fig. S4). Out of the 113 gut contents from May 2022, three were identified as empty, four were filled with unidentifiable crustacean exoskeletons, and about two-thirds of the copepods could not be identified at genus level due to high degradation. Similarly, out of the 54 samples from October 2022, three were empty, and 49 guts contained unidentifiable copepods or exoskeleton. The relative diet proportion for crustaceans and rotifers was found to be similar using microscopy and DNA metabarcoding (Spearman's $\rho \geq 0.27$; Fig. 5a–c). However, weaker correlations between relative microscope counts and read abundances were observed for the prey availability in the water column (Fig. 5d–f).

Stable isotopes analysis

Values of δ^{15}N and δ^{13}C differed among fish species during both surveys (Fig. 6). In May, mean fish δ^{15}N values ranged from 9.67 to 10.1‰ with no difference among fish species (ANOVA: $F_{2,23} = 0.58$, $P = .569$), contrarily to δ^{13}C that differed among fish species with three-spined stickleback having lower δ^{13}C values (mean ± standard deviation: –21.7 ± 0.17‰) than herring (–20.8 ± 0.10‰) and sprat (–20.9 ± 0.23‰; Tukey's HSD: $P \leq .0096$). In October, isotopic values differed among all fish species, both for δ^{15}N (ANOVA: $F_{2,24} = 11.6$, $P = .0003$) and δ^{13}C (ANOVA: $F_{2,24} = 3.51$, $P = .046$). Herring and sprat had different δ^{15}N values (Tukey's HDS: $P = .0002$), with the highest (9.20 ± 0.30‰) and lowest (7.37 ± 0.14‰) δ^{15}N, respectively, and three-spined stickleback had intermediate δ^{15}N values but not different from the two clupeids (Tukey's HSD: $P \geq .058$). Sprat had lower δ^{13}C values (−24.4 ± 0.25‰) than three-spined stickleback (−23.1 ± 0.47‰; Tukey's HSD: $P = .0484$) but were similar to herring δ^{13}C values (−23.4 ± 0.36‰; Tukey's HSD: $P = .14$).

Discussion

We used DNA metabarcoding, bulk stable isotope analyses, and microscopy to assess the diet composition of three dominating small pelagic fish species under different regimes of prey availability and diversity before and after the summer zooplankton peak production in the central Baltic Sea. All

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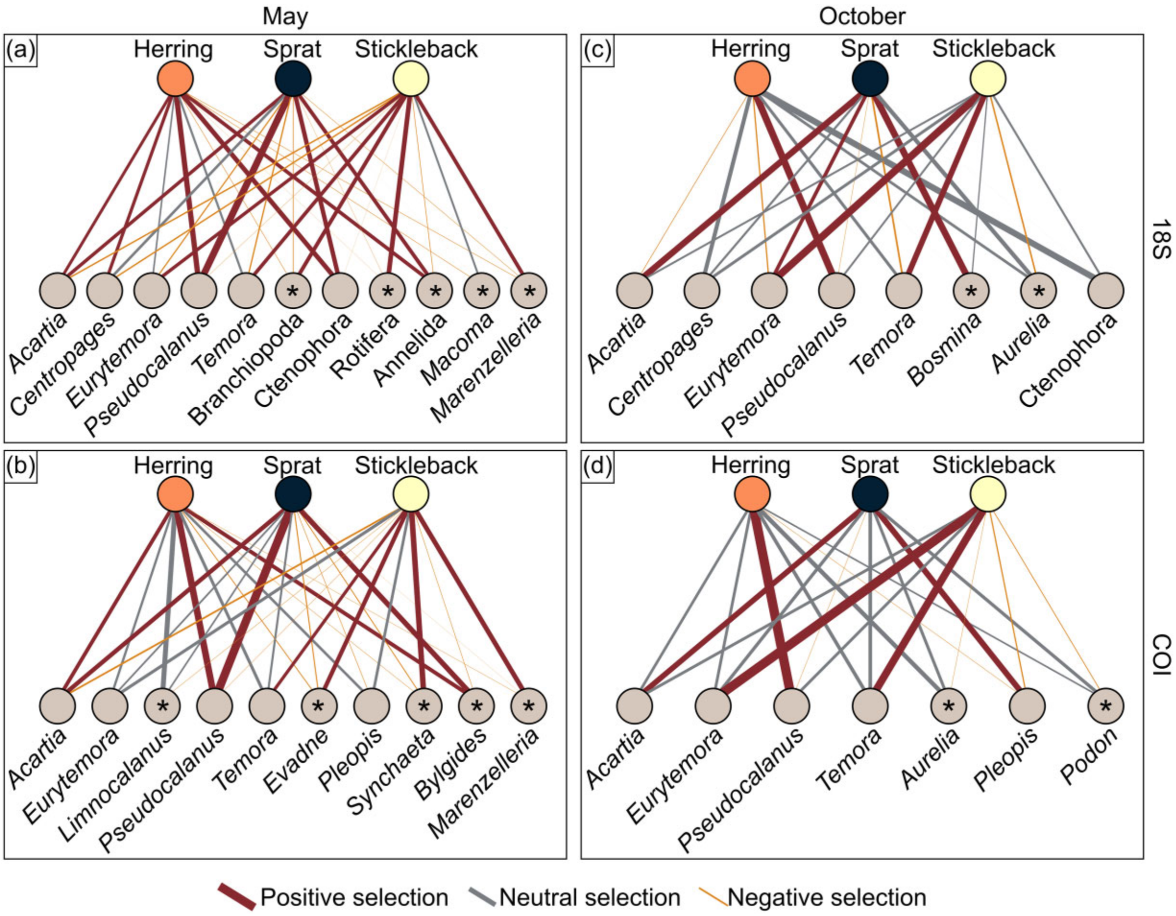

Figure 4. Prey selectivity for herring, sprat, and three-spined stickleback. The link widths are proportional to the average Chesson index for each fish species in May (a, b) and October (c, d) based on the 18S (a, c) and COI (b, d) reads. Asterisks represent prey taxa observed only in one season for each barcode.

three approaches consistently revealed that resource use differed among these forage fish species, suggesting that competition for resources is limited among sprat, herring, and three-spined stickleback. The two clupeids, sprat and herring, had the strongest resource use overlap in May, but their diet composition diverged with increasing zooplankton diversity in October. Three-spined stickleback had a distinct diet composition compared to the clupeids throughout the sampled months with a preference for the rotifer *Synchaeta* and the copepod *Eurytemora*. Our findings show that identification of prey at high taxonomy level and the inclusion of overlooked prey, such as soft-bodied and gelatinous plankton, are important to detect differences in species-specific trophic interactions between fish and their plankton prey.

We found overall limited diet overlap among the three forage fish species, although all three fish species relied on mesozooplankton. This challenges the assumption that forage fish compete for their resource, which has been suggested for herring, sprat, and three-spined stickleback in the Baltic Sea (Olin et al. 2022) and in other seas with coexisting small pelagic fish species (Palomera et al. 2007, Raab et al. 2012). Our contrasting results may be explained by the fact that comparisons of diet composition among these three species are sparse in the Baltic Sea, particularly in the pelagic, as the literature focuses on coastal areas (Peltonen et al. 2004, Jakubavičiūtė et al. 2017b). To our knowledge, our study is the first that systematically assessed the diet composition of all three fish species over two months in the pelagic Baltic Sea. The offshore scope of this study likely also explains the low contribution of benthic organisms to the fish diet and the absence of piscivorous behaviour in herring, which has recently been reported in areas where immature and adult life stages overlap, such as the coastal Baltic Sea (Donadi et al. 2024) and the Wadden Sea (Maathuis et al. 2024). This is also consistent with Kopp et al. (2015), whose stable isotope analyses showed a decrease in benthic-pelagic coupling with increasing depth in the English Channel. Together, this suggests that trophic networks vary across space, and particularly between coastal and offshore areas, stressing the need to estimate the overlap in resource use across the habitat range of small pelagic fish species to identify potential competition.

Three-spined stickleback was the only fish species to favour rotifers prey, which, in turn, consume dinoflagellates (Novotny et al. 2023). This direct link from the productive spring primary bloom to forage fish ultimately optimises trophic efficiency (Berglund et al. 2007). Further, *Synchaeta* show a steady biomass with a tendency to increase over the last decade (Jan et al. 2024) and have the potential to match the advancement in spring bloom timing. Although three-spined stickleback preferred *Synchaeta* prey more than the clupeids, their substantial diet contribution was largely due to their high availability in May and likely also because three-spined stickleback are smaller than the clupeids. Moreover, *Synchaeta* dominated the reads retrieved from the water

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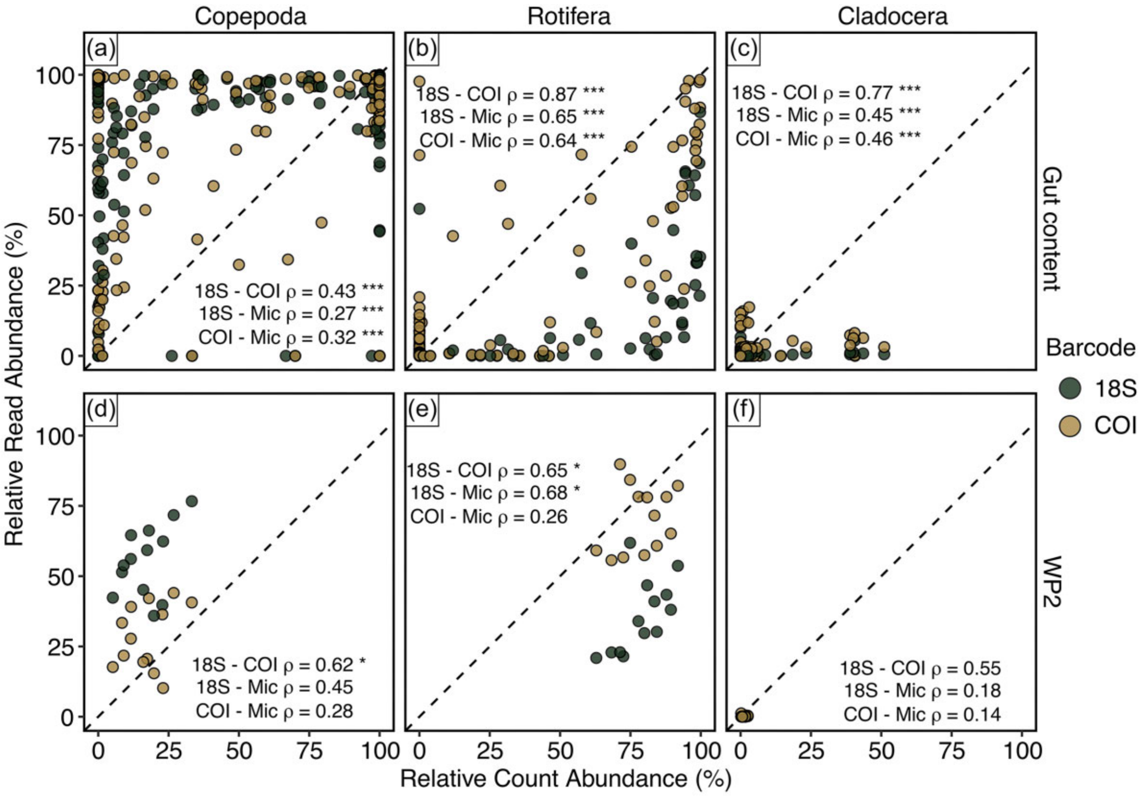

Figure 5. Relation between microscopy and DNA metabarcoding across all fish diet (gut content) and prey availability (WP2) samples. Scatterplots showing the relationships of the dominating zooplankton groups in the (a–c) fish diet composition and (d–f) prey availability assessed with microscopy count (*x*-axis) and metabarcoding (*y*-axis) in May and October 2022. The different barcodes are visualized using different colours, and the dotted diagonal line represents the 1:1 relationship. Spearman's rho correlation coefficients of fish diet composition and prey availability between the 18S and COI barcodes and microscope counts (Mic) are shown with significance levels represented with ∗∗∗ when adjusted $P \leq .001$ and ∗ when adjusted $P \leq .05$.

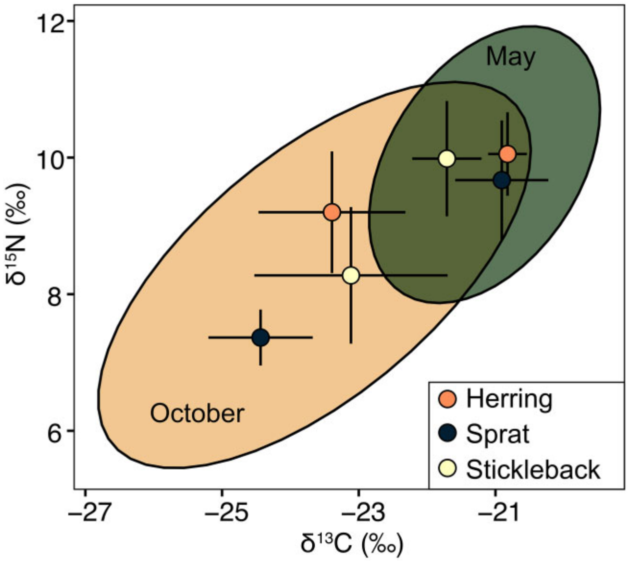

Figure 6. Biplot of carbon and nitrogen stable isotope ratios for herring, sprat, and three-spined stickleback in May and October 2022. The points represent the means, and the error bars the standard deviations. Ellipses show the 95% confidence intervals of the data following a normal distribution for each month.

columns and the microscope counts supporting previous observations that relative read abundance is related to the relative biomass of net collected zooplankton samples (Ershova et al. 2021, Novotny et al. 2025). Three-spined stickleback, especially individuals larger than 5 cm (Fig. S5), also favoured the copepod *Eurytemora* and was the only fish species consistently feeding on this copepod across locations and seasons (Lankov et al. 2010), likely due to three-spined stickleback's shallower position in water column compared to the clupeids that migrate to deeper depths during the daytime (Olsson et al. 2019). Our data further show that three-spined stickleback fed on distinct successive pulses of secondary production, *Synchaeta* in May and *Eurytemora* in October, stressing that three-spined sticklebacks are well adapted to the seasonal succession of zooplankton.

Sprat and herring overlapped in their resource use in May, independently of their size (Fig. S5), likely due to the limited available prey diversity. However, we cannot determine whether this overlap resulted in competition, as such assessment would require information on metabolic demand and energy availability together with diet composition identification. Both fish species were actively selecting for copepods, especially *Pseudocalanus* and *Acartia*, that together contributed to about a quarter of the available zooplankton biomass in May (Fig. S6), which confirms previous observations in the central and southern Baltic Sea (Möllmann et al. 2004, Bernreuther et al. 2018, Novotny et al. 2022). The distinct clupeid

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diet composition compared to the available prey composition suggests that, under low food concentrations, both fish species favoured the particulate over the filter-feeding behaviour, as shown in experiments (Gibson and Ezzi 1985). Selective foraging under conditions of low prey availability further implies that these two copepod species are high-reward prey for clupeids, meaning that the energetic gain from these relatively rare prey species exceeds the cost associated with deceleration and post-capture acceleration (Maszczyk and Gliwicz 2014). Moreover, the average water temperature between 0 and 60 m depth is about 5°C in May, suggesting that fish energy requirements are low and the limited availability of copepods may fulfil their energetic needs.

In contrast, clupeids underwent resource use differentiation later in the season. Herring had a wide prey spectrum, as also reported in the North Sea (Van Ginderdeuren et al. 2014), and continued to favour *Pseudocalanus* but also preyed opportunistically on *Temora* in October. Moreover, herring targeted ctenophores throughout the seasons, highlighting the overlooked role of gelatinous plankton in supporting fish production (Jaspers et al. 2015, Dischereit et al. 2024). These findings support the increasing evidence that gelatinous prey is consumed by higher trophic levels, ranging from seabird to fish (Diaz Briz et al. 2017, Cavallo et al. 2018, Clarke et al. 2020). Sprat diet composition shifted from a *Pseudocalanus* dominated spring diet towards a diet dominated by *Acartia* and *Eurytemora*, two copepod species that usually occupy shallower water layers than *Pseudocalanus* in the Baltic Sea (Renz and Hirche 2006, Holliland et al. 2012). This vertical niche separation of the copepod species and the different diet composition between the two clupeid species may indicate that, in addition to three-spined stickleback, also herring and sprat forage on different depth layers, similar to suggestions by Möllmann et al. (2004). This also implies that resource use partitioning may result not only from differences in prey selectivity but also from fish feeding at distinct water depth layers, a hypothesized mechanism that requires further investigation.

The observed resource use partitioning was confirmed by stable isotope values, which suggests that despite the absence of samples from the summer months, the resource partitioning likely also occurred over this period of high feeding activity (Möllmann et al. 2004) and high prey abundance and diversity (Fig. S6). While summer sampling is needed to confirm this pattern, this can be assumed as stable isotopes provide a time-integrated value of the food web structure. However, lacking baseline values, we cannot disentangle whether the lower fish $\delta^{15}N$ values in October are related to factors influencing baseline shifts, such as the atmospheric nitrogen fixation by the summer cyanobacteria bloom fuelling the food web in summer (Lesutienė et al. 2014), or by food web structure (Post 2022). Moreover, the decreasing resource use overlap with higher prey availability and diversity aligns with the optimal foraging theory (MacArthur and Pianka 1966). A wide range of abundant prey enables coexisting consumers to differentiate their diets more effectively, whereas in regimes where high quality prey is scarce, consumers are likely to target the same prey, increasing the overlap in resource use. Consequently, changes at the base of the food web can have implications on the entire system, as illustrated by the cyclic dynamics of sardines and anchovies in upwelling regions that appear to be mainly bottom-up driven (Chavez et al. 2003, Checkley et al. 2017). This shows that seasonal prey dynamics may influence the foraging behaviour of small pelagic fish, which can alter the rate of interspecific competition by shaping resource use partitioning.

DNA metabarcoding and microscope counts revealed similar patterns that were supported by stable isotope values. Thus, combining these methods allowed for a complete and robust diet assessment of small pelagic fish. Stable isotope analyses offer an integrated measure of diet composition over time (Boecklen et al. 2011), while DNA metabarcoding and microscope counts identify a snapshot of the fish diet composition. Despite the high taxonomy resolution of DNA metabarcoding, cannibalism, which was reported for herring (Skaret et al. 2002) and sprat (Köster and Möllmann 2000) feeding on their eggs, cannot be identified here, as all reads belonging to fish (18S) or the host genus (COI) were removed to avoid misinterpretation of the data. Moreover, this study is no exception in the fact that taxonomy coverage and resolution vary between barcodes (Berry et al. 2015, Clarke et al. 2020), emphasizing the need to combine gene markers for observing the full diversity of fish diet. The high versatility of the 18S allowed to identify taxa across a wide array of phylum, but the more specific COI allowed for a more resolved taxonomy. Compared to DNA metabarcoding, count data have a less resolved taxonomy and may miss important taxa. Even for taxa identified with both barcodes and microscopy, their relative abundance varied with the approach used, as illustrated by copepods that were overrepresented and rotifers underrepresented using 18S compared to COI. We also demonstrate that the relative contribution of the dominant taxonomic group was consistent between microscopy and metabarcoding, particularly for COI. This stresses that the approaches used in our study are complementary. Microscopy is needed for identification of prey life stages, 18S is a good gene marker for exploring the entire fish diet composition beyond metazoan taxa, COI is useful for obtaining more resolved taxonomy in the animal kingdom, and stable isotopes confirm the incorporation of prey in the diet.

Our results suggest that potential competition for resources among the dominating small pelagic fish may not fully explain the recent population decrease in sprat and herring in the Baltic Sea, as diet composition differed when prey availability and diversity were highest. The observed resource partitioning of the dominating forage fish may have, however, facilitated the increase of three-spined stickleback that showed a distinct prey composition by utilizing an efficient zooplankton resource coupled to the spring bloom. Besides the implications of our findings for the Baltic Sea ecosystem, our study has implications for other systems suggesting that change in zooplankton species composition and biomass has the potential to drive small pelagic fish dynamics with consequences for higher and lower trophic levels. Our findings show that species interactions need to be assessed over the seasons and at high taxonomic resolution to better understand implications of prey dynamics and resource use overlap on fish populations.

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Author contributions

Kinlan M.G. Jan (Conceptualization [equal], Data curation [lead], Formal analysis [lead], Investigation [lead], Methodology [lead], Project administration [equal], Software [lead], Validation [lead], Visualization [lead], Writing – original draft [lead], Writing – review & editing [lead]), Jonas Hentati-Sundberg (Data curation [supporting], Resources [equal], Writing – review & editing [equal]), Niklas Larson (Data curation [supporting], Resources [equal], Writing – review & editing [equal]), Monika Winder (Conceptualization [equal], Funding acquisition [lead], Project administration [equal], Resources [equal], Supervision [lead], Writing – original draft [supporting], Writing – review & editing [lead]).

Supplementary data

Supplementary data is available at *ICES Journal of Marine Science* online.

Conflict of interest: None declared.

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Data availability

Fastq file containing raw sequences are publicly available on the European Nucleotide Archive (ENA) under the project accession number PRJEB85974. R scripts, produced data and metadata (Jan et al. 2025) are publicly available on GitHub at https://github.com/KMGJan/FishDiet and in a zenodo repository (doi: 10.5281/zenodo.14886602). Shapefile data for producing the map was retrieved from ICES (2005).

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