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Environmental abundances of the non-native round goby *Neogobius melanostomus* influence feeding of native fish predators

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Abstract

The authors assessed the importance of the round goby *Neogobius melanostomus* as prey for three native predatory fish species, Atlantic cod *Gadus morhua*, European perch *Perca fluviatilis* and northern pike *Esox lucius*, in a northern and southern area of the Baltic Proper, using a combination of visual analysis and DNA metabarcoding of predator stomach contents. To explore the influence of environmental abundances of *N. melanostomus* on predation, they related the occurrence of *N. melanostomus* in predator diets to its abundance in survey fishing. *Gadus morhua* and *E. lucius* in the southern area showed the highest tendency to feed on *N. melanostomus* when it was abundant, as *N. melanostomus* occurred in up to 100% of stomachs and constituted up to 88% of the total diet volume proportion. The diet contribution of *N. melanostomus* was associated with *N. melanostomus* abundances for *G. morhua* and *E. lucius*, and when *N. melanostomus* was abundant, these predators exhibited lower prey richness and a higher degree of piscivory. *G. morhua* and *P. fluviatilis* also fed less on crustacean prey when *N. melanostomus* was abundant. The high importance of *N. melanostomus* in diets of native fish predators may modify indirect interactions between *N. melanostomus* and native prey species in invaded coastal communities.

KEYWORDS

feeding strategy, introduced species, molecular methods, non-indigenous prey, predator-prey interactions, trophic interactions

1 | INTRODUCTION

The round goby *Neogobius melanostomus* Pallas 1814, originating from the Black, Caspian and Azov Seas, is one of the most widely distributed non-native fishes in both fresh and brackish waters in the

Northern Hemisphere (Kornis *et al.*, 2012). In the Baltic Sea, it was first detected in 1990 (Skóra & Stolarski, 1996) and has since then spread to most coastal areas in the southern and central parts (Kotta *et al.*, 2016). After *N. melanostomus* establishment, fish predators like Atlantic cod *Gadus morhua* L. 1758, European perch *Perca fluviatilis* L. 1758 and turbot *Scophthalmus maximus* L. 1758 have shown changes in diet composition from pelagic to benthic prey, as well as

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from invertebrate to fish prey (Almqvist *et al.*, 2010; Rakauskas *et al.*, 2020), and *N. melanostomus* has seasonally and locally become the most abundant prey in the diet of ecologically important fish predators in the Baltic Sea (Almqvist *et al.*, 2010; Hempel *et al.*, 2016; Liversage *et al.*, 2017; Oesterwind *et al.*, 2017; Rakauskas *et al.*, 2020).

N. melanostomus importance as prey for predators may vary depending on factors like habitat characteristics (Liversage *et al.*, 2017), predator species and size or biomass of co-occurring predator species (Reyjol *et al.*, 2010). The effects of *N. melanostomus* on native fish predators are also context-dependent in terms of, for instance, life stage-dependent competition with and predation by *N. melanostomus* (synthesized by Hirsch *et al.*, 2016). Further, effects are ecosystem-specific as they can vary across ecosystems for the same predator species (Hirsch *et al.*, 2016). Thus, the consequences of *N. melanostomus* establishment likely differ between invaded regions and to understand the impact of *N. melanostomus* on local coastal ecosystems, it is essential to elucidate the mechanisms causing variation in species interactions. This is especially important for the Baltic Sea, which exhibits large differences in local environmental conditions such as salinity, temperature, habitat types and therefore also variation in native species' composition and richness (Bonsdorff, 2006, Snoeijs-Leijonmalm, 2017). Previous studies on native fish predation on *N. melanostomus* have been focused on coastal areas in the southern and eastern Baltic Sea (Germany; Hempel *et al.*, 2016; Oesterwind *et al.*, 2017; Poland; Almqvist *et al.*, 2010; Lithuania; Rakauskas *et al.*, 2013; Rakauskas *et al.*, 2020 and Estonia; Järv *et al.*, 2011; Liversage *et al.*, 2017). Although several studies have investigated the importance of *N. melanostomus* as prey for multiple fish species within one region (Almqvist *et al.*, 2010; Oesterwind *et al.*, 2017; Rakauskas *et al.*, 2020), none have carried out comparative analyses between different regions, and over time, to understand whether patterns of variation in predator diets are consistent across species and regions in the Baltic Sea.

The purpose of this study was to investigate spatio-temporal diet variation and the contribution of *N. melanostomus* as prey for three fish predators, *G. morhua*, *P. fluviatilis* and northern pike *Esox lucius* L. 1758. To be able to draw conclusions about causes of variation in predator diets across space, time and species, the authors collected predator diet data during two consecutive years, 2018 and 2019, in two geographically distant areas in the Baltic Sea, the Åland Archipelago in the northern Baltic Proper and Karlskrona Archipelago in southern Baltic Proper (Figure 1). Previous studies of *N. melanostomus*' importance in predator diets from these study areas are lacking, hampering conclusions about the implications of predation on *N. melanostomus* on a larger scale as effects of predation on *N. melanostomus* are ecosystem specific (Hirsch *et al.*, 2016). *G. morhua* is a marine species adapted to the brackish water conditions in the Baltic Sea (Kullander *et al.*, 2012). Smaller size classes feed primarily on benthic invertebrates, and the proportion of fish in the diet, mainly clupeids, gradually increases from around 20 cm in length (Haase *et al.*, 2020), although the ontogenetic shifts depend on habitat use and environmental conditions (Funk *et al.*, 2021; Haase

et al., 2020; Neuenfeldt *et al.*, 2020). *P. fluviatilis* and *E. lucius* are originally freshwater species that have adapted to brackish water conditions and are the most common piscivorous predators in shallow coastal areas (Olsson, 2019). As a generalist predator, *P. fluviatilis* diet varies depending on habitat, prey availability and ontogenetic stage (Mustamäki *et al.*, 2014; Jacobson *et al.*, 2019). *E. lucius* is a primarily piscivorous predator which starts preying almost exclusively on fishes (>90% of the diet) at >10 cm (Jacobson *et al.*, 2019). All three species are ecologically important and of interest for both small-scale commercial fisheries and recreational fisheries.

To better understand the impacts of species invasions, it is crucial to determine the role of increasing densities of non-native species on native species and ecosystems (Bradley *et al.*, 2019). Many non-native species can reach high densities in their recipient ecosystems (Simberloff and Gibbons, 2004), including *N. melanostomus* in the Baltic Sea, which has locally been reported to reach up to 9 individuals m^{-2} , with occasional density estimates of >20 fish m^{-2} (HELCOM, 2018). Predator consumption rates are governed by success rate and handling time, and together with prey densities, they determine the functional response of predators as higher prey densities generally lead to increased consumption rates until a threshold is reached (Jeschke *et al.*, 2022). Further, as generalist predators have broad dietary niches (Gerking, 1994), they can adapt their feeding strategy based on prey availability (Laske *et al.*, 2018) and temporarily specialize on abundant prey (Amundsen, 1995). *N. melanostomus* population abundances could thus influence not only the level of predation on *N. melanostomus* but also the feeding strategy of predators. Many studies have documented temporal shifts in predator diets following the establishment and increased population abundances of *N. melanostomus* (Crane & Einhouse, 2016; Hempel *et al.*, 2016; Rakauskas *et al.*, 2020; Taraborelli *et al.*, 2010), or associated higher levels of predation on *N. melanostomus* with areas where *N. melanostomus* abundances are presumably higher (Reyjol *et al.*, 2010). Nonetheless, the specific relationship between the environmental abundances of *N. melanostomus* and predation on *N. melanostomus* or predator feeding strategies has received little attention so far (but see Liversage *et al.*, 2017, finding that *N. melanostomus* densities only explained 11.1% of *N. melanostomus* quantities in *P. fluviatilis* diet).

The authors of this study used two diet assessment methods, visual identification and DNA metabarcoding of stomach contents, to obtain more comprehensive information about predator diet composition (Nielsen *et al.*, 2018). Combined, these methods make up a new approach in the study of Baltic Sea predatory fish diets. The aim of this study was to (a) estimate the contribution of *N. melanostomus* as prey in the diets of *G. morhua*, *P. fluviatilis* and *E. lucius*; (b) relate *N. melanostomus* occurrence in predator diets to its environmental abundances; and (c) describe changes in overall diet composition and feeding strategies of these three predator fish species in relation to *N. melanostomus* environmental abundances. The authors expected to gain knowledge about the extent to which *N. melanostomus* is used as prey by native predators as well as how the incorporation of

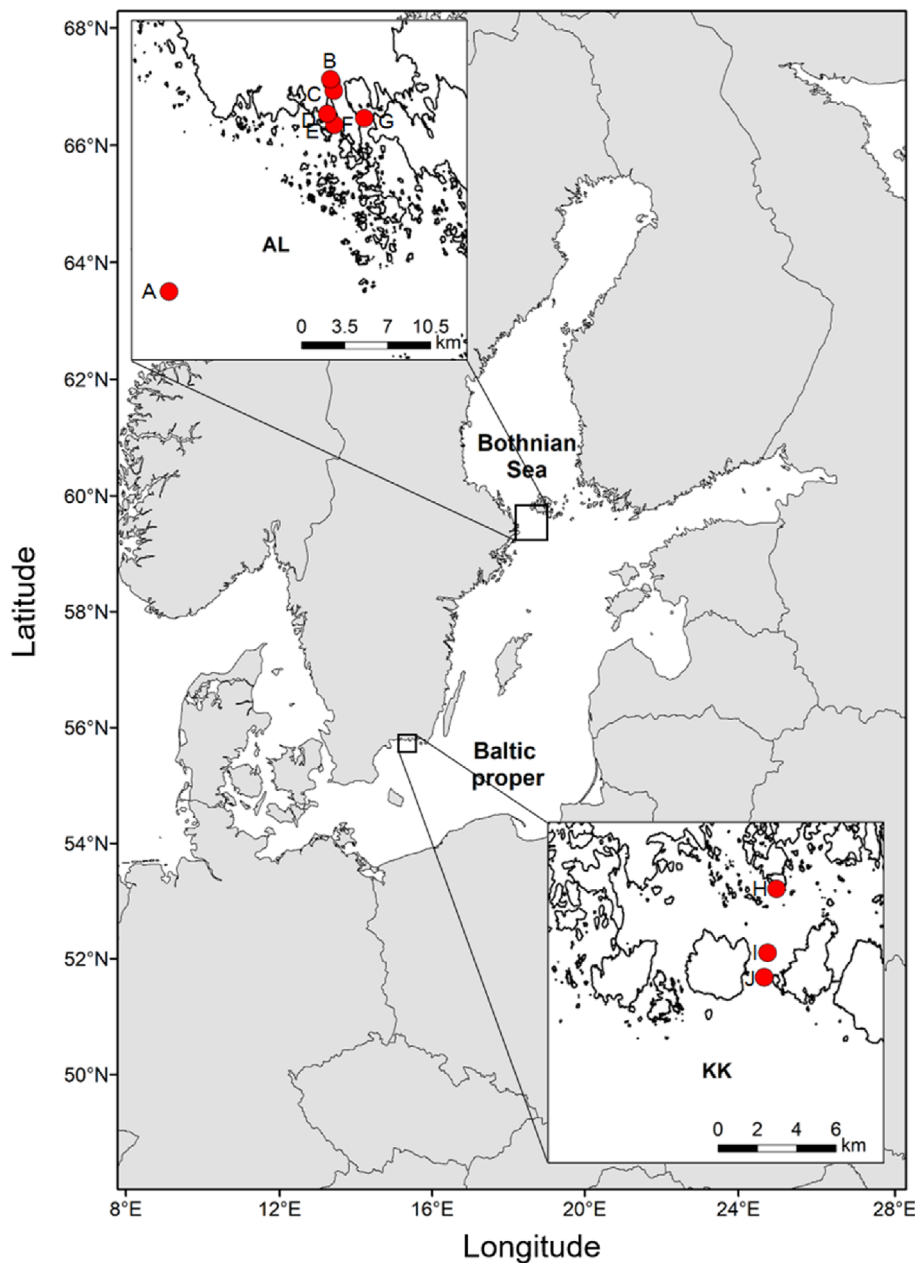


FIGURE 1 Map of sampling locations for *Gadus morhua*, *Perca fluviatilis* and *Esox lucius* in Åland (AL) and Karlskrona (KK) in 2018 and 2019. For a full description of the sampling locations (A–J), see Supporting Information Appendix S1

N. melanostomus as prey alters predator diet composition and feeding strategies in space and time.

2 | MATERIALS AND METHODS

The study was carried out in the Åland Islands (AL) in the northern Baltic Proper (ICES SD 29) and the Karlskrona Archipelago (KK) in the southern Baltic Proper (ICES SD 25, Figure 1) in 2018 and 2019. *N. melanostomus* was first observed in 2011 in AL and in 2008 in KK (Government of Åland, 2021; Swedish University of Agricultural Sciences, 2021). *N. melanostomus* is widely distributed in KK, whereas in AL it is mainly restricted to the bay of the main passenger harbour (locations B–G, Figure 1), but the population is expanding c. 500 m per year (Government of Åland, 2021).

2.1 | Data collection and sample processing

2.1.1 | Predator sampling

G. morhua, *P. fluviatilis* and *E. lucius* were sampled during May–June 2018 and March–June 2019, corresponding to spring and early summer conditions. Fishing time was one night (12–16 h) on 1–5 fishing occasions per month for each species and area. In AL, *G. morhua* was caught with gillnets (mesh-size 80 mm) in commercial fisheries c. 20 km offshore between the depths of 80 and 200 m (location A, Figure 1). *G. morhua* and *N. melanostomus* around AL are expected to overlap spatially primarily during winter, when *N. melanostomus* migrates to deeper waters (Behrens *et al.*, 2022), but as there are no studies about *G. morhua* diet around AL the authors deemed their diet study, conducted in spring and early summer, justified for comparison

between areas. *P. fluviatilis* and *E. lucius* in AL were fished in both commercial gillnet fisheries and in separate sampling campaigns with multi-mesh coastal survey gillnets (nine panels, mesh-sizes 10, 12, 15, 19, 24, 30, 38, 47 and 60 mm) and regular gillnets (mesh-size 35–60 mm) at six locations at 2–6 m depth (B–G, Figure 1; Supporting Information Appendix S1). In KK, predatory fishes were caught with gillnets in commercial fisheries (mesh-size 55–60 mm for *G. morhua*, 45–47 mm for *P. fluviatilis* and 50 mm for *E. lucius*). *G. morhua* was fished at three locations (H–J, Figure 1), whereas *P. fluviatilis* and *E. lucius* were fished at one location (H, Figure 1; Supporting Information Appendix S1) at 6–20 m depth. Commercial fishers provided either fishes frozen whole (*P. fluviatilis* and *E. lucius* in AL) or only the gastrointestinal tract (*G. morhua* in AL and all three species in KK) individually frozen (−20°C) until later processing with records of total length (L_T , cm) and total body mass (wet mass, M_{TW} , g). Only one *E. lucius* was caught in AL 2018 and was excluded from the analyses.

2.1.2 | Diet sampling

The authors aimed to estimate the contribution of *N. melanostomus* in predator diets as well as describe the full diet of the predators. Therefore, they used two methods, visual stomach content analysis (VSCA) and DNA metabarcoding. VSCA was used for all predator diet samples, whereas DNA metabarcoding was used as a complement for a sub-set of the samples. The two methods supplement each other regarding taxonomic accuracy and quantification of stomach contents (Nielsen *et al.*, 2018). Visual inspection of fish stomach contents provides taxonomic information as well as information about prey quantities and prey life stages and sizes but may yield results biased towards larger prey items or prey items with hard structures like otoliths or exoskeletons, while underestimating prey diversity due to unidentifiable, highly digested material (Nielsen *et al.*, 2018). DNA-based methods, in contrast, have shown considerable promise in the detection of cryptic species (Groen *et al.*, 2022) and prey from highly digested material (Carreon-Martinez *et al.*, 2011), increasing the taxonomic resolution in diet assessments (Nielsen *et al.*, 2018) and thereby contributing to increased knowledge about the diversity of predator diets. Nevertheless, there are many technical considerations and limitations to DNA metabarcoding, as well as quantification issues (Deagle *et al.*, 2019; Nielsen *et al.*, 2018). Methodological constraints are further addressed in the discussion (Section 4.3). In the present study, the authors used DNA metabarcoding to ensure that they did not miss *N. melanostomus* as prey and to potentially capture a broader range of prey species/groups compared to the VSCA.

In total, they sampled 595 fishes for diet analysis (Supporting Information Appendix S2). When whole fish predators were provided, they measured their total length (L_T) and total wet mass (M_{TW}) after thawing. Each stomach was dissected, and the contents were poured or scraped out and weighed for total stomach content wet mass (M_{SC} ; to the nearest 0.01 g). Gut fullness was estimated on the NOAA (2015) scale, ranging from empty (Almqvist *et al.*, 2010) to full

(Bergström *et al.*, 2022). Whenever possible, 10 fish per species, area, year and month were randomly chosen *a priori* and sampled for DNA analysis. For samples that were assigned to both VSCA and DNA metabarcoding ($n = 106$, Supporting Information Appendix S3), the DNA samples were taken before VSCA to minimize the risk of DNA contamination. For details about sample selection for DNA metabarcoding and DNA sampling methods, see Supporting Information Appendix S4.

2.1.3 | Visual stomach content analysis

The authors studied the stomach contents under a stereo microscope and determined prey items to the lowest possible taxonomic level using reference material and taxonomic keys (*e.g.*, Härkönen, 1986 and Bone Base Baltic Sea, v. Busekist, 2004). *N. melanostomus* and the native black goby *Gobius niger* L. 1758 are difficult to distinguish from one another in stomach contents, and the authors conducted dissections to identify differences in skeletal structures between the two species (Supporting Information Appendix S5). They selected samples for DNA barcoding and DNA species assignment through dPCR after visual inspection of the stomach contents, if species determination of possible *N. melanostomus* specimens proved difficult (Supporting Information Appendix S4). All prey items were counted, and they visually estimated the contribution of each prey type as a proportion (0–1) of the volume of all prey items in the stomach, excluding non-prey items such as stones or algal material (Hyslop, 1980).

2.1.4 | DNA metabarcoding

DNA metabarcoding is a process to identify multiple species in a mixed sample (*e.g.*, samples of soil, water, faeces, stomach contents; Taberlet *et al.*, 2012). All following steps in the DNA metabarcoding were conducted by SeAnalytics, Sweden (<https://www.seanalytics.se>) and Eurofins Genomics, Germany (<https://eurofinsgenomics.eu/>). DNA was extracted from the samples using DNeasy blood and tissue kit by QIAGEN following the blood protocol, amplified using PCR and sequenced on the Illumina MiSeq platform (Supporting Information Appendix S4).

The authors used the 12S rRNA marker for identifying fish prey (Miya *et al.*, 2020; Weigand *et al.*, 2019). For *G. morhua* and *P. fluviatilis*, feeding on both fishes and invertebrates, they also used the mitochondrial cytochrome c oxidase subunit I (COI) marker to identify invertebrates, as it has an extensive reference database of both freshwater and marine invertebrates (Weigand *et al.*, 2019). Separate PCR runs and sequencing were conducted for the respective markers.

After excluding samples with DNA concentrations $<0.1 \text{ ng } \mu\text{l}^{-1}$ and samples that did not yield any sequences, the final number of samples was 122 (Table 1). Rare sequences that comprised $<1\%$ of the total sequences in each sample were excluded. The prey data sets for 12S and COI were combined for further data processing and analyses. The separate PCR runs for each marker resulted in differences

TABLE 1 Predator size (mean, min-max of total length, L_T , in cm) and the number of non-empty stomach samples (n VSCA) per predator species (*Gadus morhua*, *Perca fluviatilis* and *Esox lucius*) and year from Åland (AL) and Karlskrona (KK) included in diet analyses based on VSCA

	<i>G. morhua</i>					<i>P. fluviatilis</i>					<i>E. lucius</i>				
	Mean	Min-max	n VSCA	Empty (%)	n DNA (excl.)	Mean	Min-max	n VSCA	Empty (%)	n DNA (excl.)	Mean	Min-max	n VSCA	Empty (%)	n DNA (excl.)
AL	65.5	35–91	106	17 (14)	31 (0)	28.2	23–35	132	35 (21)	16 (0)	52.7	43–64	23	2 (8)	9 (1)
2018	65.9	41–91	59	5 (8)	11 (0)	28.8	24–35	50	16 (24)	5 (0)					
2019	65.0	35–91	47	12(20)	20 (0)	27.8	23–34	82	19 (19)	11 (0)	52.6	43–64	23	2 (8)	9 (1)
KK	46.3	32–60	104	1 (1)	16 (0)	30.4	25–43	56	11 (16)	32 (0)	66.4	52–85	39	12(24)	18(2)
2018	47.4	39–57	30	-	8 (0)	31.0	27–37	14	5 (26)	16 (0)	64.7	52–79	14	6 (30)	9 (1)
2019	45.8	32–60	74	1 (1)	8 (0)	30.1	25–43	42	6 (13)	16 (0)	67.3	56–85	25	6 (19)	9 (1)

Note: Number of empty stomachs and their share (empty, %) out of all samples shown in parentheses. Number of DNA samples (n DNA) in total after filtering (excl. = excluding samples with DNA concentrations <0.1 ng μl^{-1} or samples that did not yield any sequences, shown in parenthesis). All *G. morhua* and *P. fluviatilis* samples were analysed using both 12S and COI markers. Only the 12S marker was used for *E. lucius*.

in the number of sequences between markers (Supporting Information Appendix S4). This, in combination with potential variation in primer affinity between markers and taxa (Deagle *et al.*, 2019; Taberlet *et al.*, 2012), means that the relative number of sequences between prey categories (fishes vs. invertebrates) should be interpreted with caution. In DNA metabarcoding, the risk of DNA contamination or secondary consumption (prey-of-prey) cannot be excluded (Deagle *et al.*, 2019), although secondary consumption might also be a problem in VSCA. The authors have undertaken measures to minimize contamination (see Supporting Information Appendix S4 for details about sampling and data processing).

2.1.5 | *N. melanostomus* survey fishing

To estimate *N. melanostomus* abundances in the environment, survey fishing was carried out in the same coastal areas as predatory fishes were collected during May–June 2018 and April–June 2019. The only exception was location A, where *G. morhua* was fished in AL (Figure 1; Supporting Information Appendix S1), at depths where *N. melanostomus* is generally not found during spring and summer (Behrens *et al.*, 2022). The fyke nets used in this survey of *N. melanostomus* are used in monitoring of species composition and relative abundance of fish species in coastal areas (Swedish Agency for Marine and Water Management, 2015). Fyke nets are particularly well suited for monitoring benthic fishes, or fishes that occasionally reside near the bottom (Nilsson *et al.*, 2022). The fyke nets were thus deemed suitable for tracking relative changes in *N. melanostomus* abundances between areas and years, and the authors used the catch per unit effort (CPUE) of *N. melanostomus* to compare its relative abundances between sampling occasions (*i. e.*, not relative to other species). They use the terms “environmental abundances” or “*N. melanostomus* CPUE” when referring to the relative abundances of *N. melanostomus* in fyke net survey fishing. They fished with two fine mesh twin fyke nets (in total four houses, house mesh-size 8–9 mm and 11–12 mm in leaders and netting bags) laid out in a line at each location for one night each month (1.8–4.5 m depth; Supporting Information Appendix S1). As the exact fishing time varied (9–14 h), the catches were standardized to 12 h whenever needed, to correct for differences in catches due to varying fishing time. CPUE thus equalled standardized *N. melanostomus* catches in one paired fyke net (four houses) over 12 h. Three locations per area were fished each month (equalling three nights), except in April 2019 in AL when one sampling location was fished continuously over 5 days (a total of 120 h). As *N. melanostomus* is mainly caught during dark hours (*e.g.*, Diana *et al.*, 2006; Erös *et al.*, 2005), the authors assumed that catches during the day were negligible and thus, 120 h equalled five nights. They standardized the catch by multiplying total catch with 0.6, corresponding to three fishing nights of 12 h to make the catch in April 2019 comparable to the other fishing months. For considerations about fishing depletion, see Supporting Information Appendix S1.

Fishing and animal handling were conducted following local fishing regulations (ÅLR 2018/3983, Finland; Government of Åland and SLU.aqua.2018.5.4-194, Sweden; Swedish University of Agricultural Sciences and Swedish Agency for Marine and Water Management) and under the ethical permit 5.8.18-07747/2018 (Sweden; Swedish Board of Agriculture, the Uppsala region Ethics Committee on Animal Experiments).

2.2 | Data analyses

All data analyses and visualization were conducted in R, versions 4.1.1 and 4.0.2 (R Core Team, 2020, 2021). All plots were produced using the package *ggplot2* (Wickham, 2016).

2.2.1 | Predator diet composition

Overall diet composition, as well as the contribution of *N. melanostomus* to predator diets, was summarized as relative volume proportion (V, %) and relative sequence number (S, %) for VSCA and DNA metabarcoding data, respectively. These were calculated for each predator species in each area and year as follows:

$$V = P_i/P_t \times 100 \quad (1)$$

$$S = S_i/S_t \times 100 \quad (2)$$

where P_i and S_i refer to the sum of volume proportions and number of sequences per prey group i , whereas P_t and S_t refer to the total volume and total number of sequences of all prey in all non-empty stomachs and DNA samples, respectively. The number of sequences serves as an indication of the relative biomass proportions of prey (Deagle *et al.*, 2019). Relative sequence number (S, also referred to as relative read abundance, RRA, in Deagle *et al.*, 2019) is also less affected by potential contamination of DNA samples compared to occurrence (presence/absence) data, often used in DNA studies. If contaminating DNA is present in many samples, although in low sequence numbers, it skews the results of a presence/absence measure, but remains of low importance on a population level in terms of S (Deagle *et al.*, 2019).

In addition, frequency of occurrence (FO, %) was calculated based on VSCA data for each prey type i per predator species, area and year. FO gives the percentage number of samples containing a given prey (N_i) out of all non-empty stomach samples (N_t):

$$FO = N_i/N_t \times 100 \quad (3)$$

Prey difficult to assign to species in VSCA were grouped at a higher taxonomic level (e.g., Gasterosteidae, Clupeidae and Caridean shrimp), and all prey groups in VSCA with V and FO <5% for all predators in both areas were combined to larger groups (e.g., other fishes

and other invertebrates). This resulted in 16 prey groups (unidentified excluded). For the sake of clarity, the same groups were used for analysis of DNA metabarcoding data. Fishes with empty stomachs were excluded from the VSCA data set ($n = 78$, 13%) used for describing diet composition, but 16 fishes with empty stomachs were used for DNA metabarcoding despite being discarded from VSCA (Table 1; Supporting Information Appendices S2–S4). To focus on the primarily piscivorous stages of *P. fluviatilis*, *P. fluviatilis* < 23 cm ($n = 49$) were omitted as individuals below this size feed primarily on invertebrates (Jacobson *et al.*, 2019).

Relative prey weight (RPW, %) of a predator is a measure of gut fullness and gives the amount of prey in a stomach (M_{SC}) relative to the predator body mass (M_{TW}). It was used to describe variation in the amount of ingested prey and was calculated as follows:

$$RPW = M_{SC}/M_{TW} \times 100 \quad (4)$$

2.2.2 | Diet contribution of *N. melanostomus* in relation to *N. melanostomus* abundances

The analysis of *N. melanostomus* in diets in relation to *N. melanostomus* abundances in the environment was conducted on VSCA data only, as the sample size for DNA data was limited compared to VSCA data. *N. melanostomus* mean and median CPUE per month was calculated for five occasions in each area; May and June 2018 and April, May and June 2019 in AL and KK, respectively. On all five occasions in KK and four occasions in AL, mean and median CPUE was based on one fishing night in three different locations per month. Nonetheless, in AL in April 2019, mean and median CPUE was based on three fishing nights in the same location instead of three locations of one fishing night each (see Section 2.1.5 and Supporting Information Appendix S1).

To compare *N. melanostomus* abundances between years in each area, the authors tested median CPUE of all sampling occasions per year (Mann–Whitney *U*-test). To analyse how predator diets related to *N. melanostomus* CPUE, they used linear mixed effect models (LMM) and general linear mixed models (GLMM) in the package *lme4* in R (Bates *et al.*, 2015). They used median CPUE rather than mean CPUE as the median yielded lower AIC values than mean CPUE. In the analyses they only used predator diet data for the months from which they had data of median *N. melanostomus* CPUE (Supporting Information Appendix S1). *G. morhua* and *E. lucius* from AL were excluded from the analysis, as there were no or very few (<5) overlapping predator diet samples.

There were only five independent estimates of median *N. melanostomus* CPUE in each area, resulting in low statistical power. Therefore, they chose to analyse which variables contributed to the model fit using changes in AIC (Burnham & Anderson, 2002), rather than hypothesis testing. Although changes in AIC are sensitive to small sample sizes and outliers, they indicate which variables may explain variation in predator diets. Model AIC values were compared

to intercept model AIC values. The intercept model included the respective dependent variables and the random factor. Sampling occasion, *i.e.*, 2 months 2018 and 3 months 2019 in each sampling area, was used as a random factor in all models. Therefore, the year effect was included in the random factor instead of a fixed factor, as *N. melanostomus* abundances differed between years, risking to mask the influence of *N. melanostomus* abundance. That means we cannot control for any confounding factors potentially related to between-year variation other than *N. melanostomus* abundances.

For each predator species the authors ran separate models for the three dependent variables: (a) *N. melanostomus* abundance in predator diets (log-transformed number of *N. melanostomus*, $\log_e(N_i + 1)$ per stomach sample) using LMM, (b) proportion of *N. melanostomus* in predator diets per stomach sample using GLMM (binomial distribution) and (c) relative prey weight (log-transformed relative prey weight $\log_e(RPW + 1)$ per stomach sample) using LMM. In models (a) and (b) both empty and non-empty stomachs were included ($n = 359$), whereas in model (b) only non-empty stomachs ($n = 300$) were included. For *G. morhua* and *E. lucius*, the authors used *N. melanostomus* median CPUE and total length (L_T) of predators as explanatory variables. For *P. fluviatilis*, for which they had samples from both areas and years, they also added catch area (AL and KK) as an explanatory variable and included interactions between catch area and *N. melanostomus* median CPUE. They studied differences in AIC, denoted ΔAIC , by removing or adding explanatory variables to the intercept model, considering the model with the lowest AIC to be the best model.

2.2.3 | Predator feeding strategy

Feeding strategies were visualized for each predator species in each area and year with Costello-Amundsen graphs (Costello, 1990, modified by Amundsen *et al.*, 1996). For this, the authors used the frequency of occurrence (FO) and calculated the prey-specific proportion, PSP (*i.e.*, the percentage contribution of each prey type *i* to the diet of predators that consumed prey *i*):

$$PSP = P_i / P_{ti} \times 100 \quad (5)$$

where P_i is the sum of volume proportions of prey group *i* and P_{ti} is the total volume proportion of stomachs containing prey group *i*. These graphs depict feeding strategies, *i.e.*, if the predator shows generalist or specialized feeding (Amundsen *et al.*, 1996). All the studied predators are feeding on many types of prey. Thus, the visualization here depicts adaptive feeding of more or less generalist predators, as feeding strategies can vary due to *e.g.*, temporal variation in prey abundances (Amundsen, 1995; Smith *et al.*, 2011) or intra-specific competition (Svanbäck & Persson, 2004). Therefore, both individual- and population-level specialization can occur (Bolnick *et al.*, 2003). Specialization in this case refers to either individuals that specialize on different prey or to the whole observed population feeding on a narrow range of prey (lower observed prey diversity). PSP is

not a measure of prey preference or selection *per se*, as there are no data on prey availability. Prey groups characterized by both high (>50%) PSP and FO indicate specialization on a specific prey on a population level, whereas prey characterized by high PSP but low FO indicate individual specialization within the predator population. Many prey groups with low PSP but moderate to high FO indicate a generalist feeding strategy (Amundsen *et al.*, 1996). PSP and FO were calculated and visualized for 12 taxonomic groups, as Caridean shrimp were grouped in other crustaceans and all gobiid species except *N. melanostomus* grouped in other Gobiidae. The Costello-Amundsen plot was done for VSCA data only, as DNA metabarcoding data only represent a sub-set of diet samples.

In addition, mean prey richness, *i.e.*, mean number of identified prey items in non-empty stomachs, was calculated for each predator species in each area and year to support the visualization results, indicating realized diet diversity.

3 | RESULTS

3.1 | *N. melanostomus* in predator diets and predator diet composition

N. melanostomus was a more common prey for predators in KK as FO of *N. melanostomus* in non-empty predator stomachs ranged between 32% and 100%, whereas in AL FO of *N. melanostomus* varied between 0% and 54% (Table 2). Based on VSCA, *N. melanostomus* was consumed by all three predator species in KK, but only by *P. fluviatilis* and *E. lucius* in AL. Nonetheless, it also constituted a minor share of *G. morhua* diet in AL according to DNA metabarcoding ($S = 1\% - 2\%$; Table 2).

In 2018, *N. melanostomus* constituted in total 88% and 78% in relative volume proportions for *G. morhua* and *E. lucius* in KK, whereas *N. melanostomus* contribution to *P. fluviatilis* diets was 46% and 28% in volume proportions in AL and KK, respectively (V; Figure 2a; Table 2). In 2019, the volume proportion of *N. melanostomus* dropped to only 3% for *P. fluviatilis* in AL, whereas for *G. morhua* and *E. lucius* in KK, the contribution of *N. melanostomus* decreased by 67% and 76%, although it remained one of the most important prey species ($V = 29\%$ and $V = 18\%$; Table 2). Conversely, for *P. fluviatilis* in KK, the diet contribution of *N. melanostomus* increased to 36% in 2019 (V; Table 2). In AL, *N. melanostomus* constituted 7% of the relative volume proportion for *E. lucius* in 2019. Overall, the authors found a 3% to 26% decrease in the total proportion of fishes in diets for all predator species in KK and *P. fluviatilis* in AL, and a simultaneous 200% to 2600% increase in the contribution of macroinvertebrates was observed from 2018 to 2019 for these predators (V; Figure 2a; Table 2). For *G. morhua* in AL, however, macroinvertebrates ($V = 65\% - 68\%$), primarily the Baltic isopod *Saduria entomon* L. 1758 ($V = 52\% - 56\%$), constituted the most important prey category in both years, whereas fishes constituted less important prey ($V < 30\%$; Figure 2a, Table 2) and no *N. melanostomus* were detected in VSCA.

TABLE 2 Diet composition of *Gadus morhua*, *Perca fluviatilis* and *Esox lucius* from Åland (AL) and Karlskrona (KK) in 2018 and 2019, respectively

	AL				KK																														
	2018		2019		2018		2019																												
	<i>P. fluviatilis</i>		<i>E. lucius</i>		<i>G. morhua</i>		<i>P. fluviatilis</i>																												
	FO	V	S	V	FO	V	S	V																											
Fishes	41	23	99	49	27	99	100	98	99	90	73	98	100	92	100	100	94	99	100	76	99	100	96	99	95	86	97	100	100	100	100	100	100		
<i>Neogobius melanostomus</i>	0	0	2	0	0	1	54	46	1	5	3	1	9	7	<1	100	88	56	53	29	20	57	28	16	52	36	8	86	78	2	32	18	<1		
Other Gobiidae	0	0	2	0	0	3	6	2	11	27	9	47	43	14	17	3	33	11	2	<1	7	1	24	10	8	5	7	7	<1	24	10	17	17		
Clupeidae	8	4	1	15	9	12	34	29	72	15	12	4	57	52	42	0	0	0	22	13	2	7	0	4	0	4	7	1	38	52	40	58	58		
Gasterosteidae	2	2	9	0	0	4	10	6	15	57	40	31	9	5	10	3	1	9	36	15	77	71	65	55	38	25	80	7	<1	12	9	22	22		
<i>Zoarces viviparus</i>	3	2	5	9	2	33	10	7	0	4	0	12	0	0	1	3	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	<1	<1
<i>Perca fluviatilis</i>	0	0	7	0	0	1	0	0	0	1	1	0	9	8	13	0	0	0	30	12	0	0	0	0	2	2	0	0	0	3	8	3	<1	<1	
Cottidae	7	4	56	13	10	31	0	0	0	0	0	0	0	0	<1	0	0	0	1	1	<1	0	0	0	0	0	0	0	0	0	0	56	0	0	<1
Other fishes	3	3	17	0	0	14	2	2	0	2	1	3	4	1	17	7	1	0	1	0	0	0	0	0	2	0	0	7	0	0	0	0	0	1	1
Unidentified fishes	17	8		15	5	14	6	18	6	18	6	13	5		10	2	11	3	14	1	14	1	26	14	0	0	0	0	0	0	20	16	16	16	
Macroinvertebrates	88	65	1	89	68	1	10	1	1	65	27	2	9	4	0	47	5	1	64	23	1	14	4	1	36	14	3	0	0	0	8	2	0	0	
<i>Saduria entomon</i>	78	52	<1	81	56	<1	0	0	0	0	0	0	0	0	0	3	1	0	26	8	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Mysidae	37	11	<1	40	11	<1	2	0	0	23	7	1	4	0	0	17	2	<1	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Other Crustacea	5	2	0	2	0	<1	4	1	1	95	19	<1	0	0	0	30	2	<1	70	14	<1	14	3	<1	43	11	2	0	0	0	0	0	0	0	0
Other invertebrates	0	0	<1	0	0	<1	6	0	0	15	1	<1	4	4	0	3	0	<1	12	1	<1	29	1	<1	17	3	1	0	0	0	0	0	0	0	
Unidentified	29	12	6	6	4	4	1	4	4	0	0	4	4	4	4	4	1	1	1	1	0	0	0	2	0	0	0	0	4	1	4	1	1	1	
Prey richness (mean ± S.E.)	1.6 ± 0.11	1.7 ± 0.11		1.7 ± 0.11	1.7 ± 0.11	1.4 ± 0.12	2.6 ± 0.21	1.5 ± 0.15	1.5 ± 0.15	2.6 ± 0.21	1.8 ± 0.15	2.8 ± 0.18	2.0 ± 0.35	1.9 ± 0.20	1.1 ± 0.10	1.1 ± 0.10	1.9 ± 0.20	1.6 ± 0.17																	

Note: Diet composition (%) expressed as frequency of occurrence (FO) and relative volume proportion (V) of each prey group in VSCA and their relative sequence number (S) in DNA metabarcoding. Summarized values are given for prey categories (fishes and macroinvertebrates). Mean prey richness (mean number of different prey species per stomach ± standard error) is given for VSCA data.

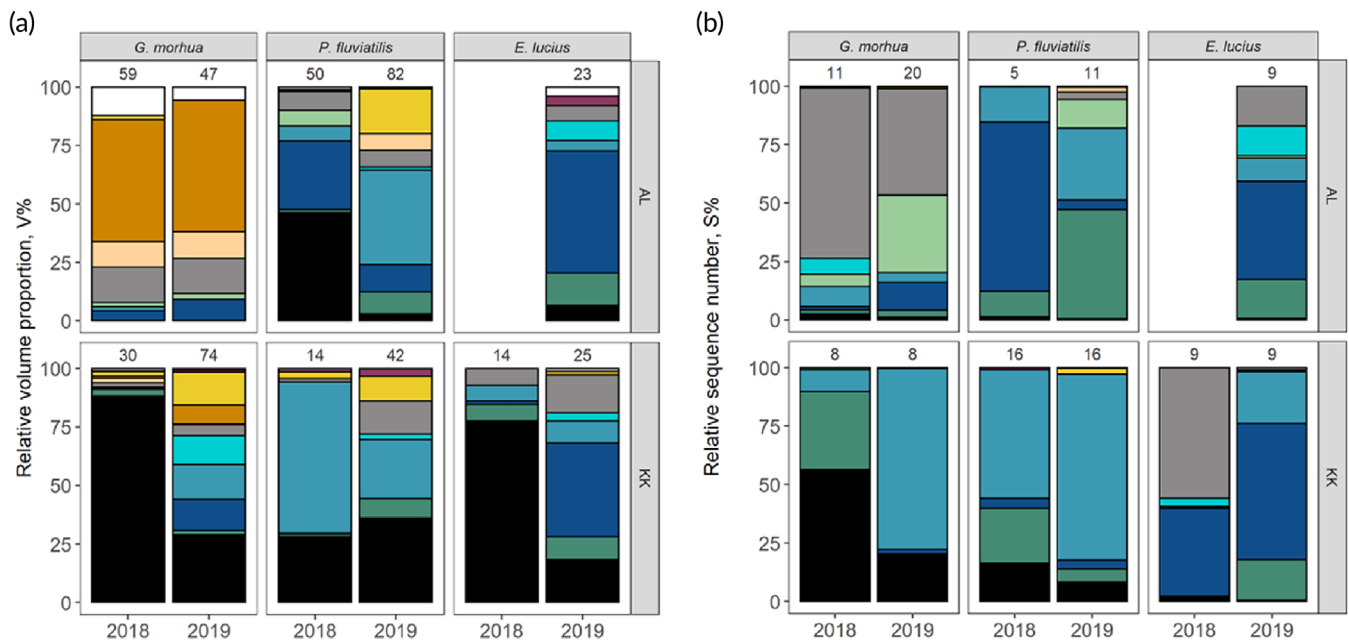


FIGURE 2 Diet composition of *Gadus morhua*, *Perca fluviatilis* and *Esox lucius* in 2018 and 2019 according to (a) VSCA (relative volume proportion, V%) and (b) DNA metabarcoding (relative sequence number, S%) in AL (Åland) and KK (Karlskrona). The number above each bar gives the number of samples per group. See Supporting Information Appendices S6 and S7 for species belonging to each prey group. □ Unidentified. ■ Other invertebrates. ■ Other Crustacea. ■ *S. entomon*. ■ Mysidae. ■ Other fishes. ■ *P. fluviatilis*. ■ *Z. viviparus*. ■ Gasterosteidae. ■ Clupeidae. ■ Other Gobiidae. ■ *N. melanostomus*

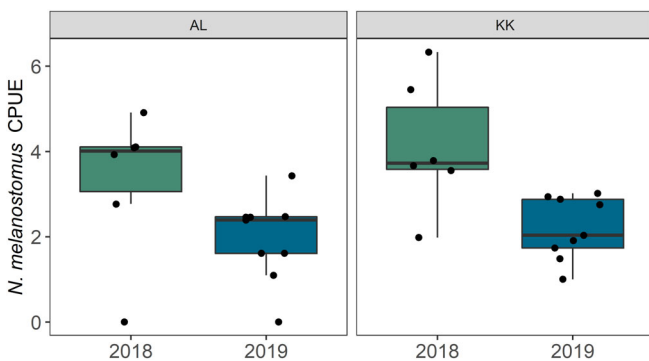


FIGURE 3 Standardized *Neogobius melanostomus* fyke net catches ($\log(\text{CPUE} + 1)$) in Åland (AL) and Karlskrona (KK) in 2018 and 2019. Each dot represents a sampling occasion (Supporting Information Appendix S1). The black vertical lines within boxes show the median values. The corresponding Mann-Whitney *U*-test was carried out with non-transformed CPUE values. Year: □ 2018, ■ 2019

In DNA metabarcoding, *N. melanostomus* made up the largest share of the prey sequences for *G. morhua* in KK in both years, which was consistent with the VSCA results ($S = 56\%$ and 20% in 2018 and 2019, respectively; Figure 2b; Table 2), compared to *P. fluviatilis* ($S = 8\%$ – 16%) and *E. lucius* ($S \leq 2\%$). In AL, *N. melanostomus* made up less than 3% of the sequences for all predators in both years ($S\%$, Table 2). According to DNA metabarcoding, *N. melanostomus* constituted prey for all studied predators in both areas, but contributed in total 31%–99% less (mean difference: $-72 \pm 29\%$ SD) to predator

diets in relative sequence numbers than in volume proportions for all predators except *G. morhua* in AL, where no *N. melanostomus* were detected using VSCA (Figure 2b; Table 2). Contrary to VSCA, macroinvertebrates constituted only 1%–3% of the prey sequences for *G. morhua* and *P. fluviatilis* in both areas and years ($S\%$; Table 2).

Overall, 36 different prey groups were detected in VSCA, of which 24 were identified to species level, 7 to genus and 5 to a higher taxonomic level (Supporting Information Appendix S6). In total, 27 prey groups were identified in AL, whereas 29 were identified for predators in KK. A total of 55 different prey groups were detected through DNA metabarcoding; fifty were identified to species level, whereas only five were identified to genus or higher taxonomic level (Supporting Information Appendix S7). In total, 44 prey species were identified in AL, whereas 37 were identified for predators in KK.

3.2 | Diet contribution of *N. melanostomus* in relation to *N. melanostomus* abundances

Median *N. melanostomus* CPUE was higher in 2018 compared to 2019 in both areas ($W = 23$, $P = 0.03$; Figure 3; Supporting Information Appendix S1).

N. melanostomus abundance in *G. morhua* and *E. lucius* diets was best explained by the respective intercept models, as no explanatory factor (*N. melanostomus* median CPUE, predator body length or the two factors combined) improved the model fit (LMM; Table 3; Figure 4a). For *P. fluviatilis*, however, *N. melanostomus* abundance in the diet was best explained by catch area, with a higher abundance in the diet in KK (Table 3).

TABLE 3 Summary of model selection results

	Model variables	Abundance	Proportion	Relative prey weight
<i>G. morhua</i>	Intercept AIC	100.02	81.14	143.2
	<i>N. melanostomus</i> median CPUE	+2.7	−3.39(+)	+5
	Predator body length	+9.6	+1.96	+7.5
	Predator body length + <i>N. melanostomus</i> median CPUE	+12.4	−1.8	+12.2
<i>E. lucius</i>	Intercept AIC	93.2	47.76	57.66
	<i>N. melanostomus</i> median CPUE	+2.97	−1.77 ^a (+)	+6.46
	Predator body length	+8.7	+1.68	+8.85
	Predator body length + <i>N. melanostomus</i> median CPUE	+11.77	−1.83 ^a (−, +)	+15.26
<i>P. fluviatilis</i>	Intercept AIC	251.18	179.71	491.82
	Catch area	−2.43(KK+)	−0.75	+3
	<i>N. melanostomus</i> median CPUE	+4.41	+1.04	+4.47
	Predator body length	+9.36	−2.8(+)	+4.3
	Predator body length + <i>N. melanostomus</i> median CPUE	+13.8	−2.18	+8.7
	Catch area + <i>N. melanostomus</i> median CPUE	+2	+0.17	+7.36
	Catch area * <i>N. melanostomus</i> median CPUE	+6.5	+1.4	+9.9

Note: Δ AIC, i.e., model AIC compared to intercept model AIC, are shown for the different linear model structures for *Gadus morhua*, *Esox lucius* and *Perca fluviatilis*. Values in bold font indicate the best model, i.e., lowest AIC. (+/−) indicates the direction of change in the parameter estimates.

^aVery small differences between models make the interpretation of the best model difficult.

In the analysis of *N. melanostomus* proportion in predator diets, *N. melanostomus* median CPUE best explained *N. melanostomus* proportion in *G. morhua* diet (GLMM; Table 3; Figure 4b). A combination of *N. melanostomus* median CPUE and *G. morhua* body length also explained *N. melanostomus* proportion in *G. morhua* diet better than the intercept model, but less so compared to *N. melanostomus* median CPUE alone. *G. morhua* body length alone did not contribute to the model fit. For *E. lucius*, interpretation was difficult due to small differences between the best models, as *N. melanostomus* proportion in *E. lucius* diet was best explained by a combination of *E. lucius* body length and *N. melanostomus* median CPUE (Table 3), closely followed by *N. melanostomus* median CPUE alone (Table 3). Body length of *E. lucius* alone did not contribute to the model fit. For *P. fluviatilis*, *N. melanostomus* proportion in diet was best explained by *P. fluviatilis* body length (Table 3). Catch area alone explained more compared to the intercept model but less than *P. fluviatilis* body length, whereas neither *N. melanostomus* median CPUE alone nor an interaction between catch area and *N. melanostomus* median CPUE improved the model fit (Table 3).

In the analysis of relative prey weight, none of the explanatory factors contributed to the model fit for either *G. morhua*, *E. lucius* or *P. fluviatilis*, as the intercept models including only the random factor had the lowest AIC for all species (LMM; Table 3; Figure 4c).

3.3 | Predator feeding strategy

All predators showed signs of specialized feeding (PSP > 50%), although most prey species or groups were rare in the diet (FO < 25%). This indicates individual specialization within a generalist

population, while only a few prey were dominating on a population level (FO > 75%; Figure 5). High PSP and FO combined (both measures > 50%), indicating predator population-level prey specialization, was seen for, e.g., *S. entomon* for *G. morhua* in AL and *N. melanostomus* for *G. morhua* and *E. lucius* in KK. Nonetheless, the degree of individual vs. population specialization, as well as the identity of the dominant prey, varied between predators and years.

In 2018, when *N. melanostomus* environmental abundances were high, all *G. morhua* (FO = 100%) and nearly all *E. lucius* (FO = 86%) in KK consumed almost exclusively *N. melanostomus* (PSP = 88% and 90% for *G. morhua* and *E. lucius*; Figure 5), indicating population-level specialization. All other prey occurred occasionally and were thus important only at an individual level (FO < 25% and PSP > 50%; Figure 5). *P. fluviatilis* showed larger inter-individual variation compared to *G. morhua* and *E. lucius*, as no single species dominated in occurrence (FO < 75%; Figure 5), although for *P. fluviatilis* in KK, Gasterosteidae was the only prey group with PSP and FO > 50%. In AL, *N. melanostomus* and clupeids were the most important prey for *P. fluviatilis* in 2018 (PSP > 75%; FO = 25%–50%), whereas all other prey occurred occasionally (FO < 25%; Figure 5).

In 2019, the lower contribution of *N. melanostomus* in both FO and PSP for *G. morhua* and *E. lucius* in KK and *P. fluviatilis* in AL (Figure 5) coincided with an increased contribution of crustacean prey and other fish prey, such as sticklebacks (Gasterosteidae), other gobiids (in AL), *P. fluviatilis* and clupeids (Clupeidae) (in KK) (Figure 5). Accordingly, predators in KK, especially *G. morhua* and *E. lucius*, and *P. fluviatilis* in AL had a more variable diet in 2019 than in 2018, as more prey groups had intermediate PSP and FO values (25%–75%; Figure 5). These changes between years were also reflected in the mean number of prey species per stomach. *E. lucius* and *G. morhua* in

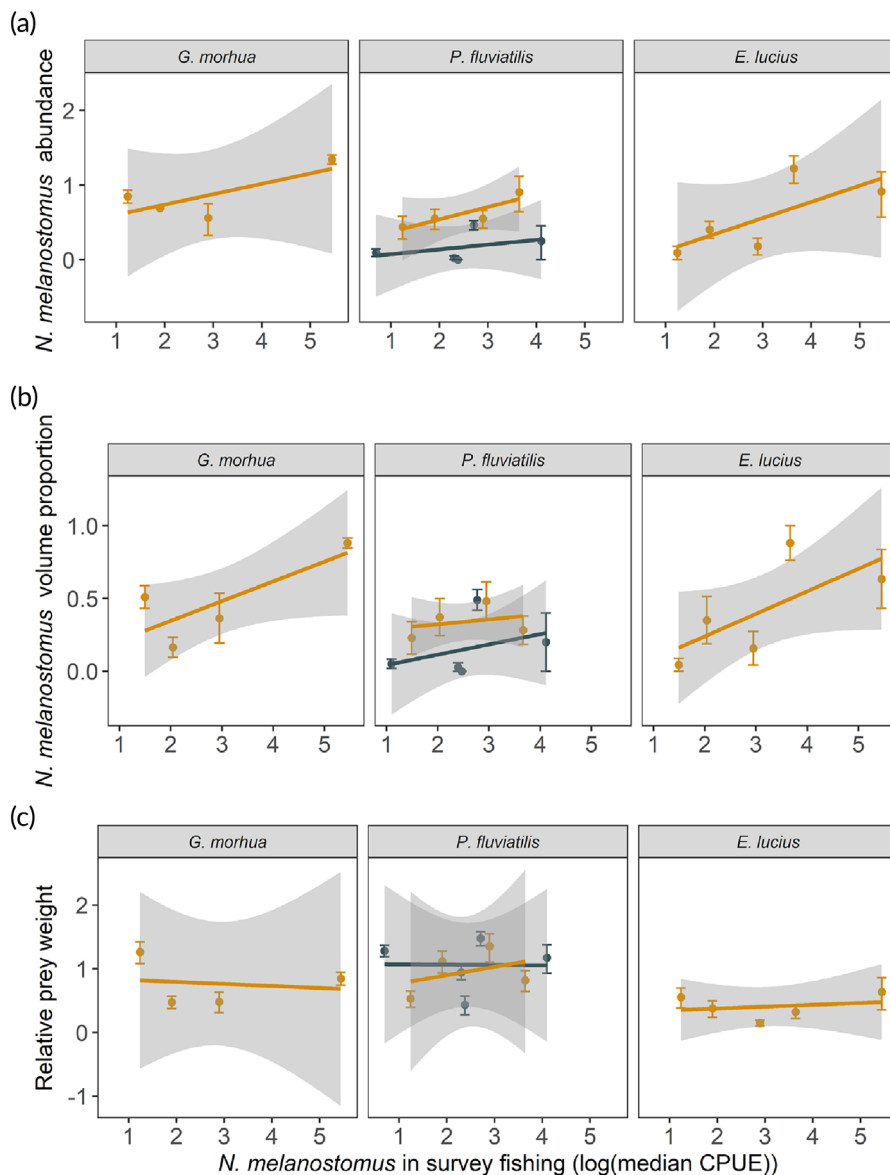


FIGURE 4 Linear models of (a) *Neogobius melanostomus* abundance ($\log_e(N_i + 1)$) and (b) volume proportion in the diets of *Gadus morhua*, *Perca fluviatilis* and *Esox lucius*, as well as (c) the relative prey weight ($\log(\text{RPW} + 1)$) as a function of *N. melanostomus* environmental abundances ($\log(\text{median CPUE})$). Each point represents the mean and standard error (S.E.) of the dependent variables, but all observed values (not means) were used in the linear models. Regression lines show best linear fit but do not represent significant associations. See Supporting Information Appendix S1 for the number of predator diet samples in each group. Catch area: AL; KK

KK and *P. fluviatilis* in AL showed a 37%, 53% and 85% increase in mean prey richness from 2018 to 2019 (Table 2).

The diet of *G. morhua* in AL differed from all other predators, as *S. entomon* was the dominating prey followed by mysid shrimp, whereas all other prey were rare in both years ($\text{FO} < 25\%$).

4 | DISCUSSION

The authors found that *N. melanostomus* is an important prey for native predators, and as expected, there were differences between predator species and areas. The predators feeding most on *N. melanostomus*, *G. morhua* and *E. lucius* in KK showed the largest inter-annual diet differences. They fed nearly exclusively on *N. melanostomus* in 2018, and the mean number of prey species in the diet was 53% and 37% lower compared to 2019 for *G. morhua* and *E. lucius*, respectively. The environmental abundances of

N. melanostomus best explained the proportion of *N. melanostomus* in the diets of the two largest predators, *G. morhua* and *E. lucius*, whereas for *P. fluviatilis*, predator body length best explained the proportion of *N. melanostomus* in the diet. Predators also showed between-year variation in their feeding strategy, as *G. morhua* and *E. lucius* in KK specialized on *N. melanostomus* in 2018 when it was abundant. The relative prey weight was not clearly explained by any of the analysed factors. This indicates that high abundances of *N. melanostomus*, in the environment and as proportion in stomachs, has no obvious effect on the total amount of prey consumed by any of the predator species. Together with the lower prey richness for predators feeding most on *N. melanostomus*, this suggests that *N. melanostomus* is replacing rather than supplementing native prey when it is abundant in the environment, at least for *G. morhua* and *E. lucius*. Nonetheless, on a longer timescale, *N. melanostomus* may act as a supplemental dietary resource, as *N. melanostomus* environmental abundances can be expected to fluctuate between seasons (Behrens

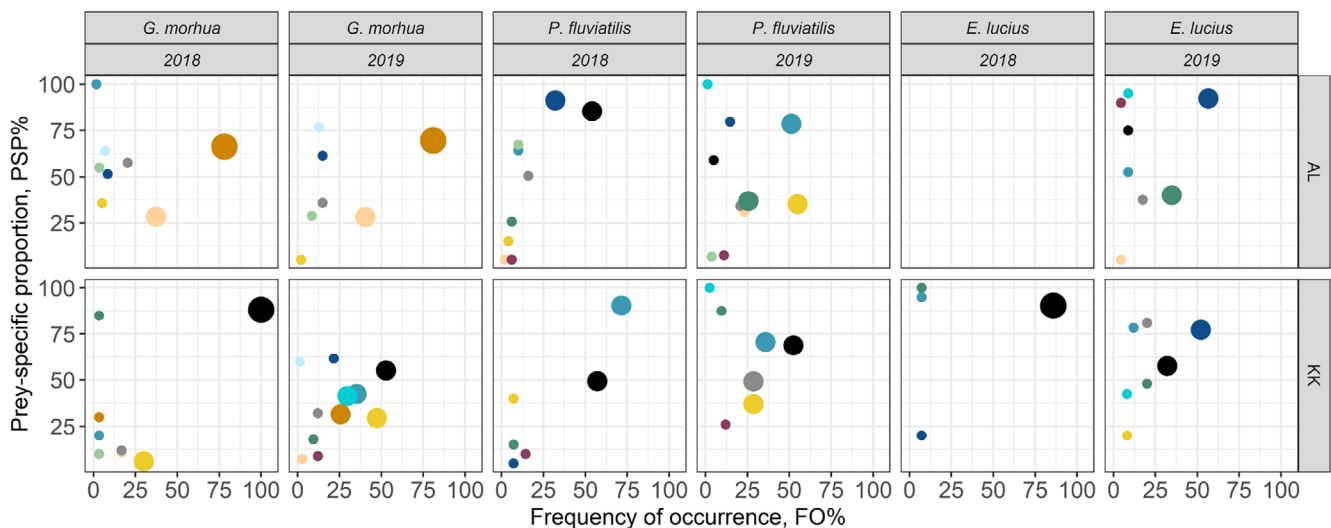


FIGURE 5 Costello-Amundsen graph depicting predator feeding strategies for *Gadus morhua*, *Perca fluviatilis* and *Esox lucius* from Åland (AL) and Karlskrona (KK) in 2018 and 2019 according to VSCA data. Prey-specific proportion (PSP) is shown on the vertical axis, where prey groups positioned in the upper half (PSP > 50%) indicate specialized feeding and generalist feeding in the lower half (PSP < 50%). Frequency of occurrence (FO), i.e., the proportion of non-empty stomachs a prey group was found in, is shown on the x-axis. Prey importance, from rare to dominant prey types, is shown on the diagonal axis (lower left to the upper right corner), whereas individual- to population-level variation is shown from the upper left to the lower right corner (Amundsen *et al.*, 1996). Prey groups are indicated by different colours. To highlight the most important prey groups on the population level, the authors scaled the size of the points so that they are relative to the FO each year; that is, the larger the point, the higher the proportion of samples the prey was found in. The group “Unidentified” is excluded. Empty panel (*E. lucius* from AL 2018) is due to no samples from corresponding year. ● *N. melanostomus*. ● Other Gobiidae. ● Clupeidae. ● Gasterosteidae. ● Cottidae. ● *Z. viviparus*. ● *P. fluviatilis*. ● Other fishes. ● Mysidae. ● *S. entomon*. ● Other Crustacea. ● Other invertebrates. ● FO < 25%. ● FO 25–75%. ● FO > 75%

et al., 2022; Brauer *et al.*, 2020) and years (Brooking *et al.*, 2022; this study). The dominance of *N. melanostomus* as prey for *G. morhua* and *E. lucius* in KK seen in this study could indicate potential for predator control of *N. melanostomus* when abundant, but the influence of predator regulation on *N. melanostomus* population dynamics in the Baltic Sea remains a topic for future studies. DNA metabarcoding provided information supplementary to VSCA, as it detected *N. melanostomus* in the diet of *G. morhua* in AL. Compared to VSCA, DNA metabarcoding also increased the taxonomic resolution and observed diet diversity of predators (see Supporting Information Appendices S6 and S7). This is the first study, to the authors’ knowledge, to show predator specialization on *N. melanostomus* at high *N. melanostomus* abundances. This is also the first to identify *N. melanostomus* as an important prey for *E. lucius* in Europe, as well as for *P. fluviatilis* in the northernmost part of the Baltic Proper.

4.1 | *N. melanostomus* importance as prey for native fish predators

In previous studies from the south-eastern Baltic Sea, *N. melanostomus* has been shown to constitute a smaller share of the diet of *G. morhua* (max. 19.7%–53.6%) compared to the diet of *P. fluviatilis* (max. 51.2%–96.3% by weight, March–June; Almqvist *et al.*, 2010; Rakauskas *et al.*, 2020). In the Gulf of Finland and the Gulf of Riga, similarly, 78%–83% of the biomass in *P. fluviatilis* diet was comprised by *N. melanostomus* (April–October; Liversage *et al.*, 2017). In contrast, this

study shows higher contribution of *N. melanostomus* to *G. morhua* diet in KK, especially in 2018 ($V = 88\%$), and lower contribution to *P. fluviatilis* diets in both areas ($V = 3\%$ –46%). At lower environmental abundances, *N. melanostomus* made up a similar proportion of *G. morhua* and *P. fluviatilis* diets in KK ($V = 29$ vs. 36%; Table 2). *E. lucius* predation on *N. melanostomus* has not been previously studied in the Baltic Sea, but this study shows that *N. melanostomus* is preyed upon and constitutes a substantial proportion of the diet, especially in KK, $V = 78\%$ (2018) and 18% (2019), vs. 7% in AL (2019). In St. Lawrence River, Great Lakes, *N. melanostomus* constituted between 10.9% and 100% of the prey biomass for *E. lucius* (Reyjol *et al.*, 2010). This study’s results from KK show higher importance of *N. melanostomus* as prey, at least in terms of frequency of occurrence (32%–86% compared to 26% in St. Lawrence River; Reyjol *et al.*, 2010). In this study, *G. morhua* and *E. lucius* in KK were the main fish predators of *N. melanostomus*, whereas in AL, no between-species comparisons could be made. Nonetheless, *N. melanostomus* was the predominant prey for *P. fluviatilis* in AL in 2018, while contributing in similar, low amounts (<10% FO and V), to the diet of *E. lucius* and *P. fluviatilis* in 2019.

4.2 | Effects of *N. melanostomus* environmental abundances on predator feeding

The effect of *N. melanostomus* environmental abundances on predator diets was best seen on the proportion of *N. melanostomus* in the diet

of the two larger predators, *G. morhua* and *E. lucius*. In the analysis of abundance of *N. melanostomus* in predator diets, no variables contributed to a better model fit relative to the intercept model for *G. morhua* and *E. lucius*. Still, the abundance of *N. melanostomus* in *G. morhua* and *E. lucius* diets was clearly highest when *N. melanostomus* abundances in the environment were highest (May and June 2018; Figure 4a; Supporting Information Appendix S1), which indicates a higher consumption of *N. melanostomus* at higher *N. melanostomus* environmental abundances. Nonetheless, the limited sample size and large variation between and within sampling occasions makes it difficult to draw definitive conclusions about the influence of environmental abundance of *N. melanostomus* on *N. melanostomus* abundance in predator diets. The fact that the main trend between *N. melanostomus* abundance and diet contribution can be seen between years, but not between months, indicates that there are likely other factors determining predator feeding responses at smaller spatial and/or temporal scales. These may include environmental factors such as macrophyte density (Liversage *et al.*, 2017) or availability of other prey items. Nevertheless, the overall decreased importance of *N. melanostomus* in predator diets seen in VSCA (FO and V) in both study areas between 2018 and 2019 also mirrored the change in *N. melanostomus* abundances in the environment, with an 80%-90% decrease in mean CPUE between years in both areas (Supporting Information Appendix S1). The authors do not know the cause for the decline in *N. melanostomus* abundances between years, but it may be related to the marine heat wave in the Baltic Sea in 2018 (Paalme *et al.*, 2020) in combination with a disease outbreak (own observations and local reports in Sweden and Finland), negatively affecting abundances in 2019.

N. melanostomus occurrence in predator diets has been shown to increase with predator size and decrease with higher interspecific competition (Reyjol *et al.*, 2010). Thus, the higher importance of *N. melanostomus* for *G. morhua* and *E. lucius* in KK compared to *P. fluviatilis* could be related to the larger sizes of these species (Table 1), indicating that *N. melanostomus* of all sizes constitute potential prey, whereas *P. fluviatilis* is restricted to feeding on smaller *N. melanostomus*, especially when the two larger and more piscivorous predators are present in the same areas (see Supporting Information Appendix S8). Moreover, for *P. fluviatilis*, predator length explained the proportion of *N. melanostomus* in the diet better than *N. melanostomus* CPUE, with larger *P. fluviatilis* feeding more on *N. melanostomus*. For *P. fluviatilis*, piscivory often increases with body size (Jacobson *et al.*, 2019), which is partly related to gape-size limitations, and this pattern is therefore not unexpected. Liversage *et al.* (2017) also found that *P. fluviatilis* predation on *N. melanostomus* was not affected by *N. melanostomus* densities to any large extent, and this study's results thus partly corroborate this. Nonetheless, the authors saw area-specific differences in both diet composition and response to *N. melanostomus* abundances for *P. fluviatilis*, as it consumed a larger number of *N. melanostomus* in KK than AL. The size of consumed *N. melanostomus* individuals was smaller in KK compared to AL, especially in 2018 (Supporting Information Appendix S8), which

indicates that *P. fluviatilis* in AL consumed fewer but larger individuals. This conclusion is supported by the fact that *N. melanostomus* occurred slightly more often in the stomachs of *P. fluviatilis* in KK (FO = 57%), but contributed less in volume proportions (V = 28%), compared to AL in 2018 (FO = 54%, V = 46%).

The authors further show that fish predators can change their feeding strategy in response to changes in non-native prey abundances. This response was also predator- and area-specific. *G. morhua* and *E. lucius* in KK showed a higher tendency to specialize on *N. melanostomus* at high abundances of *N. melanostomus* compared to *P. fluviatilis*, and *P. fluviatilis* response to the variation in *N. melanostomus* abundances differed between areas. In AL, there was a clear inter-annual difference in mean prey richness and level of predation and specialization on *N. melanostomus* for *P. fluviatilis*. In KK, in contrast, *P. fluviatilis* showed less inter-annual variation in prey richness, diet composition and feeding on *N. melanostomus*. A change in predator feeding strategy towards population-level specialization on *N. melanostomus* may be due to *N. melanostomus* being an abundant and easily caught prey, or a demonstration of prey preference. For *G. morhua* and *E. lucius* in KK, the authors show that *N. melanostomus* is preyed more on at high *N. melanostomus* abundances which supports the first explanation, although it does not exclude the second explanation. Generalist predators often feed opportunistically, which can lead to temporally limited predation on a narrow range of prey at high prey abundances, as in this case for *G. morhua* and *E. lucius* in KK and to some extent for *P. fluviatilis* in AL. Species-level responses may also be explained by behavioural attributes, such as hunting tactics or ability to capture and consume prey. *G. morhua* is an opportunistic predator (Kullander *et al.*, 2012), actively searching for prey and occasionally undertaking feeding migrations (Björnsson *et al.*, 2018). It is also a facultative schooler, meaning that it can forage alone or form large shoals (Björnsson *et al.*, 2018). *E. lucius*, on the contrary, is a solitary ambush predator which rarely migrates >5 km (Bergström *et al.*, 2022). Nonetheless, despite their inherent differences, these two predators displayed similar feeding patterns in KK, indicating that both predators fed opportunistically on a temporarily abundant prey resource. *P. fluviatilis* is known to show high levels of trophic flexibility depending on, e.g., habitat type and body size (Mustamäki *et al.*, 2014), availability of prey items or variation in inter- and intraspecific competition (Bolnick *et al.*, 2003; Svanbäck and Persson, 2004). In contrast, *G. morhua* and especially *E. lucius* are more piscivorous (Jacobson *et al.*, 2019; Haase *et al.*, 2020) and due to their larger body size may also be superior competitors for larger fish prey, as described earlier (Supporting Information Appendix S8). Thus, differing levels of interspecific competition likely also play a role, especially in KK where all three predators are present in the same area, and *P. fluviatilis* may adjust its feeding to avoid competition with the two larger predators. In AL, in contrast, where *P. fluviatilis* was the most abundant fish predator, it may feed more opportunistically primarily based on prey availability.

From the statistical models and figures, there are no clear indications that relative prey weight in predator stomachs (*i.e.*, amount of ingested food) is explained by *N. melanostomus* environmental

abundances (Figure 4c). Nonetheless, temperature, which the authors did not control for, may influence relative prey weight as digestion rates are temperature dependent (Volkoff & Rønnestad, 2020). Sampling during spring and early summer coincides with rising temperatures, which in turn may lead to difficulties in detection of patterns in relative prey weight. Nevertheless, the lack of a clear relationship between *N. melanostomus* environmental abundances and relative prey weight, together with the reduced prey richness and overall diet diversity for predators feeding most on *N. melanostomus* when abundances were high, indicates that *N. melanostomus* is replacing other prey at high environmental abundances. Both *G. morhua* and *E. lucius* consumed *N. melanostomus* nearly exclusively in KK in 2018, which also indicates high dietary overlap between predators when *N. melanostomus* is abundant. At lower *N. melanostomus* abundances, *G. morhua* and *P. fluviatilis* had a more diverse diet with a larger share of crustacean prey, whereas *E. lucius* fed more on clupeids. The authors further saw an increase in predation on Gasterosteidae and other gobiid species when *N. melanostomus* decreased in predator diets. The change in feeding patterns mirrors the decrease of crustacean prey in *G. morhua* diet following *N. melanostomus* invasion in the south-eastern Baltic Sea (Rakauskas et al., 2020). A meta-analysis showed a reduced availability of crustacean prey after *N. melanostomus* invasion (Liversage et al., 2019), likely due to *N. melanostomus* predation (Ustups et al., 2016). A decrease in the abundance of suitable crustacean prey due to predation by *N. melanostomus* could contribute to higher predator reliance on fish prey at high *N. melanostomus* abundances also in this study, although it may also simply be the result of high *N. melanostomus* abundances in itself. The lower feeding on, e.g., crustaceans, other gobiid species and Gasterosteidae may also lead to predation release on these prey groups in times of high *N. melanostomus* abundances, as suggested in other studies (Hempel et al., 2016; Liversage et al., 2017), but needs to be investigated further.

In general, feeding predominantly on *N. melanostomus* resulted in predators relying less on benthopelagic (e.g., Gasterosteidae), pelagic (e.g., Atlantic herring *Clupea harengus* L. 1758) and littoral (many crustaceans) prey. For especially *G. morhua* and *P. fluviatilis*, feeding primarily on *N. melanostomus* also meant a higher degree of piscivory, and all three predators can thus be assumed to occupy a similar trophic level when *N. melanostomus* dominates their diets, as seen in Almqvist et al. (2010). This study's results thus show a similar change in diet as seen in previous studies from the Baltic Sea, where predators have shifted from feeding on pelagic and benthopelagic planktivorous fishes and crustaceans to *N. melanostomus* after its invasion (Almqvist et al., 2010; Rakauskas et al., 2020). In fact, predatory fishes have shown similar shifts in diet, from pelagic fishes and epibenthic crustaceans to benthic *N. melanostomus* after *N. melanostomus* establishment compared to pre-invasion times, regardless of ecosystem or predatory fish species (Crane & Einhouse, 2016; Hempel et al., 2016; Rakauskas et al., 2020). This suggests that the differences in predator diets seen in this study at high *N. melanostomus* environmental

abundances may reflect long-term changes. Nonetheless, as *N. melanostomus* abundance and distribution can vary significantly between and within years (Behrens et al., 2022; Brauer et al., 2020; this study), the authors consider a complete replacement of native prey by *N. melanostomus* unlikely for the predators in this study. Rather, *N. melanostomus* constitutes a supplemental prey resource, which can dominate the diet of predators when *N. melanostomus* abundances are high.

4.3 | Method considerations

The authors used two methods, VSCA and DNA metabarcoding, to maximize the potential for detection of all prey species and groups, including *N. melanostomus*. Nonetheless, only an *a priori* chosen subset of the VSCA samples was used for DNA metabarcoding ($n_{\text{VSCA}}=460$ vs. $n_{\text{DNA}}=122$; Table 1), and the smaller sample size may have contributed to the lower detection rates of *N. melanostomus* in DNA metabarcoding compared to VSCA due to chance (Table 2; Figure 2). The higher contribution of *N. melanostomus* to predator diets in VSCA compared to DNA metabarcoding is also partly related to the larger number of detected species in each DNA sample compared to VSCA (Supporting Information Appendices S6 and S7), meaning that the relative contribution of each species is smaller in each DNA sample.

DNA metabarcoding results are also affected by marker choice and the affinity of marker primers to different prey organisms (Taberlet et al., 2012; Deagle et al., 2019). In this study, the COI marker did not work well for amplifying invertebrate prey sequences, as the numbers of invertebrate sequences obtained using COI were much lower than could be expected from VSCA (Supporting Information Appendices S3 and S4). Because the number of sequence reads can reflect the total and relative biomass of species or ingested prey in a sample (Elbrecht & Leese, 2015; Verkuil et al., 2022), the low yield of macroinvertebrate sequences using the COI marker could be due to lower biomass of invertebrate prey than estimated in VSCA due to shell and exoskeleton retention, which would indicate that invertebrate importance is overestimated in VSCA. Another potential explanation is that the COI blocking primers that were used to inhibit amplification of host (i.e., predator) DNA did not work well in the final assays, despite working well in preliminary tests. As a result, predator DNA was amplified in substantial amounts (Supporting Information Appendix S4; Panova et al., 2021), likely inhibiting the amplification of invertebrate prey DNA. In addition, the COI marker is less taxonomically specific than 12S, and it therefore also amplified other non-target DNA, apart from host DNA, to a large extent (fishes, parasites and micro-organisms; Supporting Information Appendix S4, Panova et al., 2021). DNA amplification is also dependent on prey morphology, as, e.g., prey with hard exoskeletal structures may yield lower amounts of DNA than soft-bodied organisms (Martins et al., 2020). Combined, these factors indicate that DNA metabarcoding using COI for detection of invertebrates was, in this study, not suitable for

quantification of sequences, which may explain the lower relative sequence number of *S. entomon* in DNA metabarcoding compared to in VSCA (Table 2; Supporting Information Appendices S6 and S7). Another marker, e.g., 16S or 18S, might have been a better choice for detection of macroinvertebrates, despite the more comprehensive COI reference database (Deagle *et al.*, 2014).

Based on a comparison of matched samples (Supporting Information Appendix S3), the contribution of some fish taxa, e.g., Gasterosteidae, clupeids, sculpins and eelpout *Zoarces viviparus* L. 1758, may have been underestimated in VSCA. This discrepancy could be explained by the often highly digested state of parts of the stomach content, i.e., unidentified prey in VSCA which were assigned to species in DNA metabarcoding. Moreover, as DNA sampling was conducted prior to VSCA, the samples were not homogenized and, as such, the markers may have primarily amplified DNA from highly digested prey (more DNA due to cell lysis; Martins *et al.*, 2020). Therefore, in this study, DNA metabarcoding provides a supplementary picture of diet composition as it detects prey that were unidentified in VSCA due to heavy digestion. Both VSCA and DNA metabarcoding provide snapshots of diet composition. The authors therefore suggest stable isotope analysis to complement the short-term, taxonomically more detailed diet perspective and investigate differences between the predators with regard to, e.g., trophic position and benthic-pelagic resource use.

The number of prey taxa detected in DNA metabarcoding was 53% higher than in VSCA (55 vs. 36), despite the lower number of samples. The authors identified species through DNA metabarcoding that were not detected in VSCA, especially fishes with the 12S marker (compare Supporting Information Appendices S6 and S7). *G. morhua* in AL provides a good example, as the DNA results indicate a small contribution of *N. melanostomus* to the diet, along with other coastal fish species (sand goby *Pomatoschistus minutus* Pallas 1770, *P. fluviatilis*, *E. lucius*; Supporting Information Appendix S7). Although contamination or secondary consumption cannot be entirely excluded, *G. morhua* in AL is occasionally caught in shallow coastal areas. The results indicate occasional feeding raids of *G. morhua* to coastal areas. Thus, DNA metabarcoding provided additional information on prey diversity and feeding interactions, but as discussed earlier, DNA-based methods are restricted by factors associated with sampling design, contamination, marker choice and primer affinity. Therefore, in this study, DNA metabarcoding should be regarded as a supplement rather than a substitute for VSCA.

Fyke net catches of *N. melanostomus* were used as a measure of *N. melanostomus* relative abundances in the sampled areas and periods, as no total population density estimates were available. Fyke nets are size-selective gear with the mesh-size used (minimum of 8 mm) restricting the catch to *N. melanostomus* larger than 5 cm (smallest individuals were 5–10 cm and largest 20–25 cm in both areas). All predators consumed smaller *N. melanostomus* than those caught in the fyke nets to some extent (size range measured from stomach contents: 2.4–14.3 cm for *P. fluviatilis*, 3.7–14.5 cm for *E. lucius* and 4.0–17.6 cm for *G. morhua*; Supporting Information Appendix S8). This indicates that the fyke net catches do not perfectly match the size range of *N. melanostomus* eaten by the predators. Nevertheless,

as the predators used in the linear models were caught in the same shallow water areas (mostly <10 m) during the same period as the fishing with fyke nets was conducted, it can be considered that the fyke net catches give a good indication of the general trends of *N. melanostomus* abundances available to the predators in the study areas and periods, even though the smallest size-classes were underrepresented.

4.4 | Conclusions

The non-native *N. melanostomus* has become an important prey for three ecologically important coastal fish species in two, previously unstudied, Baltic Sea areas 7–11 years after the first observations. The authors found differences in the contribution of *N. melanostomus* between species, areas and years. Differences in diet between areas and years can partly be explained by *N. melanostomus* abundance in the predators' feeding environment, both between years (*G. morhua* and *E. lucius* in KK) and areas (*P. fluviatilis*). *G. morhua* and *E. lucius* in KK display similar feeding strategies, specializing on *N. melanostomus* at high *N. melanostomus* abundances. The authors thus conclude that *N. melanostomus*, when it occurs at high abundances, constitutes an important supplementary prey source for native coastal predatory fishes in the Baltic Sea.

AUTHOR CONTRIBUTIONS

H.H., I.W.K., K.A., A.B.F., K.L. and J.M. were responsible for research design. H.H., I.W.K. and A.L. with assistance from K.L. carried out sampling and sample processing. H.H., I.W.K. and Ö.Ö. conducted the statistical analyses, and H.H. and I.W.K. drafted the main text and figures. All authors were involved in discussions and editing of the text.

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available at: <https://zenodo.org/record/7687180#.ZDOOJXZBwuU>.

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