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Research Article

Detection of multiple fish species in the diet of the invasive round goby reveals new trophic interactions in the Baltic Sea

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Abstract

The mesopredatory round goby (*Neogobius melanostomus*) is an important fish invader in fresh and brackish waters of the northern hemisphere. Trophic interactions of invasive species can generate ecological impacts across the food web in invaded ecosystems. Here we investigated major diet components, spatiotemporal variation in diet and the effect of round goby densities on diet composition in two geographically distinct round goby populations in the Baltic Sea. The round goby is a generalist feeder but previous diet studies, based on visual prey identification, have likely over-emphasized the importance of hard-shelled, invertebrate prey in round goby diet, as shells degrade and evacuate slowly relative to soft-bodied prey that break down rapidly in the stomach. We therefore, in addition to visual stomach content analysis, used DNA metabarcoding, which is less biased towards hard body structures of prey and can be used for species assignment of highly degraded prey. The results demonstrated that round goby diet composition varied between areas and years. Visual stomach content analysis indicated that blue mussel was the main prey in the southern area, whereas hydrobiid gastropods were the major diet component in the northern area. Metabarcoding revealed that several fish species, likely the egg or larval stages of e.g. stickleback, cod and herring, were also part of the round goby diet. Analyses suggested that round goby feeding on fishes was positively associated with round goby densities. Our study shows that round goby, in addition to benthic invertebrates, preys on several fish species of ecological and commercial importance. Thus, there is potential for predator-prey reversal and negative effects of the invasive round goby on large, predatory fishes.

Key words: *Neogobius melanostomus*, invasive species, diet analysis, DNA metabarcoding, spatiotemporal comparison, predator-prey interactions, density-dependent feeding

Introduction

Predator-prey interactions shape populations, communities and ecosystems (Carlsson et al. 2009), and diet is an important factor in determining the impact of invasive species on food webs of recipient ecosystems (Schmitt et al. 2018). Predation effects are heavily influenced by predator origin. Invasive predators may have greater impact on native prey than do native predators due to lack of co-evolutionary developed strategies, generally benefitting the invasive predator (Paolucci et al. 2013). Impacts of invasive predators in marine ecosystems have the potential to cascade through the food web, but impact varies considerably depending on local conditions, the life-history of the organisms involved and their degree of dispersal (Rilov 2009). Invasive predators can restructure food webs of recipient ecosystems through disruption of ecosystem function and productivity (Schmitt et al. 2018), e.g. by competitive exclusion of native predators (Crowder and Snyder 2010) or invasional meltdown, where invasive species facilitate each other's establishment, reproduction or spread (Rilov 2009).

Successful aquatic invaders share a number of life-history and functional traits. Rapid growth rate, early maturation, high fecundity and tolerance to variation in environmental conditions are traits associated with invasion success (reviewed by Papacostas et al. 2017). The round goby (*Neogobius melanostomus* Pallas, 1814) displays all of these traits and has become one of the most wide-spread invasive fishes in the northern hemisphere (Kornis et al. 2012). Originally from the Black, Caspian and Azov Seas (Kornis et al. 2012), it was first observed in the south-eastern Baltic Sea (Gulf of Gdansk) in 1990 (Skóra and Stolarski 1993). The subsequent spread of the round goby has been slow in comparison to other invaded areas (Almqvist 2008), but it is now established in most coastal areas in the Baltic Sea (Adrian-Kalchhauser et al. 2020).

The round goby is a generalist feeder, consuming both invertebrates and fishes (Skóra and Rzeznik 2001; Kornis et al. 2012; Puntila 2016; Herlevi et al. 2018; Rakauskas et al. 2020). In the Baltic Sea, round goby <50 mm mainly feeds on zooplankton and small benthos (Skóra and Rzeznik 2001; Skabeikis and Lesutienė 2015; Ustups et al. 2016), and later switches to larger invertebrate prey like isopods, amphipods, polychaetes, bivalves and gastropods (Almqvist 2008; Skabeikis and Lesutienė 2015; Puntila 2016; Ustups et al. 2016; Hempel 2017). In experiments it has been shown that round goby also feeds on fishes in early life-stages (Schrandt et al. 2016; Wiegleb et al. 2019; Lutz et al. 2020), whereas field studies using visual diet analysis report the proportion of fishes in round goby diet to be low (<1–11%, Järv et al. 2011; Vašek et al. 2014; Skabeikis and Lesutienė 2015; Matern et al. 2021).

In invasive fish, attack rates, prey consumption and prey mortality, as well as predator movement and time spent foraging, have been shown to increase with predator densities (Benkwitt 2016; DeRoy et al. 2020). It is also hypothesized that intraspecific competition may drive invasive fish predators towards diet diversification at high predator densities (Schmitt et al. 2018). In the Baltic Sea, round goby can locally reach up to 20 ind/m^2 (HELCOM 2018). Round goby densities have been shown to influence round goby feeding in the Great Lakes area; Kornis et al. (2014) demonstrated that round goby consumed most prey at moderate round goby densities, while feeding decreased at high round goby densities, potentially due to intraspecific competition. Contrary, Paton et al. (2019) showed that round goby from high density sites displayed higher per capita prey attack rates than conspecifics from low density sites. Thus, round goby in the Baltic Sea could potentially influence the prey community through variation in feeding related to round goby density fluctuations.

Visual diet analysis provides information about food quantities and prey life-stages and sizes, but may be biased towards large prey items or prey items with hard structures, while underestimating prey diversity due to unidentifiable, highly digested stomach contents (Hyslop 1980; Buckland et al. 2017; Nielsen et al. 2018). When the diet constitutes of a mixture of hard-shelled and soft-bodied prey, the proportion of hard-shelled prey is often overestimated in visual diet analysis (Brush et al. 2012). Soft-bodied prey breaks down rapidly in the gut, especially at high temperatures, and hard-part prey like mussel shells and chitinous pieces remain, as they may have a low evacuation rate (Schrandt et al. 2016; Hempel 2017; Oesterwind et al. 2017; van Deurs et al. 2021). As the occurrence and proportion of fishes in the diet can be difficult to quantify in visual diet analysis (Brush et al. 2012; Lutz et al. 2020), molecular methods might be better for detection of soft-bodied prey like fish eggs and fry and have proven successful in the study of round goby predation on native endangered fishes (Lutz et al. 2020). Currently, most molecular approaches rely on high throughput DNA sequencing (HTS; Nielsen et al. 2018). HTS comprises species-specific approaches in which unique DNA sequences are detected, barcoding approaches that rely on amplification of specific genes, and shotgun approaches where all DNA fragments in a sample are sequenced and matched against existing databases (Lutz et al. 2020). DNA metabarcoding, i.e. the identification of all species in an environmental sample or tissue mixture, e.g. soil, water, stomach content or faeces (Taberlet et al. 2012), increases the taxonomic resolution in diet assessments compared to visual diet analysis and enables species assignment of e.g. highly degraded prey (Nielsen et al. 2018). However, there are technical limitations to be considered, for example choice of target genes, primer biases and sequencing artefacts, in addition to quantification issues (Nielsen et al. 2018; Deagle et al. 2019).

As soft-bodied prey may have been underestimated in previous round goby diet studies, we used both visual stomach content analysis (VSCA) and DNA metabarcoding (hereafter metabarcoding) to provide a more comprehensive picture of round goby feeding and diet composition. Our aim was to study spatiotemporal variation of round goby diet between two geographically distinct populations of similar time since establishment during two consecutive years to understand how diet, including soft-bodied prey, differed between areas and years. In addition, we related diet to round goby densities and prey environmental densities to infer causes for spatiotemporal variation in round goby diet.

Materials and methods

Study sites and sampling

We sampled round goby during 2018 and 2019 in two locations in the Åland islands (AL) in the northern Baltic Proper, and in three locations in the Karlskrona archipelago (KK) in the southern Baltic Proper (Fig. 1, Suppl. material 3: table S1). We fished round goby in May and June at 1–4 occasions per month (Suppl. material 3: table S2). Two fine mesh twin fyke nets, in total four houses, were used at all sampling locations (house mesh size 8–9 mm and 11–12 mm in leaders and netting bags). In addition to fyke nets, multi-mesh coastal survey nets were used in one of the locations in AL (10–60 mm mesh size, with round goby caught in mesh sizes of 10, 12, 15, 19, 24 and 30 mm). Fishing time for both gears was one night (8–12 h) per location and occasion and the fish were frozen after landing. Sampling for VSCA was size stratified with the aim of 10 individuals of each 50 mm length class (50–100, 100–150, 150–200 and >200 mm) per sampling occasion and location. As we were unable to catch enough round goby of each size class per sampling location, month and year, sample size differed between areas and years. In total, we used 345 round goby for analyses in VSCA (Table 1). DNA samples for metabarcoding were size stratified with the aim of 5 individuals per length class and sampling occasion. However, as sample size varied in VSCA, also the *a priori* chosen subset of fish from VSCA used for metabarcoding varied. We used 104 round goby for metabarcoding (Table 1). The size spans of fish used in VSCA

(Suppl. material 2: fig. S1) and metabarcoding were similar, 75–215 mm in VSCA *vs*. 76–192 mm in metabarcoding, although the largest specimens were lacking in metabarcoding due to very low sample size.

Diet analysis

Visual stomach content analysis

We used VSCA for all sampled round goby. Total length of thawed fish was measured to the nearest millimeter. The whole gastrointestinal tract (hereafter called stomach) was used, since round goby lacks a clearly defined stomach (Trzeciak et al. 2012), and gut fullness was determined visually. Fish with empty stomachs (n=13,

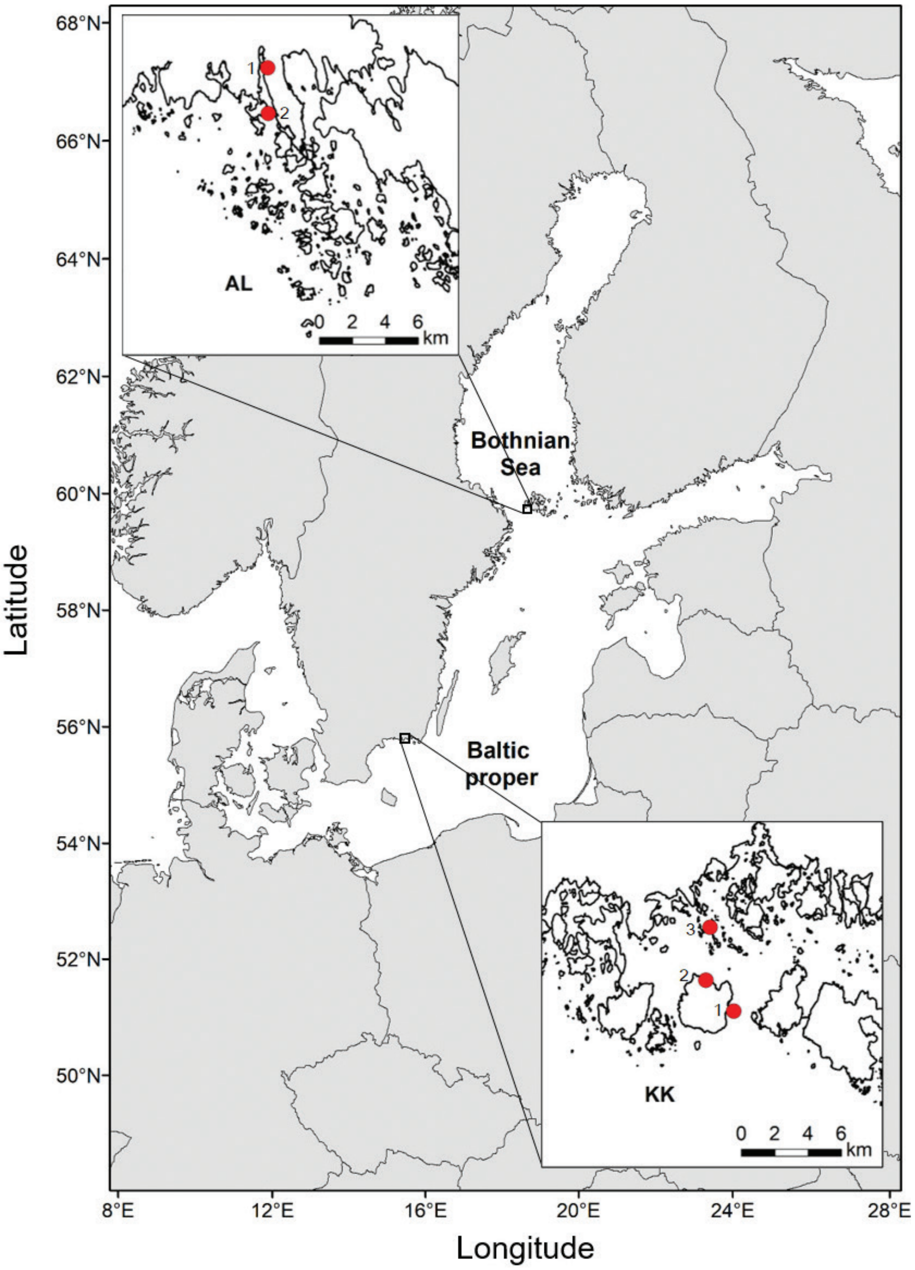

Figure 1. Round goby sampling locations in AL and KK. For coordinates, see Suppl. material 3: table S1.

3.7%) were excluded from further analysis. The stomach content was scraped out with a spatula. We determined prey items to the lowest possible taxonomic level using a stereo microscope (magnification × 6.3). We could not identify zooplankton with certainty, and as they are mainly prey for round goby <50 mm (Skóra and Rzeznik 2001; Skabeikis and Lesutienė 2015; Ustups et al. 2016), i.e. much smaller fish than used in this study (minimum length 75 mm), screening for zooplankton was disregarded in VSCA. We standardized the diet volume of each sample to 100%, visually estimated the volume proportion of each taxonomic group found in the diet to the nearest 5% (Hyslop 1980) and counted the number of prey items of each prey species/group. In cases where taxonomic assignment of prey to species or genus was impossible due to digestion, prey were aggregated at family level or higher.

Metabarcoding

DNA samples were taken prior to VSCA to minimize contamination. Round goby were thawed in room temperature or refrigerator to minimize potential contamination from DNA residue in water-holding containers. Dissection tools were washed and heat-sterilized with ethanol and flame between every sampling. Three null-samples, carried out as regular DNA samples but without an actual fish, yielded no or very little DNA (<0.02 ng/μl), and thus, we deemed the sterilizing procedures to be sufficient. Buffer solution (stool collection tube with DNA stabilizer from Invitek Molecular) was poured over the stomach content and carefully mixed by tilting the petri dish for approximately 5 seconds. Large pieces of organic material that could potentially skew the results were removed by sieving the buffer solution through a clean 65 μm mesh. Samples were stored at -20 °C until analysis.

DNA extraction, library preparation and bioinformatic analysis was carried out by SeAnalytics AB (Sweden, https://www.seanalytics.se/), and sequencing on the Illumina MiSeq platform was conducted by Eurofins Genomics (Germany, https://eurofinsgenomics.eu/; see Suppl. material 1: appendix 1 for method description). We used the 12S rRNA marker (average length 260 base pairs) for identification of vertebrates (Weigand et al. 2019; Miya et al. 2020) and the COI marker (313 base pairs) for identification of invertebrates as it has an extensive database for both marine and freshwater invertebrates (Weigand et al. 2019). 12S was amplified using the universal 12S primers from Miya et al. (2020) commonly used for fish metabarcoding, while COI was amplified with the mlCOIintF primer, which can be used for detection of both invertebrates and vertebrates (Leray et al. 2013). Separate PCR runs and sequencing were conducted for the respective markers. We used blocking primers to decrease amplification of host DNA and allow detection of prey species. All samples, except the null-samples, had sufficient DNA concentrations (>0.1 ng/μl) that were comparable between areas and years (Panova and Ring 2022). The 2018 round goby samples were analyzed together with samples from cod, pike, perch, pike-perch (*Gadus morhua, Esox lucius, Perca fluviatilis*, *Sander lucioperca*; all Linnaeus, 1758) and grey seal (*Halichoerus grypus* Fabricius, 1791). This resulted in some contamination of the round goby samples, most likely at the PCR step, and subsequent filtering of sequences to minimize the impact of contamination (Suppl. material 1: appendix 1). The 2019 samples were analyzed separately and thus this filtration step was unnecessary; otherwise, the protocol was identical in both years. Species contributing to <1% of the sequences per sample were filtered out from all samples (Suppl. material 1: appendix 1). In the final 12S data set, we filtered out *Diptera* sp. (Linnaeus, 1758), equivalent to 0.82–1.03% of the 12S sequences, as macroinvertebrates were not the target for the 12S analysis and *Diptera* sp. potentially represented a bioinformatics mistake.

On average, 85% and 89% of the 12S sequences survived the laboratory quality control in 2018 and 2019, respectively (M. Panova pers. comm.; Panova and Ring 2022). The COI primers, amplifying DNA from both invertebrates and fish, did not work well for all invertebrate groups. Common prey groups detected in VSCA, e.g. *Mytilus* sp. (Linnaeus, 1758), only made up a minor share of the COI sequences. The COI sequences were generally of low quality and few sequences survived the laboratory quality control: on average 26% of the COI sequences in 2018 and 13% in 2019 (M. Panova pers. comm.; Panova and Ring 2022). The low quality implied that analysis of COI sequences from specific prey species/groups were unsuitable, and therefore we only used COI sequences to assess the sequence distribution between fish and invertebrate prey (see Harper et al. 2020 for a similar approach). We used 12S data for analysis of fish species/groups.

Statistical analyses

Spatiotemporal diet variation

We analyzed differences in diet composition between area, year and sampling location, controlling for round goby length, with redundancy analysis (RDA) with the package *vegan* in R (Oksanen et al. 2022). As each study area consisted of several sampling locations, sampling location was nested under study area. We tested significance of explanatory variables using the function *anova.cca*. Pseudo F-values and p-values were calculated from Monte-Carlo simulations (1000 permutations). Prey in VSCA were grouped at genus level or higher, except for Baltic macoma and softshell clam (*Macoma balthica* and *Mya arenaria*; both Linnaeus, 1758), which could be identified to species level (Suppl. material 3: table S3). Fish species detected in 12S metabarcoding were assigned to the group ‘Other fishes’ if they were considered rare in our sampling, i.e. not detected in the diet in both areas and years, or if total sequence number was low (<~1000 sequences; Suppl. material 3: table S4). In analysis of VSCA data we used diet volume proportion of stomach content, while for metabarcoding data we used number of sequences as a proxy for prey proportions. The relationship between number of sequences and biomass is not linear, but serves as a relative indication of biomass proportions (Deagle et al. 2019).

To better meet the assumption of normal distribution of residuals and reduce impact of dominant prey groups, we log-transformed diet volume proportions instead of arcsine transformation in the VSCA redundancy analyses according to log(%prey+0.05), 0.05 being the smallest diet volume proportion larger than zero in the dataset. All sequence values were transformed according to log(y+1), where 1 was the smallest sequence number larger than zero in the dataset.

Diet in relation to round goby densities, prey densities and round goby length

To analyze how round goby diet related to densities of round goby, we used linear mixed effect models (LMM) and general linear mixed models (GLMM) in the package *lme4* in R (Bates et al. 2015). Round goby densities were estimated as the median catch of standardized (12 h) round goby catches in fyke nets per sampling location for each area, year and month. Two and three locations were sampled in AL and KK in May and June 2018 and 2019, except for May 2019 when data was available only for one location at AL. Hence, round goby densities was calculated at four occasions in KK and three occasions at AL, while absolute catch was used as a proxy for round goby densities in AL in May 2019. Due to the relatively few independent estimates of round goby density, resulting in low statistical power, we studied which

Table 1. Round goby sample size in the respective areas and years (fish with empty stomachs in VSCA excluded).

Area	Year	VSCA (n)	Metabarcoding 12S (n)	Metabarcoding COI (n)
AL	2018	83	24	24
KK	2018	80	30	34
AL	2019	64	25	25
KK	2019	105	25	25

variables contributed to the model fit using changes in Akaike's Information Criteria (AIC). Using stepwise selection, we selected the best model with lowest AIC and studied changes in AIC, denoted ΔAIC, when removing or adding variables to the best model. Sampling occasion in each area was used as a random factor, i.e. diet of round goby sampled at the same occasion (area, year and month) were considered to be dependent on each other, but independent between occasions. Round goby density, area and round goby length were used as explanatory factors, also including the interactions between area and median or absolute catches and area and length. We conducted separate analyses for macroinvertebrate prey (numbers per stomach sample) from VSCA, and fish prey sequences (log-transformed numbers per stomach sample+1) from 12S metabarcoding as dependent variables. As fish prey in VSCA were very rare (n_{prey}=19), we chose not to analyze the number of fish prey in relation to round goby densities. We fitted count data from the VSCA to a negative binomial model, whereas sequence data was fitted to an ordinary linear mixed effect model to avoid singular fit of model. Both round goby density and length were log transformed to improve fit of residuals to the model.

To investigate if differences in diet composition were related to variation in prey availability, we used data of environmental densities of prey sampled once per area and year (see Suppl. material 1: appendix 2 for description of prey sampling and data standardization). Data of macroinvertebrate densities were available from both AL and KK, while data of fish fry densities were available only from KK. The exact sampling locations differed from the round goby sampling locations (Suppl. material 2: fig. S2, Suppl. material 3: table S1). It was not possible to directly associate environmental densities of prey to round goby diet, and instead we investigated if there were significant differences in environmental densities of prey that could explain variation in round goby diet. We used linear models with data of log-transformed environmental densities for each prey species/group as dependent variable, and year and species group as well as the interaction between them as explanatory variables using general linear models. To investigate variation in round goby catches between years, we used log-transformed round goby catches in fyke nets as dependent variable, and area and year as explanatory variables in general linear models.

Results

Spatiotemporal diet variation in visual stomach content analysis

Round goby diet composition, expressed as diet volume proportion in VSCA, showed significant differences between the study areas (RDA $F_{1,325}$=23.45, p<0.001), years (RDA $F_{1,325}$=17.62, p<0.001; Fig. 2, Suppl. material 3: table S5) and sampling locations within areas (RDA $F_{3,325}$=2.65, p<0.001). To a lesser extent, diet was influenced by round goby length (RDA $F_{1,325}$=1.95, p=0.04). The main differences in diet between areas were explained by higher proportions of hydrobiid gastropods (22–27%; Hydrobiidae Stimpson, 1865), barnacles (4–11%; Balanidae Leach, 1817) and Baltic macoma (5–13%) in AL, whereas the diet consisted more of blue mussel (33–38% *Mytilus* sp.), cardiid bivalves (2–15%; Cardiidae Lamarck, 1809) and isopods (7–16%; *Idotea* sp. Fabricius, 1798) in KK (Figs 2, 3, Suppl. material 3: table S6). Cardiid bivalves constituted more important prey in 2019 compared to 2018 in both areas (10 *vs.* 0.4% in AL and 15 *vs.* 2% in KK, respectively). There was a tendency towards larger round goby in AL and in KK Location 2, which fed more on cardiid bivalves and hydrobiid gastropods, while crustaceans, mainly *Idotea* sp., were more common prey for smaller individuals (Fig. 2). Fishes were detected in the diet estimated from VSCA, but constituted only a minor proportion (1.5% in total; Fig. 3, Suppl. material 3: table S6).

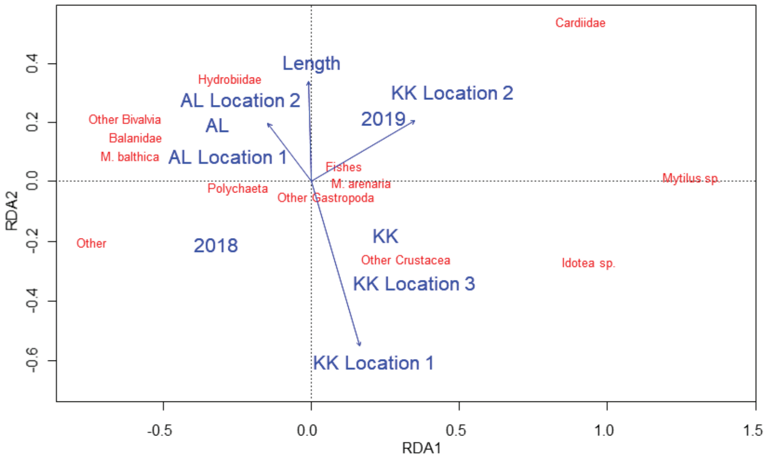

Figure 2. Spatiotemporal variation in round goby diet composition in VSCA. The axes represent linear combinations of the explanatory variables in terms of which explain the most variation of the species matrix (RDA1 0.104, RDA2 0.013).

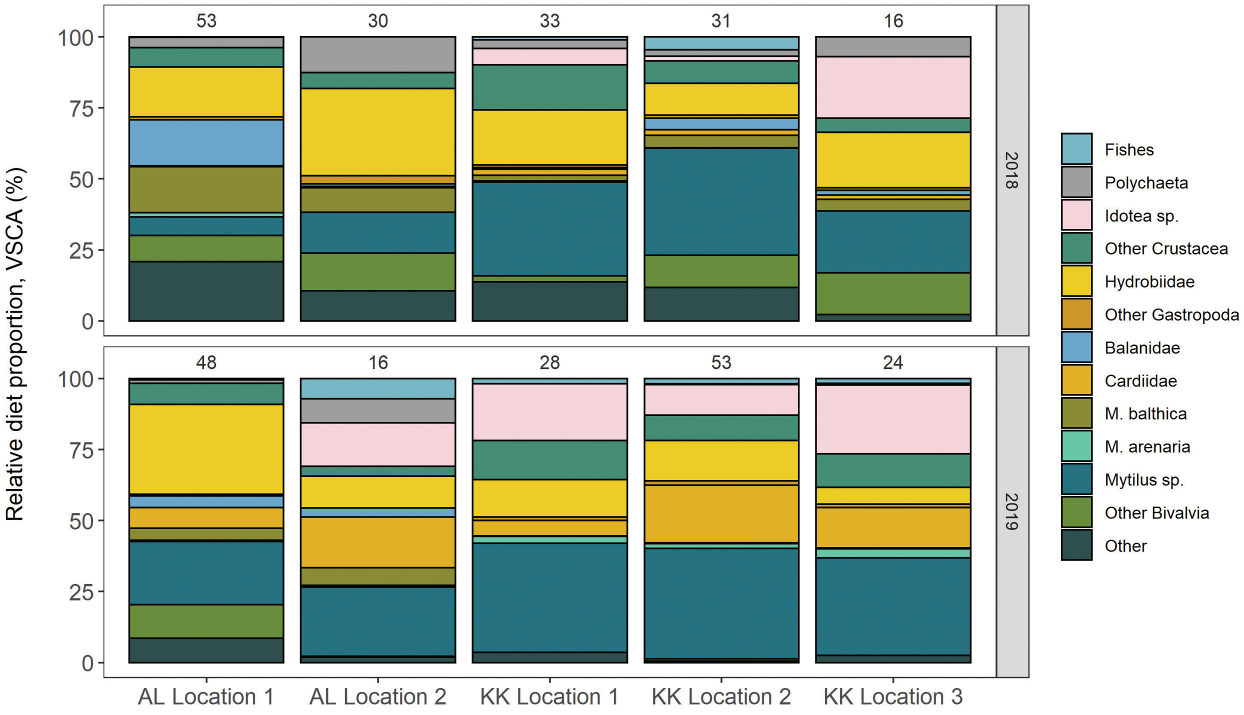

Figure 3. Spatiotemporal variation in diet volume proportion of round goby prey in VSCA. Number of samples are indicated above each bar.

Spatiotemporal diet variation in metabarcoding

In contrast to VSCA, fish sequences from COI constituted 24 and 28% in AL and KK in 2018, respectively, but only 0.3 and 0.01% in 2019 (Fig. 4). Several fish prey species/groups were detected by 12S metabarcoding. The fish part of the diet, based on 12S, varied between areas (RDA $F_{1,97}$=3.89, p<0.01) and years (RDA $F_{1,97}$=25.19, p<0.001; Fig. 5, Suppl. material 3: table S5), but was not influenced by

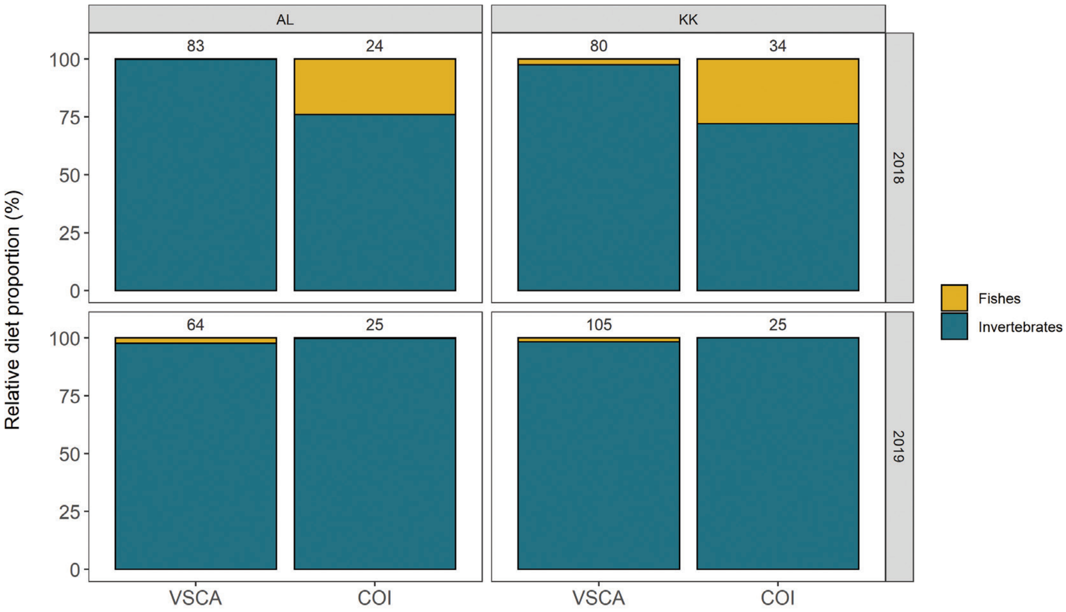

Figure 4. Relative diet proportion of fishes and invertebrates in VSCA and DNA metabarcoding using the COI marker. Four COI samples from KK 2018 (n=34) lack matching 12S samples (n=30).

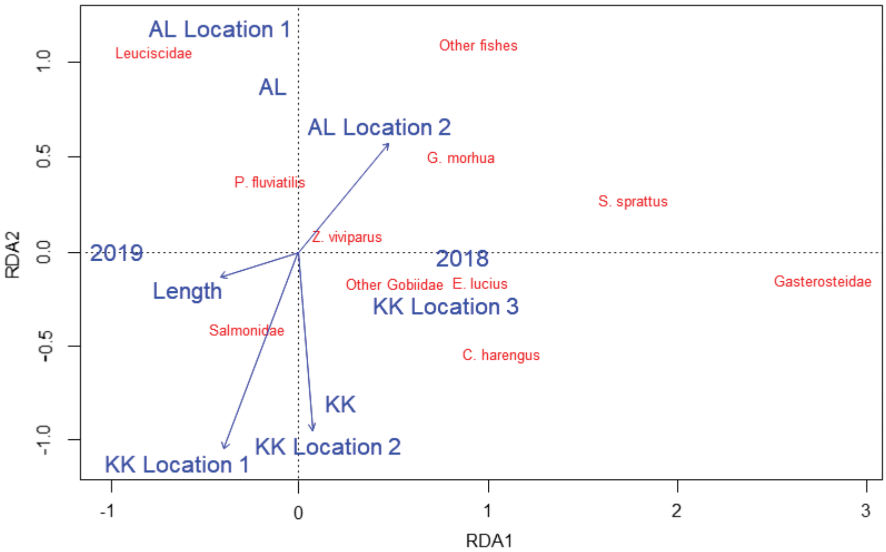

Figure 5. Spatiotemporal variation in round goby diet composition in DNA metabarcoding using 12S. The axes represent linear combinations of the explanatory variables in terms of which explain the most variation of the species matrix (RDA1 0.196, RDA2 0.035).

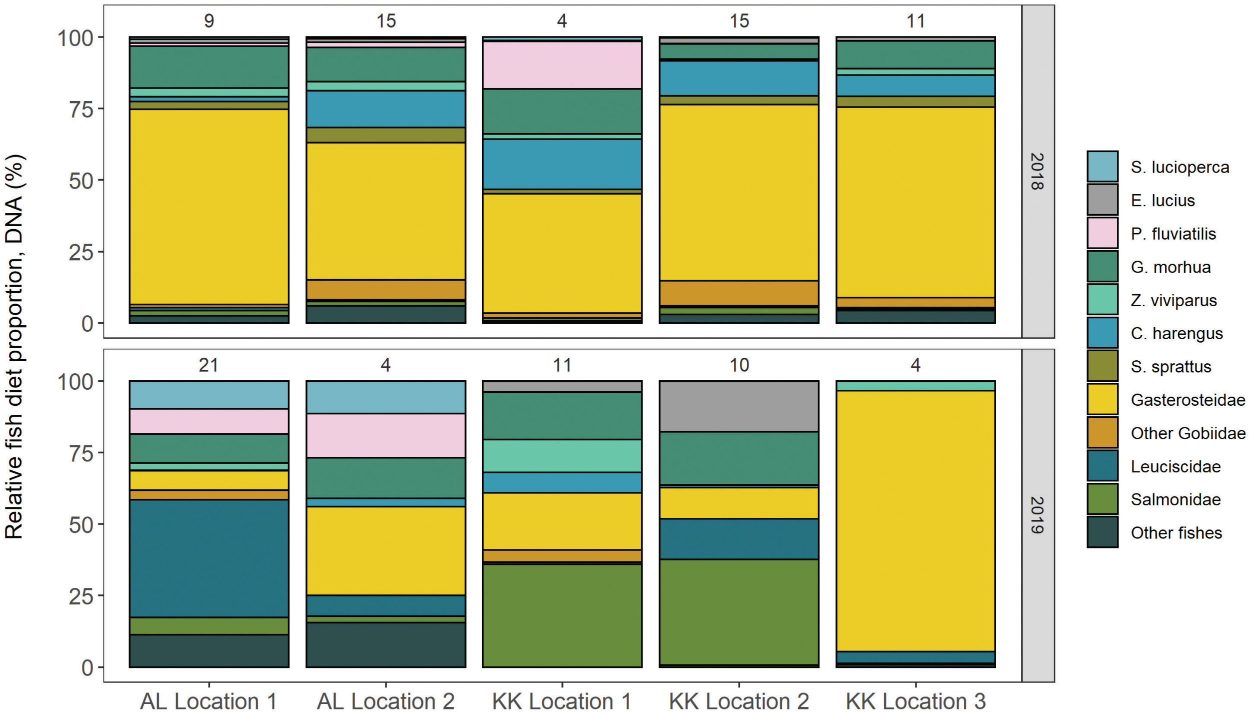

Figure 6. Spatiotemporal variation in sequence proportion of round goby fish prey in in DNA metabarcoding using 12S. Number of samples are indicated above each bar.

round goby length or sampling location within areas. The differences between study areas were mainly explained by sticklebacks and herring (Gasterosteidae and *Clupea harengus*; both Linnaeus, 1758), which were more common prey in KK, while cod, sprat (*Sprattus sprattus* Linnaeus, 1758), cyprinids (Leuciscidae Bonaparte, 1835) and the group 'Other fishes' were more common prey in AL (Fig. 5). The diet variation between years was primarily explained by sticklebacks, cod, herring, sprat and the group 'Other fishes', which were more common prey in 2018, while salmonids and cyprinids increased in importance between 2018 and 2019. Sticklebacks dominated the fish part of the diet in AL 2018 (58% of total number of fish sequences, compared to 14% 2019) and both years in KK (60 and 71%, respectively), but in AL 2019 cyprinids constituted the largest proportion of fish sequences (31%; Fig. 6, Suppl. material 3: table S7). Several commercially interesting fish species/groups were detected in metabarcoding, for example cod (5–13% of sequences per area and year), herring (0.9–11%), perch (0.01–11%), pike (0–3%), pike-perch (0–10%) and salmonids (2–10%). These species were detected in lower sequence numbers relative to non-commercial species (Fig. 6, Suppl. material 3: table S4), but in a high proportion of the stomachs (frequency of occurrence, FO%, Fig. 7). For example, cod was detected in 48% of the samples in both areas in 2019 (Fig. 7).

Diet in relation to round goby densities, prey densities and round goby length

Round goby densities were significantly higher in 2018 than 2019 in both study areas (GLM: $F_{1,16}=10.12$, $p<0.01$; Suppl. material 2: fig. S3). In VSCA, the interaction between round goby length and area explained most variation in number of macroinvertebrate prey in the diet (ΔAIC=-14.95; Suppl. material 3: table S8). The interaction was due to a positive relationship in KK but negative in AL (Suppl. ma-

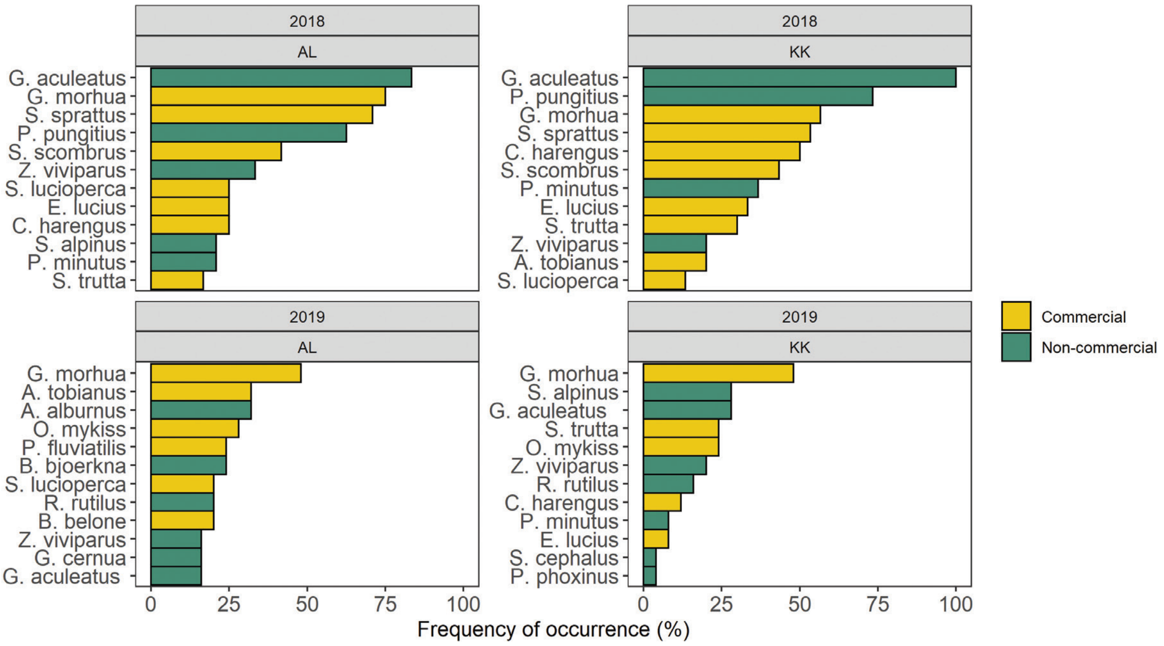

Figure 7. Spatiotemporal variation in frequency of occurrence (FO%) of fish prey DNA metabarcoding using 12S, showing the 12 fish prey with the highest FO in each area and year.

terial 2: fig. S4). Round goby densities explained marginal variation (ΔAIC=-1.7), with a negative association of the number of macroinvertebrate prey in the diet with round goby densities in KK but no relationship in AL (Fig. 8). Round goby densities best explained variation in number of sequences of fish prey in 12S metabarcoding (ΔAIC=-3.68; Suppl. material 3: table S8), with positive associations in both areas (Fig. 9). Round goby length did not contribute to the model fit (ΔAIC=+0.46).

The environmental densities of fish prey in KK and of macroinvertebrate prey in AL did not differ between years (GLM: $F_{1,20}$=0.04, p>0.05, Suppl. material 2: fig. S5.1; GLM: $F_{1,43}$=2.83, p>0.05, Suppl. material 2: fig. S5.2). However, for macroinvertebrates in KK, densities differed between species and years, as densities of Chironomidae (Newman, 1834) and Baltic macoma were higher in 2019 relative to 2018, while densities of Clitellata (Michaelsen, 1919) and hydrobiid gastropods were lower in 2019 relative to 2018 (GLM: $F_{3,16}$=3.2, p=0.05; Fig. 10).

Discussion

Spatiotemporal diet variation in visual stomach content analysis

Our study shows round goby diet variation between areas and years, which consolidates the picture of round goby as a generalist predator (Skóra and Rzeznik 2001; Kornis et al. 2012; Nurkse et al. 2016; Puntila 2016; Rakauskas et al. 2020). One reason potentially contributing to the area-related differences seen in VSCA is the Baltic Sea salinity gradient. Salinity decreases towards the east and north, impacting species richness and composition (Snoeijs-Leijonmalm 2017). The main difference in diet between areas according to VSCA was the result of more hydrobiid gastropods in AL *vs.* more blue mussel in KK. Blue mussel is more common and grows faster in the southern Baltic Proper where salinity is higher (Westerbom et al. 2002), which likely explains the higher prevalence in round goby diet in KK. The diet variation in VSCA was also explained by round goby length, with hydrobiid gastropods and cardiid bivalves being more common prey for larger individuals, and

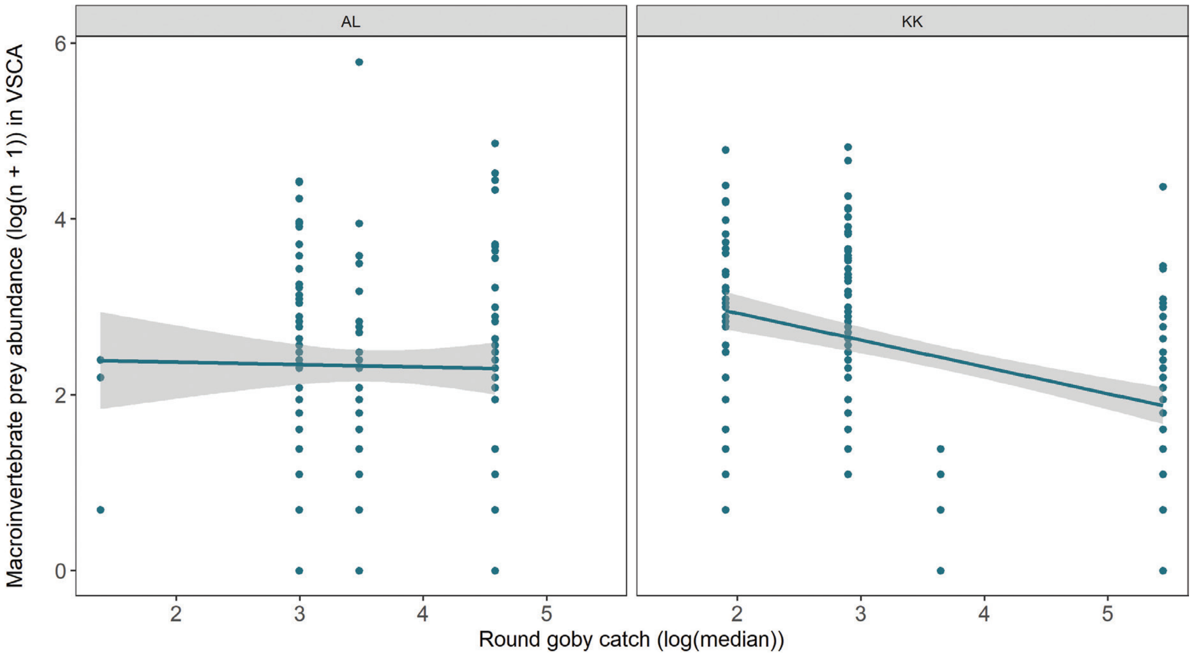

Figure 8. Relationship between number of macroinvertebrate prey in VSCA and round goby densities in AL and KK.

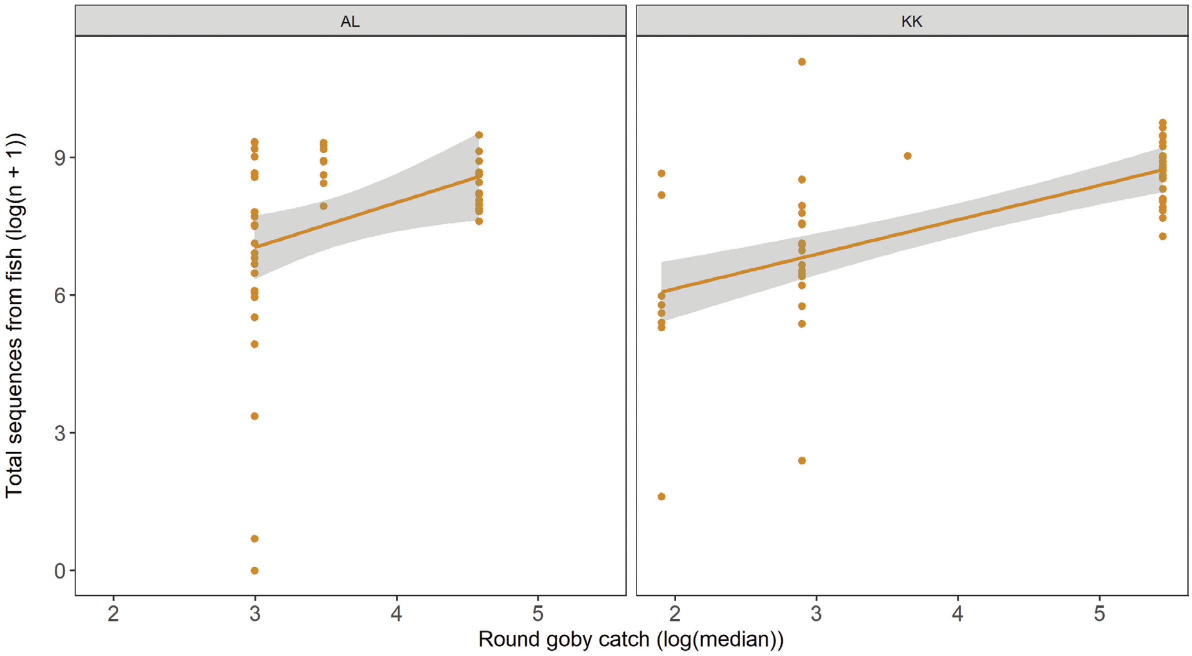

Figure 9. Relationship between number of DNA metabarcoding sequences from fish prey and round goby densities in AL and KK.

crustaceans, mainly isopods, being more common prey for smaller individuals. This may partly be confounded by on average larger round goby in AL and KK Location 2, where hydrobiid gastropods and cardiid bivalves were more common in the diet. It is still consistent with earlier findings, reporting an ontogenetic shift at 100–150 mm length from soft-bodied benthic organisms to larger shelled prey (Puntila 2016; Ustups et al. 2016; Oesterwind et al. 2017). The increased feeding on larger, shelled prey with age is likely related to increased gape width and height (Skabeikis and Lesutienė 2015), the ability of larger round goby to pick and break the shells of molluscs and a more sedentary life-style of adult fish (Skóra and Rzeznik 2001).

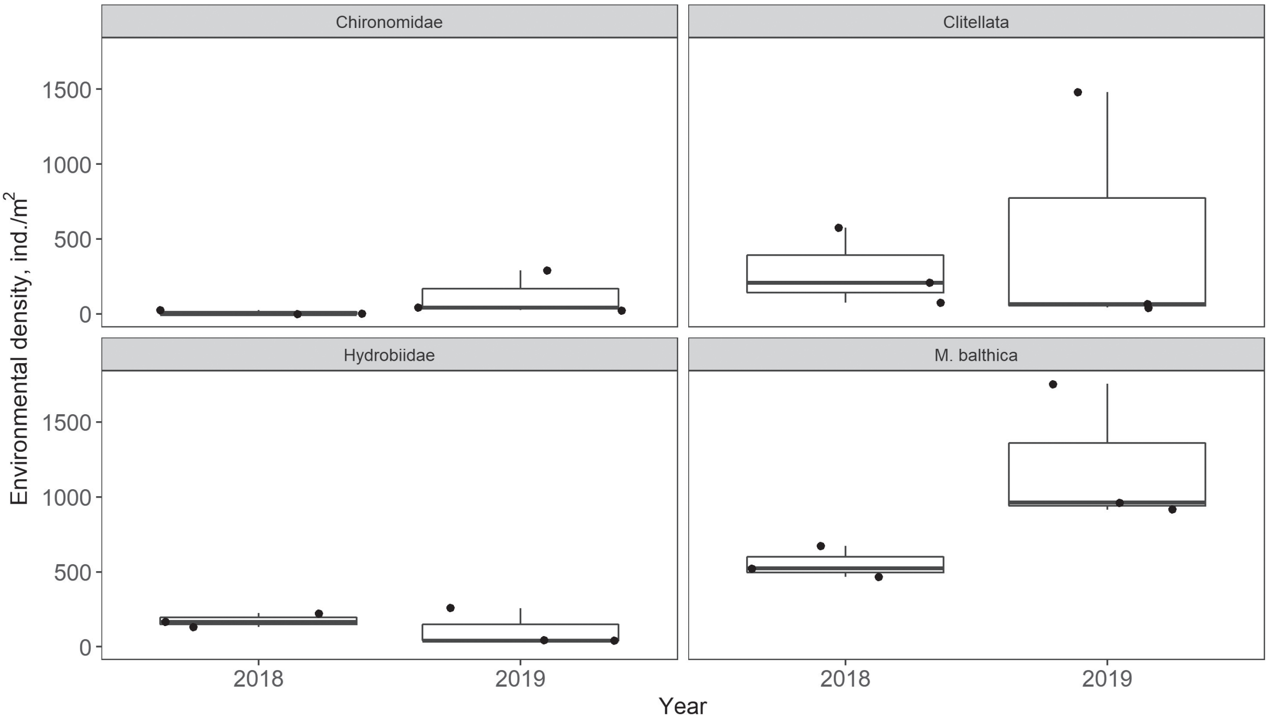

Figure 10. Macroinvertebrate environmental densities in KK in 2018 and 2019.

Spatiotemporal diet variation in metabarcoding

The differences in proportion of COI sequences varied greatly between years, although the differences need to be interpreted with caution, as some macroinvertebrates did not seem to be amplified relative to their abundance in VSCA. Yet, the fish proportion in round goby diet was ~100 times higher 2018 compared to 2019 in both areas, which we find hard to believe would only be due to methodological artefacts. Round goby fish diet composition, assessed from 12S metabarcoding, differed between areas and years (Figs 5, 6). The round goby in our study were sampled in May and June, when many fish species in the Baltic Sea spawn or have newly hatched larvae (Swedish Agency for Marine and Water Management 2020). It is likely that differences in locally spawning species was reflected in the diet of round goby in this study. Sticklebacks dominated the fish prey in both areas 2018 and in KK 2019 (Fig. 6, Suppl. material 3: table S7) and are common throughout the Baltic Proper (Olin et al. 2022). The greater importance of cyprinid prey in AL (Figs 5, 6) might be related to the lower salinity around AL, which is favourable for this originally limnetic family (Snoeijs-Leijonmalm 2017), whereas the marine originated herring was more common in KK (Figs 5, 6) where salinities are higher. Predation on cod in AL is interesting, as it is uncertain if salinity is high enough for cod reproduction around AL (Nissling and Westin 1997; Bergström et al. 2015). However, the detection of cod in the stomachs of round goby in both AL and KK suggests either an inflow of pelagic larvae to AL or scavenging.

Diet in relation to round goby densities, prey densities and round goby length

The number of sequences from fish prey in 12S metabarcoding was best explained by higher round goby densities. Thus, fish prey increased in the diet at higher round goby densities, i.e. 2018, in both study areas. The environmental densities of fish prey in KK (Suppl. material 2: fig. S5.1) and of macroinvertebrate prey in AL (Suppl. material 2: fig. S5.2) did not differ significantly between years, albeit sparse and uncertain data, while two macroinvertebrate prey groups in KK, Chironomidae and Baltic macoma, differed between years with higher densities in 2019 (Fig. 10). Thus, there is little support that the increased intake of fish prey 2018 was related to higher environmental densities of fish prey or lower environmental densities of macroinvertebrate prey. Admittedly, the weak statistical power exclude any stronger conclusions, but from our results we hypothesize that round goby is more prone to include fish prey in the diet at high round goby densities due to intraspecific interactions, or that macroinvertebrates, as the preferred or more easily available prey, may be depleted in areas of high round goby densities. The indications of increased feeding on fishes at high round goby densities also agree with the findings of Paton et al. (2019), showing that round goby from a high density site attacked motile prey more frequently compared to round goby from a low density site. The change in feeding patterns with round goby densities indicated in our study may imply that the amount of round goby in the ecosystem could determine which type of prey is more impacted.

There was a marginal contribution of round goby density to the model fit for VSCA data, and instead, the interaction between round goby length and area best explained the variation in number of macroinvertebrate prey. Also for VSCA data we need to interpret results with care due to few independent samples, but the results indicate that the number of macroinvertebrate prey decreased with length in AL but increased in KK (Suppl. material 2: fig. S4). Previous studies have shown ontogenetic shifts in round goby diet (Puntila 2016; Ustups et al. 2016; Oesterwind et al. 2017), but we have no immediate hypothesis why food intake would increase with body size in KK but not in AL.

Method evaluation

VSCA and metabarcoding yielded very different results. Fast degradation of soft material like fish eggs and larvae in stomach contents likely leads to underestimation of fish prey in VSCA, and retention of prey hard parts in the stomach may cause overestimation of e.g. hard-shelled prey groups in VSCA (Hyslop 1980; Buckland et al. 2017; Nielsen et al. 2018). The fishing methods could also impact the results, as fish could be trapped in the fyke nets or gill nets for >12 h before landing and freezing, further degrading the stomach content. To that end, we complemented VSCA with DNA-based methods. The COI marker is regarded a "standard" barcode with primers amplifying multiple taxa (Leray et al. 2013). In our study, however, the COI marker seemed to work better, in general, for crustaceans compared to bivalves, potentially due to higher degradation of shelled prey if they had been retained in the stomach for a long time (Panova et al. 2021; Panova and Ring 2022). Thus, analysis of specific prey species/groups detected using COI was deemed unsuitable. We instead used COI for estimation of the proportion of fish *vs*. invertebrate prey. The affinity of the COI primers to different fish species has not been thoroughly evaluated and species determination is uncertain, meaning that COI primers in this study may have failed to detect some fish species (Panova and Ring 2022). Thus, species of both invertebrates and fishes may have been underestimated in the COI analysis. Therefore, the distribution of sequences between fishes and invertebrates using COI provides only a coarse indication of their respective contribution to round goby diet (Fig. 4). However, the difference between years was hundred-fold in both study areas, which makes us confident that fish prey was more common 2018 than 2019. The same pattern was seen in 12S, which strengthens the indication of a change in fish diet between years. Still, experimental follow-ups, using different markers and primers, are required for more reliable results of amplification of different taxa in fish diet.

In metabarcoding, there is also the possibility that some sequences may stem from environmental DNA (eDNA). However, DNA concentrations from eDNA are much lower compared to DNA concentrations from ingested prey and should result in low sequence numbers (Traugott et al. 2021). As species contributing to <1% of the sequences per sample were filtered out from all samples (Suppl. material 1: appendix 1), we expect that a large proportion of the sequences stemming from eDNA were excluded prior to analysis. Consequently, the relatively high sequence proportion of e.g. cod (5–13%; Suppl. material 3: table S7) would not be an expected outcome if the source was eDNA alone (Panova and Ring 2022). Some fish sequences could also stem from scavenging (Traugott et al. 2021) as round goby is capable to feed on dead fish (Polačik et al. 2015), a phenomenon that would not be revealed by VSCA. Scavenging would not affect the diet contribution of fishes *vs*. invertebrates, but rather the interpretation of potential ecosystem effects of round goby feeding. Further, previous studies have shown that round goby is cannibalistic in experimental settings (Meunier et al. 2009) as well as under natural conditions (French and Jude 2001; Števove and Kováč 2013; Skabeikis and Lesutienė 2015). It is likely that this phenomenon occurs also in our study areas. However, detection of cannibalism is challenging due to difficulties to detect fish prey in general in VSCA and the use of host-blocking primers in metabarcoding. It is thus probable that the proportion of fish prey in round goby diet is underestimated if cannibalism goes undetected.

Species identification in 12S metabarcoding produced some implausible results. The insect genus *Diptera* sp., probably representing a bioinformatics chimaera, was filtered out prior to analysis (see Materials and methods). However, fish species from neighboring water bodies not regularly found in the Baltic Sea because of salinity constraints were included in the dataset, i.e. the fishes Arctic char (*Salvelinus alpinus* Linnaeus, 1758) and Atlantic mackerel (*Scomber scombrus* Linnaeus, 1758; Suppl. material 3: table S4). These species probably represent bioinformatics chimaeras, but as the sequences survived extensive laboratory quality control (Suppl. material 1: appendix 1) they were kept because they, although unlikely, may represent potential trophic interactions. Rainbow trout (*Oncorhynchus mykiss* Walbaum, 1792) is an introduced species in the Baltic Sea (SLU Artdatabanken *Oncorhynchus mykiss* n.d.). Its reproductive success in our study areas is unknown (SLU Artdatabanken *Oncorhynchus mykiss* n.d.) and round goby scavenging, rather than egg or larvae predation, is a more plausible explanation in the case of rainbow trout.

We conclude that, despite the respective issues described above, VSCA and metabarcoding may complement each other well. A trained expert appears to be able to visually more correctly identify macroinvertebrate prey groups and their respective proportions, as quantitative results from COI analysis of invertebrates were unreliable, potentially due to highly degraded invertebrate prey. Contrary, the estimation of contribution of fish prey to round goby diet would not have been possible without metabarcoding using COI and 12S. The results from our study propose that metabarcoding of the 12S rRNA gene should be used to asses fish prey as a complement to VSCA of macroinvertebrates.

Ecological implications of round goby predation

Dietary breadth or flexibility in feeding of invasive species can generate ecological impacts across the food web in invaded ecosystems (McKnight et al. 2016). Round goby predation on filter feeders (e.g. barnacles in AL and blue mussel in KK) and grazers (e.g. hydrobiid gastropods in AL and isopods in KK) has the potential to exacerbate eutrophication symptoms (Liversage et al. 2019; Nõomaa et al. 2022) and increase growth of filamentous algae, which could lead to habitat degradation in shallow coastal areas. In KK, round goby diet composition indicates resource competition with flatfish and long-tailed duck (*Clangula hyemalis* Linnaeus, 1758) for blue mussel, as seen in the south-eastern parts of the Baltic Sea (Skóra and Rzeznik 2001; Karlson et al. 2007; Järv et al. 2011; Ustups et al. 2016; Skabeikis et al. 2019). Competition for food has the potential to worsen the situation for European flounder (*Platichthys flesus* Linnaeus, 1758), which is currently declining in the Baltic Sea (Momigliano et al. 2019). Round goby diet as depicted in this study also indicates a dietary overlap of crustaceans, bivalves, gastropods and various fishes with eelpout (*Zoarces viviparus* Linnaeus, 1758; Ojaveer and Järv 2003), with potential negative consequences for eelpout. Round goby predation on crustaceans (6–10% in AL and 18–27% in KK) further indicates potential competition with perch, as crustaceans can constitute between ~ 40–60% of perch diet in the Baltic Sea (Mustamäki et al. 2014; Jacobson et al. 2019).

Metabarcoding revealed that non-commercial fish species like sticklebacks and cyprinids dominated the fish part of the diet, but also commercially interesting species like cod, perch and pike-perch were detected (Figs 5–7). As these species are predators on round goby (Almqvist et al. 2010; Hempel et al. 2016) our findings could indicate predator-prey reversal, which would point towards new trophic relationships after the establishment of round goby in the Baltic Sea. Round goby predation on early life-stages of other fish species could negatively impact recruitment and stock abundance, which is primarily determined by mortality in early life-stages (e.g. Archambault et al. 2014). Predation pressure from round goby can potentially be an additional stressor for coastal predatory fishes of ecological and commercial interest, already under pressure from e.g. habitat degradation, recreational fishing, boating and environmentally harmful substances (Olsson 2019). Round goby predation may also hinder conservation efforts, as round goby predation on vulnerable fish species might counteract such efforts (Lutz et al. 2020). Further field sampling and experimental set-ups are required to assess the importance of the round goby as predator on early life-stages of predatory fish.

Conclusions

Round goby has had large ecological consequences in invaded ecosystems (reviewed by Kornis et al. 2012). Previous studies from the Baltic Sea have suggested predation primarily on benthic fauna (e.g. Skabeikis and Lesutienė 2015; Puntila 2016; Nõomaa et al. 2022). We add to this knowledge by showing that round goby can also prey on other fish species, most likely their eggs and larvae, but potentially also by scavenging. This suggests that round goby is part of previously unknown trophic links within the Baltic Sea food web, with possible complex feedback mechanisms as round goby is both competitor and prey to other fish species. Predatory fishes occurred in low diet proportions, but the detection of such species in the diet shows that predator-prey reversal might be occurring. The magnitude of this potential predator-prey reversal remains to be estimated, but strong populations of native fish predators to exert biological control of round goby (Ojaveer et al. 2015) could be important to keep the round goby population at lower, potentially less harmful levels.

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Author contribution

IWK: research conceptualization, sampling design and methodology, investigation and data collection, data analysis and interpretation, ethics approval, funding provision, writing original draft.

ABF: research conceptualization, sampling design and methodology, data interpretation, ethics approval, funding provision, review and editing of the manuscript.

KL: research conceptualization, sampling design and methodology, data interpretation, funding provision, review and editing of the manuscript.

ÖÖ: data analysis and interpretation, review and editing of the manuscript.

Ethics and permits

Fishing in Åland was conducted under "Permit for fishing with gear of smaller mesh size than stated in the Åland law of fisheries, ÅLR 2018/3983" (Finland; Government of Åland). Fishing in Karlskrona was conducted under "Dispensation from fisheries regulations 2018, SLU.aqua.2018.5.4-194" and "Dispensation from fisheries regulations 2019, SLU.aqua.2019.5.4-26" (Sweden; Swedish University of Agricultural Sciences and Swedish Agency for Marine and Water Management). All animal handling was conducted under the ethical permit "Ecological effects of round goby, 5.8.18-07747/2018" (Sweden; Swedish Board of Agriculture, the Uppsala region Ethics Committee on Animal Experiments).

Data availability

The data that support the findings of this study are available at: https://doi.org/10.5878/m5m1-br15

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Supplementary material 1

Method descriptions

Authors: Isa Wallin Kihlberg, Ann-Britt Florin, Karl Lundström, Örjan Östman
Data type: Microsoft Word document (.docx)
Explanation note: appendix 1: Method descriptions of metabarcoding of round goby samples; appendix 2: Sampling and standardization of fish fry and macroinvertebrate environmental density data

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Supplementary material 2

Supplementary figures

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Data type: figures, Microsoft Word document (.docx)

Link: https://doi.org/10.3391/ai.2023.18.2.104960.suppl2

Supplementary material 3

Supplementary tables

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Data type: tables, Microsoft Excel-file (.xlsx)

Link: https://doi.org/10.3391/ai.2023.18.2.104960.suppl3