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Causes and consequences of the round goby invasion in the Baltic Sea and beyond

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Cover: Swimming round goby surrounded by scales and fins. Illustration by Kerstin Kempe Lange and Selma Eriksson.

Errata for
Causes and consequences of the round goby invasion in the Baltic Sea and beyond

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Page 23	Location:	Section 3.1, second paragraph
	Is now:	starting in 2009
	Should be:	starting in 2008

Page 33	Location:	Section 4.1, second paragraph
	Is now:	In addition, only four species displayed body and fin types similar to round goby (longspined bullhead, black goby, eelpout *Zoarces viviparus* and burbot *Lota lota*; paper I).
	Should be:	In addition, only three species displayed body and fin types similar to round goby (fourhorn sculpin, black goby and eelpout *Zoarces viviparus*; paper I).

Page 38   Location: Figure 9
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Causes and consequences of the round goby invasion in the Baltic Sea and beyond

Abstract

Species invasions have increased rapidly over time and pose negative effects on ecosystems and societies worldwide. The round goby, one of the most widespread invasive fishes in the northern hemisphere, established in the Baltic Sea in 1990. My results show that the round goby, in contrast to most native species in the coastal fish community, exhibits territorial behaviour and parental care, which may have given it a competitive advantage and facilitated its establishment in the Baltic Sea. I found the major diet components of the round goby to be macroinvertebrates, but DNA metabarcoding revealed feeding also on several fish species, likely in their early life stages. Further, the round goby constituted prey for cod, perch and pike. When round goby was abundant in the environment, the reliance on fish prey increased for both round goby and its predators. As a potential consequence of round goby expansion to freshwater, I further studied round goby interactions with spawning salmon. Presence of round goby delayed salmon spawning, and male and female salmon resided closer together in the presence of higher densities of round goby compared to lower densities, which could be interpreted as a protective behaviour. In order to minimise the negative effects of the round goby in and around the Baltic Sea, I recommend management measures to reduce its abundances and prevent further spread, for example by strengthening native coastal predatory fish populations, developing a round goby fishery and developing a system for early detection and eradication of the round goby in rivers and streams.

Keywords: *Neogobius melanostomus*, non-indigenous species, functional traits, competition, predator-prey interactions, DNA metabarcoding, spatiotemporal comparison, reproductive disturbance, range expansion

Orsak och verkan av invasionen av svartmunnad smörbult i och runt Östersjön

Sammanfattning

Införsel av främmande invasiva arter har ökat kraftigt över tid, vilket har lett till negativa ekosystem- och samhällseffekter världen över. Den svartmunnade smörbulten är en av de mest spridda invasiva fiskarterna på norra halvklotet och har funnits i Östersjön sedan 1990. Mina resultat visar att svartmunnad smörbult, till skillnad från de flesta inhemska arter i kustnära fisksamhällen, hävdar revir och sköter om äggen medan de utvecklas, vilket kan ha gett arten en konkurrensfördel som har underlättat dess etablering i Östersjön. Den största delen av födan för svartmunnad smörbult utgjordes av ryggradslösa djur, men DNA-analyser visade att svartmunnad smörbult också äter andra fiskarter, troligen mest ägg och yngel. Svartmunnad smörbult visade sig också utgöra byte för abborre, gädda och torsk. Födosammansättningen hos både svartmunnad smörbult och dess predatorer påverkades av mängden svartmunnad smörbult i miljön, eftersom alla fyra arterna åt mer fisk när det fanns mycket svartmunnad smörbult i miljön. Som en möjlig effekt av spridning av svartmunnad smörbult till sötvatten undersökte jag också interaktioner mellan svartmunnad smörbult och lekande lax. Laxleken fördröjdes i närvaro av svartmunnad smörbult, och laxarna i lekparen höll sig närmare varandra när tätheterna av svartmunnad smörbult var högre, vilket kan tolkas som ett skyddsbeteende. För att minska de negativa effekterna av svartmunnad smörbult i och runt Östersjön rekommenderar jag att man vidtar förvaltningsåtgärder för att hålla nere tätheterna och bromsa dess spridning, till exempel genom att stärka populationerna av inhemska kustnära rovfiskar, utveckla ett fiske på svartmunnad smörbult och ta fram ett system för tidig upptäckt och utrotning av svartmunnad smörbult i rinnande vatten.

Nyckelord: *Neogobius melanostomus*, främmande arter, funktionella egenskaper, konkurrens, predator-bytesinteraktioner, DNA metabarcoding, tidsmässiga och rumsliga jämförelser, störning under lek, utökning av utbredningsområde

Dedication

To my family

Contents

List of publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I. Antoni Vivó Pons, Isa Wallin Kihlberg, Jens Olsson, Peter Ljungberg, Jane Behrens & Martin Lindegren. The devil is in the details: exploring how functionally distinct round goby is among native fish in the Baltic Sea. (In revision)

II. Isa Wallin Kihlberg, Ann-Britt Florin, Karl Lundström & Örjan Östman (2023). Detection of multiple fish species in the diet of the invasive round goby reveals new trophic interactions in the Baltic Sea. *Aquatic Invasions*, 18 (2), 141-162.

III. Heidi Herlevi, Isa Wallin Kihlberg, Katri Aarnio, Erik Bonsdorff, Ann-Britt Florin, Andreas Ljung, Karl Lundström, Johanna Mattila & Örjan Östman. (2023). Environmental abundances of the non-native round goby *Neogobius melanostomus* influence feeding of native fish predators. *Journal of Fish Biology*, 102 (6), 1340-1357.

IV. Isa Wallin Kihlberg, Ann-Britt Florin, Ioannis Efstathiadis, Tomas Brodin & Gustav Hellström. Indications of reproductive disturbance by the invasive round goby on Atlantic salmon. (Submitted manuscript)

Papers II and III are open access publications (CC BY 4.0). Heidi Herlevi and Isa Wallin Kihlberg are joint first authors on paper III.

1. Introduction

1.1 Ecological perspectives on species invasions

'If we look far enough ahead, the eventual state of the biological world will become not more complex but simpler – and poorer.'

C.S. Elton, *The ecology of invasions of animals and plants*, 1958

Biodiversity and the ecosystem processes related to it are currently facing multiple pressures worldwide, as species are going extinct at an unprecedented rate (IPBES 2019). The loss of species, genetic diversity and functional diversity has the potential to alter the conditions for life on Earth, with tremendous impairment of ecosystem services that humankind relies on for its survival (ibid.). Invasive species are defined as introduced species with negative effects on ecosystems, economics or society (Keller et al. 2011). Such species can cause biodiversity loss through a multitude of direct and indirect processes (e.g. Elton 1958; reviewed by Pyšek et al. 2020).

   Species invasions are driven by increasing trade and transport, habitat degradation and climate change (IPBES 2019), and paleontological and molecular data show that the current invasion rates are several orders of magnitude higher than in prehistoric times (Simberloff & Ricciardi 2020a). However, many species transferred to new areas may not be able to establish, and of those that establish, certainly not all become invasive (Bax et al. 2003). Following successful establishment, some species have neutral or even positive effects on the recipient ecosystem (Katsanevakis et al. 2014),

but others may have detrimental effects, as species introductions in some cases lead to population declines or extinctions of native species through competition or predation (reviewed by David et al. 2017 and Pyšek et al. 2020). Many invasive species further suppress native species through feedback mechanisms, causing regime shifts or trophic cascades (Olden et al. 2004; reviewed by David et al. 2017; Simberloff & Ricciardi 2020b). In addition, invasive species can drive ecosystems towards functional homogenisation, e.g. by replacing native ecological specialists with invasive generalists (Olden et al. 2004), and biotic homogenisation, either through addition of species to ecosystems or through reduction in the number of native species (Petsch et al. 2022). Biotic homogenisation as a means of biodiversity loss can lead to simplification of food-web structures (Olden et al. 2004), deterioration of ecosystem resilience and function and increased community vulnerability to diseases (Petsch 2016). Biotic homogenisation may also pave the way for subsequent species introductions, and affect genetic and evolutionary processes (Olden et al. 2004). Apart from direct and indirect effects on native biota, invasive species can affect abiotic ecosystem components like hydrology and disturbance regimes, and the cycling of nutrients and contaminants (reviewed by Pyšek et al. 2020). Moreover, there are many examples of invasive species as transmitters of pathogens and parasites, acting as threats to ecosystem or human health (Elton 1958; Mazza et al. 2014; reviewed by Pyšek et al. 2020).

Population growth following species introductions is often rapid and the invasive species may reach very high densities in the recipient ecosystems (Elton 1958; Simberloff & Gibbons 2004; Strayer & Malcom 2006). The phenomenon may be related to enemy release if natural predators are absent or present only in low numbers, or lack of competition in the recipient ecosystem (reviewed by Daly et al. 2023). However, population crashes, or 'boom-and-busts', of invasive species in their introduced ranges are common (Elton 1958; Simberloff & Gibbons 2004). Boom-and-bust can modify species interactions like competition via density-dependent processes (Simberloff & Gibbons 2004). While some populations are able to recover after such crashes and display a cyclic pattern of population increase and decline, others experience population crashes so dramatic that the invasive species are subsequently eradicated from the invaded ecosystem (Simberloff & Gibbons 2004; Strayer & Malcom 2006). Yet others recover but stabilise at much lower abundances than prior to the crash (Strayer & Malcom 2006).

It is often difficult to infer causes for the dramatic declines, but proposed reasons include predator-prey interactions, competition with or parasitism by subsequently introduced species, resource depletion or outbreaks of pathogens (Simberloff & Gibbons 2004; Strayer et al. 2017).

The impacts of invasive species are in many cases related to their activities, making the invasive species drivers of change (reviewed by Pyšek et al. 2020). However, the impacts are also related to anthropogenic disturbances like climate change or pollution, which may enhance a species invasion success or negatively affect native communities (ibid.). Consequently, species invasions can drive and amplify ecosystem effects by their own means as well as in concert with other pressures (IPBES 2019; reviewed by Pyšek et al. 2020), accentuating the negative effects on biodiversity on the local, regional and global levels (Ros et al. 2023).

1.2   To invade or to be invaded

The ecological niche of an organism is shaped by all biotic and abiotic interactions that define its living space (Begon et al. 1990; Fig. 1). Ultimately, the ecological niche determines the role of the organism in the ecosystem (ibid.; Fig. 1). The functional traits of a species, defined as the morphological, physiological and phenological characteristics that influence its growth, reproduction and survival (Violle et al. 2007), determine both the species' biotic interactions and how it responds to environmental (abiotic) conditions (Siefert & Laughlin 2023). Species or individuals with traits better adapted to prevailing environmental conditions will experience higher fitness (ibid.). Invasive species are believed to establish in new areas by filling empty niches (Elton 1958; Fig. 1) and theory states that it is difficult to establish in a community dominated by functionally similar species because of greater niche overlap (reviewed by Daly et al. 2023). Trait similarity between species/groups on the community level indicate niche overlap via similar habitat and feeding requirements, which, in theory, leads to competition (reviewed by Cerwenka et al. 2023). Moreover, invasive species commonly share a number of functional traits, although such traits do not necessarily determine successful establishment, proliferation or spread (ibid.). Functional traits related to invasion success often involve tolerance to various stressors such as variation in temperature and salinity, or exposure to hypoxia, contaminants and sedimentation (reviewed by

Papacostas et al. 2017). The ability to cope with different environmental conditions can aid species in particular during early phases of the invasion process (ibid.). Functional behavioural traits such as boldness, interspecific aggression, nesting and parental care are also important features in invasion success, but more so for the later stages of invasion that involve establishment and spread (Chapple et al. 2012). However, behavioural traits such as boldness may differ with population age, i.e. time since introduction, although the direction of change varies (see Thorlacius 2015; Galli et al. 2023).
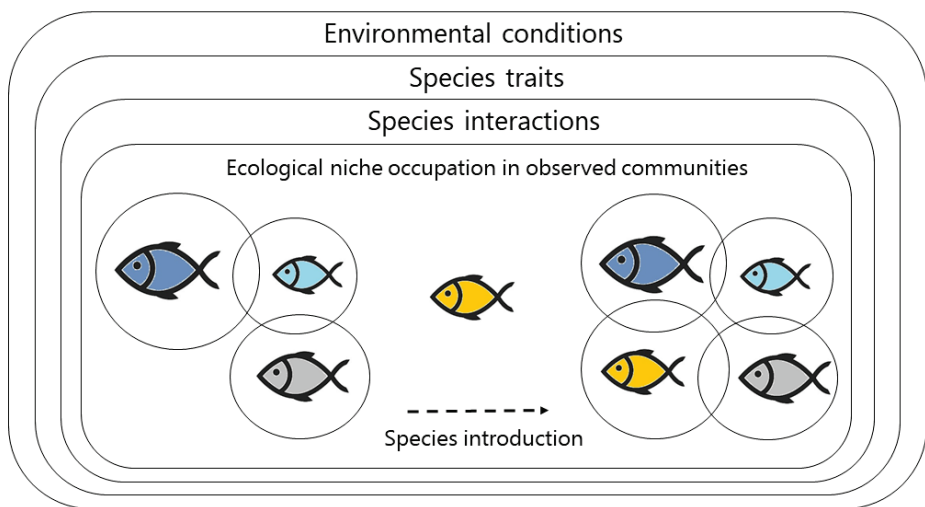

Figure 1. Environmental conditions affect which species traits are desired in specific communities. Environmental conditions and species traits affect species interactions, which in turn determine ecological niche occupation in observed communities. Species introductions can affect niche occupation of native species via trait overlap.

Biotic and abiotic ecosystem characteristics combined determine the susceptibility of an ecosystem to species invasions, i.e. its invasibility (Davis et al. 2005; Catford et al. 2012). Ecosystems of high biodiversity are supposed to be more resistant to species invasions as a majority of the ecological niches are expected to be filled by native species, putting up a threshold of competition for the invader (Elton 1958; Beaury et al. 2020, but see also e.g. Simberloff & Ricciardi 2020c and Brown & Barney 2021). Consequently, simple ecosystems with few trophic links are expected to be more vulnerable to species invasions due to vacant ecological niches

(Snoeijs-Leijonmalm 2017), and in addition, invasive species are in many cases found to be favoured by anthropogenic disturbances (Hobbs & Huenneke 1992; Simberloff & Ricciardi 2020c; reviewed by Daly et al. 2023). For example, climate change has the potential to facilitate establishment of invasive species (IPBES 2019) as it may enhance the chance of environmental matching between a potentially invasive species and the recipient ecosystem (Kriticos 2012), enhance the invasibility of ecosystems (reviewed by Pyšek et al. 2020) and exacerbate the impacts of invasive species (Ros et al. 2023).

1.3 The Baltic Sea

The Baltic Sea is a geologically young ecosystem (~8,000 years; Laamanen et al. 2017), defined by its north-south salinity gradient and stratification of the water mass by a thermocline and halocline (Snoeijs-Leijonmalm & Andrén 2017). Salinity is governed by saline water inflows from the Kattegat together with fresh water river runoff and precipitation (ibid.). The brackish conditions constrain species of both marine and limnetic origin, limiting the number of species that can inhabit the Baltic Sea (Snoeijs-Leijonmalm 2017). The low native biodiversity results in a simple ecosystem with comparatively few species interactions, and accordingly few links in the Baltic Sea food web (ibid.). However, despite its simple food web, the productivity of the Baltic Proper is similar to that of the much more diverse North Sea (ibid.).

The irregular saline inflows, low biodiversity and slow water exchange make the Baltic Sea vulnerable to anthropogenic disturbances (Lehtonen et al. 2017; Snoeijs-Leijonmalm 2017). Its catchment area is home to around 85 million people (HELCOM 2018a). Nutrient load and subsequent eutrophication from agriculture, inferior sewage treatment and NOx from combustion gases have resulted in seasonal growth of cyanobacteria, and hypoxic and anoxic conditions in the deeper areas with consequences primarily for the benthic community and its predators (Laamanen et al. 2017). In shallow coastal areas, eutrophication has resulted in excess growth of filamentous algae and increased turbidity, reducing habitat quality (Radziejewska et al. 2017). The Baltic is also heavily polluted by emissions from industry, agriculture, households and dumpsites, among other sources (Laamanen et al. 2017). In addition, fishing puts significant pressure on the

Baltic Sea fish ecosystem, as indicators for fishing mortality and spawning stock biomass show inferior status for 2/3 of the assessed species (HELCOM 2018b). Since mainly large, predatory species are targeted by the fishery, top predators vital for ecosystem structure and function are continuously removed, resulting in complex feedback mechanisms with far-reaching ecological consequences (Laamanen et al. 2017). The Baltic is also subject to climate change effects and is warming up at a higher pace than many other sea areas, although there are large differences between sub-basins (Viitasalo & Bonsdorff 2022). In the Baltic Sea, opportunistic species with warm-water preferences inhabiting soft sediments are likely to be favoured under climate change, implying that some coastal areas may be particularly vulnerable to species introductions (ibid.).

The pattern of low native species richness associated with a high degree of invasibility is obvious in the Baltic Sea (Snoeijs-Leijonmalm 2017). 173 non-indigenous species, including species of unknown origin, have been reported in the Baltic Sea, with an increasing number of introductions since 2000 (ICES 2022a). Of the reported species, 72 are established, and of these approximately 1/3 are considered to be widespread, with negative ecological effects (Ojaveer et al. 2021). The major known vectors are shipping, where species are commonly carried in ballast water or on ship hulls, and natural spread from adjacent water bodies (ICES 2022a). Most species introduced to the Baltic Sea originate from the North American east coast, the Ponto-Caspian region and East Asia (ibid.). The contribution to the Baltic Sea species pool is likely affected by the evolutionary adaption of Ponto-Caspian species to salinity fluctuations, making many species from the Black, Azov and Caspian Seas well suited for the brackish conditions in the Baltic Sea (reviewed by Pauli & Briski 2018 and Dobrzycka-Krahel et al. 2023).

1.4 Ecology and ecological effects of the invasive round goby

The round goby (*Neogobius melanostomus* Pallas 1814; Fig. 2), originating from the Ponto-Caspian area, is one of the most widespread invasive fish species in the northern hemisphere (reviewed by Kornis et al. 2012). Ballast water is believed to be the vector of transport of the round goby to the Baltic Sea, where it was first found in the Gulf of Gdansk in Poland in 1990 (Skóra & Stolarski 1993). The round goby tolerance for pollutants and toxins has

likely enhanced its survival in ballast tanks in low-quality water and aided establishment, reproduction and spread in and from heavily polluted harbours where it is released (reviewed by Cerwenka et al. 2023). It thrives in both fresh and brackish water (reviewed by Kornis et al. 2012 and Cerwenka et al. 2023) and also displays high physiological performance across a broad range of temperature and oxygen conditions (Behrens et al. 2017; Christensen et al. 2021; Dickey et al. 2021). Fully marine conditions appear to restrict the spread of the round goby (Behrens et al. 2017), but it is currently expanding its range in the nearly marine conditions on the Swedish west coast (ICES 2022b; Fig. 3), indicating that adaptation to even higher salinities may be possible in the future. It is also slowly expanding its range to freshwaters around the Baltic Sea (Verliin et al. 2017; Puntila et al. 2018; Rakauskas et al. 2018; Carl et al. 2019; Florin et al. 2021).

Figure 2. Round goby from Karlskrona in Blekinge, Sweden (photos by Jens Hedlund). (A) Gravid female (left) and male exhibiting spawning coloration (right), (B) parental male, (C) catch in fyke nets 2018, (D) embryos in eyed stage.
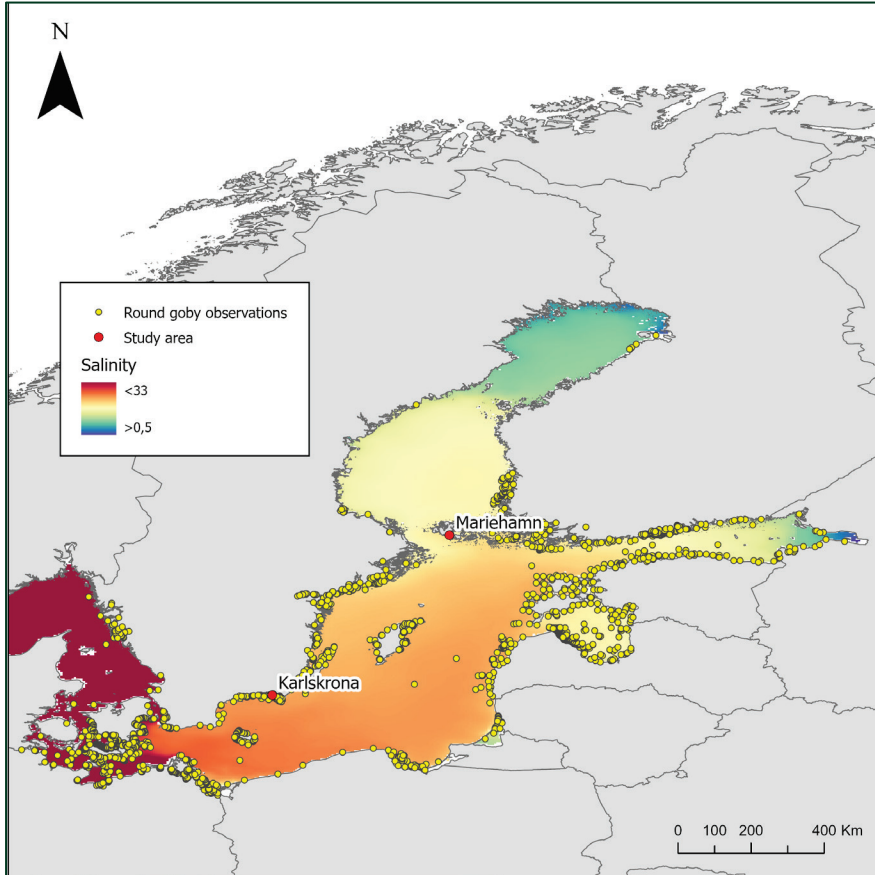
Figure 3. Map of the Baltic Sea showing reported round goby observations 1990-2022, indicated as yellow dots. Red dots show the study areas in paper II and III (credit R. Naddafi 2023).

The round goby prefers sheltered areas with low wave exposure (Kotta et al. 2016; Florin et al. 2018; Holmes et al. 2019) and rocky bottoms (Skabeikis 2019), and grows to a maximum of 22-23 cm in the Baltic Sea (Brauer et al. 2020 and references therein). In the spring, it gathers in large numbers in shallow coastal areas to spawn (Almqvist 2008; Behrens et al. 2022) and can locally reach densities of up to ~20 individuals/m² (Puntila et al. 2018). The round goby is a benthivorous generalist feeder (Skóra & Rzeznik 2001; Kornis et al. 2012; Skabeikis & Lesutienė 2015; Puntila 2016; Herlevi et al. 2018; Nõomaa et al. 2022) that competes for prey with native Baltic Sea fishes and birds (Karlson et al. 2007; Rakauskas et al. 2013; Ustups et al.

2016; Skabeikis et al. 2019; Rakauskas et al. 2020). There are indications that abundances may govern the round goby prey consumption, but results are contradictory as to whether intraspecific interactions could mediate (Kornis et al. 2014) or enhance (Paton et al. 2019) its ecological effects. There is, however, evidence that round goby functional responses (Paton et al. 2019), aggression and stress tolerance (Thorlacius 2015) increase at high round goby densities. The round goby in the Baltic Sea has also become a seasonally dominant prey for predatory fishes like cod (*Gadus morhua*), perch (*Perca fluviatilis*) and pike-perch (*Sander lucioperca*; Almqvist et al. 2010; Hempel et al. 2016; Liversage et al. 2017; Oesterwind et al. 2017; Rakauskas et al. 2020) and fish-eating birds in the southern and eastern Baltic (Jakubas & Manikowska 2011; Rakauskas et al. 2013; Oesterwind et al. 2017), and occasional prey for seals in the south-western Baltic (Scharff-Olsen et al. 2018). In invaded freshwaters in central-European rivers and canals, round goby has been found to change the abundance and composition of benthic invertebrate communities (Mikl et al. 2017; Franta et al. 2021) and compete with native demersal fish species (Roje et al. 2021). Reported from the Great Lakes is also round goby interference with native species during spawning, reducing their reproductive success (Dubs & Corkum 1996; Janssen & Jude 2001; Balshine et al. 2005; Steinhart et al. 2005; Leblanc et al. 2020). Similar ecosystem effects of round goby expansion to Baltic Sea rivers and streams cannot be excluded.

It is becoming increasingly clear that the round goby plays an important role in a multitude of food web processes at different trophic levels in the Baltic Sea, and affects native species on both the individual and population level (Ojaveer et al. 2021). As the impacts of round goby are ecosystem and context dependent (Hirsch & N'Guyen et al. 2016), it is fundamental that we increase our knowledge of its behaviour and interactions with other species under various conditions, and identify the potential threats it poses to native species and ecosystem functions (HELCOM 2021).

2. Aim of the thesis

For many invasive species, major knowledge gaps exist about their respective origins and ecology, the vectors that transported them and the quantification of ecological impacts in their new ecosystems (reviewed by Ros et al. 2023). Despite a continuously growing number of scientific studies of the round goby invasion in the Baltic Sea (Ojaveer et al. 2021), we know little about round goby functional ecology in relation to native species in this area. Further, studies of round goby in its role as predator and prey have been lacking a multispecies spatiotemporal perspective, and the importance of abundance dynamics has generally been overlooked in the study of ecological effects following the round goby invasion. The use of molecular methods also holds great promise regarding diet analysis but has, so far, rarely been used in the study of the Baltic Sea fish community (but see Jakubavičiūtė et al. 2017 and Novotny et al. 2022). Lastly, perspectives on round goby impacts beyond the 'who eats whom' angle are scarce from the Baltic Sea (Ojaveer et al. 2021), underlining the importance of investigating also other types of interactions between round goby and native species.

The overall aim of my thesis was to broaden the understanding of the round goby ecology and interactions with native species in and around the Baltic Sea. I addressed three phases over the course of invasion, namely establishment, residency and expansion. I specifically aimed to fill the knowledge gaps described above by answering the following questions:

Which functional traits distinguish the round goby from native species and may have contributed to its successful establishment in the Baltic Sea? (**Paper I**)

How does the round goby affect the Baltic Sea ecosystem as predator and prey at different environmental abundances? (**Paper II, III**)

What could be the consequences of round goby expansion to freshwater rivers and streams around the Baltic Sea? (**Paper IV**)

3. Materials and methods

3.1 Functional trait analysis and modelling of abiotic factors affecting distinctiveness

To identify functional traits that may have contributed to the successful round goby establishment and spread in the Baltic Sea, a theoretical approach was used to analyse round goby functional distinctiveness against the native species pool (paper I).

A dataset of abundance of round goby and native species was extracted from the Swedish national and regional coastal fish monitoring program as registered in the national coastal fish database KUL (https://www.slu.se/kul). The dataset covered 14 fishing locations distributed along a north-south gradient and starting in 2009, which was the first year round goby was caught in monitory fishing. In order to avoid very rare species being caught in the monitory fishing, species representing 99.5% of the observations were included in the dataset. This, coupled with complete trait information, resulted in 27 species in the dataset.

A total of 11 functional traits were selected, based on previous trait-based descriptions that have been used in recent studies of marine organisms and communities: habitat switching, parental care, territorial behaviour, diet, temperature preference, development mode, pharyngeal bones, habitat, fin type, body type and length class. I allocated each trait a score based on information from online trait data portals, scientific literature, grey literature or expert knowledge. Binary traits, e.g. parental care, were allocated a score of either 0 or 1. In cases where multiple modalities were applicable for a trait, e.g. feeding mode, the modalities received a score proportional to the total diet for each applicable modality that ultimately summed to 1. For example,

a mainly piscivorous species that occasionally feeds on benthic prey could receive a score of 0.8 for the modality 'piscivorous' and 0.2 for the modality 'benthivorous'. All trait and trait modality scores were summed per species, resulting in a unique value for each species.

The functional distinctiveness index (D), weighted by species biomass in monitory fishing and expressed as weight per unit effort, was used to determine the degree of functional similarity between the round goby and native species. The index expresses the mean functional distance of a species to all other species in a predefined community (Violle et al. 2017). If a species' D is high, it indicates that the species is functionally distinct from other species in the community (ibid.). In order to investigate round goby functional distinctiveness compared to the native species pool, the value of functional distinctiveness of round goby was compared to the values for all native species in the data set. Furthermore, a Principal Coordinate Analysis was performed to assess and compare the degree of niche overlap in trait space between round goby and native species. To assess the relative importance of each trait on functional distinctiveness, the difference was calculated between the distinctiveness values for each species based on all traits, compared to values obtained when removing each individual trait from the analysis. This was repeated for all traits, removing one trait at a time.

To investigate how environmental drivers affected the distinctiveness of round goby at each sampling site, round goby functional distinctiveness was modelled in relation to abiotic conditions, using Generalised Additive Mixed Models and Random Forests. Data of depth, bottom salinity and temperature during monitory fishing was extracted from the dataset of abundance of round goby and native species. Data of dissolved oxygen and chlorophyll a was extracted from the ice-ocean model NEMO-Nordic (https://doi.org/10.48670/moi-00013) from the Copernicus Marine Service (https://marine.copernicus.eu/) at the corresponding sampling dates. The level of coastal exposure was estimated in ArcGIS Pro as distance to the open sea. A full method description can be found in paper I.

3.2 Diet analysis of the round goby and its potential predators

In my investigation of potential ecological effects of the round goby as predator (paper II) and prey (paper III) for native Baltic Sea species, I used an empirical approach.

In these studies, I used diet analysis to capture potential spatiotemporal diet variation. The purpose was to depict the full diet of the round goby (paper II) and to assess the contribution of round goby to predator diets (paper III). Round goby and potential fish predators, cod, perch and pike (*Esox lucius*), were sampled in two areas, Karlskrona (KK; Sweden) in the southern Baltic Sea and Åland (AL; Finland) in the northern Baltic Sea (paper II, III; Fig. 3). Both are archipelago areas under heavy impact from human activities such as commercial shipping, fisheries, recreational boating and building of decks along the coast. The Baltic Sea salinity gradient results in higher salinity in KK, around 7-8 psu compared to 5-6 psu in AL (Snoeijs-Leijonmalm & Andrén 2017; see also Fig. 3), which affects species richness and composition (Koehler et al. 2022).

Fish sampling was carried out in spring and early summer of 2018 and 2019. Round goby was mainly caught in fine mesh fyke nets (paper II), while cod, perch and pike were caught in multi-mesh coastal survey gillnets and regular gillnets (paper III). The round goby population crash in the Baltic Sea in late summer 2018 (see Kruze et al. 2023) negatively affected the catches of round goby in 2019 (paper II, III). In Latvian waters, the population crash has been attributed to overfishing (Kruze et al. 2023), but the decline was evident also in my study areas, where round goby is not fished commercially. In 2018, large numbers of round gobies were caught dead or dying with skin lesions (own observations and local reports from Sweden and Finland). I therefore find it likely that the marine heat wave in the Baltic Sea in the summer of 2018 (Paalme et al. 2020), in combination with high environmental abundances of round goby (paper II, III), spurred an outbreak of parasites or pathogens which decimated the round goby population.

I studied the round goby and predator diets using visual stomach content analysis, VSCA, and DNA metabarcoding, i.e. automated detection of all species in a mixed sample (Taberlet et al. 2012; paper II, III). In VSCA, I dissected the fishes (Fig. 4), identified the prey to the lowest possible

taxonomic level, estimated the relative prey proportions of each sample and counted the individual prey items (Hyslop 1980).
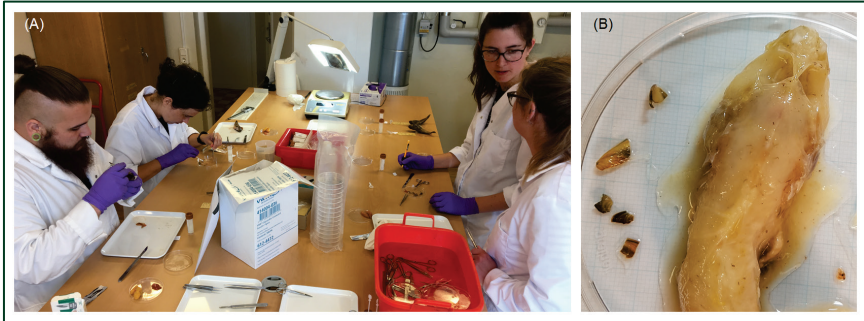

Figure 4. (A) Dissection of round goby (photo by Sofia Bureborn). (B) Round goby prey from cod caught in Karlskrona in May 2018 (photo by Isa Wallin Kihlberg).

I took DNA samples for metabarcoding of the stomach content from round goby, cod, perch and pike from a subset of the VSCA samples (paper II, III). I used two markers for metabarcoding, 12S and COI, for detection of fishes and invertebrates, respectively. The output, i.e. sequence numbers, was treated as a proxy for biomass proportions (Deagle et al. 2019), making it somewhat comparable to the relative prey proportions determined in VSCA (paper II, III).

Overall diet composition of round goby and its potential predators was summarised as relative volume proportion (V, %) and relative sequence number (S, %) for VSCA and DNA metabarcoding data (paper II, III). To depict the round goby diet variation between areas and years, I carried out redundancy analysis (paper II). By combining frequency of occurrence (the percentage of samples containing a given prey out of all non-empty stomach samples) and prey-specific proportion (the percentage contribution of a prey type to the diet of the predator individuals that consumed that specific prey type), predator feeding strategy per area and year could be visualised (Amundsen et al. 1996; paper III). To describe variation in the amount of ingested prey by potential round goby predators, I calculated relative prey weight, which gives the amount of prey in a stomach relative to the predator body weight (paper III).

To investigate how the round goby and predator diets related to relative environmental abundances of round goby, I used linear mixed effect models and general linear mixed models (paper II, III). Standardised catches of

round goby in fyke nets were used as a proxy for relative environmental abundances (in paper II termed environmental densities, in paper III termed median CPUE; hereafter termed abundances). I used stepwise model selection and studied changes in Akaike's Information Criteria (AIC) to select the best models (paper II, III). A full method description can be found in papers II and III.

3.3   Analysis of salmon behaviour during spawning

To investigate potential effects of round goby expansion to fresh water I used an empirical, experimental approach for the study of reproductive disturbance of Baltic Sea Atlantic salmon (*Salmo salar*) exposed to round goby (paper IV).

Salmon was provided by the hatchery at the SLU Fisheries Research Station by the River Dalälven in Älvkarleby in Sweden. The experiment was run in 2019 and 2021, with a break 2020 due to the COVID-19 pandemic and subsequent working restrictions. Round goby was fished with fyke nets in Muskö (2019) and Gävlebukten (2021), transported to the research station and acclimatised to fresh water upon arrival. I divided a 35,000 L stream water aquarium into two identical sections, where round goby and salmon were allowed to interact on one side (Fig. 5) and only salmon was kept on the other side as a control. Round goby densities ranged between 7.67 and 16.67 ind/m^2 in the respective years and were deemed ecologically relevant (see Puntila et al. 2018 for density estimates of round goby in the Baltic Sea). Salmon spawning behaviour (courting, digging and probing), movement patterns, aggression and time until spawning were then observed in the presence versus absence of round goby.

Figure 5. Salmon male and female in the presence of round goby during the 2019 experiment round (photo by Isa Wallin Kihlberg).

The datasets from the respective years were treated separately to account for variation in trial length between 2021 and 2019, as well as some methodological adjustments in 2021 (paper IV). Generalised linear mixed effect models were used to test whether the presence of round goby had an effect on salmon spawning behaviour, with separate models for the respective response variables (courting, digging and probing). General linear mixed effect models were further applied to analyse whether the presence of round goby had an effect on the distance between male and female of each salmon pair and to investigate the pattern of male attacks on round goby over time. In addition, a general linear mixed model was used to test if time affected the distance between male and female salmon in the presence versus absence of round goby. In the analysis of time until onset of salmon spawning in the presence versus absence of round goby, a survival analysis using the

Cox proportional hazards model was carried out. A full method description can be found in paper IV.

4. Results and discussion

4.1 Functional traits of the round goby with potentially positive effects on its establishment in the Baltic Sea

I found that round goby had a functional distinctiveness value D slightly above the median for the entire species pool (0.49 compared to 0.44; paper I). The round goby was the 9th most functionally distinct species, and in trait space located closely to black goby (*Gobius niger*), longspined bullhead (*Taurulus bubalis*), fourhorn sculpin (*Triglopsis quadricornis*), three-spine stickleback (*Gasterosteus aculeatus*), European flounder (*Platichthys flesus*) and Baltic flounder (*P. solemdali*; paper I; 'trait space' referring to the uniqueness of invader functional traits relative to the traits of native species; Brown & Barney 2021). This indicates that although round goby displays a relatively high value of distinctiveness, it is not intrinsically functionally distinct compared to the native coastal fish community in the Baltic Sea. Parental care and territorial behaviour increased round goby distinctiveness by 7.3% and 6.4%, respectively (paper I; Fig. 6). This implies that most species in the pool neither defend their territory nor protect their offspring, in contrast to round goby. Parental care increases the chances of high reproductive output (Klug & Bonsall 2014), and territorial behaviour is linked to aggression, which for invasive species can increase invasion success through successful competition (Chapple et al. 2012). Benthivory, in contrast, was one of the traits that made round goby functionally less distinct (-10.3%; paper I), indicating that benthivory is a common trait in the native species pool.
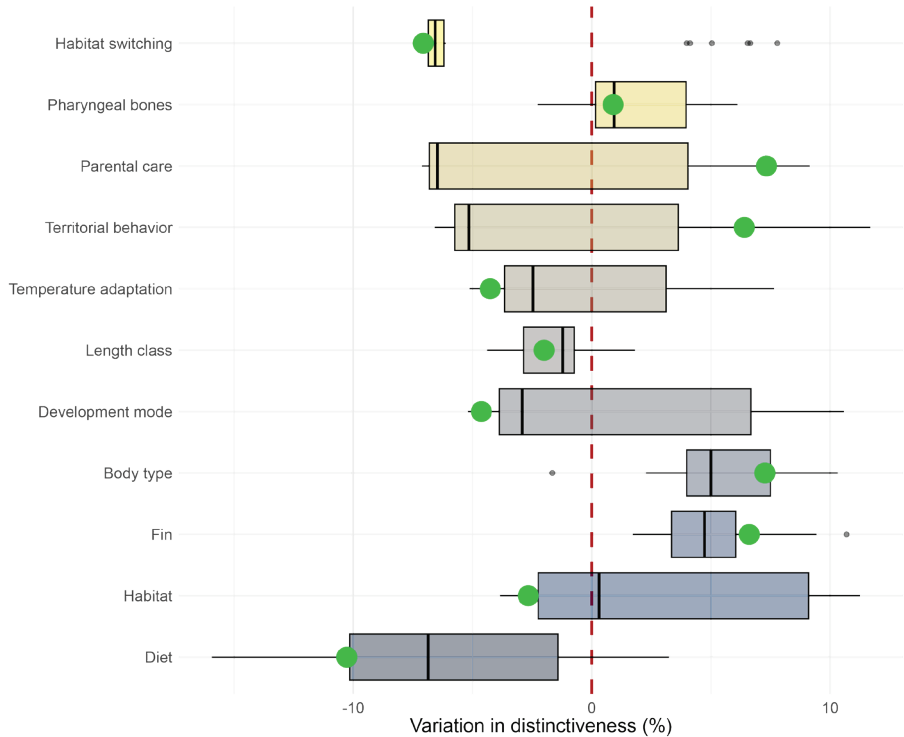
Figure 6. Variation in distinctiveness of functional traits in the Baltic Sea fish community (paper I). The round goby is represented by green circles.

Combining the trait composition with data for environmental variables (depth, bottom temperature, salinity, oxygen, chlorophyll *a* and coastal exposure; paper I), I found that round goby in the Baltic Sea shows a partial trait overlap with native fish species in its preferred habitat, i.e. more shallow, less exposed, warmer or more oxygenated areas (paper I; Kotta et al. 2016; Florin et al. 2018; Holmes et al. 2019). Such areas are dominated by species/groups like perch, cyprinids, sticklebacks (mostly in spring and summer) and other gobiids (Olsson et al. 2012; Olsson 2019; HELCOM 2018c). Round goby was closer to these species in trait space, i.e. displaying less unique traits, compared to species from colder, offshore waters like cod and clupeids (paper I; Olsson et al. 2012; HELCOM 2018c). The partial trait overlap indicates that round goby occupies a similar niche as native species in its preferred habitat (paper I). Hence, the round goby appears to have successfully established in the Baltic Sea despite niche (trait) overlap with

native species, in contrast to invasion ecology theory stating that invasive species establish in new areas by filling empty niches (Elton 1958; reviewed by Daly et al. 2023). In addition, only four species displayed body and fin types similar to round goby (longspined bullhead, black goby, eelpout *Zoarces viviparus* and burbot *Lota lota*; paper I). This implies that there may be only certain life stages of a few species for round goby to compete with e.g. for hiding places or nests, or less competition on feeding grounds that would be optimal for their elongated body shape and swimming ability derived from a rounded fin.

Invasion success depends on the performance, originality and/or plasticity of traits of the invader (Elton et al. 1958; reviewed by Daly et al. 2023), implying that an invader needs to be functionally similar to the native species to withstand local environmental conditions, but functionally dissimilar enough to give the invader a competitive advantage (Gallien et al. 2014). The round goby appears to be similar enough to native species to successfully exploit resources in the way native species do (by being benthivorous and having a demersal lifestyle), but is functionally dissimilar in traits related to reproduction (parental care) and aggression (territorial behaviour; paper I). The implied aggression may give it a competitive advantage, and my results also indicate that the number of species or groups to compete with is limited, when considering morphological traits (paper I). Thus, it is likely that the round goby combination of traits (paper I), complemented with previous knowledge of preadaptation of Ponto-Caspian species (reviewed by Pauli & Briski 2018 and Dobrzycka-Krahel et al. 2023), round goby life-history traits (reviewed by Kornis et al. 2012), physiological performance across environmental gradients (Behrens et al. 2017; Christensen et al. 2021; Dickey et al. 2021; Behrens et al. 2022; Green et al. 2023), immunological responses (Adrian-Kalchhauser et al. 2020) and possibly limited competition (paper I) in combination with high propagule pressure (Kotta et al. 2016) have contributed to the successful establishment of the round goby in the Baltic Sea.

4.2 Ecological effects of the round goby as predator and prey at different environmental abundances in the Baltic Sea

The round goby abundances in my study areas decreased by 80-90% between 2018 and 2019, as judged from fyke net catches (paper II, III). The decline was potentially the result of an outbreak of parasites or pathogens in late summer 2018 (see Materials and methods). I found environmental abundances of the round goby to be a driver of ecological effects, although the results should be treated with caution due to small sample sizes and potentially confounding factors not controlled for (paper II, III).

In my study of the round goby as predator, I found that the fish diet proportion of the round goby was high in both AL and KK, approximately 25%, when round goby abundances were high in 2018, but low, <1%, when abundances were low in 2019. The pattern was only evident in metabarcoding, but was similar for both the COI and 12S markers (paper II). Natural variation in prey densities or depletion of macroinvertebrate prey resources at high round goby abundances, with subsequent intraspecific competition and diet diversification, could potentially result in the observed increased feeding on fish prey, although I did not find any indications of substantial variation in prey densities between years in my study areas (paper II). The increased feeding on fish prey in 2018 (paper II) could also emerge from higher functional responses at higher round goby abundances (Paton et al. 2019). However, potential prey density variation and increased functional responses under different conditions are not mutually exclusive and require attention in future studies of round goby ecological impacts. The estimated variation in diet proportions between years points towards an ability of round goby to exploit various food sources, and indicates that round goby abundances may affect which types of prey are more exposed to predation.

Metabarcoding of the fish proportion of the round goby diet using the 12S marker revealed that the round goby fed on a total of 32 fish species in my study areas. The fish prey composition was slightly more diverse in AL than in KK (30 versus 24 fish species), and a larger number of species were unique to AL compared to KK (8 versus 3 fish species; paper II, Table S4). The major part of the round goby fish diet consisted of small, non-commercial fish species/groups. Sticklebacks (*Gasterosteidae*) were the main fish prey in both areas in 2018 and in KK 2019, while cyprinids (*Leuciscidae*) were the main prey in AL in 2019 (Fig. 7; paper II). However, larger species like

cod, herring (*Clupea harengus*) and perch were also detected in the round goby diet in both areas (paper II). The detection of large, predatory fish species in the round goby diet indicates predator-prey reversal, if the detection represents actual predation on early life stages and not scavenging.

In VSCA, I found the main prey items of round goby to be hydrobiid gastropods in AL and *Mytilus* sp. in KK (paper II). Furthermore, I found between-year diet variation in VSCA, much related to an increase in consumption of cardiid bivalves between years in both areas and an increase in consumption of isopods in KK in 2019 (paper II). It is possible that round goby predation in my study areas has the potential to deplete macroinvertebrate prey communities, as seen in the south-eastern Baltic Sea (Skabeikis et al. 2019; Nõomaa et al. 2022). Such a depletion could have negative effects on other mussel-eating fishes and birds, and lead to potential cascade effects when filter feeders and grazers are removed.
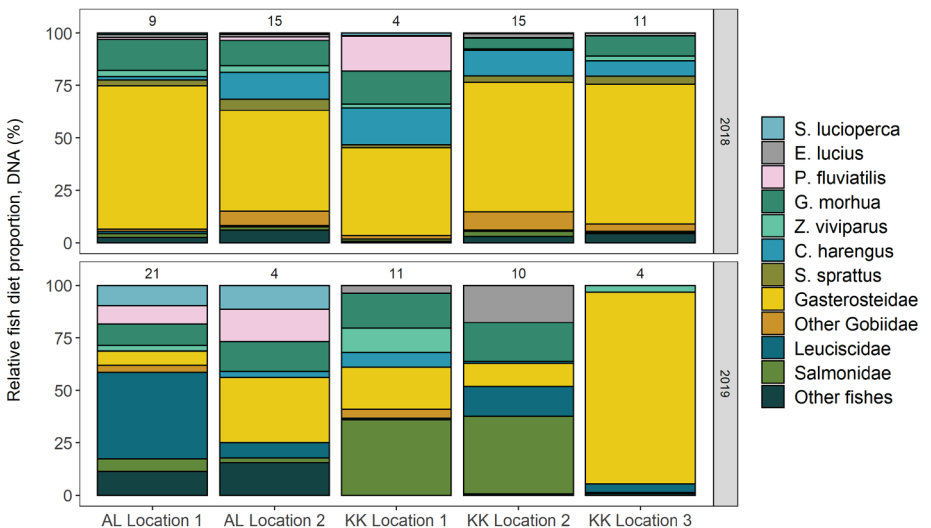

Figure 7. Spatiotemporal variation in fish diet composition of round goby in DNA metabarcoding. The relative sequence numbers of fish prey are shown for the respective round goby fishing locations in the study areas AL and KK, referring to Åland (northern Baltic Sea) and Karlskrona (southern Baltic Sea) in 2018 and 2019. Number of samples are indicated above each bar.

Also in my study of the round goby as prey, I found that round goby abundances influenced the feeding of cod and pike in KK and perch in AL, with indications of specialised feeding on round goby in 2018 when round goby abundances were high (paper III). In VSCA, round goby dominated the diets of cod and pike in KK and represented 46% of the volume proportion of the perch diet in AL in 2018 (Fig. 8; paper III). The feeding on fishes then dropped by 3-26% between 2018 and 2019 for all predator species in KK and perch in AL, while the contribution of macroinvertebrates increased by 200-2,600%. Also fish species such as perch, sticklebacks and clupeids were much more common prey in 2019, when round goby abundances were low (paper III). Relative prey weight, i.e. the amount of ingested prey in relation to predator body weight, was, however, not influenced by round goby abundances, indicating that the round goby temporarily replaces other prey in the predator diets rather than adds to the prey base (paper III).

The findings for cod corroborate results from the south-eastern Baltic Sea, showing that round goby replaces crustaceans and clupeids in the cod diet and induces a diet shift from pelagic to benthic prey (Almqvist et al. 2010; Rakauskas et al. 2020). For pike, I lacked data from AL 2018, but the pike diet was rather similar between areas in 2019, with clupeids being the main prey this year (Fig. 8). The replacement of round goby with clupeids in the diet in KK in 2019 (Fig. 8) indicates that pike largely relies on pelagic prey, but shifts to a more benthic diet when round goby abundances are high (paper III). The extensive use of round goby as prey for cod and pike made their diets more similar in KK in 2018, increasing the trophic level of cod and indicating competition between cod and pike in KK when round goby is abundant (paper III). For perch, the pattern of feeding on round goby differed between AL and KK, being higher but more variable in AL (paper III). I hypothesise that the differences between areas may be related to perch being the dominating predator in AL that overlaps spatially with round goby, as cod around AL inhabits offshore areas and pike abundances along the coast are very low. Thus, perch in AL may experience less interspecific competition in feeding on round goby and could potentially have been able to utilise round goby at a maximum level, while perch in KK may have experienced more interspecific competition from cod and pike in its feeding on round goby (paper III).
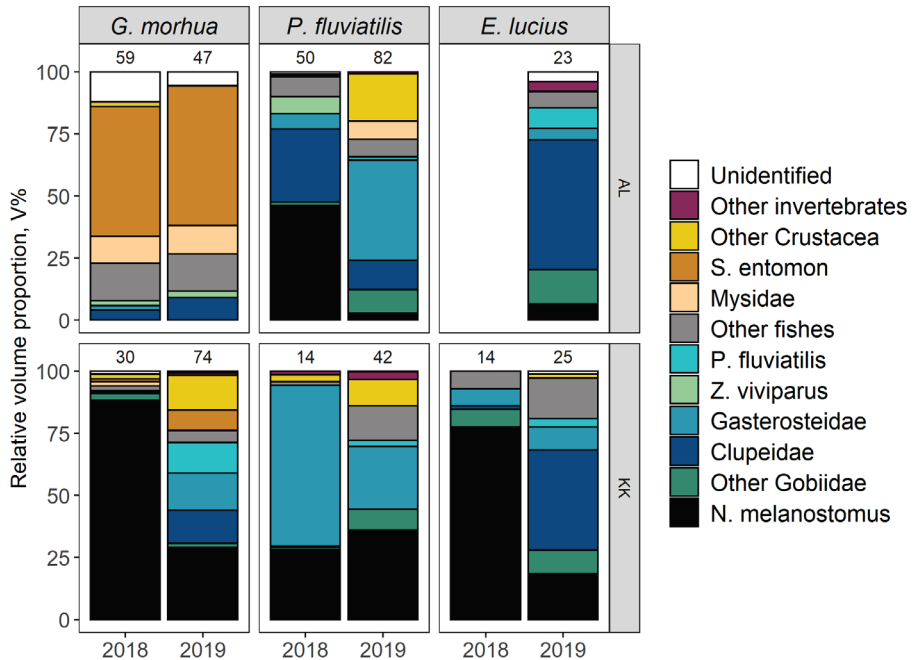
Figure 8. Spatiotemporal diet variation of cod, perch and pike shown as relative volume proportion in VSCA in 2018 and 2019. AL and KK refer to the study areas Åland (northern Baltic Sea) and Karlskrona (southern Baltic Sea). Number of samples are indicated above each bar.

High abundances of round goby skewed the diet proportions for round goby in both areas as well as for cod in KK and perch in AL, and temporarily increased the reliance on fish prey (paper II, III; Fig. 9). Diet diversification was lower for cod and pike in KK and perch in AL at high round goby abundances, but higher for the more benthivorous round goby as it fed on both fishes and invertebrates, compared to a diet consisting of almost only invertebrates at low round goby abundances (Fig. 10). I also found fewer predator-prey interactions between fishes (round goby in both areas, cod in KK and perch in AL) and macroinvertebrates at high round goby abundances, indicating temporary weakening of the trophic links between fishes and macroinvertebrates when round goby is abundant.
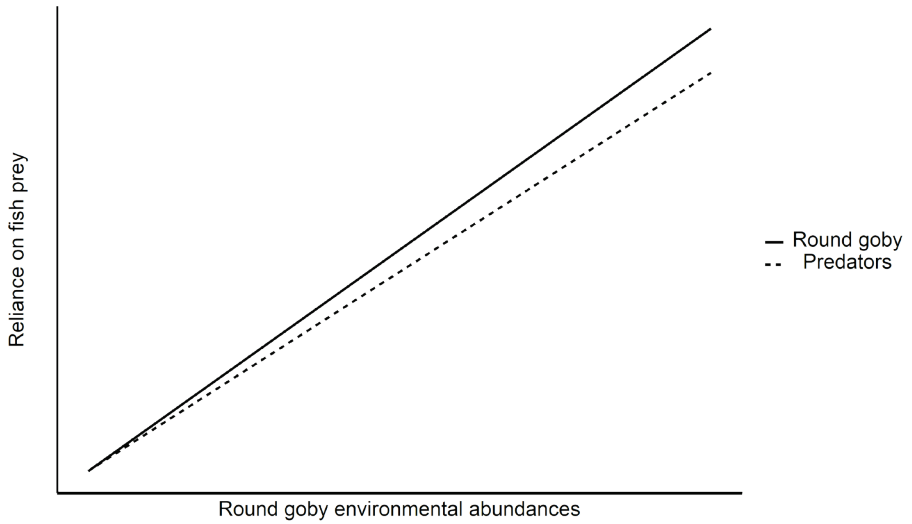
Figure 9. Conceptual figure depicting reliance on fish prey of round goby in AL and KK and predators (cod in KK and perch in AL) in relation to round goby environmental abundances.
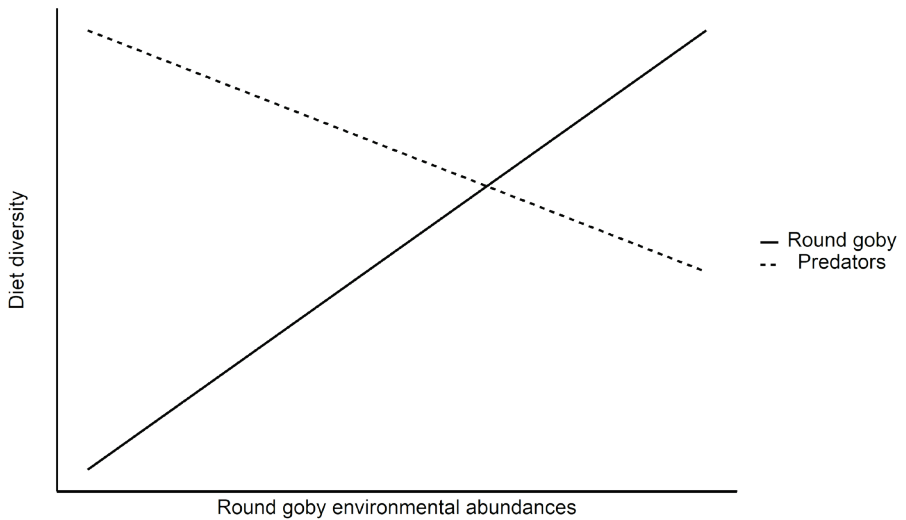

Figure 10. Conceptual figure depicting diet diversity of round goby in AL and KK and predators (cod and pike in KK and perch in AL) in relation to round goby environmental abundances.

4.3 Ecological implications of expansion of the round goby to freshwaters around the Baltic Sea

In my study of interactions between round goby and spawning salmon, I found that salmon in the presence of round goby displayed delayed spawning (Fig. 11). The chance of spawning was approximately 80% lower for salmon exposed to round goby compared to the control group at any given time (paper IV).
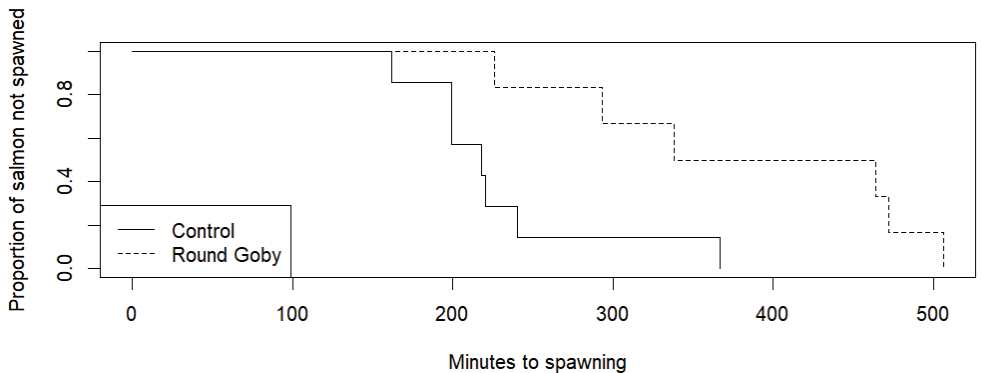

Figure 11. Comparison of time (minutes) until spawning of salmon in the presence (dashed line) versus absence (solid line) of round goby. The probability of spawning is expressed as the proportion of salmon that have not spawned at each time point.

The salmon males displayed aggression towards round goby, and, although not significant, I found indications of more frequent attacks with time over the trials (paper IV). The attacks may indicate increasing frustration of salmon males over time, but further studies are needed to investigate the mechanisms underlying this behaviour. I detected no effect of presence of round goby on salmon spawning behaviour, i.e. courting, probing and digging. However, the male and female salmon spawners resided closer to each other in the presence of round goby compared to the control in 2019. The short distance between salmon spawners in the presence of round goby 2019 may be explained by higher round goby densities this year, if high densities induce a protective behaviour in salmon that makes them stay closer together. I also found an interaction effect of time on salmon position that depended on treatment in 2019, showing that the salmon male and female spawners in the treatment initially resided closer to each other compared to

in the control, but that the distance decreased less over time compared to in the control (paper IV).

A clear limitation of the study was that neither round goby nor salmon had the option to move away, as the reproductive behaviour and aggression of fishes can be affected by competition for space both with conspecifics and heterospecifics (reviewed by Quinn 1999). Under natural conditions, salmon trying to avoid round goby may spend more time in search of a suitable spawning ground, i.e. without round goby, or are forced to utilise suboptimal spawning grounds, i.e. with round goby present. Round goby, on the other hand, may under natural conditions move away to avoid salmon aggression. However, if round goby abundances are high enough, it may be impossible for salmon to avoid both intra- and interspecific competition for space, with potential effects on salmon reproductive behaviour and/or aggression. Round goby at invasion fronts and in freshwater may also display different life history, functional and/or behavioural traits compared to in established populations under other salinity conditions (e.g. Gutowsky & Fox 2012; Thorlacius 2015; Kornis et al. 2016; Brandner et al. 2018; Masson et al. 2018; reviewed by Cerwenka et al. 2023; Galli et al. 2023). Such features may affect interactions between round goby and native species differently under natural conditions.

I interpret the delayed spawning in the presence of round goby to be an effect of salmon aggression rather than disrupted spawning behaviour. I hypothesise that the detected interactions between round goby and spawning salmon, i.e. delayed spawning in salmon in the presence of round goby and a potentially protective behaviour (paper IV), may pose negative effects on salmon fitness. It remains to be investigated whether this could have negative consequences for salmon population development in and around the Baltic Sea if the round goby continues its expansion to freshwater.

5. Management advice

Over the last three decades, the round goby has become a prominent member of coastal communities in the Baltic Sea ecosystem, but there are currently no explicit management measures in place targeting round goby in the Baltic Sea (ICES 2022b). The opportunity for early detection and subsequent eradication of round goby is long gone, but reduction of local abundances and hindering of further expansion of the established population (Ojaveer et al. 2018) is feasible. Based on my findings in this thesis, I suggest a number of management measures.

On a local scale, an efficient management measure would be to keep round goby abundances down, as I see in my studies that ecological effects are exacerbated at high round goby abundances (paper II, III). This could be achieved, for example, via reduction fishery carried out by the county administrative boards, or angling competitions arranged in cooperation with anglers' associations. To maximise the impact, such measures should be carried out in spring and early summer, when round goby abundances peak in coastal areas and other fish species are the most vulnerable to predation on eggs and larvae.

Fish predators can regulate long-term dynamics of invasive prey (Carlsson et al. 2009), and as such, predation on round goby could under certain circumstances be important in order to slow down its population growth and expansion on the local and regional scales. However, the abundance of piscivorous fish in the Baltic Sea is low in several coastal regions due to intense exploitation, making effective control of the round goby population through predation unlikely under current conditions (Ojaveer et al. 2015). If management of round goby is desired, strengthening of the large predatory fish populations in the Baltic Sea could help reach the

goal. On a regional scale, developing a round goby fishery would also likely be an efficient management measure.

No analytical stock assessment is carried out for round goby in the Baltic Sea and its total biomass is unknown (ICES 2022b). I agree with Cerwenka et al. (2023) that national monitoring programs for round goby are motivated to be able to follow population trends and get reliable abundance estimates. In addition, monitoring data is needed to quantify the ecological effects of round goby on the Baltic Sea ecosystem. Furthermore, I suggest development of a system for early detection and eradication of round goby in rivers and streams on a pan-Baltic scale to try to hinder or delay its expansion to freshwater, as I found evidence for potentially negative effects of round goby on salmon during spawning (paper IV). I also see the need for continuous research on potential ecosystem effects of round goby, as my results indicate threats towards native Baltic Sea species through round goby predation (paper II) and reproductive disturbance (paper IV).

6. Conclusions and future perspectives

In my thesis, I have studied causes and consequences of the round goby invasion in the Baltic Sea and beyond.

I found that the round goby displays similar functional traits in comparison to native species, particularly related to feeding. However, the round goby differs from many native species in the sense that it displays territorial behaviour and parental care. These traits presumably play a part both in round goby invasion success and its direct interactions with native species (paper I).

I further found that both the round goby and predator diets were affected by round goby abundances, with a larger round goby diet proportion consisting of fish at high round goby abundances, while some of the predators fed extensively on round goby when it was abundant (paper II, III). The round goby predation on filter feeders and grazers could potentially cause cascade effects in Baltic Sea coastal areas, and the detection of large predatory fish species in the round goby diet points towards predator-prey reversal (paper II). As predation by cod, perch and pike on crustaceans, other gobiids and sticklebacks increased when round goby abundances decreased but the relative prey weight of predators remained unaffected, I see indications of predation release of native prey at high round goby abundances, with potential for complex feedback mechanisms (paper III).

Lastly, I found that salmon spawning was delayed when salmon was exposed to round goby. It is further possible that the observed shorter distance between salmon spawners in the presence of round goby at high densities stems from a protective behaviour in salmon. However, I see no evidence for disrupted spawning behaviour of salmon in the presence of round goby. The results indicate that salmon reproduction could be affected

by an expansion of round goby to fresh water streams around the Baltic Sea (paper IV).

I see potential for negative effects on fish species on the same trophic level as the round goby due to competition or predation (paper I, II). I further find that lower trophic levels, i.e. first order consumers like benthic macroinvertebrates, may be negatively impacted via predation by round goby (paper II), but that the establishment of round goby is advantageous to higher trophic levels, as native predators appear to benefit from this occasionally abundant and easily caught prey (paper III). This is in line with earlier literature, showing that invasive species on lower trophic levels may have positive bottom-up effects (reviewed by David et al. 2017) or mixed effects (reviewed by Bradley et al. 2019). The negative effects of round goby as predator and positive effects of round goby as prey increased with round goby abundances (paper II, III). However, if the large predatory fishes detected in the round goby diet in metabarcoding actually represent predation and not scavenging, negative effects of round goby on higher trophic levels cannot be excluded. The round goby thus has the potential to negatively affect native species on lower, similar and higher trophic levels in the Baltic Sea.

Further, I see several interactions and ecosystem effect parallels between round goby and sticklebacks throughout ontogeny. Round goby and sticklebacks are functionally similar (paper I), indicating competition especially between sticklebacks and small round goby. Both round goby and stickleback predation on invertebrates has the potential to exacerbate eutrophication symptoms, like increased growth of filamentous algae, potentially contributing to habitat degradation along the Baltic Sea coasts (paper II; Olin et al. 2022). Sticklebacks also exhibit predator-prey reversal (Nilsson et al. 2019; Eklöf et al. 2020), and likewise, I see the potential for such ecosystem effects through round goby predation (paper II). When round goby abundances decreased, predators fed more on sticklebacks, indicating predation release of sticklebacks when round goby abundances are high (paper III), while at the same time round goby predates on sticklebacks to a large extent in some years (paper II). These ecological interactions should be explored further as they may have profound consequences for the coastal communities of the Baltic Sea (see also ICES 2022b).

In the future, the round goby will likely benefit in a number of ways from the consequences of climate change. The round goby in the Baltic Sea

commonly produces two egg batches per season (Almqvist 2008), which is less than in other areas (3-6 egg batches per season; e.g. Marsden et al. 1997; MacInnis & Corkum 2000). As round goby spawning is cued by temperature, and temperature also determines spawning intervals (Marsden et al. 1997), climate change could potentially prolong the round goby spawning season in the Baltic Sea and enable it to increase the number of egg batches per season. Higher total reproductive output could result in more years of high round goby abundances, enhancing the potential ecosystem effects and feedback mechanisms (paper II, III). Climate change in combination with higher total reproductive output per spawning season could also, in theory, fuel further northward expansion of the round goby in the Baltic Sea. Higher temperatures would probably also benefit invasive and potentially invasive invertebrates in the Baltic Sea such as zebra and quagga mussels (*Dreissena polymorpha* and *D. bugensis*), dark false mussel (*Mytilopsis leucophaeata*), gulf wedge clam (*Rangia cuneata*) and killer shrimp (*Dikerogammarus villosus*; SLU Artdatabanken n.d. a-e). Round goby expansion in freshwater has commonly been preceded by or synchronised with invasion of zebra or quagga mussel (reviewed by Cerwenka et al. 2023), a potentially important lesson when considering future round goby expansion to freshwater rivers and streams around the Baltic Sea (paper IV). If populations of the aforementioned invertebrates increase because of higher temperatures and/or less saline water in the future, these prey resources would likely be advantageous for the round goby, with potential spill-over of effects and subsequent negative impacts on local and regional biodiversity.

In conclusion, the potential implications of the round goby in its different ecological roles probably have far-reaching consequences for the vulnerable Baltic Sea ecosystem and beyond. There may also be complex feedback mechanisms operating, perhaps on different spatial and temporal scales, with effects yet impossible to overlook. This makes it our shared responsibility to try to abate the known impacts of the round goby, now and in the future.

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Popular science summary

I remember endless summer mornings on the deck by the bay on Björkö in the Stockholm archipelago, with the gulls screaming high above and the waves slowly lapping. When I dove into the water, I could easily open my eyes beneath the surface; the salinity was the same as that of tears. Massive bladder wrack swayed in the distorted sunrays in the blue-green-yellow world, giving shelter to perch and stickleback.

But the Baltic Sea is a vulnerable ecosystem. Invasive species have profoundly changed its structure and functioning over the last century, in combination with a multitude of anthropogenic stressors such as eutrophication, emissions of hazardous substances and overfishing. The round goby, one of the most widespread invasive fish species in the northern hemisphere, has been established in the Baltic since 1990. In my thesis, I used modelling to study which traits the round goby shares with native fish species, and sampled round goby and three potential predator species – cod, perch and pike – to study their respective diets over the course of two years in the northern and southern Baltic. I also experimentally studied how salmon were affected by the round goby during spawning, to investigate the potential consequences of a round goby expansion to freshwater rivers and streams around the Baltic.

I found that the round goby is functionally similar to a number of species in the coastal Baltic Sea fish community, but in contrast to most species, it exhibits territorial behaviour and parental care. It is likely that these traits give the round goby a competitive advantage that has facilitated its establishment and proliferation in the Baltic. Further, I found that the round goby displays higher functional trait overlap with fish species in warm, shallow and sheltered areas, which is the round goby's preferred habitat, compared to in colder, deeper areas. This indicates potential competition in

this preferred habitat with native fish species or groups such as perch, sticklebacks and cyprinids. In my study of the round goby as a predator, I found its major diet components to be blue mussels in the southern Baltic and hydrobiid gastropods in the northern Baltic. In addition, by using DNA analysis on the round goby's stomach content, I found that they also fed on fishes – primarily small, non-commercial species like sticklebacks and other gobiids. However, I also detected large predatory species like cod in their diet, although in lower proportions, which could indicate predator-prey reversal. My results indicate that the round goby primarily feeds on early life stages of other fish species, but the probability of scavenging cannot be excluded. I also found that the round goby constitutes prey for cod, perch and pike. In addition, both the round goby and predator diets were affected by round goby environmental abundance. When the round goby was abundant, they had a larger diet proportion of fish, and in the southern area cod and pike fed extensively on round goby when round goby was abundant. However, the predators did not feed more in total at high round goby abundance, which indicates that the round goby occasionally replaces native prey in the predator diets. If cod, perch and pike under specific circumstances feed on round goby instead of native prey, it could have substantial ecological effects. In my study of interactions between round goby and spawning salmon, I found that the presence of round goby delayed salmon spawning. The males and females of the spawning pairs also resided closer together in the presence of round goby at high densities, which could be interpreted as a protective behaviour. I did not, however, detect any effects of the presence of round goby on salmon spawning behaviour.

Based on my work in this thesis, I conclude that the potential implications of the round goby in its different ecological roles may have far-reaching effects on the vulnerable Baltic Sea ecosystem and beyond. It is also likely that climate change may further facilitate its expansion and potentially aid a number of invasive invertebrates in their establishment or spread, which could provide the round goby with additional food sources. In a couple of years, when I lie on the deck on Björkö, I may well see round gobies in the water beneath me. In order to minimize the negative effects of the round goby, I recommend management measures to reduce its abundance and prevent further spread to freshwater. Such management measures could comprise strengthening native coastal predatory fish populations, developing

a round goby fishery and developing a system for early detection and eradication of round goby in rivers and streams around the Baltic.

Populärvetenskaplig sammanfattning

Jag minns oändliga sommarmorgnar på bryggan på Björkö i Stockholms skärgård, med fiskmåsarna skriandes i skyn och vågorna stilla kluckandes under mig. När jag dök kunde jag lätt öppna ögonen under ytan; salthalten i vattnet var densamma som i tårvätska. Abborre och spigg gömde sig i stora blåstångsruskor som sakta vajade i de förvridna solstrålarna i den blågröngula världen.

Men Östersjön är ett känsligt ekosystem. Under det senaste århundradet har det förändrats i grunden av människan, som har gjort Östersjön övergödd, förorenad och överfiskad. Människan har också fört in ett stort antal invasiva arter som påverkar ekosystemet. Svartmunnad smörbult är en av de mest spridda invasiva fiskarterna på norra halvklotet och har funnits i Östersjön sedan 1990. I min avhandling har jag använt modeller för att undersöka vilka egenskaper svartmunnad smörbult delar med inhemska arter, och också samlat in svartmunnad smörbult och tre möjliga rovfiskar, abborre, gädda och torsk, för att undersöka vad de åt under två år i norra och södra Östersjön. I ett experiment studerade jag också hur närvaro av svartmunnad smörbult påverkade lax under laxens lek, för att undersöka vad som kan hända om svartmunnad smörbult sprider sig uppåt i vattendrag runt Östersjön.

Mina resultat visar att svartmunnad smörbult har liknande egenskaper som flera inhemska arter i kustnära fisksamhällen, men att den, till skillnad från de flesta inhemska arter, hävdar revir och sköter om äggen medan de utvecklas. Det kan ha gett arten en konkurrensfördel som har underlättat dess etablering i Östersjön. Svartmunnad smörbult visade sig också dela flera egenskaper med andra arter i grunda, varma och skyddade områden, där svartmunnad smörbult trivs bäst, i jämförelse med arter som framförallt lever i kallare, djupare områden. Det kan tyda på att den konkurrerar med inhemska arter som abborre, spigg och olika karpfiskar om föda eller revir.

När jag undersökte svartmunnad smörbult i dess roll som rovfisk fann jag att den framförallt åt blåmusslor i södra Östersjön och tusensnäckor i norra Östersjön. Dessutom visade DNA-analyser av dess maginnehåll att svartmunnad smörbult också äter fisk. Mest åt den små, icke-kommersiella arter som spigg och andra smörbultar, men jag fann också rester av större rovfiskar, bland annat torsk i mindre mängder. Det skulle kunna innebära att svartmunnad smörbult är rovfisk på stora inhemska rovfiskarter. Mina resultat tyder på att svartmunnad smörbult framförallt äter ägg, larver och yngel av andra arter, men jag kan inte utesluta att den också äter döda fiskar. Vidare visade mina resultat att svartmunnad smörbult utgör byte för abborre, gädda och torsk. Dessutom påverkades både vad svartmunnad smörbult och rovfiskarna äter av hur mycket svartmunnad smörbult det fanns i miljön. En större andel av svartmunnad smörbults föda utgjordes av fisk när det fanns mer svartmunnad smörbult i miljön. Av rovfiskarna åt framförallt gädda och torsk i södra Östersjön väldigt mycket svartmunnad smörbult när den förekom i stora mängder. Rovfiskarna åt däremot inte mer totalt sett när de åt mycket svartmunnad smörbult, vilket tyder på att svartmunnad smörbult under vissa perioder ersätter inhemska arter som byte, och att dessa arter därmed slipper hamna i en rovfiskmage. Om abborre, gädda och torsk under vissa förhållanden äter svartmunnad smörbult istället för inhemska byten kan det få stora ekologiska konsekvenser. När jag undersökte samspelet mellan svartmunnad smörbult och lekande lax fann jag att närvaro av svartmunnad smörbult förlängde tiden det tog för laxen att börja leka. Laxhanarna och -honorna i lekparen höll sig också närmare varandra när tätheterna av svartmunnad smörbult var högre, vilket kan vara tecken på ett skyddsbeteende. Däremot hittade jag inga effekter av närvaro av svartmunnad smörbult på laxens lekbeteende.

Baserat på mina resultat från avhandlingen drar jag slutsatsen att effekten av svartmunnad smörbult i dess olika ekologiska roller troligen kommer att ha långtgående följder både för Östersjöns känsliga ekosystem och dess omkringliggande vattendrag. Det är dessutom troligt att klimatförändringarna kommer att underlätta vidare spridning av svartmunnad smörbult, och möjligen också etablering och spridning av vissa invasiva ryggradslösa djur den skulle kunna äta. Om några år kommer jag antagligen att se svartmunnad smörbult när jag tittar ner i vattnet från bryggan på Björkö. För att minska de negativa effekterna av svartmunnad smörbult rekommenderar jag att man vidtar förvaltningsåtgärder för att hålla

nere tätheterna och förhindra spridning till sötvatten. Det kan göras till exempel genom att stärka populationerna av inhemska kustnära rovfiskar, utveckla ett fiske på svartmunnad smörbult och ta fram ett system för tidig upptäckt och utrotning av svartmunnad smörbult i rinnande vatten runt Östersjön.

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Only one person is credited in the end, but a PhD project is in fact made possible by the contributions of a vast number of people. This is my attempt to say thanks.

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(although I was usually too busy to join…), for kontorsgympa, Thursday fikas, dog walks and everything else that makes a workplace you actually want to go to on Monday mornings. If everyone had colleagues like you, the world would be a better place!

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I have heard that not everyone everywhere sees the value in a PhD, but **to my family and extended family** – thank you for seeing it, and for all the

emotional and practical support that came with it. I could not have done it without you! A special thanks to **mamma, Christina and Eva**. I am lucky to have three female PhDs with a vast amount of knowledge and experience in my inner circle.

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Öregrund, September 2023.

II

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

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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/m² (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,

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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

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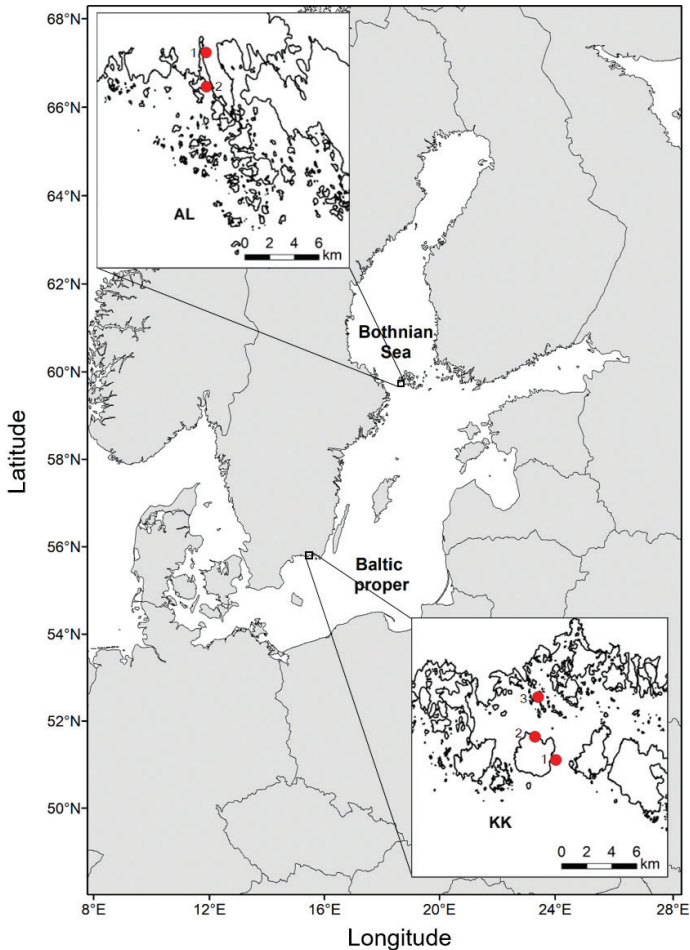
Figure 1. Round goby sampling locations in AL and KK. For coordinates, see Suppl. material 3: table S1.

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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.

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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

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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%; Bala-

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nidae 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).
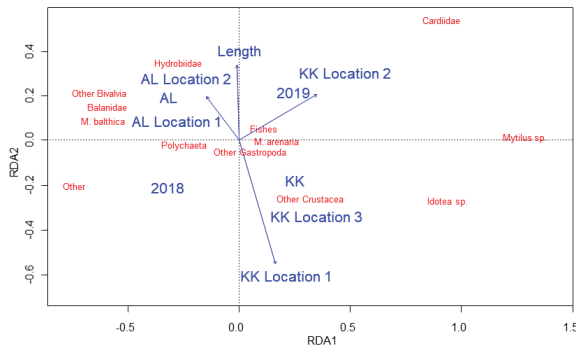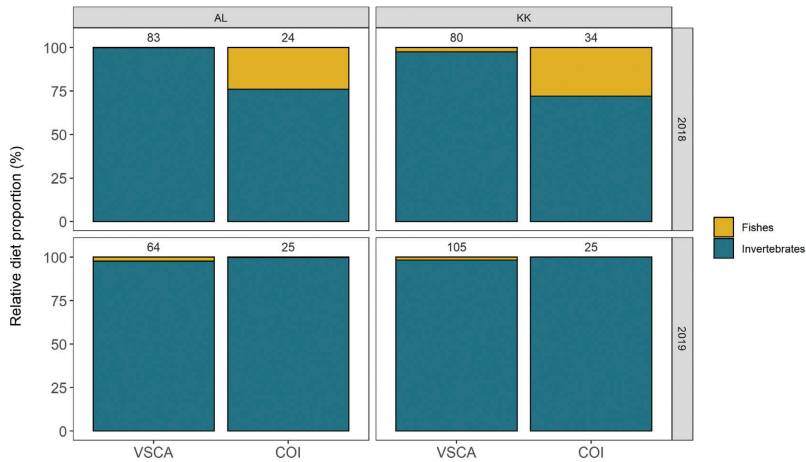

Figure 2. Spatiotemporal variation in round goby diet composition in VSCA. The axes represent linear combinations of the explanatory variables in terms of which explain the most variation of the species matrix (RDA1 0.104, RDA2 0.013).
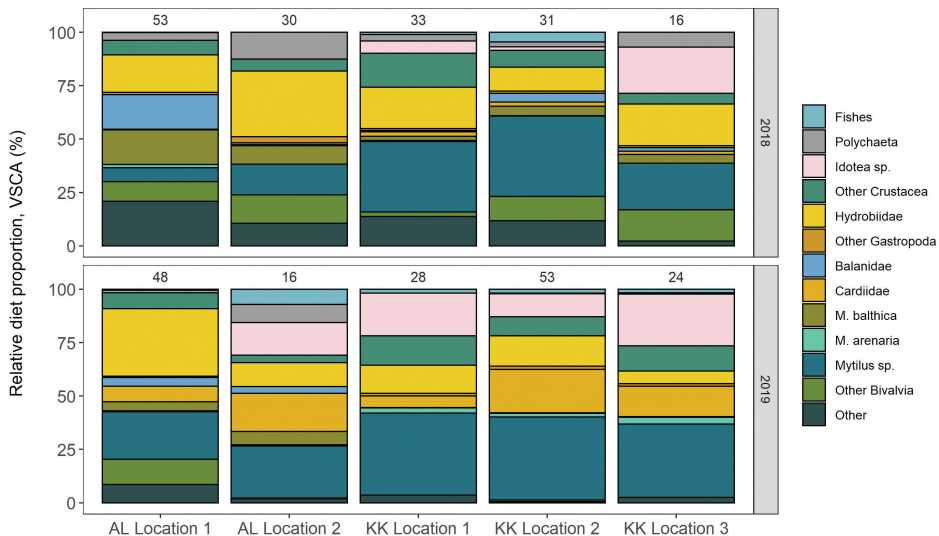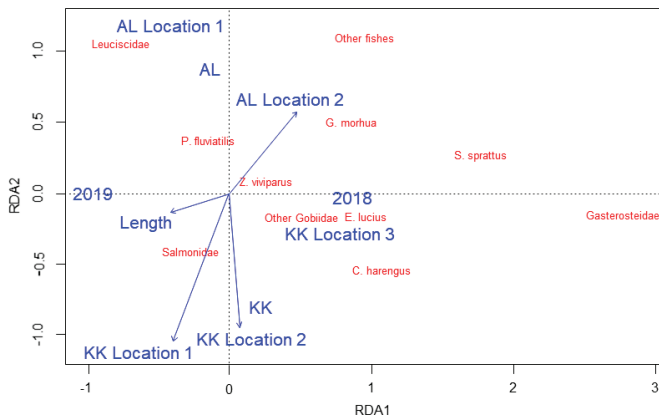

Figure 3. Spatiotemporal variation in diet volume proportion of round goby prey in VSCA. Number of samples are indicated above each bar.

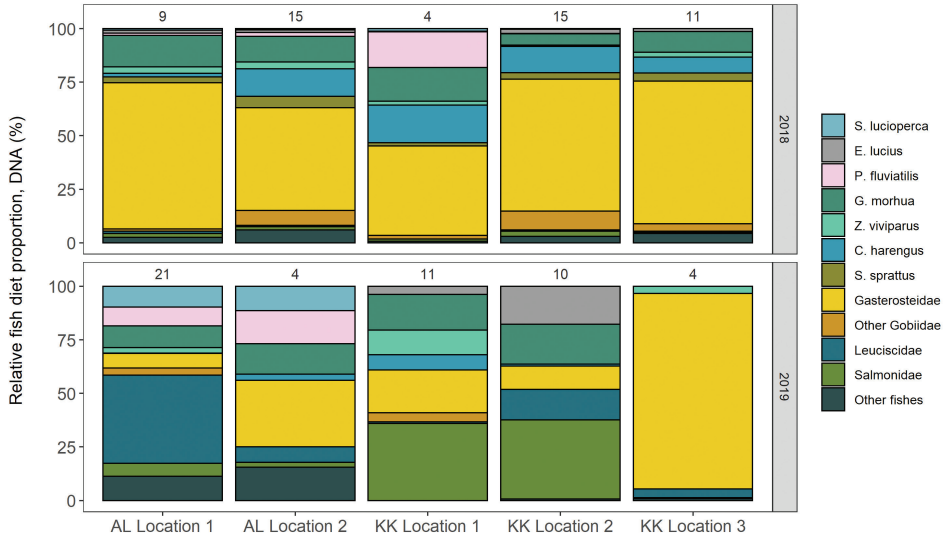## Spatiotemporal diet variation in metabarcoding

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).

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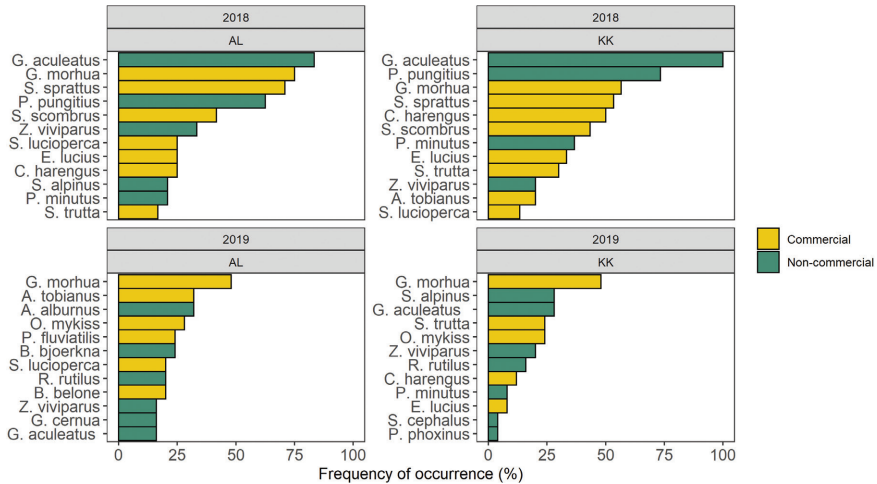
Figure 7. Spatiotemporal variation in frequency of occurrence (FO%) of fish prey DNA metabarcoding using 12S, showing the 12 fish prey with the highest FO in each area and year.

terial 2: fig. S4). Round goby densities explained marginal variation ($\Delta AIC=-1.7$), 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 ($\Delta AIC=-3.68$; Suppl. material 3: table S8), with positive associations in both areas (Fig. 9). Round goby length did not contribute to the model fit ($\Delta AIC=+0.46$).

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
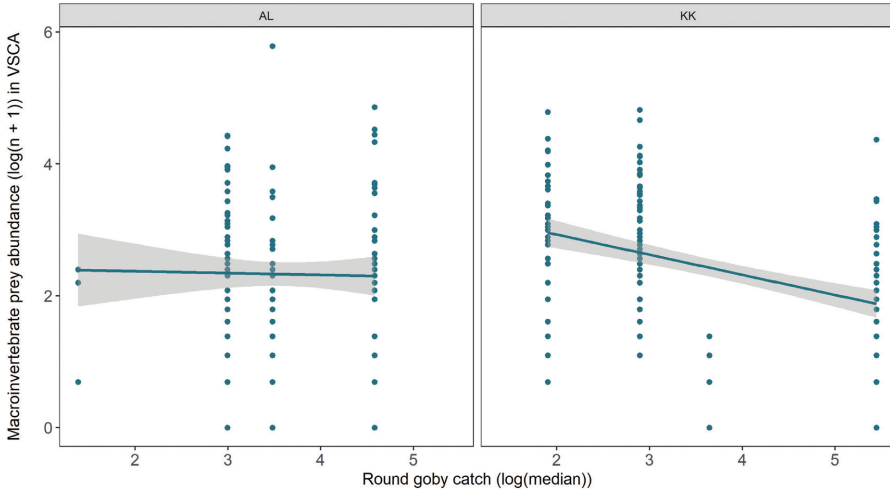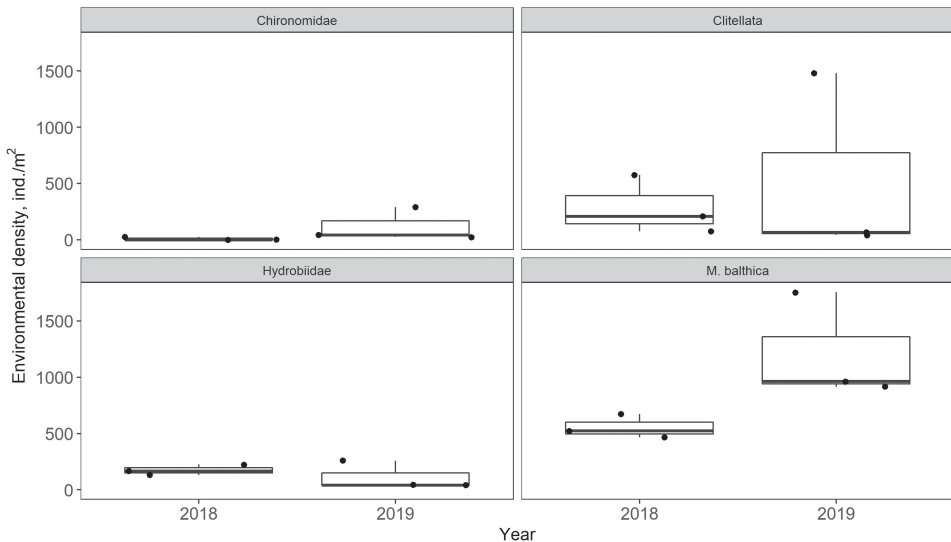
Figure 8. Relationship between number of macroinvertebrate prey in VSCA and round goby densities in AL and KK.
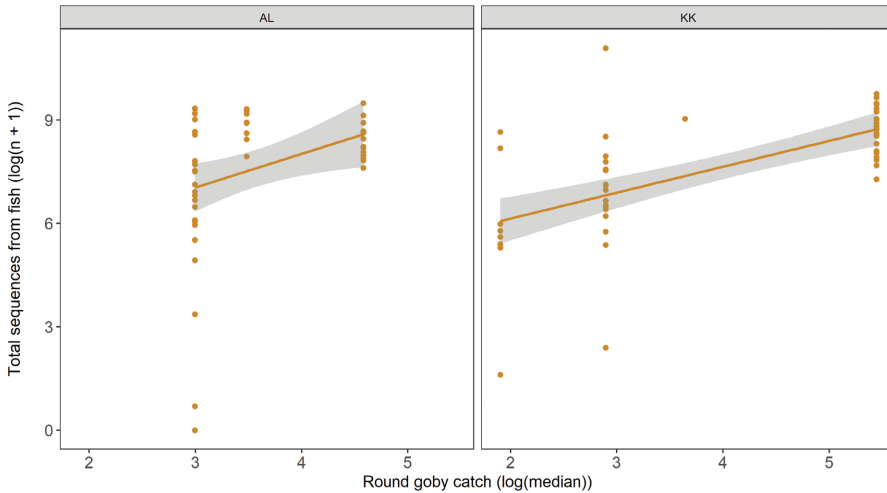
Figure 9. Relationship between number of DNA metabarcoding sequences from fish prey and round goby densities in AL and KK.

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).

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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

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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, experimen-

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tal 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

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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

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

Authors: Isa Wallin Kihlberg, Ann-Britt Florin, Karl Lundström, Örjan Östman
Data type: figures, Microsoft Word document (.docx)
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Supplementary material 3

Supplementary tables

Authors: Isa Wallin Kihlberg, Ann-Britt Florin, Karl Lundström, Örjan Östman
Data type: tables, Microsoft Excel-file (.xlsx)
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III

REGULAR PAPER

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

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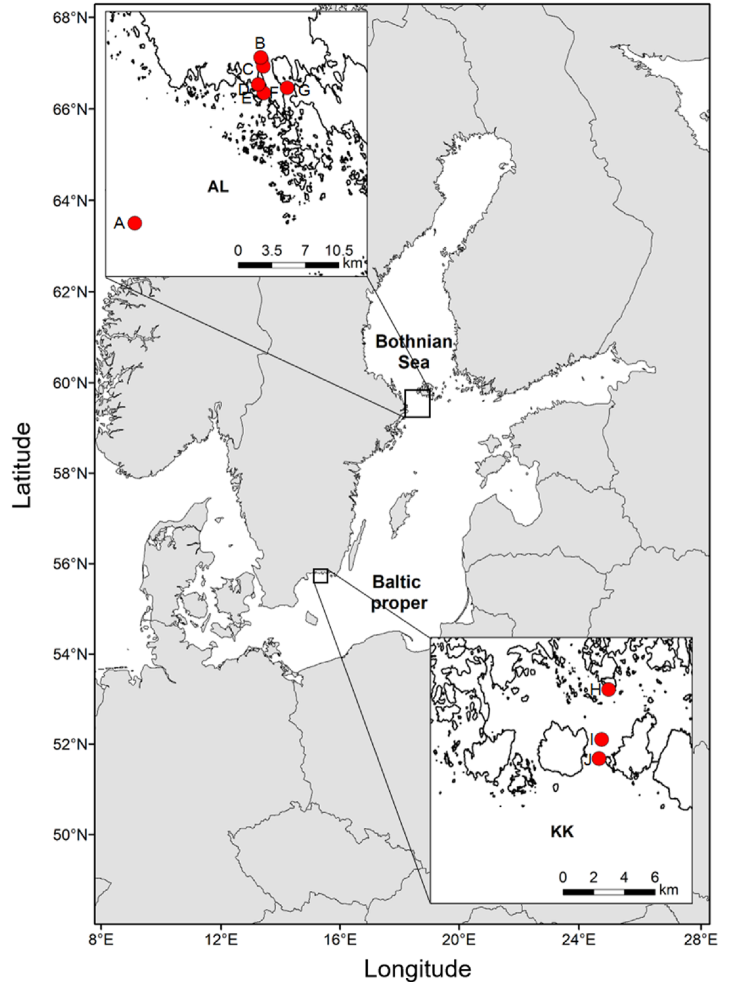
N. melanostomus as prey alters predator diet composition and feeding strategies in space and time.

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 ng μl^{-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

	G. morhua					P. fluviatilis					E. lucius				
	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)	52.6	43–64	23	2 (8)	9 (1)
2019	65.0	35–91	47	12 (20)	20 (0)	27.8	23–34	82	19 (19)	11 (0)	66.4	52–85	39	12 (24)	18 (2)
KK	46.3	32–60	104	1 (1)	16 (0)	30.4	25–43	56	11 (16)	32 (0)	64.7	52–79	14	6 (30)	9 (1)
2018	47.4	39–57	30	–	8 (0)	31.0	27–37	14	5 (26)	16 (0)	67.3	56–85	25	6 (19)	9 (1)
2019	45.8	32–60	74	1 (1)	8 (0)	30.1	25–43	42	6 (13)	16 (0)					

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; Erős 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:

$$V = P_i/P_t \times 100 \tag{1}$$

$$S = S_i/S_t \times 100 \tag{2}$$

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):

$$FO = N_i/N_t \times 100 \tag{3}$$

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:

$$RPW = M_{SC}/M_{TW} \times 100 \tag{4}$$

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):

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

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 intraspecific 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																	
	G. morhua						*P. fluviatilis*						*E. lucius*			*G. morhua*						*P. fluviatilis*						*E. lucius*					
	2018			2019			2018			2019			2019			2018			2019			2018			2019			2018			2019		
	FO	V	S	FO	V	S	FO	V	S	FO	V	S	FO	V	S	FO	V	S	FO	V	S	FO	V	S	FO	V	S	FO	V	S	FO	V	S
Fishes	41	23	99	49	27	99	100	98	99	90	73	98	100	92	98	100	94	99	100	76	99	100	96	99	95	86	97	100	100	100	100	97	100
Neogobius melanostomus	0	0	2	0	0	2	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	2	6	2	11	27	9	47	43	14	47	3	3	33	11	2	<1	7	1	24	10	8	5	7	7	<1	24	10	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	0	4	7	1	38	52	40	58
Gasterosteidae	2	2	9	0	0	9	10	6	15	57	40	31	9	5	10	3	1	9	36	15	77	71	65	55	38	25	80	7	7	<1	12	9	22
Zoarces viviparus	3	2	5	9	2	5	10	7	0	4	0	12	0	0	12	0	0	1	0	0	0	0	0	0	0	0	0	0	0	<1	0	0	<1
Perca fluviatilis	0	0	7	0	0	7	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
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	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	7	0	0	0	1
Unidentified fishes	17	8		15	5		14	6		18	6		13	5		10	2		11	3		14	1		26	14		0	0		20	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
Saduria entomon	78	52	<1	81	56	<1	0	0	<1	0	0	0	0	0	0	3	1	0	26	8	0	0	0	0	0	0	0	0	0	0	0	0	0
Mysidae	37	11	<1	40	11	<1	2	0	<1	23	7	1	4	0	0	17	2	<1	3	0	<1	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	8	2	0
Other invertebrates	0	0	<1	0	0	<1	6	0	<1	15	1	<1	4	4	0	3	0	<1	12	1	<1	29	1	<1	17	3	1	0	0	0	0	0	0
Unidentified	29	12		6	6		4	1		0	0		4	4		4	1		1	1		0	0		2	0		0	0		4	1	
Prey richness (mean ± S.E.)	1.6 ± 0.11			1.7 ± 0.11			1.4 ± 0.12			2.6 ± 0.21			1.5 ± 0.15			1.8 ± 0.15			2.8 ± 0.18			2.0 ± 0.35			1.9 ± 0.20			1.1 ± 0.10			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.
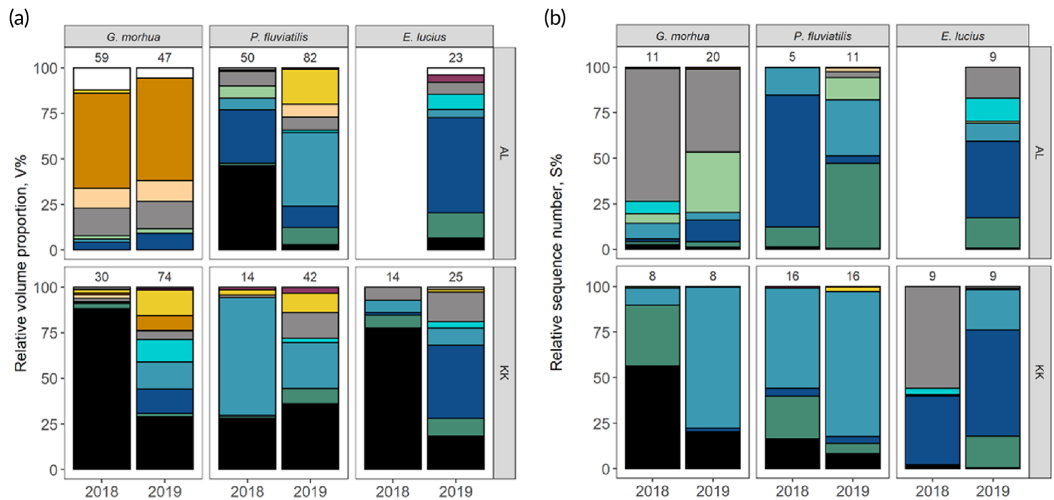
FIGURE 2 Diet composition of *Gadus morhua*, *Perca fluviatilis* and *Esox lucius* in 2018 and 2019 according to (a) VSCA (relative volume proportion, V%) and (b) DNA metabarcoding (relative sequence number, S%) in AL (Åland) and KK (Karlskrona). The number above each bar gives the number of samples 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
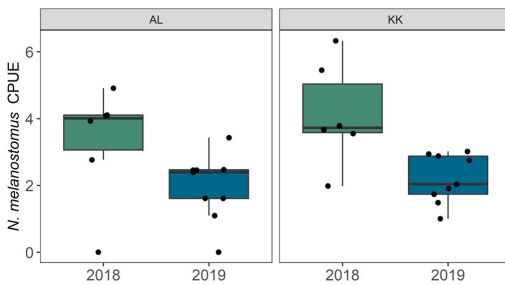
FIGURE 3 Standardized *Neogobius melanostomus* fyke net catches (log(CPUE +1)) in Åland (AL) and Karlskrona (KK) in 2018 and 2019. Each dot represents a sampling occasion (Supporting Information Appendix S1). The black vertical lines within boxes show the median values. The corresponding Mann–Whitney 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 ≤ 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
	Intercept AIC	**100.02**	81.14	**143.2**
G. morhua	*N. melanostomus* median CPUE	+2.7	**−3.39**(+)	+5
	Predator body length	+9.6	+1.96	+7.5
	Predator body length + *N. melanostomus* median CPUE	+12.4	−1.8	+12.2
E. lucius	Intercept AIC	**93.2**	47.76	**57.66**
	N. melanostomus median CPUE	+2.97	**−1.77**[a] (+)	+6.46
	Predator body length	+8.7	+1.68	+8.85
	Predator body length + *N. melanostomus* median CPUE	+11.77	**−1.83**[a] (−, +)	+15.26
P. fluviatilis	Intercept AIC	251.18	179.71	**491.82**
	Catch area	**−2.43**(KK+)	−0.75	+3
	N. melanostomus median CPUE	+4.41	+1.04	+4.47
	Predator body length	+9.36	**−2.8**(+)	+4.3
	Predator body length + *N. melanostomus* median CPUE	+13.8	−2.18	+8.7
	Catch area + *N. melanostomus* median CPUE	+2	+0.17	+7.36
	Catch area * *N. melanostomus* median CPUE	+6.5	+1.4	+9.9

Note: ΔAIC, *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.
[a]Very 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(RPW + 1)) as a function of *N. melanostomus* environmental abundances (log(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
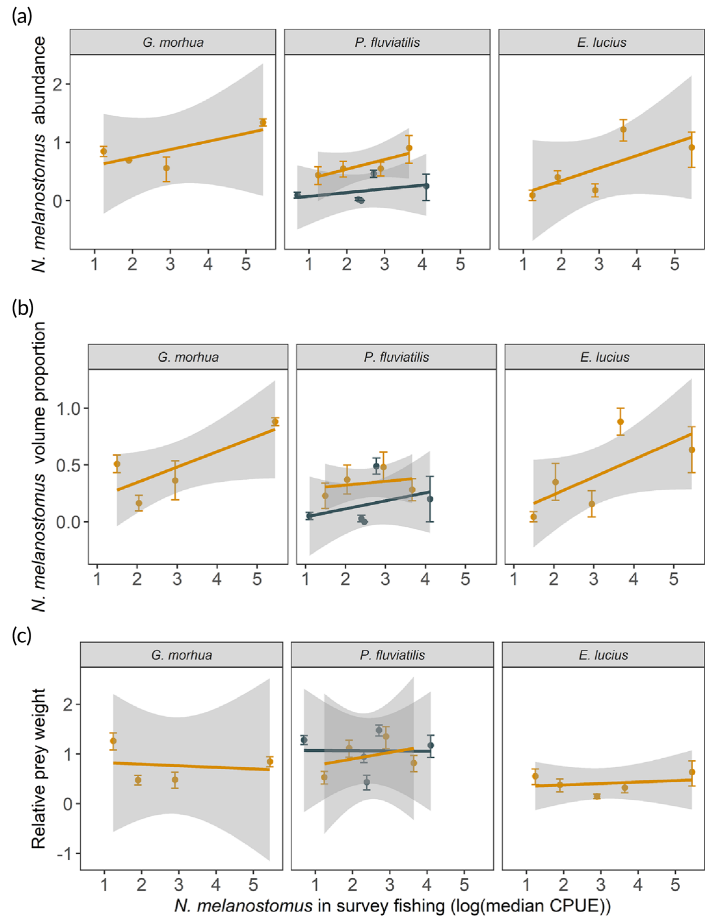
KK and *P. fluviatilis* in AL showed a 37%, 53% and 85% increase in mean prey richness from 2018 to 2019 (Table 2).

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 (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
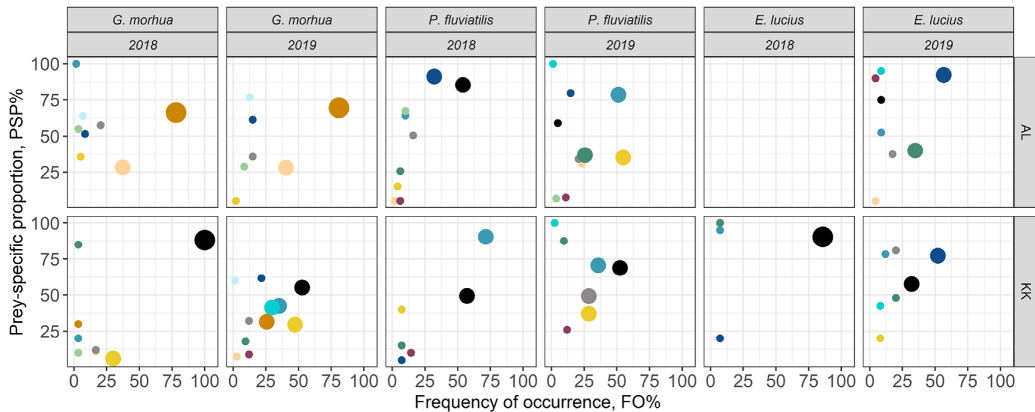
FIGURE 5 Costello-Amundsen graph depicting predator feeding strategies for *Gadus morhua*, *Perca fluviatilis* and *Esox lucius* from Åland (AL) and Karlskrona (KK) in 2018 and 2019 according to VSCA data. Prey-specific proportion (PSP) is shown on the vertical axis, where prey groups positioned in the 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 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 temporally 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_{VSCA}=460 vs. n_{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.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available at: https://zenodo.org/record/7687180#.ZD0OJXZBwuU.

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Doctoral Thesis No. 2023:75

Species invasions pose negative effects on ecosystems and societies worldwide. The Ponto-Caspian round goby was first found in the Baltic Sea in 1990. In this thesis, I show that round goby functional traits like male parental care and territorial behavior may have facilitated its establishment. I further demonstrate that environmental abundances of round goby modify its ecological effects as predator and prey, and show that the round goby has the potential to adversely affect salmon reproduction.

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