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THREE-SPINED STICKLEBACK (*GASTEROSTEUS ACULEATUS* L.) IN THE BALTIC SEA: FEEDING ECOLOGY AND IMPLICATIONS FOR STOCK IDENTIFICATION

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TRISPYGLIŲ DYGLIŲ (*GASTEROSTEUS ACULEATUS* L.) EKOLOGINĖ REIKŠMĖ BALTIJOS JŪROJE: MITYBA BEI IŠTEKLIŲ GRUPIŲ IDENTIFIKAVIMAS

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INTRODUCTION

The impact of human activities on the marine environment is steadily increasing, and in this respect the Baltic Sea is one of the most heavily affected marine ecosystems world-wide (Halpern et al., 2008). In addition to this, due to its natural complexity we often do not realize to what extent we are impacting the ecosystem. Sometimes a relatively slight change in the environment might lead to an unexpected and unwanted chain of events. A tiny fish, the three-spined stickleback (*Gasterosteus aculeatus*, hereafter referred to as 'stickleback') which inhabits the Baltic Sea, provides an example where the importance of ecological perturbation has been underestimated.

The Baltic Sea ecosystem has undergone profound changes in its structure and functioning over the past decades (Möllmann et al., 2008). Exploitation and climate-induced changes have caused a decline in abundance of predatory fish species, such as cod (Möllmann et al., 2008). Consequently, mesopredators like sprat were released, and top-down processes affected both zooplankton and phytoplankton communities (Casini et al., 2008). The decrease of cod in the offshore and pike and perch in some coastal areas may have also been beneficial to sticklebacks (Eriksson et al., 2011).

During its life cycle the stickleback utilizes offshore areas for wintering before migrating towards the coast to spawn in the spring, thereby potentially functioning as a vector linking, transferring and mediating effects between these two systems (Ljunggren et al., 2010; Eriksson et al., 2011). Over the past two decades, stickleback populations have been increasing dramatically in the Baltic Sea, evident both in coastal and offshore waters (Bergström et al., 2015). Stickleback populations now seem to be able to affect the structure and functioning of the Baltic Sea ecosystem. Recent studies indicate that sticklebacks may have a substantial impact on coastal food webs in the Baltic by exerting a trophic cascade, i.e. a chain reaction in the food web, leading to excess production of filamentous algae (Ljunggren et al., 2010; Eriksson et al., 2011;

Sieben et al., 2011a; Östman et al., 2016). The overproduction of these fast-growing algae may lead to habitat degradation, locking the food web in a mesopredator - dominated regime and reducing water quality in coastal regions of the Baltic Sea to an undesired state. It is likely that the role of sticklebacks in the ecosystem will become even more significant via their future expansion. Much less is known, however, about the role played by sticklebacks in the offshore food-web.

Sticklebacks are planktivores, as with herring and sprat, and might interfere with these commercial fish species by competing for food. In the Central Baltic, abundance of sprat and herring has declined, and the condition of these fish has deteriorated (Casini et al., 2011), which could possibly be a consequence of competition for food. Since changes in the pelagic food web may have consequences for the whole Baltic Sea ecosystem (Andersson et al., 2017), a study of the diet of sticklebacks is necessary to acquire a better understanding of their interactions in the offshore region and the extent and mechanisms by which these may be affecting commercial fisheries for sprat and herring.

Although information on fish diet composition is fundamental to understanding trophodynamics among sympatric fish species, there remain large gaps in the data available for analysis. This is especially the case for commercially unimportant, but ecologically relevant (for ecosystem functioning) fish species like sticklebacks. There is a lack of information about the stickleback's diet from a seasonal perspective and in relation to other species in the Baltic Sea. Knowledge about food webs is prerequisite for ecosystem-based fisheries management or other conservation strategies (Garcia and Cochrane, 2005). Despite numerous studies on trophic ecology in aquatic ecosystems (Belgrano et al., 2005), challenges in obtaining comprehensive, detailed and reliable information still exist. Many methods have been employed for diet determination of marine organisms: visual stomach content analysis (e.g., Lankov et al., 2010), hard parts analysis in faecal remains (e.g., Kirkman et al., 2000), and analysis of stable isotopes (e.g., Ravinet et al., 2014) or fatty acid signatures (Jo et al., 2013).

The variety of approaches that have been used implies that none of them is perfect. Since none of the conventional techniques are free from weaknesses, alternative - molecular - methods have been developed, and it is DNA metabarcoding which is now about to supplant other approaches (Pompanon et al., 2012; Kress et al., 2015). Advances in sequencing have enabled the development of DNA based identification, which has an immense potential to speed up analysis and increase the precision of prey identification. The study presented in this thesis was the first attempt to apply a DNA-based method for determining the stickleback's diet, and thus could serve for further development of the method.

Stock identification is a key part in fisheries management (Cadrin et al., 2005). An absence or deficiency in information that would otherwise identify the existence of several separate stocks of the same species, and the associated extent of heterogeneity in stock structure, can lead to overfishing or undesirable changes in biological characteristics (Begg and Waldman 1999 and references therein). Due to the substantial increase in stickleback abundance observed, potential target fishery in the Baltic Sea is now under consideration (Bergström et al., 2015), which, in turn, requires information about the population structure of sticklebacks. Knowledge of population differentiation could also facilitate an evaluation about how sticklebacks impact particular ecosystems. Morphological analysis - a tool for stock discrimination – can complement stickleback genetic studies already conducted (DeFaveri et al., 2013), since no single method can fully address stock separation and applying several approaches is highly recommended (Begg and Waldman, 1999; ICES, 2014).

Thus, although stickleback is a well-studied species in terms of its behavioural and evolutionary ecology (Huntingford and Ruiz-Gomez, 2009), its role and interactions within the Baltic Sea is poorly known. Moreover, in the context of eutrophication, global warming, invasive species and other pressures in the Baltic Sea, ecosystem might have limited resilience to buffer increasing stickleback abundance (Eklöf et al., 2012; Olsson et al., 2013). Since there is

already some evidence that sticklebacks can accelerate the negative effects of the trophic cascades (Ljunggren et al., 2010; Eriksson et al., 2011; Sieben et al., 2011a; Östman et al., 2016), there is an urgent need to obtain a better understanding of stickleback feeding ecology as well as population differentiation, and the extent to which this matters for commercial fisheries development in the Baltic.

AIM AND OBJECTIVES

The aim of this thesis was to assess the ecological role of the three-spined sticklebacks in the Baltic Sea, with emphasis on the species feeding patterns and stock delineation. Briefly, there were three main objectives: to investigate stickleback diet in the offshore region (I) and at the coast (II), and to reveal possible stickleback population differentiation (identify stocks) in the Baltic Sea by morphological analysis (III). Each objective falls into a corresponding paper (indicated by Roman numbers, see section Structure of the thesis).

Specifically, in each part we asked:

I. What do sticklebacks feed on in the offshore region of the Baltic Sea during different seasons? Does the stickleback diet overlap with that of other mesopredators like herring and sprat? What prey species do sticklebacks prefer?

II. What do sticklebacks feed on at the coast? How does diet change with fish size? Is visual stomach content analysis reliable enough? What advantages does DNA metabarcoding have in comparison with traditional visual stomach content analysis?

III. Can morphology help to distinguish stickleback stocks in the Baltic Sea? Are there differences in body shape, otolith shape or body plate number, among sticklebacks from the Baltic Sea?

SCIENTIFIC NOVELTY

There are very few studies on stickleback feeding ecology in the offshore Baltic Sea, and none which would include both seasonality and selectivity, so this study fills a knowledge gap. For the first time, an advanced DNA metabarcoding method was successfully adapted and implemented to elucidate a highly diverse stickleback diet. This study was also the first one to comprehensively and simultaneously look at the traits of different plasticity (body plates, body shape and otolith shape) in order to differentiate stickleback stocks in the Baltic Sea.

Specifically, the results may be useful: 1) in fisheries management plans for the offshore stocks of herring and sprat as well as for potential future management of stickleback; 2) for implementing an ecosystem-based approach to management in the Baltic, each of which depends on knowledge about important ecosystem components and their interactions.

STRUCTURE OF THE THESIS

This thesis consists of the following chapters: Literature Review; Material and Methods; Results; Discussion; Conclusions; Acknowledgements; Bibliography; and Papers (I-III). Based on the subject considered, the Material and Methods, Results, and Discussion chapters are each divided into three sub-sections:

I. Diet in the offshore;
II. Diet at the coast;
III. Morphology for stock identification in the Baltic Sea;

The thesis is based on the following papers, which are referred to by Roman numerals:

I. **Jakubavičiūtė, E**., Casini, M., Ložys, L., Olsson, J., 2017. Seasonal dynamics in the diet of pelagic fish species in the southwest Baltic Proper. ICES Journal of Marine Science J. du Cons. 74, 750–758. doi:10.1093/icesjms/fsw224

II. **Jakubavičiūtė, E**., Bergström U., Eklöf J., Haenel Q., Bourlat S.J., 2017. DNA metabarcoding reveals diverse diet of the three-spined stickleback in a coastal ecosystem. PLoS One, 12, e0186929. doi: 10.1371/journal.pone.0186929

III. **Jakubavičiūtė, E**., De Blick, Y., Dainys, J., Ložys, L., Olsson, J. Morphological divergence of three-spined stickleback in the Baltic Sea – implications for stock identification. Fisheries Research (submitted)

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The results of the current study were also presented at two national and two international scientific conferences, as well as three HELCOM FISH-PRO meetings.

1 STUDY SPECIES

The three-spined stickleback (*Gasterosteus aculeatus*) is a common short-lived mesopredatory fish of marine origin. It is widespread all over the northern hemisphere in various types of habitats including coastal seas, estuaries, freshwater lakes and streams, occurring both in circumarctic and temperate regions (Banister, 1986; Bell and Foster 1994a; Froese and Pauly, 2007). The stickleback can be considered as species complex, comprised of thousands of diverse marine, anadromous, and resident freshwater populations (Bell and Foster, 1994).

Sticklebacks reach about 11 cm in length, possess a streamlined body covered with plates instead of fish scales and have three dorsal and two pelvic spines. They possess an interesting mating behaviour, in which the males build a nest that they guard and oxygenate the eggs released by females. Males become reddish when they reach reproductive condition, while females show minor body colour changes (Bell and Foster, 1994).

Due to its unique life history and huge diversity, the stickleback became an eco-genomic model organism, well-studied in terms of behavioural and evolutionary ecology (Bell and Foster, 1994; Huntingford and Ruiz-Gomez, 2009; Des Roches et al., 2013; Hendry et al., 2013). For many years it has been serving as an outstanding model species, research on which shaped the understanding of genomic and phenotypic variation, speciation and eco-evolutionary dynamics (Hendry et al., 2013). However, its role in the functioning of ecosystems is much less investigated, especially in the sea.

Sticklebacks can migrate quite far in the open ocean, they have been reported 110 km (in the Atlantic ocean, Cowen et al., 1991) or even 800 km from the shore (in the Gulf of Alaska, Morrow, 1980). Sticklebacks (usually anadromous forms) generally die after spawning due to exhaustion (Wootton, 1984). Only

freshwater residents experience more than one breeding season (Kottelat and Freyhof, 2007).

Over recent decades, the abundance of the stickleback has increased considerably in the Baltic Sea, especially in the central Baltic proper (ICES rectangles SD27-29) and along the western coast (Bergström et al., 2015). Although reasons for this increase are not yet established, warming climate, release from both coastal and offshore predators, and increased prey abundance may be explanatory factors (Ljunggren et al., 2010; Eriksson et al., 2011; Lefébure et al., 2014). In terms of their respective roles in the Baltic Sea food web, species like cod, herring, and sprat in the Baltic Sea have received much more attention, while knowledge gaps related to commercial unimportant fish, like sticklebacks, are yet to be filled.

2 DIET OF THE STICKLEBACK

Records of the stickleback diet extend back to the XIX century, when sticklebacks were claimed to be voracious eaters: worms, insects and larvae, crustaceans, fish eggs and fry, even infusoria have been recorded as their prey (Smitt, 1892; Leiner, 1930). Sticklebacks inhabit many different ecosystems and habitats, and their diet varies accordingly (see summary in Table S3, Paper II). In freshwater systems, they are known to prey on both planktonic and benthic organisms. In streams, they feed on chironomids, copepods, oligochaetes, cladocerans, ostracods, even on the smallest individuals, rotiferas and diatoms (Hynes, 1950). In freshwater oligotrophic lakes, sticklebacks actively choose to consume cladocerans; amphipods, chironomids, molluscs, ostracods, Trichoptera larvae make a large part of their menu as well (Campbell, 1991). In a highly vegetated river, for example, sticklebacks were found to prefer the largest prey available at the time: cladocerans *Daphnia* sp. in May, Simuliidae larvae in June, and chironomids in July (Dukowska et al., 2009). In marine waters of the White Sea, with Zostera seagrass beds, copepods *Temora longicornis*, *Microsetella norvegica*, ciliates, oligochaetes and chironomids

Orthocladiinae were the main prey for juvenile sticklebacks (Demchuk et al., 2015).

In the pelagic areas of the brackish Baltic Sea, where sticklebacks spend a large part of their life, they feed primarily on cladocerans like *Bosmina* spp., *Podon* spp., *Cercopagis pengoi* and calanoid copepods *Eurytemora affinis*, *Temora longicornis*, *Acartia* spp. (Leinikki, 1995; Peltonen et al., 2004; Lankov et al., 2010). Whereas at the coast, the main prey items are Diptera larvae, harpacticoids and amphipods (Frande et al., 1993; Candolin et al., 2016).

Thus, it is clear that sticklebacks have a broad diet, however, usually due to limitations of the methods used (difficulties with visual taxonomic identification of a digested material), prey items are often identified to only a coarse level. Moreover, information is generally insufficient to identify which of the prey items the sticklebacks prefer to eat, i.e. stomach content compared with what is available in the environment and which species are accessible. This becomes an issue for the application results from dietary studies - in defining precise predator-prey species relations in order to deepen the knowledge about the role sticklebacks play in a food web.

2.1 Selectivity

Sticklebacks' preference for a certain prey depends not only on prey profitability (largest energetic value taking handling into account), but also on hunger level, the size of the prey, and how often the fish encounters the prey (Hart and Ison, 1991). Selection behaviour might also differ between individuals of the same species (Wootton, 1990). Ideally, individual resource specialisation should be assessed. Nevertheless, some common features are prominent.

Firstly, sticklebacks are known to selectively prey upon the cladocerans (Podonidae, *Bosmina* sp., *Daphnia* sp.), but avoid the calanoid copepod *Acartia* spp., a tendency consistent both in marine and freshwater populations (Campbell, 1991; Leinikki, 1995; Lankov et al., 2010). Cladocerans have a slower predator avoidance response compared to the fast-swimming copepods,

making the former easier and less energy-consuming to capture (Drenner et al., 1978; Viitasalo et al., 2001).

However, the seasonal feeding dynamics of sticklebacks in relation to the availability of their prey have rarely been explored. *Bosmina* spp. and *Eurytemora affinis* were found to be favoured during summer (Leinikki, 1995; Lankov et al., 2010). *E. affinis* might be preferred because of its conspicuous egg sacs and *Bosmina* spp. due to its pigmented eggs and their low escape response (Flinkman et al., 1992; Viitasalo et al., 2001).

Concerning benthic prey, sticklebacks tend to avoid gastropods (Reiss et al., 2014), and prefer amphipods (gammarids) (Sieben et al., 2011a, 2011b), a tendency proven from both laboratory experiments and field surveys.

It should be noted, however, that the stickleback mouth width and gape size obviously influence the type and size of the prey that can be eaten (Hart and Ison, 1991; Lavin and McPhail, 1986). Hart and Ison (1991) found that the size threshold of prey rejection was at 6 -7 mm, and Byström et al. (2015) suggested an upper limit of around 5 mm. Also, jaw morphology (gape size, gill raker spacing) can change food handling efficiency (Ibrahim and Huntingford, 1988). Therefore, the optimum diet might differ for different stickleback populations and/or habitats depending on their morphology. Some fish are better adapted to benthic (or littoral) prey, and others for limnetic (or pelagic) prey (Lavin and McPhail, 1986) (see section 5.3 Stickleback morphology).

2.2 Feeding behaviour

Sticklebacks consume more food in the summer than in the winter, with a peak in June (Bell and Foster, 1994), which is a pattern consistent with other temperate fish species. Sticklebacks feed only in the light (Wootton, 1984). Prey encounter rate depends on habitat complexity (e.g., physical structures in the littoral), water turbidity, size, colour, and behaviour of the prey (Eggers, 1977; Eggers, 1982; Bell and Foster, 1994). For instance, the copepod *Acartia hudsonica* was found to change its migration behaviour due to predation by sticklebacks (Bollens and Frost, 1989). Sticklebacks were found to prefer red

over darker colours of prey, fast over slow movement, and larger over smaller size (Ibrahim and Huntingford, 1989). However, prey choice is a much more complex process.

How sticklebacks exploit resources, which are often patchy, depends on prey availability, hunger level, presence of other sticklebacks, and the threat of predation. When hungry, sticklebacks tend to attack dense aggregations of prey (e.g., zooplankton), but when hunger decreases, they choose prey with lower density, where conditions to avoid predators are better since they still have to be vigilant against their own predators (Ohguchi, 1981).

Thus, stickleback prey choice is determined by many factors, including but not limited to fish and prey properties: fish and prey size, prey availability, habitat structure, visual capacities of the fish, presence of predators and their conspecifics, even infection with parasites (Bell and Foster, 1994). For instance, to avoid predation from adult sticklebacks, juveniles stick to the littoral zone and their foraging behaviour changes under certain circumstances (Foster et al., 1988).

Since many of the features mentioned above vary with season, seasonality is important to consider when estimating selectivity for a particular prey.

3 THE STICKLEBACK'S ROLE IN ECOSYSTEM FUNCTION – BALTIC SEA

Recent studies indicate that sticklebacks may have substantial impact on coastal food webs in the Baltic Sea by precipitating a trophic cascade, i.e. a chain reaction in the food web, leading to excess production of filamentous algae (Ljunggren et al., 2010; Eriksson et al., 2011; Sieben et al., 2011a). High densities of sticklebacks can change community composition by consuming considerable amounts of grazers such as amphipods (Sieben et al., 2011b), and increase primary production (Candolin et al., 2016). Consequently, the overproduction of fast-growing algae may lead to habitat degradation, locking the food web and shifting the water quality in coastal regions of the Baltic Sea towards a by humans undesired state.

The strong preference of sticklebacks for cladocerans, from a food-web perspective, might indicate competition with juvenile stages of other fish. Ljunggren et al. (2010) suggested that recruitment of coastal predatory fish in the Baltic Sea (pike and perch) was impaired by limited food availability (zooplankton) for their larvae, due to competition with sticklebacks. The three-spined stickleback has indeed been shown to be able to deplete zooplankton communities in brackish water lagoons (Jakobsen et al., 2003).

Moreover, fish eggs (albeit including their own) have been reported in the stomachs of sticklebacks (Hynes, 1950; Greenbank and Nelson, 1959; Delbeek and Williams, 1987; Dukowska et al., 2009; Kotterba et al., 2014; Byström et al., 2015). Sticklebacks have also been found to feed on small pike and perch larvae, which would constitute a more direct effect on populations of large predators, than competition (Byström et al., 2015).

Top-down effects are of no less importance as bottom-up controls in the coastal ecosystems of North Atlantic region (Östman et al., 2016). Mesopredators (including stickleback) increase the biomass of ephemeral algae via trophic cascades and consequently magnify eutrophication symptoms. To restore coastal food webs and increase resilience of coastal ecosystems, management measures have been suggested, one of which is a fishery targeting those mesopredators (Östman et al., 2016).

4 METHODS FOR DIET ELUCIDATION

In this chapter, the main focus is on two methods that are used for diet elucidation: 1) the most common - traditional visual stomach content analysis based on morphology keys, and 2) more advanced, and undergoing rapid development - DNA metabarcoding. Other methods are only briefly mentioned as they were beyond the scope of this study.

4.1 Visual

Visual stomach content analysis has been the most common method for investigating the feeding ecology of fishes so far and in many cases, it remains a standard practice (Hyslop, 1980; Manko, 2016). It is a relatively simple and straightforward procedure, which can be routinely implemented. With this tool, one can obtain a valuable information on the numbers, sizes and types of prey as well as the total meal size (Hyslop, 1980). However, it relies heavily on taxonomic expertise and provides only a snapshot of the diet i.e., represents only those prey that were ingested very recently. To counterbalance this drawback of low temporal resolution, and to get a reliable picture (to draw reliable conclusions about the diet), many samples are needed. Moreover, based on morphology, very often it is impossible to assign prey item to any prey category due to the extent of their digestion, and attempts to do so introduces bias that cannot be quantified (Baker et al., 2014). Prey differ in digestion rates, which may explain under (over) representation of a certain prey items (Hyslop 1980, and references therein). Another weakness is related to the enumeration methods employed – frequency of occurrence, percentage by numbers, or percentage by weight – all can lead to very different conclusions when determining diet (Hyslop, 1980; Baker et al., 2014).

4.2 Other methods

Other methods such as stable isotope and fatty acid analysis can provide valuable information on energy flow through the food web, however, these methods do not enable precise determination of the contributions of different prey species to the diet (Hardy et al., 2010).

Stable isotope analysis - a common method in trophic ecology - relies on ratios of stable isotopes of nitrogen ($^{15}N/^{14}N$), carbon ($^{13}C/^{12}C$), and sulphur ($^{34}S/^{32}S$) which assimilate in tissue in different rates (Michener and Kaufman, 2008). The technique has an advantage of providing information on what was actually consumed and assimilated into a predator's body, not just what was ingested (Chen et al., 2012). Contrary to conventional stomach content analysis, stable

isotope analysis provides a long-term signal from the diet (Perga and Gerdeaux, 2005).

Another biomarker used to get dietary data is fatty acid composition. This approach is built on the fact that species differ in fatty acid composition and signatures of these can be detected in predators' tissue (Iverson et al., 2004). Stable isotope analysis when combined with fatty acid analysis can provide good insight into material and energy flows through the complex food web (Jo et al., 2013). They cannot, however, precisely define contributions of different prey species to the diet and typically cannot deliver species-level taxonomic resolution (Iverson et al., 2004; Hardy et al., 2010).

4.3 Molecular approaches – the beginning

Molecular techniques were developed as an alternative to conventional methods (Symondson, 2002). They have emerged as a very promising tool since they can overcome many limitations of the older methods: it is possible to detect prey at a very high resolution (species or even stage level), accelerate sample analysis, and to get information from what were traditionally considered to be uninformative samples. Initially, molecular diet studies applied immunological approaches, or enzyme electrophoresis to identify prey specific proteins (Solomon, 1996). Later on, due to development of polymerase chain reaction technology (PCR, Mullis et al., 1986), DNA-based studies came to the fore-front of diet research (Symondson, 2002; Deagle, 2006; King et al., 2008; Pompanon et al., 2012; Sousa et al., 2016).

Asahida et al. (1997) were the first to successfully amplify prey DNA extracted from predator gut samples. Both nuclear rRNA (Hoogendoorn and Heimpel, 2001) and mitochondrial genes (mtDNA) have been used as targets in predator–prey studies, however, mitochondrial genes (mtDNA) dominate (Sheppard and Harwood, 2005; King et al., 2008; Harms-Tuohy et al., 2016; Thalinger et al., 2016). Mitochondrial gene cytochrome c oxidase subunit I (CO1) rapidly became a standard in barcoding animal species (Hebert et al., 2003a, 2003b). Barcoding is species identification using a standardized DNA region - barcode

(Hebert et al., 2005). DNA barcode sequences are recovered and then matched against a reference database (such as Barcode of Life Database, BOLD) for accurate taxon identification. Nowadays, DNA barcoding is widely used in many fields of ecology, conservation and evolution, including studies of trophic interaction and food webs (Valentini et al., 2009; Joly et al., 2014; Kress et al., 2015).

What makes CO1 gene a desirable target, is that, firstly, it is highly conserved across species and thus can be amplified from unknown organisms, and secondly, there are many copies of CO1 in a single cell (Hoy, 1994), making it possible to successfully extract and then amplify (during PCR) the desired region. Moreover, there is a good balance between gene conservancy and variability: sequences change slowly enough over time that they are likely to be identical in the same species, but fast enough that they differ between species (Folmer et al., 1994; Hebert et al., 2003a; King et al., 2008). Last but not least, for taxonomic identification extensive libraries of reference sequences are necessary, without which, obviously, taxonomic identification cannot be done. So far, CO1 is the most widely available sequence region in public reference libraries – no other genetic region has databases of sequences covering so many taxa (Leray et al., 2013b; Deagle et al., 2014).

4.4 DNA metabarcoding

Eventually development of high-throughput sequencing enabled the rise of DNA metabarcoding – combination of next generation sequencing (NGS) and barcoding to identify multiple species in a sample simultaneously (Taberlet et al., 2012). Universal PCR primers are used to mass-amplify DNA barcodes from bulk samples, such as environmental samples or gut contents. Thus, while barcoding usually refers to single species identification, DNA metabarcoding is capable of identifying multiple species in a sample (Taberlet et al., 2012; Kress et al., 2015).

Metabarcoding has now proven to be a valuable tool for trophic interaction investigation (Pompanon et al., 2012; Soininen et al., 2013; Berry et al., 2015;

Diaz-Real et al., 2015; Sousa et al., 2016). It has also recently been introduced to fish feeding ecology studies (Leray et al., 2013b, 2015). With a suitable reference library, metabarcoding can offer high taxonomic resolution, and detect prey that might otherwise be unidentifiable, e.g., eggs, larvae, morphologically convergent or heavily digested prey. In providing reliable and high-resolution diet data, metabarcoding can benefit ecosystem modelling, ecosystem-based management and monitoring strategies (Bourlat et al., 2013; Berry et al., 2015). Beside these advantages, DNA-based methods, however, still possess the same shortcoming as conventional visual stomach content analysis - i.e., provide only a snapshot in time.

5 MORPHOLOGY

5.1 Morphology as a basis for fish stock determination

Plenty of techniques exist to define fish stocks– from molecular (DNA, proteins, RNA), to phenotypic traits (e.g., body size, parasite load) or demography (e.g., age/size distribution) investigations (Begg and Waldman, 1999; Cadrin et al., 2005; Östman et al., 2017). No single approach is capable of fully addressing stock separation, hence using more than one method for the same population is recommended (ICES, 2014). Morphology analysis - one of the phenotype approaches – can complement, for instance, genetic studies (Begg and Waldman, 1999; Karahan et al., 2014). It serves as a tool for stock discrimination which in turn is essential information for better understanding of population structure. Techniques for morphological analysis are briefly reviewed in the following sections.

5.2 Morphometrics

Morphometry is the investigation of shape and its covariation with other factors (Bookstein, 1991). Differences in shape may be driven by many processes, including, but not limited, to ontogenesis, evolutionary diversification or disease (Zelditch et al., 2012). As a result, morphometric tools are employed in various types of studies, from investigations of local adaptations to taxonomic

assignments. The main approaches for making quantitative descriptions of the morphology of an organism include traditional and geometric morphometrics.

Traditional morphometrics (TM) comprise linear measurements of length, depth, and width, such as the standard length of fish, and body depth. It can also include angles or counts - meristic attributes, which are discontinuous variables, e.g., number of body plates, number of gill rakers, or fin rays (Reyment, 1996). However, TM does not provide much information about shape: although ratios among lengths are used as a proxy for the shape, it is often extremely complicated to separate information about shape from size (Atchley and Anderson, 1978). Moreover, some information obtained is actually redundant because some measurements partly overlap, and, since measurements often derive from the same attribute, they are not independent.

To overcome these shortcomings, a shift in methodology has taken place. Alternative methods were developed to investigate shape, and this "revolution in morphometrics" opened a new field of geometric morphometrics (Rohlf and Marcus, 1993), which has been rapidly elaborated and successfully used for various scientific purposes (Adams et al., 2004; Dean et al., 2013).

Geometric morphometrics (GM) is the quantitative representation and analysis of morphological shape using geometric coordinates instead of measurements. Information about shape stems from arrays of coordinates rather than simple linear distances between points. GM enables one to visualise and quantitatively compare the differences between the shapes (Zelditch et al., 2012). One of the biggest advantages of employing GM is that it is possible to separate size information from shape. Two main approaches in GM exist: 'landmark-based' and 'outline' methods. 'Landmark-based' GM methods collect coordinates of landmarks – biologically homologous points on the structure. After assembling coordinates, a standard procedure of landmark data analysis follows, i.e. Procrustes paradigm is implemented (Dean et al., 2013). It removes differences in location, orientation and size (see also Materials and Methods section, and Paper III). 'Outline' methods in GM digitize points along the outline, and fit

them with a function (Adams et al., 2004). An otolith outline, for example, can be examined using Fourier analysis (Tracey et al., 2006), or Wavelet functions (e.g., Sadighzadeh et al., 2014).

Very often differences in shape are associated with differences in size (ratios among dimensions) of an organism, a relationship that is referred to as allometry (Klingenberg, 1998; Dean et al., 2013). In GM, shape is 'all geometric information that remains when location, scale and rotational effects are filtered out from an object' (Kendall, 1977). Thus, in order to compare the shapes among groups of interest (e.g. body shapes between different fish populations), there is a need to account for allometry (e.g., Webster et al., 2011).

5.3 Stickleback morphology

Morphological traits are especially useful to study environmentally induced differentiation (Kinsey et al., 1994). Numerous studies have investigated morphological variation of sticklebacks as adaptation to diverse freshwater environments (Bell and Foster, 1994; Walker, 1997; Barrett, 2010; Aguirre and Bell, 2012). After isolation in freshwater environments, sticklebacks have undergone considerable diversification, and various aspects of their morphology, like body size (e.g., Snyder, 1991), body shape (e.g., Walker, 1997), or body armoring (e.g., Reimchen, 2000) have diversified and evolved within and among populations. Both biotic and abiotic factors may influence stickleback morphology. Concentration of calcium ions in the water (Spence et al., 2012), salinity (Barrett, 2010) and temperature (Reimchen, 2000) may affect the body armor development of sticklebacks. Higher salinity can induce shallower bodies in sticklebacks (Mazzarella et al., 2015) as a means to improve swimming performance in open habitats (Blake et al., 2005). In a complex habitat - highly vegetated freshwater environment - body plates may be lost in order to increase manoeuvrability and thereby improve their capability to escape from predators (Barber and Nattleship, 2010).

Among biotic factors, predation is by far most well-documented to influence stickleback morphology, especially in small freshwater habitats (Bell and Foster,

1994; Reimchen, 2000; Walker and Bell, 2000; Cano et al., 2008; Barber and Nattleship, 2010; Leinonen et al., 2011). As a response to different predation pressures and types of predation, lateral body plate development and body depth of sticklebacks can alter. Selection towards a higher number of body plates may be triggered by piscivorous fish (Gross, 1978; Reimchen, 1983), while the response to macroinvertebrate or bird predation may cause a reduction of body plates (Reimchen, 1983; Bergstrom, 2002; Zanella et al., 2015). Meanwhile, slender bodies among freshwater sticklebacks have been suggested to be a result of low predation (Walker and Bell, 2000).

The most prominent division in stickleback feeding patterns is driven by their adaptation to different habitats (pelagic vs benthic). Morphology is a key here. Several morphotypes of the stickleback are distinguished: benthic, limnetic and intermediate (Lavin and McPhail, 1986). Those having smaller inter-raker distances, i.e. more gill rakers, are more effective in feeding on zooplankton than benthos (Ibrahim and Huntingford, 1988). Limnetic and intermediate types tend to prey in the water column, whereas benthic – at the bottom of the water body.

Differences in morphology (body shape) have also been observed between anadromous and freshwater stickleback populations (Walker and Bell, 2000), and between stream and lake populations (Berner et al., 2008), and within lakes (McPhail, 1984; Walker, 1997; Willacker et al., 2010). Less is known, however, about differentiation among sticklebacks within the marine environment - whether they diverge in morphology in less heterogenic marine environments.

5.4 Stickleback differentiation in the Baltic Sea

Spatial structure of stickleback population in the Baltic Sea has been investigated based mainly on genetic sub-division (DeFaveri et al., 2013; DeFaveri and Merilä, 2013) or spatial synchrony in demography (Östman et al., 2017). Genetic studies (DeFaveri et al., 2013) revealed two major genetic clusters of stickleback in the Baltic Sea: one in the eastern, and the other in the western Baltic Sea. Previous studies also revealed some phenotypic differentiation of the species along the coast (DeFaveri and Merilä, 2013), but studies from the offshore

region are still lacking. Demographic studies revealed that sticklebacks have global demographic changes, i.e. change in abundance from year to year correlates among sites (Östman et al., 2017).

The main methods are presented in this section. For a more detailed method description, please refer to the relevant paper (I-III).

6 DIET IN THE OFFSHORE (I)

6.1 Sampling

To study the diet of sticklebacks in the offshore (their role in the pelagic food web), in relation to other mesopredators (herring and sprat), samples were collected in Kalmar Sound, south-western Baltic Proper (Figure 1). Although Kalmar Sound is a semi-enclosed area, it is dominated by pelagic fish species and thus suitable for pelagic food web studies. Fish (herring, sprat and sticklebacks) were caught with a pelagic trawl in three different seasons: spring, summer and fall, in 2009-2011. A random sample of ca. 10-60 fish, depending on the size of the catches, was taken from each haul for stomach content analysis.

Zooplankton (mesozooplankton > 200 μm and a fraction of microzooplankton 20–200 μm) were sampled monthly in 2009 – 2010 by vertical tows from 50 m depth to the surface or from the seabed to the surface at depths <50 m using a WP2 zooplankton net with 90 μm mesh size. Zooplankton samples were analysed according to the HELCOM manual (HELCOM, 2015), and identified to the lowest taxonomic level possible (for a detailed description of the zooplankton sampling and analyses see Diaz-Gil et al., 2014).

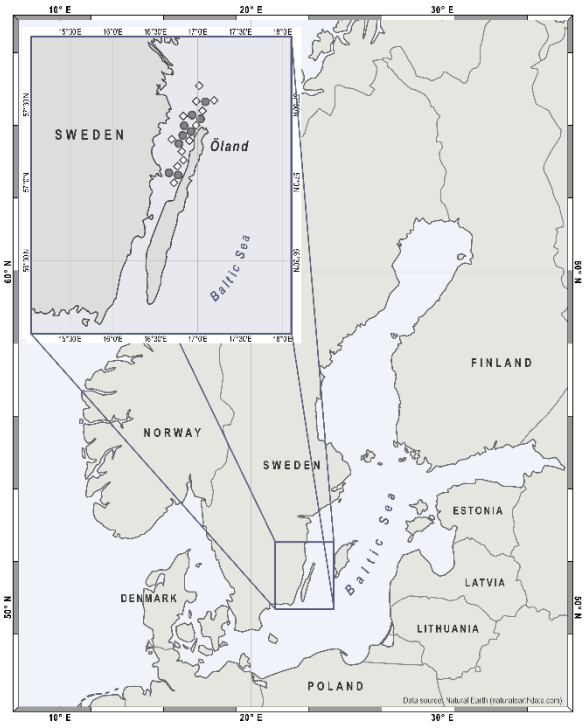

Figure 1. Study area, Kalmar Sound, southwest Baltic Proper. Filled circles indicate fish sampling stations; open rectangles zooplankton sampling sites.

6.2 Diet analysis

Prey items in the stomachs of the fish were sorted under a stereo-microscope and identified to the lowest taxonomic level possible. Larger food organisms (Mysidae, Insecta) were counted separately. The remaining part (zooplankton) of each sample was diluted in water, and a subsample of at least 100 zooplankton individuals from each stomach of herring, sprat, and stickleback was counted under stereo-microscope in a Bogorov chamber. The development stage was determined for all copepods in the subsample (nauplii, copepodits C1–C3, C4–C5, and adults C6 male/ female), and the length for all other zooplankton items in the subsample was measured (µm). In total, 498 fish stomachs were analysed (N stickleback=163, N herring=186, N sprat=149).

The diet composition of the different prey types for the three fish species was expressed using a numerical index (N_i), which is the average proportion of individuals of the *i*th prey type with respect to the total number of prey consumed of a single fish (Hyslop, 1980).

To assess diet overlap between the fish species, a simplified Morisita index (C_H) was calculated (Horn, 1966):

$$C_H = 2\sum p_{ij}p_{ik}(\sum p_{ij}^{\ 2} + \sum p_{ik}^{\ 2})^{-1} \quad (1)$$

where *j* and *k* are the fish species, and p_{ij} and p_{ik} are the proportions of the prey *i* of the total prey consumed by the two species (i = 1, 2, 3 ... , n).

Selectivity for a certain prey type was investigated using the V-selectivity index (Pearre, 1982) which has been used previously in studies of clupeid diet (Casini et al., 2004):

$$V = \pm(\mathrm{Chi}^2/n)^{½} \quad (2)$$

where n is the number of observations (total abundance of zooplankton in the sea sample and in the stomachs). The average proportion by number of certain prey species in the zooplankton samples (standardized to 1 m^3) and in the fish stomach was used in the V estimation (see Pearre, 1982 for detailed description). The index ranges from -1 (absolute rejection) to +1 (absolute preference), and a zero value indicates no-selection.

To assess the precision of the diet estimation, a bootstrapping technique was used (Tirasin and Jørgensen, 1999), while sample size–sufficiency for assessing the diet was tested using cumulative prey curves (Hurtubia, 1973) (see Paper I).

7 DIET AT THE COAST (II)

7.1 Sampling

To investigate the diet of sticklebacks in neritic the coastal waters, sampling was performed in 16 bays situated along a 350 km stretch of the central Swedish Baltic Sea coast, in May 2014 (Figure 2). Sampling of stickleback stomachs was performed as part of a larger survey targeting the entire food webs of shallow vegetated bays (Donadi et al., 2017). Sticklebacks were caught using Nordic survey gillnets (European Union 112 standardized method EN 14757:2005).

After sticklebacks were removed from the nets they were immediately immersed in 95% ethanol.

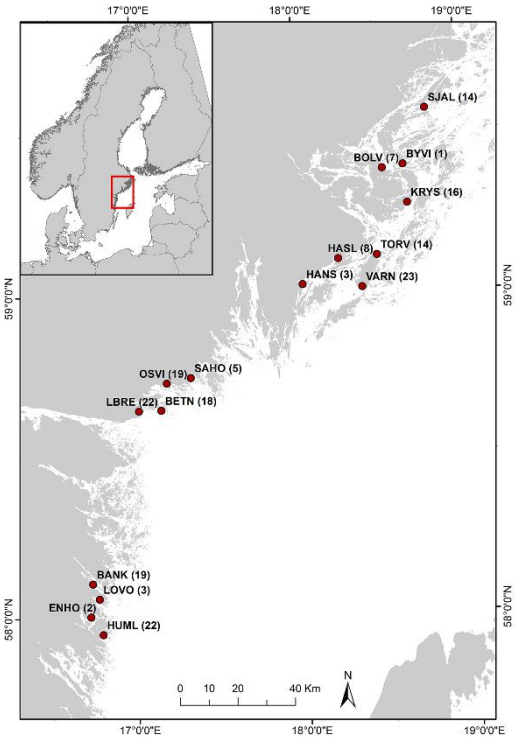

Figure 2. Sampling sites for diet study at the coast. Numbers in brackets indicate number of sticklebacks analysed from each bay.

7.2 Diet analysis. Visual stomach content analysis

A combination of classic (visual stomach content analysis) and emerging (DNA metabarcoding) methods was used for diet elucidation. The same 192 individual sticklebacks were analysed using both visual stomach content analysis and metabarcoding; an additional 4 stickleback individuals were used in a pilot study for DNA metabarcoding. The stomachs were dissected and flushed with 80% EtOH to remove all stomach contents. To avoid cross-contamination, the dissection tools were rinsed with soap, bleach, and Milli-Q water before each individual dissection. Prey items visually distinguishable in the flushed stomach contents were identified to the highest taxonomic resolution possible, using a stereo microscope (magnification 20-80x). Frequency of occurrence for each prey item was estimated (%F_{vis}, the percentage of stomachs in which a prey was

present). Thereafter, all stomach contents were stored at -20° C in 80% EtOH for subsequent DNA extraction.

7.3 DNA metabarcoding

7.3.1 Sample processing

DNA was extracted from the 196 sticklebacks' gut contents using the UltraClean® Tissue and Cells DNA Isolation Kit (MO BIO Laboratories). The dual PCR amplification method was used for Illumina MiSeq library preparation (Bourlat et al., 2016). The cytochrome oxidase 1 (CO1) marker was first amplified using locus specific primers including an Illumina adapter overhang (amplicon PCR). The primers were based on (Leray et al., 2013b) 'mini-barcode' yielding a 313 bp fragment (CO1mini_mICOIintF_MiSeq: TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG**GGWACWGGWTGAACWGTWTAYCCYCC**, CO1_dgHCO2198_MiSeq: GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG**TAAACTTCAGGGTGACCAAARAAYCA,** CO1 specific sequence is shown in bold, and Illumina adapter in regular font). A blocking primer was used in the amplicon PCR, to prevent amplification from *G. aculeatus*, following (Leray et al., 2013a).

For each sample, two independent PCR reactions were performed and later pooled, ensuring greater coverage of prey items amplified.

Amplicon PCRs were performed as 30 µl reactions with 20pm of each primer and 100pm of blocking primer and using Pfu proofreading DNA polymerase (Promega). Cycling conditions were as follows: 2 min at 95°C (1x); 1 min at 95°C, 45s at 55°C, 1 min at 72°C (40x); 5 min at 72°C (1x); hold at 4°C. Amplicons were checked on a 2% agarose gel. Agencourt® AMPure® XP paramagnetic beads (Beckman Coulter) were used to purify the PCR products. DNA quantification was carried out using a Qubit Fluorometer (Invitrogen) and the average fragment size was verified using Tapestation (Agilent Technologies). Pooled libraries were sequenced as paired-ends using Illumina MiSeq Reagent v3, producing 30 103 790 paired-end reads of 300 bp in length.

7.3.2 Bioinformatic data processing

The processing steps were performed using Qiime (Quantitative Insights into Microbial Ecology) version 1.9.1 (Caporaso et al., 2010) and custom python scripts. Paired-end joining was done using the Qiime fastq-join tool. A 48% sequence loss was observed after the paired-end joining step due to poor sequence quality at read ends, resulting in 15 706 724 joined reads (the raw data are available from the NCBI sequence read archive under accession number SRP101702, BioProject number PRJNA378633). Dual indexes and Illumina overhangs were removed by the sequencing platform. Primer sequences were removed using a custom python script, resulting in 10 982 728 reads (a 30% loss). Due to its stringency, the script quality filters sequences by removing incomplete reads or chimeras. Additional quality filtering with Qiime removed 3% of the reads, resulting in 10 641 526 reads. Finally, remaining chimeric reads were excluded using UCHIME (Edgar et al., 2011), producing a final dataset of 10 586 546 reads (0.5% loss).

The Bayesian clustering algorithm CROP was used to cluster the sequences into operational taxonomic units (OTUs) based on the natural distribution of the data, using a Gaussian model (Hao et al., 2011).

For taxonomic assignment of CO1 sequences, a custom database was created, consisting in a taxonomy file associated with a reference sequence file, of Metazoan sequences retrieved from BOLD (http://www.boldsystems.org/ downloaded in March 2016), combined with own reference databases of Chironomidae, Nemertea, Xenacoelomorpha and Oligochaeta and barcodes of Swedish Echinodermata, Mollusca, Cnidaria and Arthropoda from the Swedish Barcode of Life database (SweBol).

Taxonomic assignment was done using a 97% similarity threshold using the Uclust software implemented in Qiime with the default parameters (Edgar, 2010).

7.3.3 Data analysis

After sequencing, an OTU (operational taxonomic unit) table was obtained, showing the number of reads per taxon found in the stomach of each fish. For diet derived from the barcoding identification, frequency of occurrence was estimated ($\%F_{bar}$) - the percentage of stomachs in which a prey (OTU) was present. Also, to estimate the relative abundance of a certain prey in the stomach, and to make data from different fish individuals comparable, numbers of reads were normalized to the total number of reads in each sample (individual), and proportions of different taxa in each stomach were estimated (hereafter termed as '$\%N_{bar}$').

To investigate the effect of fish body size (mm TL) on the diet and account for the hierarchical data structure, we performed permutational multivariate analysis of variance (PERMANOVA, *adonis* function in the vegan package for R, Oksanen et al., 2016) on the Bray–Curtis distance matrix with 'bay' (16 levels) as strata, fish size group as fixed predictor, and diet composition (counts of stomach with a certain prey present) as a response. Fish were divided into two size groups (TL): ≤6.5 cm (S), and >6.5 cm (L).

7.4 Comparison of the methods

The results from the visual analysis and the metabarcoding analysis were compared with respect to both number of taxa identified and to the taxonomic resolution of the data. To compare the methods with respect to their taxonomic resolution, ranks were given to each prey item in each stomach and then mean taxonomic rank of the stomach was used (Berry et al., 2015). Taxonomic resolution was ranked as follows: species = 1, genus = 2, family = 3, infra-order = 4, order = 5, infra-class = 6, class = 7, phylum = 8. Paired t-tests were used to compare the resolution between the methods.

7.5 Prey selectivity estimation

At each station within the 16 bays (there were 3-5 stations in each bay), zooplankton assemblages were sampled using vertical tows from 0.5 m depth to the surface. The samples were fixated in a 4% formalin solution. Zooplankton

analysis was carried out under HELCOM manual (HELCOM, 2013). Simultaneously, benthos was also surveyed as it was a part of a larger study (for details see Donadi et al., 2017).

An analysis of stickleback prey selection was performed based on the results from metabarcoding, since in visual stomach content analysis neither quantification nor taxonomic resolution was reliable enough due to digestion. The proportion of OTU reads per sample was used as quantitative measure for diet items in the stomach. Feeding selectivity was measured using Jacob's index (Jacobs, 1974):

$$D=(r-p)/(r+p-2pr) \quad (3)$$

where r is the proportion of number of OTU reads of certain prey species in stomach ($\%N_{bar}$), and p is the proportion of certain prey abundance in the environment (zooplankton/ benthos). This is a modified Ivlev's electivity index, corrected for item depletion (Jacobs, 1974). It has a range between -1 and 1, the former indicating the strongest negative selection, and the latter indicating the strongest positive selection, and 0 corresponds to random utilization. To test whether selectivity (Jacob's index) significantly deviated from 0, a one-sample non-parametric Wilcoxon Signed Rank test was used.

8 MORPHOLOGY (III)

8.1 Sampling

Stickleback samples were collected to represent two major genetic clusters of the Baltic Sea (for the sake of simplicity, we call them the eastern and the western) as distinguished by DeFaveri et al. (2013). Hereafter we term them as 'east' and 'west' divisions. Since the west division is more spatially extensive covering all major basins of the Baltic Sea and substantial environmental gradients, it was further divided into the smaller spatial units of Kalmar Sound, Baltic Proper and Bothnian Sea (hereafter termed as 'locations'). Sticklebacks were collected by trawling in Kalmar Sound, Baltic Proper, and Bothnian Sea

(west division) and by beach seining or using trap-nets in the Curonian Lagoon (east division) in 2010-2014 (Figure 3). From each survey, a randomly taken sub-sample was used for morphological analysis. Once caught, fish were frozen and later, slowly thawed in the laboratory.

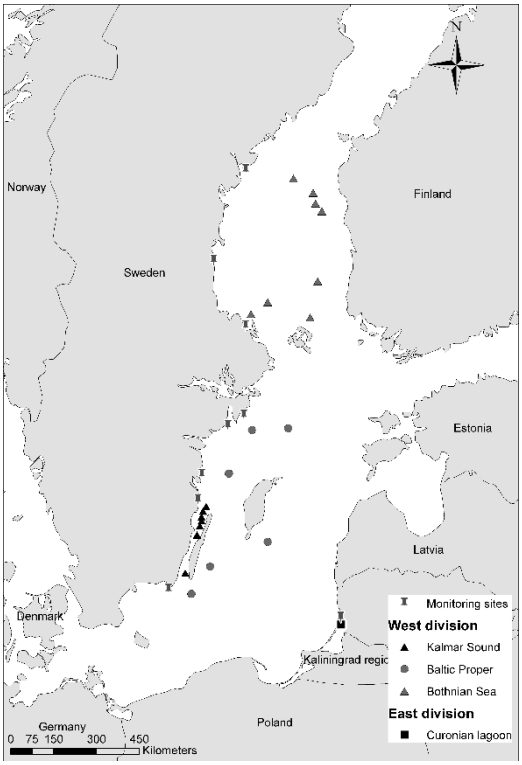

Figure 3. Sampling locations of the samples from the east and west division. The west division was further divided into four locations, Kalmar Sound, Baltic Proper and Bothnian Sea. The coastal monitoring sites corresponds locations from where coastal predation estimates was derived.

8.2 Number of body plates

The left side of each individual was positioned for observation of the number of lateral body plates. Fish of a total length (TL) of <40 mm were excluded from analysis to ensure that bone plate development was completed (Wootton, 1976; Bell, 1981). An ANCOVA (Analysis of Covariance) was used to test for differences in body plate numbers between locations and divisions, using fish length as a covariate to control for fish size. The number of body plates were analysed for 397 fish (N east division=130, N west division=267).

To relate the number of body plates of the sampled sticklebacks to the predation pressure in the area, we used an abundance index of piscivorous fish in the area where samples were collected. The index was estimated using publicly available coastal fish monitoring data (HELCOM, 2017) as the catch per unit effort of piscivorous fish in each monitoring area (see Paper III).

8.3 Body shape analysis

Landmark based geometric morphometrics (Bookstein, 1991; Zelditch et al., 2012) was used for comparison of body shape of fish across divisions and locations. First, fish were positioned within a groove in a polystyrene block to prevent deformation of the body and images of the left side of each specimen were captured with a Canon EOS 700D. Then 22 landmarks were digitized for each fish using the tpsDig v 2.3 software (Rohlf, 2017) (see Paper III).

Using tpsRelw v 1.54 software (Rohlf, 2017) landmarks were superimposed to remove the non-shape part of variation (General Procrustes analysis, Rohlf and Slice, 1990). Finally, shape variation as obtained from the Procrustes shape coordinates (Mitteroecker and Bookstein, 2011) across divisions and locations was analysed using Principal Components Analysis (PCA) and Canonical variates analysis (CVA).

A subset of fish (only adult specimens of 5 – 7 cm TL) was chosen in this analysis to mitigate allometric effects on the body shape (Walker, 1993). In total, 270 fish were digitized. A permutational MANCOVA on the shape coordinates was used to test the effect of location on differences in body shape. Discriminant Functions analysis was used to compare body shapes of fish from the east and west divisions, and the degree of correct classification into the divisions was evaluated using jackknife cross-validation.

8.4 Otolith shape analysis

Both left and right sagittal otoliths (Sagittae) were used to analyse differences in otolith shape between divisions and locations. Once removed, digital image captures of each otolith were taken under an Olympus BX41 transmitted light microscope using an Olympus MicroPublisher 3.3 RTV camera. The otolith outline was then analysed with Fourier and Wavelet analysis, using the open source software package ShapeR (Libungan and Pálsson, 2015). To reduce the ontogenetic effects on otolith shape, the analysis was performed on adult fish only (size range - 5.5 – 7.6 cm total length). Otolith shapes were compared using an ANOVA-like permutation test. The otolith shape was analysed for 71 fish (N east division=32, N west division=39).

RESULTS

The main results are presented in this section (for a summary see also Table 4 below). For a more comprehensive results description, please refer to the relevant paper (I-III).

9 DIET IN THE OFFSHORE (I)

Substantial seasonal differences in the diet of all three fish species studied was observed. In the spring, the majority of the herring and sprat diet consisted of *T. longicornis*, whereas the diet of sticklebacks mainly consisted of *E. affinis* in the same season. *E. affinis* made the most substantial contribution to the diet of all three fish species in the summer, while in the fall the cladoceran *Bosmina* spp. was the most important prey for all fish species (Figure 4).

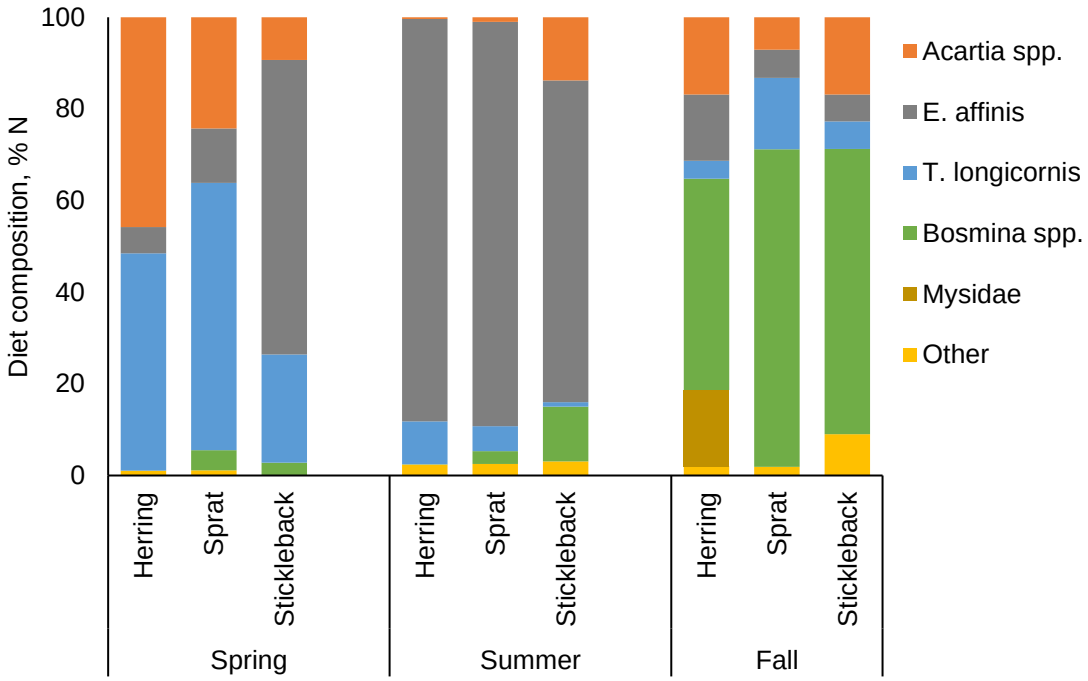

Figure 4. Summary of diet composition of herring, sprat and stickleback during different seasons in Kalmar Sound, south-western Baltic Proper. Only prey items that made >5% of the diet are shown.

Other cladoceran species (*Podon* spp.) were only notable (>1%) in the autumn in the diet of sticklebacks (3%). The invasive *Cercopagis pengoi* occurred in small quantities (1–2%) in all fish species stomachs in summer. Some rotifers (*Keratella* sp.) were found in the stomachs of sticklebacks in autumn (3%) and summer (<1%). Generally, very few (<1%) calanoid *Centropages hamatus* appeared in the diet, comprising >1% only in the diet of herring and sticklebacks in autumn. It was evident that the diet of herring, sprat and sticklebacks differed mainly across seasons rather than among fish species (see Paper I).

Unexpectedly high overlap between diets was observed among the three species, although the extent of overlap depends on the season and fish size (Table 1). The highest dietary overlap among the three fish species was found in summer (94–99%), lower - in spring (69 - 94%). Although generally the highest overlap was present between clupeids (sprat and herring), in autumn, however, a higher overlap was observed between sticklebacks and clupeids (69 - 93%) than between clupeids (51 – 88%). Even the diet of large (>15 cm) herring exhibited a considerable overlap with the diet of the much smaller sticklebacks (Table 1).

Table 1. Mean dietary overlap (Morisita simplified indices, C_H) among fish species in different fish size groups (TL) in different seasons in Kalmar Sound, south western Baltic proper. Small sprat and herring – TL≤10 cm, medium – 10<TL≤15 cm, large – TL>15 cm. Sticklebacks were not divided into size groups, all ≤7cm TL.

		Spring	Summer	Autumn
Stickleback – herring	Small herring	0.67		0.69
	Medium herring	0.61	0.95	0.78
	Large herring	0.87	0.94	0.72
	Mean	**0.69**	**0.94**	**0.74**
Stickleback – sprat	Small sprat	0.66		0.67
	Medium sprat	0.76	0.95	0.93
	Mean	**0.71**	**0.95**	**0.8**
Herring – sprat	Small – small	0.98		
	Medium – small sprat	0.98		0.7
	Large herring –small sprat	0.94		0.51
	Medium – medium	0.91	0.99	0.88
	Large herring –medium sprat	0.86	0.99	0.61
	Mean	**0.94**	**0.99**	**0.69**

All fish species negatively selected *Acartia* spp. (except during spring). *E. affinis* was especially preferred in summer, while cladoceran *Bosmina* spp. was positively selected in autumn, especially by sticklebacks. However, wide confidence intervals in spring imply high uncertainty of the estimates (Table 2).

Table 2. Selectivity (V-index) of sticklebacks, herring and sprat for the main prey species. Means and lower (Low) and upper (Up) limits of 95% confidence intervals calculated by bootstrapping. Significant values bolded ($p<0.05$, Wilcoxon Signed Rank test).

Season	Prey	Stickleback			Sprat			Herring		
		Mean	Low	Up	Mean	Low	Up	Mean	Low	Up
Spring	*Acartia* spp.	0.57	-0.17	0.94	**0.58**	0.32	0.81	**0.76**	0.59	0.88
	Bosmina spp.	0.33	0.00	1.00	0.15	0.00	0.37			
	Eurytemora affinis				0.15	-0.04	0.40	0.04	-0.04	0.17
	Temora longicornis				0.02	-0.07	0.14	**0.12**	0.02	0.24
Summer	*Acartia* spp.	**-0.12**	-0.19	-0.03	**-0.30**	-0.31	-0.28	**-0.31**	-0.32	-0.30
	Bosmina spp.	**0.16**	0.10	0.22	0.00	-0.03	0.04	**-0.07**	-0.08	-0.06
	Eurytemora affinis	**0.39**	0.30	0.46	**0.58**	0.54	0.62	**0.58**	0.53	0.61
	Temora longicornis	**-0.21**	-0.22	-0.19	**-0.10**	-0.14	-0.07	-0.03	-0.05	0.00
Fall	*Acartia* spp.	**-0.09**	-0.15	-0.02	**-0.15**	-0.19	-0.11	**-0.20**	-0.26	-0.14
	Bosmina spp.	**0.70**	0.62	0.77	**0.65**	0.57	0.73	**0.44**	0.33	0.54
	Eurytemora affinis	0.04	0.00	0.09	**0.07**	0.03	0.10	0.13	0.05	0.21
	Temora longicornis	**-0.05**	-0.09	0.01	0.09	0.01	0.18	**-0.11**	-0.16	-0.05

10 DIET AT THE COAST (II)

Using DNA metabarcoding, unusually high prey diversity in the stickleback stomachs was revealed: 120 taxa, belonging to 15 phyla, 83 genera and 84 species (Paper II). Taxa likely to be incidental or secondary prey were excluded from further analyses. Specifically, we omitted Fungi, Macroalgae and Chromista; a few OTUs of Metazoa were also excluded as they were either

unlikely to be prey, or due to possible contamination (see S1 Table in Paper II). In total, 103 taxa (out of 120) were considered as primary prey and were used in the subsequent analyses.

Sticklebacks had a broad spectrum of prey items (Figure 5). Insecta (chironomids), Maxillipoda (harpacticoid copepods) and Branchiopoda (cladocerans) were dominating food items. At the species level, the main prey were the chironomid *Tanytarsus usmaensis*, the harpacticoid *Tachidius discipes,* and the cladoceran *Pleopis polyphemoides.*

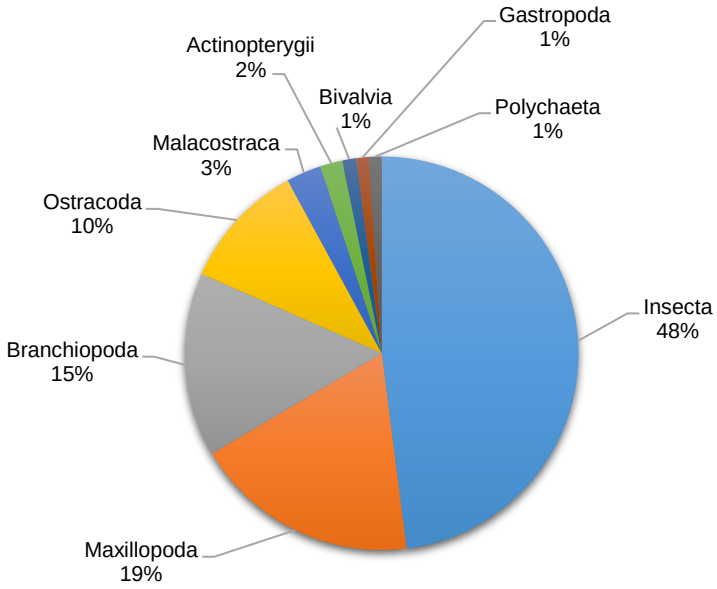

Figure 5. Proportion of different classes in stickleback stomachs based on number of OTU reads (%N_{bar}). Only classes that made >1% of OTU reads are shown.

The diet of the large sticklebacks (>6.5 cm) differed from the group of smaller fish (≤6.5 cm) ($F=1.95$, $p=0.044$). Amphipods, isopods and gastropods, as well as insects like hemipterans and coleopterans appeared to be more common in the diet of the larger fish (Figure 6).

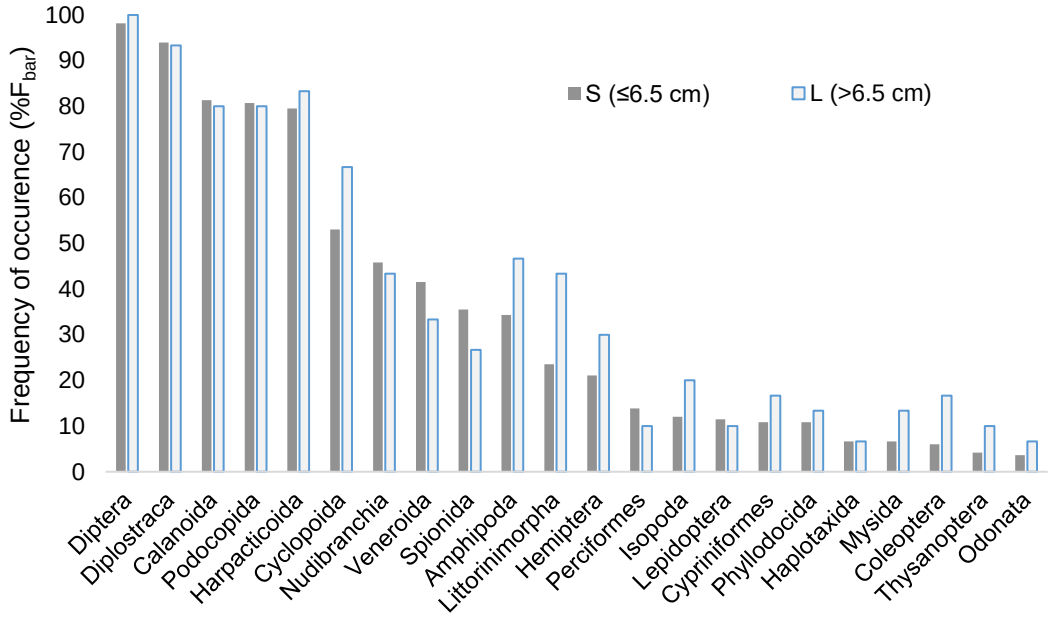

Figure 6. Diet composition of different size groups of sticklebacks (at order level). Only orders with >5% of frequency of occurrence are shown.

DNA metabarcoding gave much higher taxonomic resolution ($p<0.0001$), as well as higher number of prey taxa identified per stomach (Table 3). Number of taxa identified by means of barcoding greatly differed from that of visual analysis ($p<0.0001$, Table 3).

Table 3. Comparison of the methods used for stickleback diet elucidation.

	Visual inspection	**DNA metabarcoding**
Mean taxonomic rank	Order	Genus
Mean number of taxa identified per stomach	1.96 ± 1 (SD)	21.7 ± 8.8 (SD)
Total number of taxa identified	21	120

When comparing diet composition with epifauna and zooplankton composition across the 16 sampled areas, sticklebacks showed a preference for the cladocerans *Pleopsis polyphemoides* ($p=0.003$) and *Evadne nordmanni* ($p=0.038$) among the zooplankton, and for chironomids ($p=0.003$) among the

epifauna. Moreover, sticklebacks appeared to avoid calanoids *Acartia* spp. (p=0.0076) and cyclopoids among the zooplankton, and Gammaridae (p=0.0066) and Gastropoda (p=0.0007) among the epifauna (Figure 7).

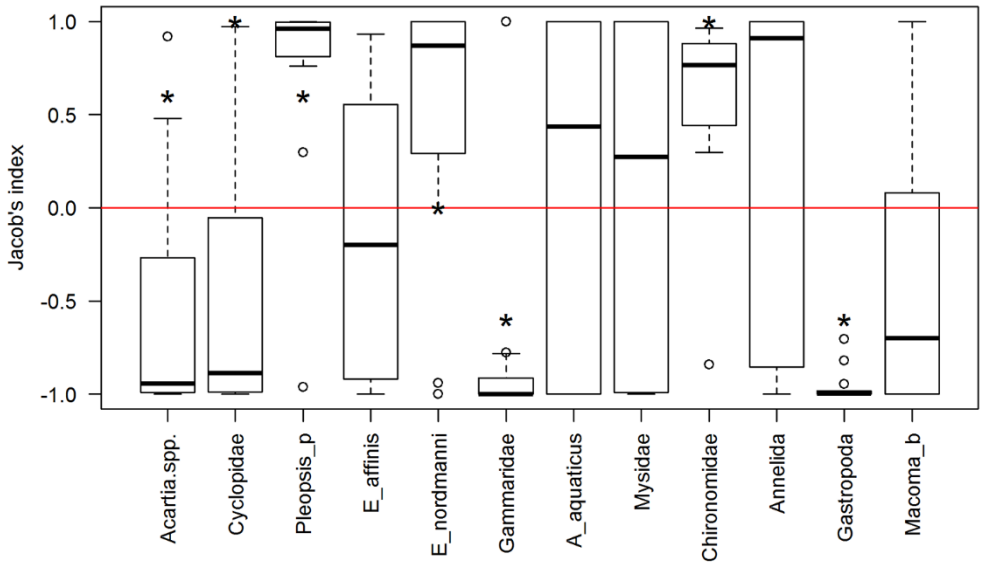

Figure 7. Diet selectivity of three-spined stickleback. Index values above zero indicate prey item to be preferred, and values below zero avoided. Black line represents median, boxes first and third quartiles, whiskers either maximum values or 1.5 times interquartile range (whichever is smaller) and points outliers. The statistically significant values are marked with asterisk ($p<0.05$, Wilcoxon Signed Rank test).

11 MORPHOLOGY (III)

The number of body plates differed considerably between the east and west divisions ($F=244.16$, $p<0.0001$). In the east division, sticklebacks had on average 22.7 ± 0.2 (SE) body plates, while in the west division - 13.5 ± 0.4. Within the west division, there was a slight but not significant difference in the number of body plates across locations ($F= 1.83$, $p= 0.16$). The average number of body plates was 15.4 ± 0.9, 14.3 ± 0.85 and 12.5 ± 0.5 in the Bothnian Sea, Baltic Proper and Kalmar Sound respectively (Figure 8).

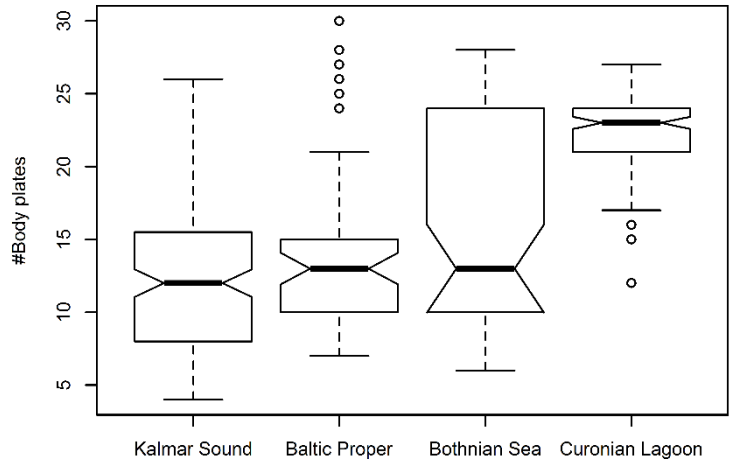

Figure 8. Number of stickleback body plates in the locations studied. Black line represents median, boxes first and third quartiles, whiskers either maximum values or 1.5 times interquartile range (whichever is smaller) and points outliers. Notches show 95% CI around the median.

The relative abundance of piscivorous fish was highest in the Curonian Lagoon (Figure 9), where the number of body plates of sticklebacks was also the highest (Figure 8).

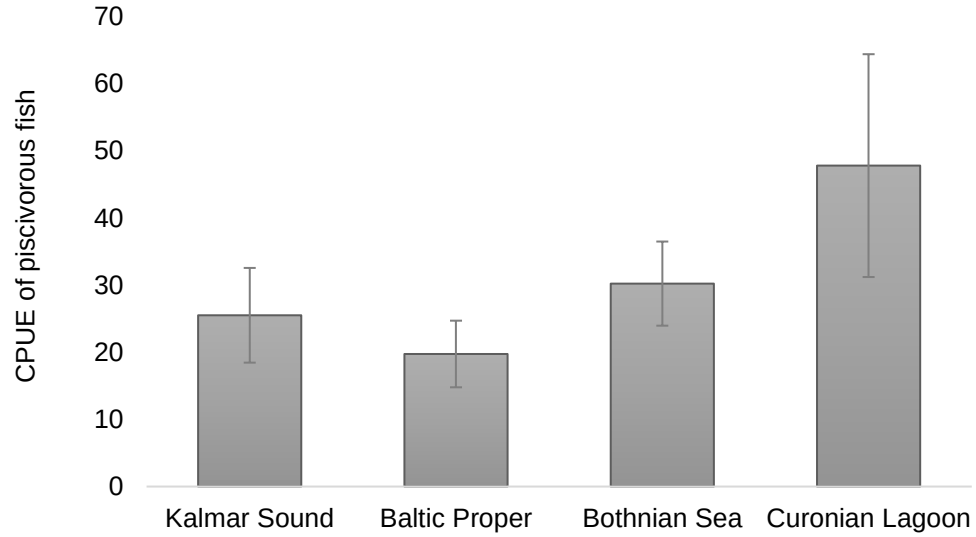

Figure 9. Abundance (catch per unit effort, CPUE) of coastal predatory fish in the areas studied (data of 2010-2014). Error bars are standard deviations (+/- 1 SD) of the annual means.

The body shape of sticklebacks differed among locations studied (MANCOVA, $p<0.05$). The most pronounced difference in body shape, however, was found between east and west divisions: sticklebacks from the east division (Curonian Lagoon) had deeper bodies compared to fish from the west division (Figure 10). The differentiation in body shape between the west and east division was confirmed by a high percentage of correctly classified fish (93 %) in the jackknife cross-validation analysis. Within the west cluster, some significant differences in the body shape were present as well. Fish from Kalmar Sound were more dorsally convex, while the fish in Bothnian Sea and Baltic Proper were more ventrally convex (see Paper III). The Kalmar Sound fish also had longer snouts.

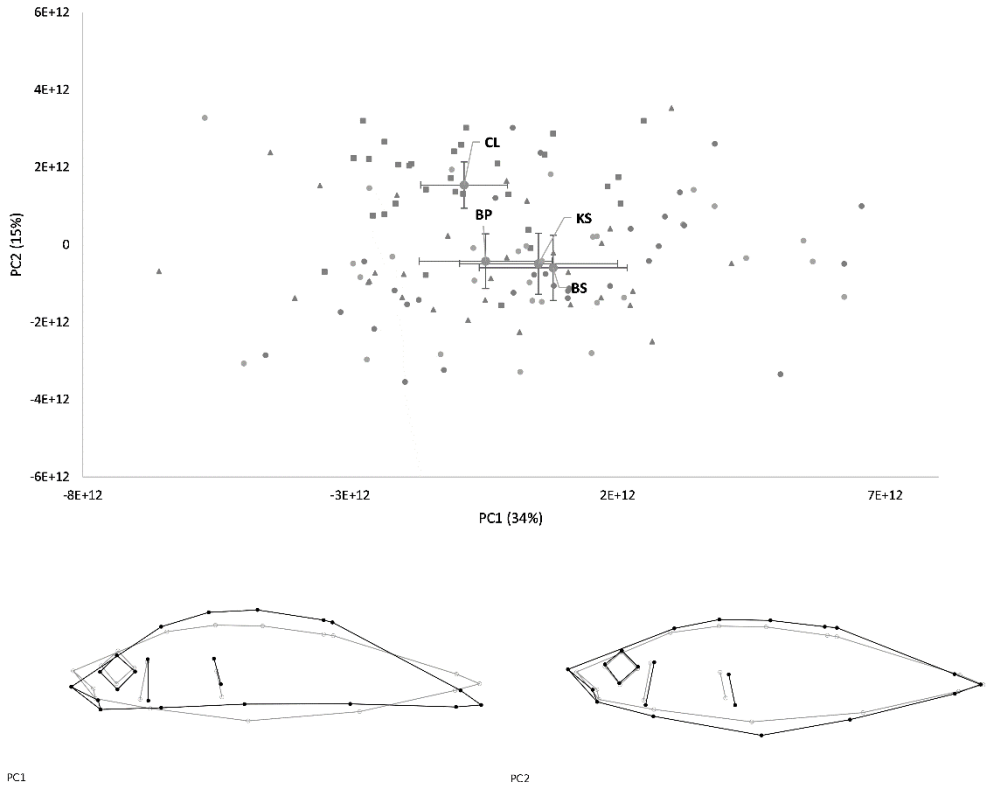

Figure 10. Principal component analysis of body shape of sticklebacks from the different locations in the study (mean ± SD). CL = Curonian Lagoon (squares) BP = Baltic Proper (triangles), KS = Kalmar Sound (circles), BS = Bothnian Sea (rhombus). Bottom: Wireframe graphs, illustrating changes in body shape (black) in relation to mean shape (grey) along the PC1 (left), and along PC2 (right). Deformations presented correspond to the range of PC axes.

No significant differences in otolith shape neither among locations nor divisions have been detected (Wavelet: F=1.56, p=0.14; Fourier: F= 0.7, p=0.7). Some minor differences were only revealed in the *excisura major* region of the otolith (Wavelet analysis, Figure 11). Number of gill rakers on the first gill arch slightly differed (F=4.7, p=0.03, ANCOVA) between fish from Curonian Lagoon (16.06 ± 0.2, N=121) and Kalmar Sound (15.09 ± 0.19, N=149).

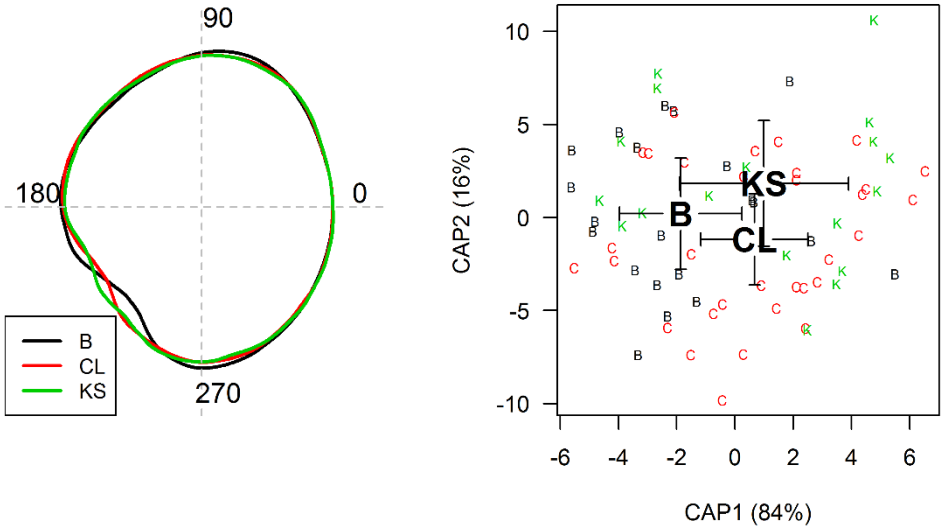

Figure 11. Left: Average otolith shape, based on Wavelet reconstruction, of three-spined stickleback from the Bothnian Sea (B), Curonian Lagoon (CL), and Kalmar Sound (KS). Right: Otolith shape of three-spined stickleback from Bothnian Sea (B), Kalmar Sound (KS) and Curonian Lagoon (CL) using Canonical analysis of Principal Coordinates with Wavelet coefficients. Black letters represent the mean canonical value for each population, and smaller letters represent individual fish showing the first letter of each population. The error bars of the mean canonical values represent the standard error (mean +/- 1SE).

Table 4. Summary of the main results of the thesis.

Part	Question	Main result	Implications
I	What do sticklebacks feed on in Baltic Sea offshore (Kalmar Sound)?	Main prey items vary depending on the season, but mainly cladocerans *Bosmina* spp. and calanoids *Eurytemora affinis*, *Temora longicornis*, *Acartia* spp.	Stickleback diet, to a large extent, overlaps with the diet of herring and sprat. All three fish species show similar preferences for the same prey. Combined with high stickleback abundances, it may be relevant for management of the commercial fish species in the Baltic Sea.
	Do sticklebacks show selective feeding behaviour?	Yes. They prefer cladocerans and avoid copepods, although with variable persistence depending on the season.	
	Does stickleback diet overlap with herring and sprat?	To a large extent, yes. But depends on the season, highest overlap observed in summer (95%).	
II	What do sticklebacks eat at the Baltic Sea coast?	>100 taxa revealed as prey items for sticklebacks, including both zooplankton and benthic organisms. Among benthos, sticklebacks prefer chironomids, among zooplankton – cladocerans.	In the context of high stickleback abundance and considering their diverse diet, many parts of both benthic and pelagic food web may be affected.
	How do visual inspection of stomach content compare with DNA-metabarcoding method?	DNA-metabarcoding gives much higher resolution and allows elucidation of diet richness compared to visual inspection. However, some taxa were only revealed by traditional visual analysis. Also, quantification from DNA-based method is still problematic.	This study suggests that methods should complement each other, not replace, at least until DNA metabarcoding is further developed.
III	Do body shapes of sticklebacks from eastern and western Baltic Sea differ?	Yes. Sticklebacks from Curonian Lagoon (eastern Baltic Sea) have deeper bodies compared to fish from Bothnian Sea, Baltic Proper and Kalmar Sound (western Baltic Sea).	Some evidence for stock differentiation and adaptation to higher predation based on highly plastic traits (body shape and body plates).
	Does body plate number of sticklebacks from eastern and western Baltic Sea differ?	Yes. Sticklebacks form eastern Baltic Sea (Curonian Lagoon) had more body plates than fish in the western part of the Baltic Sea (Kalmar Sound).	
	Does otolith shape of sticklebacks from eastern and western Baltic Sea differ?	No. Neither Fourier nor Wavelet analysis of otolith shape did provide proof for differences between populations.	

DISCUSSION

Stickleback abundance in the Baltic Sea has been increasing considerably during the last two decades, with these trends evident both at the coast and offshore (Bergström et al., 2015). Evidence for the significant role of sticklebacks in coastal and offshore systems in the Baltic Sea is increasing together with their population expansion leading to calls for future management of sticklebacks. In this context, several urgent questions arise within the scope of this study: what is the stickleback diet in relation to other species in the offshore and at the coast, and is the Baltic Sea population differentiated into separate stocks. Below each topic in question is briefly discussed, for a more detailed discussion please refer to a respective Paper (I-III).

12 DIET IN THE OFFSHORE BALTIC SEA (I)

Before coming to the coast to spawn, sticklebacks spend a major part of their life cycle in the offshore habitat of the Baltic Sea (Bergström et al., 2015). However, current knowledge about their role and feeding ecology in the offshore regions of the Baltic Sea is limited, especially in respect to seasonal scale and in relation to other planktivorous fish species. In the Kalmar Sound, offshore of the south-western Baltic Proper, this study revealed that the diet of sticklebacks depends heavily on the season (Figure 4): they mainly feed on calanoids *Eurytemora affinis* and *Temora longicornis* in spring, *E. affinis* in summer, and cladocerans *Bosmina* sp. in autumn. Interestingly, the diets of other commercially important mesopredators, like sprat and herring, were very similar.

In general, the dietary composition from this investigation accords with the findings from other studies: *Bosmina* spp. was found to be the main prey for small herring, sprat and stickleback in early September in the Gulf of Finland (Peltonen et al., 2004), while in the summer the copepods *E. affinis* and *Acartia* spp. were the main prey for herring, and the cladoceran *Bosmina* spp. was more important for sprat and sticklebacks in the Gulf of Riga (Lankov et al., 2010). It

is well known, that sprat have a higher preference for cladocerans than herring (Arrhenius, 1996). We also found both sprat and sticklebacks have a higher selectivity for *Bosmina* spp. than herring. *Bosmina* spp. have pigmented eggs and poorer escape response (Flinkman et al., 1992; Viitasalo et al., 2001), which makes them an easy target.

Despite that dietary overlap between herring and sprat has been observed in several other studies (e.g. Ojaveer, 1997; Lankov et al., 2010), as well as between clupeids and sticklebacks (Lankov et al., 2010), such a substantial overlap as found in our study even between small sticklebacks (≤7 cm) and larger (>10 cm) herring and sprat was surprising (Table 1). On average, 95% overlap was detected in summer, 72–93 % in autumn, 61 – 87% in spring (Table 1). Almost complete dietary overlap among the fish species in summer implies that if food supply is limited, interspecific competition might influence the growth of the fish, since the summer season is usually a critical period for the growth of fish. Due to density-dependent mechanisms, clupeid condition especially that of herring has deteriorated (Casini et al., 2011). Sticklebacks may comprise a significant portion of clupeid biomass (Jurvelius et al., 1996; Ljunggren et al., 2010; Olsson et al., unpublished), thus high abundances of sticklebacks and possible competition for food may also impact the condition of other fish.

It should be noted, however, that actual dietary overlap at the population or stock level might be smaller than estimated here, due to differences in spatial (vertical) distribution of the fish species. Sticklebacks tend to feed closer to the surface (Peltonen et al., 2004), and large herring, for example, dwell deeper (Cardinale et al., 2003) and thus not always vertically overlap with sticklebacks. However, in this study, large herring (>15 cm) were also found closer to the surface as with sticklebacks, suggesting at least partial spatial overlap of the species.

13 DIET AT THE COAST (II)

At the Baltic Sea coast, sticklebacks are known to foster changes in ecosystem structure and functioning via trophic cascades and intraguild predation (Ljunggren et al., 2010; Eriksson et al., 2011; Byström et al., 2015; Candolin et al., 2016). However, given rising interest from management, and the complexity the food-webs, investigations on stickleback's role in a coastal food chain are still in demand.

In this study, DNA metabarcoding revealed >100 taxa as prey items for sticklebacks at the coast, uncovering a complex picture of their diet and highlighting that many links are indeed present. No previous studies have revealed such an extensive diversity of prey items, most likely because of limitations in their methods (see Table S3 in Paper II). Such a wide variety of both pelagic and benthic organisms found in the diet indicate that sharp increase in stickleback abundance may affect many parts of the Baltic Sea coastal ecosystem.

The phenomenon of intraguild predation, or prey-to-predator loop, includes adult sticklebacks feeding on predators' eggs and/or competing with juveniles and larvae of the predators for food. The high abundance of cladocerans found in the diet (Figure 5 and 6) might indicate competition with juvenile stages of other fish species, especially since preference for cladocerans (Figure 7) is evident (Campbell, 1991; Leinikki, 1995; Lankov et al., 2010). Ljunggren et al. (2010) suggests that recruitment of pike and perch in the Baltic Sea has been impaired by limited zooplankton availability for their larvae due to competition with sticklebacks. The three-spined stickleback can actually deplete zooplankton communities in brackish water lagoons with similar densities as in the current study area (Jakobsen et al., 2003). Moreover, sticklebacks have been shown to feed on small pike and perch larvae, which would constitute a direct effect on populations of large piscivore fish (Byström et al., 2015). Perciformes was detected in the stomachs of fish (although in six stomachs only, see S1 Table in

Paper II), potentially indicating sticklebacks may have been feeding on perch eggs or larvae.

Experimental studies show that by controlling grazers, namely gammarid amphipods, sticklebacks may indirectly increase nearshore primary production (Sieben et al., 2011a, 2011b). In our study, however, sticklebacks seemed to have fed less on amphipods than expected. Underrepresentation of large individual fish in this study (see S1 Figure in Paper II) might explain the lack of amphipods found in the diet, since only larger (>6.5 cm) sticklebacks fed more on amphipods (Figure 6).

The largest sticklebacks seem to occupy the most beneficial habitats in the bays, i.e. the shallowest vegetated parts with the highest abundances of gammarids, where gillnets could not be used for sampling. Large sticklebacks (>6.5 cm) also tend to have a higher frequency of occurrences of cyclopoid copepods *Eucyclops macruroides* in their stomachs, which is the species typically inhabiting vegetation in the littoral zone. This supports possible small-scale differences in foraging habitats between stickleback size classes.

Among benthic prey, the most significant part of the diet consisted of chironomid larvae (Paper II). Chironomids are a broad taxonomic group, with a diverse diet spanning between phytoplankton, epiphytic algae, detritus, macrophytes, and crustacean zooplankton (Armitage et al., 2012). More knowledge on the role of chironomids in food webs and their interactions with sticklebacks is needed, since possible cascading effects from sticklebacks via chironomids (not only via amphipods as shown in previous studies) to lower trophic levels may be present (e.g., Rudman et al., 2015).

13.1 Selectivity

We found that sticklebacks selectively prey on the cladocerans *Pleopsis polyphemoides* and *Evadne nordmanni*, but avoid the calanoid *Acartia* spp (Figure 7); a result consistent with those found in both marine and freshwater populations (Campbell, 1991; Leinikki, 1995; Lankov et al., 2010). Cladocerans have a slower predator avoidance response compared to the fast-swimming copepods, making the former easier and less energy-consuming to capture (Drenner et al., 1978; Viitasalo et al., 2001).

In contrast to the positive selection for chironomids, sticklebacks seemed to avoid feeding on gastropods and, more surprisingly, gammarid amphipods (Figure 7). The avoidance of gastropods fits well with previous data from experiments, where sticklebacks fed mainly on gammarids and isopods, whereas gastropods were eaten primarily by roach (Reiss et al., 2014).

Stickleback preference for certain prey depends not only on prey profitability (largest energetic value taking handling into account), but also on hunger level, the size of the prey, and how often the fish encounters the prey (Hart and Ison, 1991). Selection behaviour might also differ between individuals of the same species (Wootton, 1990). In many of the 16 bays there were relatively few individuals sampled, resulting in an inability to assess individual specialisation - for that, a more detailed and intense sampling program should be conducted.

13.2 Visual vs metabarcoding

Despite some discrepancies, DNA metabarcoding and visual stomach content inspection gave consistent results. Both methods revealed the same prey taxa dominating (Paper II). However, since the stomach contents were extensively digested, and/or had very few prey items present, the visual prey species identification was in many cases obscured. In diet studies, a high proportion of unidentifiable material in the guts, which cannot be visually assigned to any prey category, is common (Baker et al., 2014). Thus, barcoding could be a solution – in our study it gave much higher taxonomic resolution and therefore produced a more accurate and detailed analysis of gut contents. However, with barcoding,

some prey species were also missed (*Temora longicornis*, Bosminidae, Hydracarina) and quantification from OTU reads could be problematic (see section below 'Methodological shortcomings'). Thus, to achieve the best resolution of diet composition, we recommend combining high-throughput DNA sequencing and traditional visual stomach content analysis; at least until metabarcoding methods become further developed.

13.3 Methodological shortcomings

Like any other method, DNA metabarcoding has its drawbacks. Below I discuss the issues of secondary consumption, difficulties in primer choice, possible biases introduced during bioinformatic analysis, and quantification from OTU reads, and how it may have affected results.

Secondary consumption, i.e., prey of the prey, parasites or accidental material consumed during feeding, may confound the results in DNA-based studies (Sheppard et al., 2005; Bowser et al., 2013; Oehm et al., 2016). Although a few unlikely prey taxa were removed from the analysis (based on expert judgement), some secondary prey may still have been incorrectly assigned as primary prey. However, DNA of secondary prey is expected to represent only a minor part of total OTU reads compared to primary prey, due to a much lower total biomass and to a higher level of degradation.

In order to determine the full taxonomic range of the prey ingested, universal primers should be used. However, although the CO1 primers are designed to be 'universal' they may not bind equally well to all prey species, and maybe not at all to some. These biases are then accumulated through DNA amplifications during the PCR reaction (Polz and Cavanaugh, 1998; Piñol et al., 2015). Bosminidae was identified during visual inspection of stomach contents, but when barcoded only a higher corresponding taxon was detected (Diplostraca). Thus, only species or group specific primers would guarantee the most accurate identification.

Host DNA may constitute up to 90% of the sequences obtained by NGS (Shehzad et al., 2012). Thus, blocking primers are necessary to significantly improve prey detection, however, they may also block prey DNA (Vestheim and Jarman, 2008). We used a blocking primer to avoid stickleback sequences, but since predator (the stickleback) and its prey are not phylogenetically close, and the blocking primer used is specific to *G. aculeatus*, this should not have impaired the results.

During the bioinformatic analysis, biases may be introduced. For example, during the clustering of sequences, where the number of OTUs or 'species' found depends on the sequence similarity cut-off used, or during taxonomic assignment, which uses a sequence identity threshold of 97%. The best lower and upper bound values to cluster metazoan CO1 sequences were selected based on a benchmarking study by Leray et al. (2013b). Identity threshold chosen (97%) was in accordance with widely accepted protocols in similar studies (see e.g., Leray and Knowlton, 2015). Also, it is obvious that comprehensive reference library is necessary: if some species are not represented in the DNA reference library, no matches for these will be found. In our study, the largest publicly available BOLD reference library as well as unpublished libraries for Chironomidae, Nemertea, Xenacoelomorpha and Oligochaeta, and Swedish invertebrates (SweBoL) were used, to ensure that as many sequences would be assigned as possible.

Although read counts can be used as a semi-quantitative proxy for diet composition (Deagle et al., 2009; Kowalczyk et al., 2011; Soininen et al., 2009, 2013), several well-known issues still impede the use of DNA metabarcoding for quantification. Quantitative estimates of certain prey in the stomach may be influenced by prey size, level of digestion, DNA preservation, as well as experimentally introduced biases from DNA extraction, primer-template mismatches, PCR amplification bias, OTU clustering, reference library quality and taxonomic assignment process (Polz and Cavanaugh, 1998; Deagle and Tollit, 2007; Troedsson et al., 2009; Kembel et al., 2012; Pompanon et al., 2012;

Blanco-Bercial et al., 2014; Kress et al., 2015; Piñol et al., 2015). One way to reduce such biases is to introduce correction factors, by creating a library of mixed prey standards and then using them to correct counts from unknown composition (Thomas et al., 2016). This was not done in our study due to the large number of prey items in the stomachs, but the application of alternative methods (visual analysis) enabled us to at least partly validate the DNA metabarcoding results (Paper II). However, results did not match fully - some prey taxa (*Temora longicornis*, Bosminidae, Hydracarina) were detected by visual inspection only. As we could visually identify these prey organisms, their DNA is unlikely to have been too degraded for barcoding to identify them. This could be due to above-mentioned issues, i.e., primer-template mismatches.

Frequency of occurrences gained by both methods ($\%F_{bar}$ vs $\%F_{vis}$) do not follow a linear relationship (Paper II). Indeed, our results were as expected – a saturation curve, with much higher $\%F_{bar}$ values than $\%F_{vis}$ values - since the nature of DNA barcoding is to detect even very little prey in the stomach (which could not be visually detected, given the partial degradation of the stomach content).

Thus, quantification from DNA data can be successfully implemented (Deagle and Tollit, 2007; Pompanon et al., 2012; Deagle et al., 2013), though with caution, using proportional read counts, and ideally whilst implementing alternative method to validate results.

14 MORPHOLOGICAL DIVERGENCE: IMPLICATIONS FOR STOCK DIFFERENTIATION (III)

Variation in the number of bony lateral plates can illustrate population differentiation in sticklebacks (e.g., Hermida et al., 2005), especially considering that changes in these traits can occur very rapidly (Kristjansson et al., 2002). Our study reflected clear-cut differences between the eastern and western Baltic Sea with respect to body plate numbers, which may be a response to local predation pressure (Paper III). Although a higher body plate number is typically associated

with higher predation pressure (Bell and Foster 1994), different types of predators can mediate armor development differently (Reimchen, 1983; Bergstrom, 2002; Zanella et al., 2015). We accounted for coastal fish predation only – our predation index misses other types of predation, such as cod in offshore waters, or cormorants in the coastal waters.

Not only did the sticklebacks from Curonian Lagoon (eastern cluster) possess the highest number of body plates, they also had the deepest bodies. Like lateral plates, a deeper body can be a part of a predation defence system (Walker and Bell, 2000).

It is well known, that limnetic (pelagic) and benthic stickleback populations can diverge while adapting to different habitats (Schluter, 1993). The benthic morph has a deeper body, and less gill-rakers making it more efficient in feeding on benthic prey, while the limnetic morph possesses a streamlined body, which increase efficiency of locomotion, and a higher number of gill-rakers suited for feeding on small planktonic prey (Schluter, 1993; Bell and Foster, 1994). However, a deeper body does not necessarily mean poorer swimming performance (Seebacher et al., 2016), and since sticklebacks from the Curonian Lagoon are anadromous in migrating to the Baltic Sea (Gaigalas, 2001), there is likely a trade-off between predation defence (deep and fully plated bodies) and ability to swim efficiently in a brackish more open system. Sticklebacks from the east division (Curonian Lagoon) had even slightly more gill-rakers than fish from the west (Kalmar Sound, see Results), implying that feeding behavior is not likely to be the cause for the body shape differences observed. This again suggests that predation might be the most important driver of divergence in stickleback morphology in the Curonian Lagoon.

Despite some slight differences within the west division (sticklebacks from the Kalmar Sound location were more dorsally convex and had longer snouts), all fish had morphological features that are typically associated with a planktivorous diet (Bjærke et al., 2010; Willacker et al., 2010).

Although otolith shape analysis can even discriminate between populations of the same species (e.g., Libungan et al., 2015), no differences in the stickleback otolith shape were found. Thus, the differentiation in the highly plastic traits as body plate number and body shape, but not in the less plastic trait otolith shape suggest that population subdivision of sticklebacks in the Baltic Sea is either rather recent or weak, something that has also been indicated by earlier studies on both the spatial synchrony in demography and molecular markers (Östman et al., 2017).

CONCLUSIONS

1. In the offshore Baltic Sea, the main prey of sticklebacks varies over the season. Calanoid copepods as *Acartia* spp. is preferred in the spring, in the summer *Eurytemora affinis* is preferred and in the autumn cladocerans *Bosmina* spp.
2. In the offshore system, the diet of sticklebacks, herring and sprat overlap considerably. The highest dietary overlap between sticklebacks and clupeids was observed in summer (94-95%), followed by autumn (67-93%), and lowest in spring (61-87%).
3. DNA metabarcoding of stickleback stomach contents revealed a selective but high diversity (>100 taxa) diet of sticklebacks in the coastal waters of the Baltic Sea. Amphipods, isopods and gastropods were more common in the diet of the larger fish (>6.5 cm). Sticklebacks positively selected chironomids and cladocerans.
4. DNA metabarcoding complements rather than replaces traditional visual stomach contents analysis, and this situation will persist until the method is further developed.
5. Sticklebacks from the eastern Baltic Sea (Curonian Lagoon) had significantly more body plates and deeper bodies compared to those from the western parts of the area (Kalmar Sound, Baltic Proper, and Bothnian Sea). The number of body plates correlated with local predatory fish abundance.
6. Otolith shape does not differ between sticklebacks from the eastern (Curonian Lagoon) and western (Kalmar Sound, Baltic Proper, Bothnian Sea) Baltic Sea, implying that stock differentiation is rather recent or weak.
7. Overall, the results of this current study emphasize the need to include consideration of sticklebacks in management plans and monitoring programs because this species is likely to be exerting significant influence on many components of pelagic and benthic food webs, in offshore and coastal habitats of the Baltic Sea.

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Original Article

Seasonal dynamics in the diet of pelagic fish species in the southwest Baltic Proper

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There is accumulating evidence of the significant role of three-spined sticklebacks (*Gasterosteus aculeatus*) for ecosystem structure and functioning in coastal areas of the Baltic Sea, but little is known about the role of the species in the pelagic foodwebs of the Baltic and its interaction with other planktivorous fish species. In this study, we assess the feeding niche, diet overlap, and prey selectivity of sticklebacks, herring (*Clupea harengus*), and sprat (*Sprattus sprattus*) in an area of the southwest Baltic Sea (Kalmar Sound) in relation to the seasonal abundance of zooplanktonic prey during 2009–2011. The main prey items for all fish species studied were the calanoid copepods *Eurytemora affinis*, *Temora longicornis*, and *Acartia* spp. during spring and summer, and the cladoceran *Bosmina* spp in autumn. The diet of all the three fish species overlapped considerably, especially during summer and autumn. A substantial diet overlap was even present between sticklebacks and larger (>10 cm) herring and sprat. We also found evidence for an overlap in feeding preferences, i.e. certain zooplankton species were selected by all the three fish species in each given season. Overall, these results indicate potential resource competition between sticklebacks and clupeids. With an increasing abundance of sticklebacks in the Baltic Sea, their role should not be neglected when investigating pelagic foodweb dynamics, and management of herring and sprat may have to account for sticklebacks by considering that sticklebacks, via food competition, might affect clupeid stocks.

Keywords: diet overlap, herring, planktivorous fish, selectivity, sprat, three-spined stickleback.

Introduction

The assessment of the feeding habits of animals is fundamental in many aspects of ecology, from understanding prey selection and competition to revealing patterns of energy transfer between or within ecosystems (Stergiou and Karpouzi, 2002; Baxter *et al.*, 2004; Svanbäck and Bolnick, 2007). Planktivorous pelagic fish species, i.e. mesopredators, play an important role in marine ecosystems as they may induce changes in ecosystem structure through bottom-up (affecting their predators) and top-down (affecting their prey) mechanisms ("wasp-waist" control, Cury *et al.*, 2000; Fauchald *et al.*, 2011) and even lock the ecosystem in an alternative state through foodweb interactions (Pace *et al.*, 1999; Casini *et al.*, 2008; Möllmann *et al.*, 2008). Hence, knowledge on foodweb interactions is of high relevance in ecosystem and fisheries management, and the demand for studies addressing this is universally recognized, with the Baltic Sea being no exception (e.g. Sieben *et al.*, 2011).

The pelagic fish community in the Baltic Sea mainly consists of the clupeids herring (*Clupea harengus*) and sprat (*Sprattus sprattus*), both commercially and ecologically important, and probably the most well-studied fish species in the open Baltic Sea (Cardinale and Arrhenius, 2000; Casini *et al.*, 2004). However, another mesopredatory fish species that is less studied in this area, the three-spined stickleback (*Gasterosteus aculeatus*, from here on referred to as stickleback), may constitute a considerable amount of the pelagic fish biomass in some areas (Jurvelius *et al.*,

1996, Ljunggren *et al.*, 2010). These three species are all zooplanktivorous and important prey for piscivorous fish in the Baltic and are hence essential for the ecosystem functioning in the pelagic system of the Baltic Sea (Arrhenius and Hansson, 1993; Rudstam *et al.*, 1994). However, the trophic interactions, i.e. the diet preferences and overlap between sticklebacks, sprat, and herring, have been relatively overlooked. In particular, the seasonal feeding dynamics of the three species have rarely been explored in relation to the availability of their prey (see Lankov *et al.*, 2010 for selectivity in summer). Evidence is now accumulating for the significant role of sticklebacks for ecosystem structure and functioning in coastal areas of the Baltic Sea (Ljunggren *et al.*, 2010; Eriksson *et al.*, 2011; Sieben *et al.*, 2011), but little is known on the ecological interactions between sticklebacks and the other main planktivorous fish species. Coupled with a clupeid condition deterioration (Casini *et al.*, 2006), information on the potential competition between sticklebacks and clupeids is highly relevant for sustainable management of the Baltic Sea ecosystem.

The stickleback is a small, short-lived mesopredatory fish, common in freshwater lakes, streams, estuaries, and coastal seas of the northern hemisphere (Banister, 1986). It is a well-studied species in terms of behavioural and evolutionary ecology (e.g. Des Roches *et al.*, 2013; Hendry *et al.*, 2013), but knowledge of its function in marine foodwebs is relatively limited. Moreover, the abundance of sticklebacks in the western parts of the Baltic Sea has increased drastically over the past two decades, both in the offshore areas and along the coast (Bergström *et al.*, 2015), suggesting an increasing role of the species in these systems. Since sticklebacks spawn in spring along the coast and migrate offshore for wintering (Ljunggren *et al.*, 2010; Bergström *et al.*, 2015), it might also act as a vector linking offshore and coastal systems and hence transfer and mediate effects between the systems (Eriksson *et al.*, 2011). DeFaveri *et al.* (2013) discriminated several genetic clusters of three-spined stickleback in the Baltic Sea, with a clear east–west division in the central Baltic. In addition, a recent exponential increase in stickleback abundance is obvious in the western, but not in the eastern, part of the Baltic Sea Proper (Bergström *et al.*, 2015; Olsson *et al.*, 2015), suggesting separate dynamics in these areas. There is evidence for sticklebacks being potential competitors for herring and sprat in the eastern parts (Peltonen *et al.*, 2004; Lankov *et al.*, 2010), but to the best of our knowledge, no studies have assessed the diet overlap between sticklebacks and the other main pelagic fish species in the western Baltic Sea Proper in different seasons and accounting for the zooplankton prey availability. In this study, we assess the feeding niche of sticklebacks, herring, and sprat in an area of the southwest Baltic Proper. More specifically, using scientific trawl and zooplankton data from Kalmar Sound over 2 consecutive years and three seasons, the following questions were addressed: (i) what are the seasonal patterns in the diet of herring, sprat, and three-spined stickleback; (ii) do the diets of herring, sprat, and stickleback overlap; and (iii) are there common preferences for certain prey species in the three fish species?

Methods

Fish and zooplankton sampling

Samples were collected in Kalmar Sound in the southwest Baltic Proper (Figure 1). Kalmar Sound is a semi-enclosed area between the island of Öland and the Swedish mainland. The area surveyed in this study is, however, dominated by pelagic fish species.

Fish (herring, sprat, and sticklebacks) were caught with a pelagic trawl in three different seasons: spring, summer, and autumn, mainly at dusk or during night-time during 2009–2011 (Tables 1 and 2). The average hauling speed was three knots, and the trawl codend was 6-mm bar length. Once caught, the fish were measured [to the nearest mm, total length (TL), see Supplementary Figure S1 for fish size distribution] and immediately frozen, except for the stomachs that were stored in 4% formaldehyde/70% ethanol solution for subsequent stomach content analysis. Zooplankton (mesozooplankton $>$ 200 µm and a fraction of microzooplankton 20–200 µm) were sampled monthly in 2009–2010 by vertical tows from 50 m depth to the surface or from the seabed to the surface at depths $<$50 m using a WP2 zooplankton net with 90 µm mesh size. This sample was assumed to represent the zooplankton community in the area and hence the food available for the fish species studied. Zooplankton samples were analysed according to the HELCOM COMBINE Manual (HELCOM, 2015), and identified to the lowest taxonomic level possible (see Diaz-Gil *et al.*, 2014 for a detailed description of the zooplankton sampling and analyses).

Stomach content analysis

We aimed to analyse fish from the hauls where all three species were present. However, in autumn 2011, only herring and stickleback were caught (Table 1). A random sample of ca.10–60 fish, depending on the size of the catches, was taken from each haul for stomach content analysis. Prey items in the fish stomachs were sorted under microscope and identified to the lowest taxonomic level possible. Larger food organisms (Mysidae, Insecta) were counted separately. The remaining part (zooplankton) was diluted in water, and a subsample of at least 100 zooplankton individuals from each stomach of herring, sprat, and stickleback was counted under stereomicroscope in a Bogorov chamber. The development stage was determined for all copepods in the subsample (nauplii, copepodits C1–C3, C4–C5, and adults C6 male/female), and the length for all other zooplankton items in the subsample was measured (µm). In total, 498 fish stomachs, collected over nine trawl hauls, were analysed (Table 1).

Data analysis

Diet composition

The diet composition of the different prey types for the three fish species was expressed using a numerical index (N_i), which is the average proportion of individuals of the *i*th prey type with respect to the total number of prey consumed by a single fish. The percentage composition by weight (W_i), defined as the mean proportion of *i*th prey weight in respect to total weight of all prey consumed, was also computed to assess the effect of larger prey (Hyslop, 1980). Prey weight was estimated using a stage (or length)–weight relationship according to Hernroth (1985). N_i and W_i were calculated for individual fish and then averaged for each prey type (Chipps and Garvey, 2007). Since W_i showed the same pattern as N_i, W_i was not analysed further. To meet the assumptions of a normal distribution for a parametric test, the data (N_i) were square-rooted for further statistical analysis, which also reduced the importance given to numerous prey items. Because of highly uneven sample sizes across years, annual comparisons were not possible. Samples from different years were, therefore, considered as seasonal resamplings from the same system. Hence, samples from different years were pooled, the diet composition estimated, and bootstrapping on N_i done for each

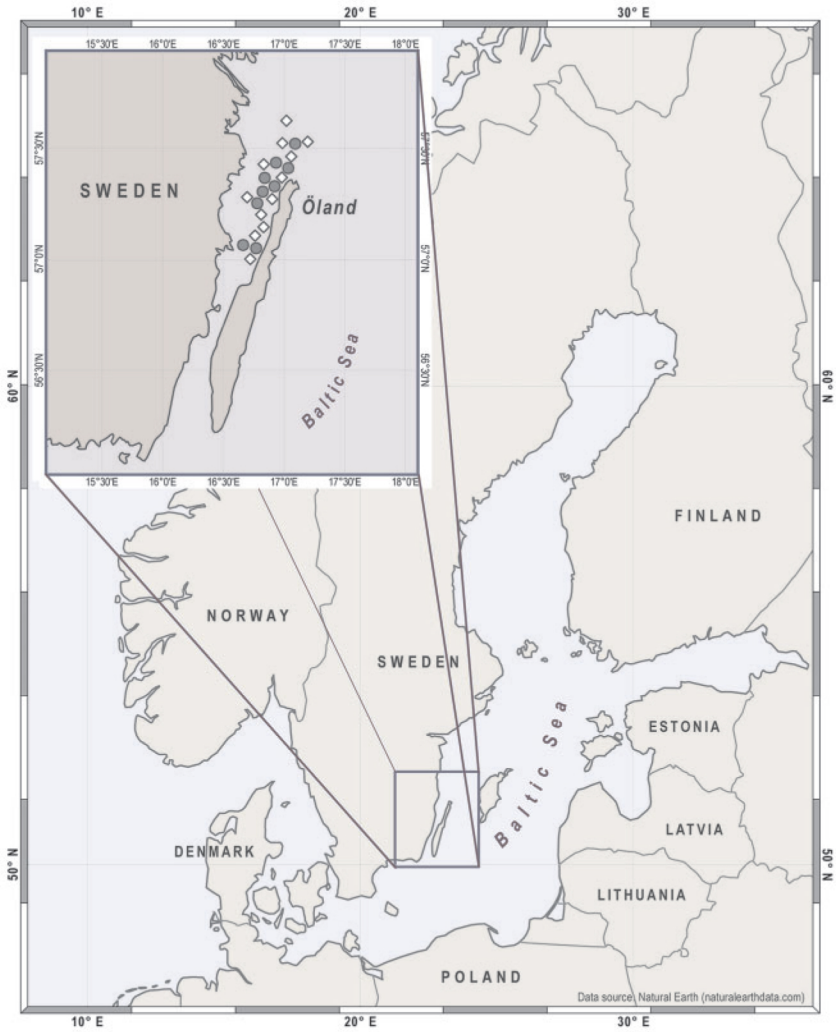

Figure 1. Study area, Kalmar Sound, southwest Baltic Proper. Filled circles indicate fish sampling stations; open rectangles zooplankton sampling sites.

Table 1. Number of stomachs (*n*) and mean length (range) of the fish (TL, cm) analysed in each season.

		Herring		Sprat		Stickleback	
Season	**Year**	***n***	**TL (cm)**	***n***	**TL (cm)**	***n***	**TL (cm)**
Spring	2010	22	11.2 (8–19.8)	15	10.4 (8–12.5)	15	5.6 (4–6.7)
	2011	18	10.5 (9–13.8)	25	10.4 (8.1–13.5)	36	5.5 (3.3–7)
	Total	40	10.9 (8–19.8)	40	10.4 (8–13.5)	51	5.6 (3.3–7)
Summer	2010	58	14.6 (11.3–19.7)	43	11.5 (9–13.3)	54	5.2 (4.1–6.7)
Autumn	2009	71	15.4 (9.8–20)	59	10.9 (8.5–13.9)	37	5.4 (3.3–6.7)
	2010	8	15.6 (11.8–20.9)	7	10.3 (7.4–12.7)	12	6 (5–6.8)
	2011	9	9.2 (8.8–9.5)	–	–	9	4.7 (3.4–5.9)
	Total	88	14.9 (8.8–20.9)	66	10.8 (7.4–13.9)	58	5.4 (3.3–6.8)
Sum/mean		186	13.8	149	10.9	163	5.4

season (see below). The software used for analysis were Brodgar (v. 2.5) and R Development Core Team (2010).

Cluster analysis using Bray–Curtis similarity index (Bray and Curtis, 1957) on N_i was used for comparison of the fish diet across seasons and fish species. The Bray–Curtis similarity index is a commonly used and robust measure of resemblance among samples in ecological datasets (Bloom, 1981). Grouping of the fish diet over season and species was then carried out by hierarchical agglomerative clustering with an unweighted pair-group method using arithmetic averages (Gauch, 1982).

Table 2. Trawling data. Fish were collected in autumn (September–October 2009, 2010, and 2011), spring (April 2010 and 2011), and summer (August 2010). UTC = time of the day when trawling was performed, bottom depth = bottom depth at trawling, fishing depth = depth where the trawl was set.

Season	Date	UTC	Bottom depth (m)	Fishing depth (m)
Autumn 2009	19 September 2009	18:44	34	5
	19 September 2009	23:39	41	5
	27 October 2009	18:02	90	47
	28 October 2009	15:40	43	39
Spring 2010	6 April 2010	19:16	36	25
Summer 2010	18 August 2010	19:11	47	12
Autumn 2010	13 October 2010	00:56	54	10
Spring 2011	7 April 2011	01:08	43	10
Autumn 2011	21 September 2011	18:48	36	1

In order to assess the influence of fish size on the diet, we included size as an explanatory variable in redundancy analysis (RDA) (described below). We also tested for any significant relationships between fish size and fish diet by simple linear regressions (i.e. TL vs. N_i).

RDAs (partial RDA) were performed to disentangle the factors (season, fish species, or fish length) which accounted for most of the variation in fish diet. The RDA models multivariate response data and using a partial RDA, it was possible to extract the influence of specific explanatory variables (Zuur *et al.*, 2007). Using the total sum of canonical eigenvalues of each RDA analysis (algorithm for variance partitioning in RDA according to Zuur *et al.*, 2007), the pure season effect, species effect, and fish size effect were explained as a percentage of the total variation.

Diet overlap

To assess diet overlap and potential competition between fish species, a simplified Morisita index (C_H) was calculated (Horn, 1966):

$$C_H = 2\sum p_{ij}p_{ik}\left(\sum p_{ij}^2 + \sum p_{ik}^2\right)^{-1} \quad (1)$$

where j and k are the fish species, and p_{ij} and p_{ik} are the proportions of the prey i of the total prey consumed by the two species ($i = 1, 2, 3 \ldots, n$). Diet overlap analysis was performed on a haul basis over seasons and size groups using N_i generated after bootstrapping (see below) as input variable. To be consistent and comparable with other similar studies, we used the size groups of clupeids as TL $\leq$ 10 cm ("small fish"), 10 $<$ TL $\leq$ 15 cm ("medium fish"), and TL $>$ 15 cm ("large fish", Casini *et al.*, 2004; Peltonen *et al.*, 2004).

Selectivity

Diaz-Gil *et al.* (2014) discriminated the Kalmar Sound zooplankton communities into season-specific geographical clusters. Based on that, a particular fish sampling station in our study was coupled with a particular cluster of the zooplankton community, which we assume, therefore, to best reflect the food available for the fish. Since zooplankton and fish sampling stations did not match exactly temporally and spatially (Figure 1), the zooplankton data used for selectivity estimation came from the station in closest proximity to fish trawling. Priority was given to the station that was sampled on the same day as the fish. If not available, data from several zooplankton stations that fell into the same cluster were used.

Selectivity for the certain prey types was investigated using the *V*-selectivity index (Pearre, 1982; Flinkman *et al.*, 1992, 1998; Casini *et al.*, 2004). The index ranges from −1 (absolute rejection) to +1 (absolute preference), with a zero value for no selection. The index is based on the Chi-square:

$$V = \pm(\mathrm{Chi}^2/n)^{1/2} \quad (2)$$

where n is the number of observations (total abundance of zooplankton in the sea sample and in the stomachs). The average proportion by number of certain prey species in the zooplankton samples (standardized to 1 m^3) and in the fish stomach was used in the V estimation (see Pearre, 1982 for detailed description). The V-index was estimated for each fish within each haul and then averaged for each season. To test whether selectivity (V index) significantly deviated from 0, a one-sample non-parametric test (Wilcoxon signed rank test) was used, as data were not normally distributed. For selectivity estimation, data from 2009 to 2010 were used ($n = 373$).

Precision of diet estimates

To assess the precision of the diet estimation, a bootstrapping technique was used (Tirasin and Jørgensen, 1999). A total of 1000 independent random samples with replacement were used to estimate means and 95% confidence intervals (2.5th and 97.5th percentiles) of N_i and V-index. Samples from different years were pooled and bootstrapping done for each season and size group.

Sample size sufficiency for assessing the diet was investigated using cumulative prey curves (Hurtubia, 1973). Randomized cumulative prey curves for each fish species in each season were constructed using the vegan package (Oksanen *et al.*, 2010) of the R statistical software (R Development Core Team, 2010). The cumulative number of prey taxa present in each sample was plotted against the randomly pooled number of samples. The order in which stomach contents were analysed was randomized 1000 times. Linear regression of the raw data generated for the last four mean values was used to quantitatively determine if the curve reached an asymptote, signifying an adequate number of samples (Bizzarro *et al.*, 2007). If the slope was $<$0.05, we assumed the curve reached an asymptote. As a conservative measure, the lowest taxonomic level to which the prey was identified was used to construct the curves, making it less likely that the curves would reach an asymptote. For comparative purposes, we also

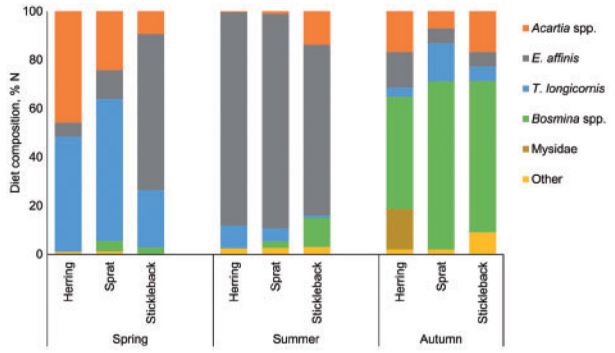

Figure 2. Summary of diet composition of herring, sprat, and stickleback during different seasons in Kalmar Sound, southwest Baltic Proper. All prey items that comprised >5% of the diet are shown in the graph. More detailed diet composition and error estimates (95% *CIs*) are presented in Supplementary Table S1.

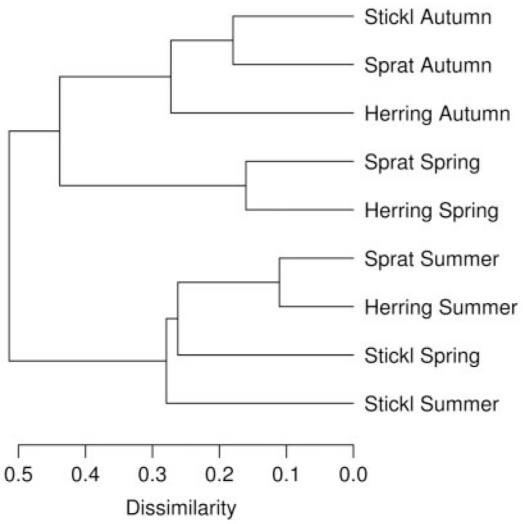

Figure 3. Cluster analysis using the Bray–Curtis similarity index on root transformed data (N_i) of three pelagic fishes in different seasons. Stickl = three-spined stickleback.

constructed the curves excluding prey items that occurred in only one stomach.

Results

Overall diet composition

The size of the fish analysed differed across seasons. The majority of the herring and sprat, especially in summer, were >10 cm (see Supplementary Figure S1), while sticklebacks were always about the same size (5.3–5.5 cm on average) independent of the season. All three species almost exclusively fed on copepods and cladocerans. The most important food items (in terms of both numbers and biomass) were the calanoid copepods *Eurytemora affinis*, *Acartia* spp., and *Temora longicornis*, and the cladoceran *Bosmina* spp. (Figure 2, Supplementary Table S1).

Seasonality

There were substantial seasonal differences in the diet of all three species. In spring, the majority of the herring and sprat diet consisted of *T. longicornis*, while the diet of sticklebacks mainly consisted of *E. affinis*. Further, *E. affinis* made the most substantial contribution to the diet of all three fish species in summer, while the cladoceran *Bosmina* spp. was the most important prey for all fish species in autumn (Figure 2). Herring also fed on mysids in autumn. Other cladoceran species (*Podon* spp.) were only notable (>1%) in autumn in the diet of sticklebacks (3%). The invasive *Cercopagis pengoi* occurred in small quantities (1–2%) in all fish species stomachs in summer. Some rotifers (*Keratella* sp.) were found in the stomachs of sticklebacks in autumn (3%) and summer (<1%). Generally, very few (<1%) calanoid *Centropages hamatus* appeared in the diet, comprising only >1% in the diet of herring and sticklebacks in autumn (Supplementary Table S1).

When diets were compared among seasons and species using cluster analyses (Figure 3), it was evident that the diets of herring, sprat, and stickleback mainly differed across seasons rather than across fish species. It was only stickleback that showed a similar diet in spring as in summer (Figure 3).

Besides season and fish species, the third potentially influential factor, fish TL, was included in variance partitioning in the RDA analyses. Again, season explained most of the variance (13%), while fish species and fish length were of about the same explanatory magnitude (2.5%). A large part of the variation in diet (82%) remained unexplained.

We found only a few significant relationships between fish size and diet (see Supplementary Figure S2). First, large herring (>15 cm) exhibited a positive relationship with the share of mysids in the diet in autumn ($r = 0.52$, $p < 0.0001$). Second, in summer, larger sticklebacks feed on *Acartia* spp. to a lesser extent than smaller ones ($r = -0.65$, $p < 0.0001$), and the same pattern was revealed for herring in autumn ($r = -0.58$, $p < 0.0001$). No other significant relationships between fish size and diet were found.

Diet overlap

Overall, the highest diet overlap between the three species was found in summer (94–99.99%), whereas a lower overlap was found in spring (Figure 4, Supplementary Table S2). Generally, the highest overlap was present between clupeids (sprat and herring). During autumn, however, a higher overlap was observed between sticklebacks and clupeids than among clupeids (Supplementary Table S2). Even the diet of large (>15 cm) herring exhibited a considerable overlap with the diet of the much smaller sticklebacks. Despite the fact that large herring in autumn were found to include mysids in their diet (fish caught in deeper waters, ca. 39 m fishing depth had higher proportion of mysids in the diet), some of them (caught mostly at the surface, ca. 5–10 m fishing depth) mainly fed on zooplankton, as did the sticklebacks.

Selectivity

All fish species negatively selected *Acartia* spp. (except during spring, Figure 5). *E. affinis* was especially preferred in summer, while *T. longicornis* did not show any clear pattern. The cladoceran *Bosmina* spp. was positively selected in autumn, especially by sticklebacks. In spring, all fish species were positively selecting their prey; however, wide confidence intervals imply high uncertainty (Figure 5, Supplementary Table S3) for this

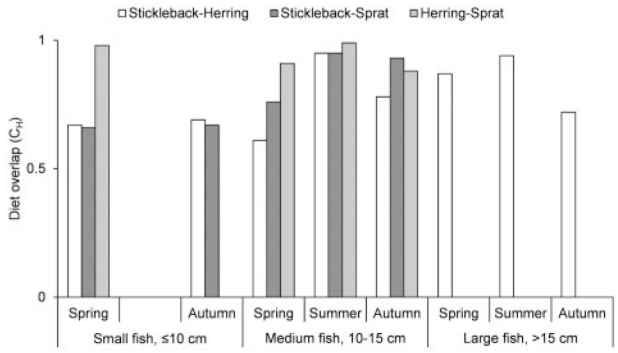

Figure 4. Mean dietary overlap (Morisita simplified indices, C_H) among fish species in different fish size groups (TL) in different seasons in Kalmar Sound, southwest Baltic Proper. Small sprat and herring = TL ≤ 10 cm, medium = 10 < TL ≤ 15 cm, large = TL > 15 cm. Sticklebacks were not divided into size groups. See Supplementary Figure S1 for stickleback size distributions.

season. The highest variation of selectivity indices was observed in spring, while the lowest was in summer. Nevertheless, variability within a season was lower compared with differences between seasons.

Precision of diet estimates

Cumulative prey curves in all cases reached an asymptote except for herring and sprat in spring (see Supplementary Figure S3) where the sample size was too low for a sound estimation of the diet at the genus level. This was mainly due to scarcity of some prey items, since all curves reached an asymptote when excluding prey items that appeared in only one stomach.

Discussion

In this study, we show that the main prey items for sticklebacks, sprat, and herring in the semi-enclosed Kalmar Sound (southwest Baltic Proper) were the calanoids *E. affinis*, *T. longicornis*, and *Acartia* spp., and the cladoceran *Bosmina* spp. The diet of all three fish species overlapped considerably, and the fish showed similar preferences for certain types of prey. Substantial diet overlap was even present between sticklebacks and large (>10 cm) clupeids. To the best of our knowledge, this study is the first to assess the diet of sticklebacks, herring, and sprat over several seasons and in relation to prey abundance. Below, we discuss these findings in relation to the existing literature.

Diet seasonality

Zooplankton taxa like *E. affinis*, *T. longicornis*, *Acartia* spp., and *Bosmina* spp. dominated the diet of all three fish species, but the proportion of each prey varied over seasons. In summer, all fish species almost exclusively included *E. affinis* in their diet, and *Bosmina* spp. was the most abundant prey item in the fish diets in autumn. Although there are studies focusing on the feeding ecology of planktivorous fish in the pelagic system of the Baltic Sea (e.g. Arrhenius, 1996; Möllmann and Koster, 1999; Casini *et al.*, 2004), only a few have considered stickleback. Similar to our study, Peltonen *et al.* (2004) found that *Bosmina* spp. was the main prey for small herring, sprat, and stickleback in early September in the Gulf of Finland, with *E. affinis* being the second-most abundant diet item of the three species in that area. In a study conducted in the Gulf of Riga, Lankov *et al.* (2010) showed that the copepods *E. affinis* and *Acartia* spp. were the main prey for herring in summer and that the cladoceran *Bosmina* spp. was more important for sprat and stickleback. Sprat is known to have a higher preference than herring for cladocerans (Szypula, 1985; Arrhenius, 1996). These findings are all in line with the results of our study where sprat and sticklebacks had a higher proportion in the diet and a higher selectivity value for *Bosmina* spp. than herring.

Pseudocalanus spp. used to be an important food item in spring for sprat (Kostrichkina *et al.*, 1980), the main prey type for both herring and sprat before the decline of the species since the 1980s (Möllmann *et al.*, 2004, 2005). Accordingly, this zooplankton species was almost absent in the stomachs analysed in our study.

Diet overlap

Large dietary overlap between herring and sprat has been observed in several other studies (e.g. Ojaveer, 1997; Lankov *et al.*, 2010), but our study also shows this for sticklebacks. Diet overlap was, however, dependent on season, haul, and fish size, but generally still high with a median of 87%. In spring, the overlap was the lowest, but increased to very high levels (up to 99%, although again depending on fish size and haul) in summer and autumn.

During spring, zooplankton diversity is the lowest in Kalmar Sound (Diaz-Gil *et al.*, 2014). One should, therefore, have expected the highest diet overlap at this time of year. Since it was not the case, we assume it might have been due to differences in the different depth distribution of zooplankton that was not considered in this study. In spring 2011 (when low diet overlap was observed), sticklebacks mainly fed on *E. affinis*, while clupeids fed on *T. longicornis*.

Selectivity

We found highly selective feeding behaviour of all three fish species studied. The copepod *E. affinis* was mostly favoured in summer, and *Bosmina* spp. was preferred mainly during autumn by all three fish species. In a previous study in the Gulf of Bothnia, *Bosmina* spp. and *E. affinis* were found to be favoured by sticklebacks in late August (Leinikki, 1995). *E. affinis* might be preferred because of its conspicuous egg sacs and *Bosmina* spp. due to its pigmented eggs and low escape response (Flinkman *et al.*, 1992; Viitasalo *et al.*, 2001). Similar to other studies (Leinikki, 1995; Möllmann and Koster, 2002; Casini *et al.*, 2004), *Acartia* spp. was also disfavoured by all three species except in spring. The reason for disfavouring *Acartia* spp. might be its high escape response (Viitasalo *et al.*, 2001), and the positive selection in spring might be explained by scarcity of food items other than *Acartia* spp. Actually, the abundance of all food items in the water column in spring was much lower (10-fold or more) compared with other seasons (Diaz-Gil *et al.*, 2014), and *Acartia* spp. is the most abundant prey item at this time of year. In the Gulf of Riga, herring selectively fed on *Limnocalanus macrurus* and *E. affinis* during spring (Livdane *et al.*, 2016). We also found clupeids selectively feeding on *E. affinis*, but for all fish, *Acartia* spp. was the main preferred item in spring.

Estimations of diet preference by nature involve uncertainty/variability, and thus zooplankton abundance can differ considerably among samples taken even in close proximity to each other

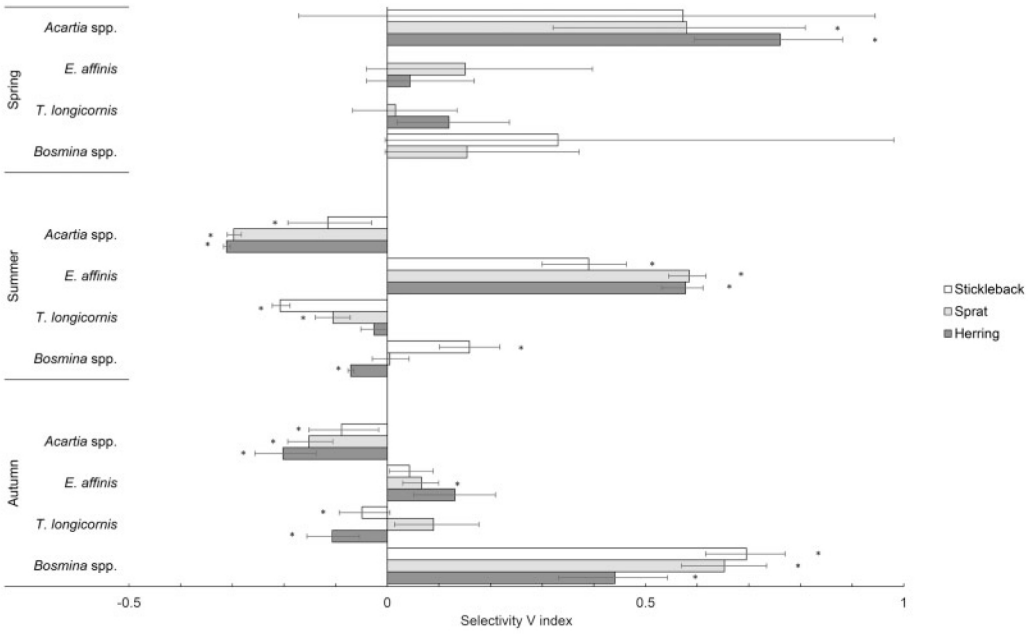

Figure 5. Selectivity (*V*-index) of stickleback, herring, and sprat for the main prey species in different seasons. Bars and whiskers denote averages and 95% *CI*s, respectively; asterisks = statistical significance (Wilcoxon signed rank test).

(Klais *et al.*, 2016). The estimates in our study represent a snapshot in time and show only relative selectivity of the species (i.e. the diet of all three species is compared with the same set of zooplankton data). However, we believe our data are sufficient to compare fish species prey preferences.

One should note that since fish were analysed on a haul basis (i.e. fish collected at the same depths), we might partly have overestimated the diet overlap between the three species. Overlap at the population or stock level might actually be smaller since large herring are known to dwell deeper and thus do not always vertically overlap with sticklebacks. When both herring and sticklebacks were sampled in deeper waters, large herring tended to have mysids in their stomachs, but closer to the surface (shallower hauls), large herring fed on zooplankton as did sticklebacks.

Fish size might be another confounding factor for our findings since it has been shown to influence fish diet (see e.g. Casini *et al.*, 2004). Surprisingly, we also found substantial diet overlap between sticklebacks and larger (>10 cm) herring and sprat (Figure 4). Our division into size groups was done to make our results comparable with other studies. In accordance with many other studies, there were considerable amounts of nektobenthos in the stomachs of large (>15 cm) herring in autumn (Szypula, 1985; Raid and Lankov, 1995; Casini *et al.*, 2004), but fish size still explained only a small fraction (2.5%) of the total variation in diet between the three fish species in our study. It could, however, be that we failed to find substantial impact of fish size on the diet due to a low sample size of small fish.

The highest consumption of zooplankton by herring and sprat populations is in July–October (Arrhenius and Hansson, 1993). We found almost complete diet overlap among the fish species in summer (August), suggesting that if food supply is limited, interspecific competition might influence fish growth. Although our dataset is unbalanced with respect to the smallest sizes of fish, the high diet overlap observed is likely to be significant also for smaller herring and sprat. Juvenile or larval fish are especially sensitive to low levels of food resulting in young fish investing more in somatic growth than older fish and being less resistant to starvation (Blaxter and Hunter, 1982; Munk, 1993). Our findings, therefore, suggest a potential negative effect of increasing stickleback abundance on juvenile herring and sprat. That the highest diet overlap overall was observed in summer further indicates a potential for high intraspecific competition since summer is likely to be a critical period for the growth of all three species. Therefore, we advocate that future studies should include more comprehensive datasets including also herring and sprat <10 cm to disentangle potential effects of intraspecific competition.

Concluding remarks

During recent decades, mesopredators like herring and sprat have been released from predation by cod and substantially increased in abundance in the Baltic Sea (Casini *et al.*, 2008). This, together with climate change and increased temperatures, might have also been beneficial for another mesopredatory fish, the three-spine stickleback (Lefébure *et al.*, 2014; Bergström *et al.*, 2015). Stickleback abundance is rapidly increasing in the western parts of the Baltic Sea (Bergström *et al.*, 2015), and its biomass can potentially constitute a substantial part of the planktivorous fish biomass in the offshore area (Ljunggren *et al.*, 2010). Our findings of a similar diet composition, a high diet overlap, and a selection of the same prey items as for herring, sprat suggest that sticklebacks might be potential competitors to other planktivorous and commercially important fish species in the Baltic Sea. However, to establish that sticklebacks are competing with clupeids for food and are impacting the condition status of these species, information is needed to verify that resource availability of zooplankton constitutes a limiting factor for the clupeids. Our study and earlier studies (Peltonen *et al.*, 2004; Lankov *et al.*,

2010) used data from different time periods (2009–2011, 2002, and 1996–2006, respectively) and also with probable differences in stickleback abundance. Therefore, it would be important to investigate whether the drastic increase in sticklebacks in the western part of the Baltic (Bergström *et al.*, 2015) has also influenced the diet of herring and sprat over time. Nevertheless, based on the results of this study, we suggest that the role of sticklebacks in the pelagic foodweb of the Baltic Sea should not be neglected when studying the structure and dynamics of the system, even though more studies are needed to assess the spatial extent and generality of our findings in other parts of the Baltic Sea.

Supplementary data

Supplementary material is available at the *ICESJMS* online version of the article.

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Seasonal dynamics in the diet of pelagic fish species in the southwest Baltic Proper

Eglė Jakubavičiūtė, Michele Casini, Linas Ložys, and Jens Olsson

Table S1. Diet composition (percentage by number, N_i) of herring, sprat, and three-spined stickleback during different seasons in Kalmar Sound, southwest Baltic Proper. Data from 2009–2011 combined. Means and lower (Low) and upper (Up) limits of 95% confidence intervals calculated by bootstrapping, as outlined in text (see Methods section). Small sprat and herring = TL ≤10 cm, medium = 10 <TL ≤15 cm, large = TL >15 cm.

Season	Prey	Stickleback			Small sprat			Small herring			Medium sprat			Medium herring			Large herring		
		Mean	Low	Up	Mean	Low	Up	Mean	Low	Up	Mean	Low	Up	Mean	Low	Up	Mean	Low	Up
Spring	*Acartia* spp.	9.34	3.86	16.92	19.3	6.8	33.6	52.0	30.9	73.8	28.7	11.7	48.0	32.4	15.2	53.8			
	Bosmina spp.	2.81	0.00	8.11	3.1	0.0	9.4				5.6	0.0	16.7						
	Centropages hamatus				1.1	0.0	2.8	1.4	0.0	4.2									
	Eurytemora affinis	64.22	56.86	70.61	1.1	0.0	3.3	10.8	2.5	23.5	21.4	8.0	37.3	0.7	0.0	2.1			
	Evadne spp.				0.6	0.0	1.9							0.8	0.0	2.3			
	Temora longicornis	23.63	19.64	27.74	74.6	59.1	88.0	35.8	17.6	57.2	43.9	25.5	63.6	66.1	47.1	85.0			
	Pseudocalanus sp.										0.4	0.0	1.2						
Summer	*Acartia* spp.	13.82	7.27	20.88							1.0	0.3	1.8	0.6	0.1	1.2	0.1	0.0	0.2
	Balanus larvae	0.07	0.00	0.22															
	Bosmina spp.	11.96	8.38	16.07							2.8	1.3	4.7	0.1	0.0	0.3	0.0	0.0	0.1
	Centropages hamatus	0.04	0.00	0.11							0.6	0.3	1.0	0.4	0.1	0.8	0.1	0.0	0.4
	Cercopagis	2.62	0.34	5.81							1.9	0.1	5.1	3.6	0.0	10.7	0.0	0.0	0.1
	Eurytemora affinis	70.21	62.82	77.09							88.4	84.8	91.3	85.1	78.1	89.6	91.2	89.1	93.1
	Evadne spp.	0.00	0.00	0.00										0.0	0.0	0.1			
	Temora longicornis	0.93	0.31	1.72							5.1	3.8	6.7	10.0	8.1	12.0	8.6	6.6	10.5
	Pseudocalanus sp.	0.16	0.00	0.47										0.0	0.0	0.1			
	Podon spp.	0.05	0.00	0.20							0.2	0.0	0.5						
	Keratella sp.	0.13	0.00	0.34															

Table S1 *Continued from previous page*

Season	Prey	Stickleback			Small sprat			Small herring			Medium sprat			Medium herring			Large herring		
		Mean	Low	Up	Mean	Low	Up	Mean	Low	Up	Mean	Low	Up	Mean	Low	Up	Mean	Low	Up
Autumn	*Acartia* spp.	14.57	9.57	20.63	7.7	3.4	12.7	99.5	98.9	100.0	6.7	3.5	10.1	5.3	0.6	11.2	6.3	0.0	15.6
	Alona sp.										0.0	0.0	0.1						
	Bosmina spp.	66.14	57.63	73.83	61.1	44.0	75.3	0.2	0.0	0.5	74.1	63.5	83.6	59.6	44.6	74.1	45.8	28.3	60.6
	Centropages hamatus	1.15	0.12	2.96	0.6	0.0	1.4				0.7	0.2	1.3	3.6	0.0	10.7	1.0	0.0	3.1
	Eurytemora affinis	5.50	3.27	8.23	10.0	4.7	15.9				3.8	1.9	6.0	22.8	11.9	35.2	11.0	3.6	21.2
	Evadne spp.	0.43	0.11	0.79	1.3	0.3	2.4				0.8	0.2	1.8	0.0	0.0	0.1			
	Mysidae													0.3	0.0	1.0	35.0	19.3	53.1
	Temora longicornis	5.79	2.42	10.24	19.2	8.8	30.2	0.1	0.0	0.4	13.5	6.5	20.9	8.4	2.7	15.1	0.9	0.0	2.6
	Podon spp.	2.89	1.28	5.02	0.1	0.0	0.2	0.2	0.0	0.7	0.4	0.0	1.0						
	Keratella sp.	3.07	0.55	6.68															

Table S2. Mean dietary overlap (Morisita simplified indices, CH) among fish species in different fish size groups (TL) in different seasons in Kalmar Sound, southwest Baltic Proper. Small sprat and herring = TL ≤10 cm, medium = 10 <TL ≤15 cm, large = TL >15 cm. Sticklebacks were not divided into size groups (see Figure S1 for stickleback size distributions).

Season	**Stickleback – herring**				**Stickleback – sprat**			**Herring – sprat**					
	Small herring	Medium herring	Large herring	Mean	Small sprat	Medium sprat	Mean	Small – small	Medium – small sprat	large herring – small sprat	Medium – medium	Large herring – medium	Mean
Spring	0.67	0.61	0.87	0.69	0.66	0.76	0.71	0.98	0.98	0.94	0.91	0.86	0.94
Summer		0.95	0.94	0.94		0.95	0.95				0.99	0.99	0.99
Autumn	0.69	0.78	0.72	0.74	0.67	0.93	0.8		0.7	0.51	0.88	0.61	0.69

Table S3. Selectivity (V-index) of stickleback, herring, and sprat for the main prey species (data from 2009 and 2010 combined). Means and lower (Low) and upper (Up) limits of 95% confidence intervals calculated by bootstrapping. Significant values bolded (p <0.05, Wilcoxon signed rank test).

Season	**Prey**	**Stickleback**			**Sprat**			**Herring**		
		Mean	Low	Up	Mean	Low	Up	Mean	Low	Up
Spring	*Acartia* spp.	0.57	–0.17	0.94	**0.58**	0.32	0.81	**0.76**	0.59	0.88
	Bosmina spp.	0.33	0.00	1.00	0.15	0.00	0.37			
	Eurytemora affinis				0.15	–0.04	0.40	0.04	–0.04	0.17
	Temora longicornis				0.02	–0.07	0.14	**0.12**	0.02	0.24
Summer	*Acartia* spp.	**–0.12**	–0.19	–0.03	**–0.30**	–0.31	–0.28	**–0.31**	–0.32	–0.30
	Bosmina spp.	**0.16**	0.10	0.22	0.00	–0.03	0.04	**–0.07**	–0.08	–0.06
	Eurytemora affinis	**0.39**	0.30	0.46	**0.58**	0.54	0.62	**0.58**	0.53	0.61
	Temora longicornis	**–0.21**	–0.22	–0.19	**–0.10**	–0.14	–0.07	–0.03	–0.05	0.00
Autumn	*Acartia* spp.	**–0.09**	–0.15	–0.02	**–0.15**	–0.19	–0.11	**–0.20**	–0.26	–0.14
	Bosmina spp.	**0.70**	0.62	0.77	**0.65**	0.57	0.73	**0.44**	0.33	0.54
	Eurytemora affinis	0.04	0.00	0.09	**0.07**	0.03	0.10	0.13	0.05	0.21
	Temora longicornis	**–0.05**	–0.09	0.01	0.09	0.01	0.18	**–0.11**	–0.16	–0.05

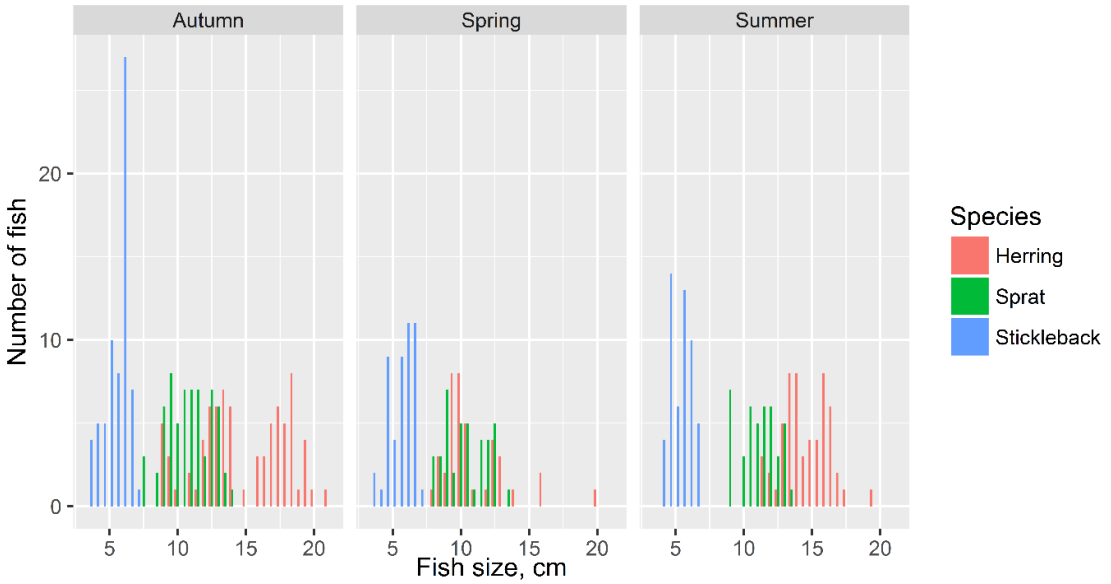

Figure S1. Fish length (TL) distribution during different seasons in the sample.

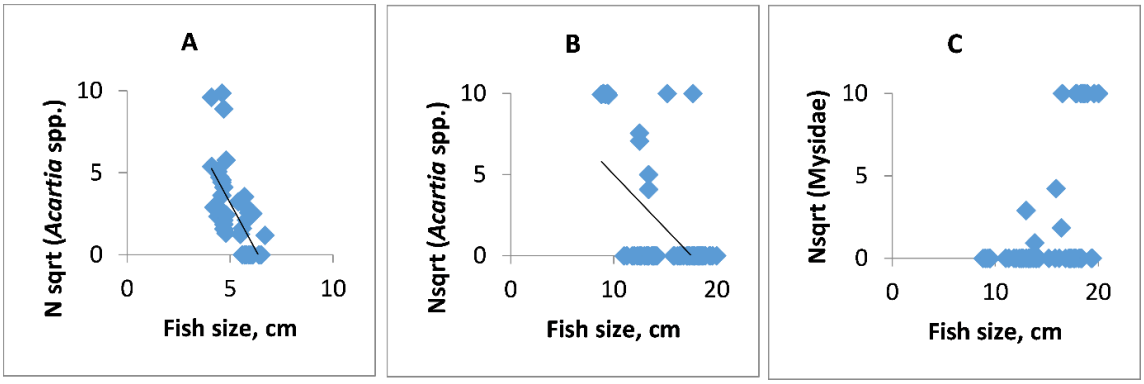

Figure S2. Significant fish size (TL, cm) and diet relationships. (A) Three-spined stickleback feeding on *Acartia* spp. in summer, $r = -0.65$, $p < 0.0001$; (B) herring on *Acartia* spp. in autumn, $r = -0.58$, $p < 0.0001$; (C) herring on Mysidae in autumn, $r = 0.52$, $p < 0.0001$.

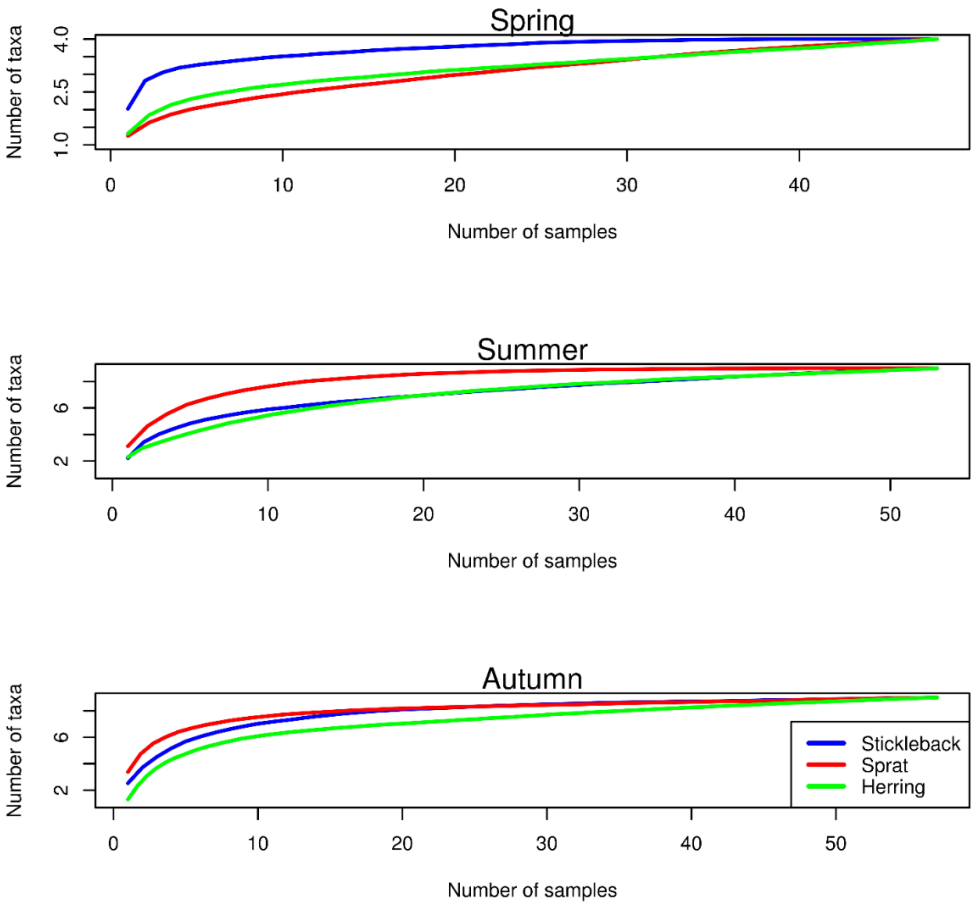

Figure S3. Cumulative prey curves for three-spined stickleback, sprat, and herring calculated for prey identified to the lowest possible taxa.

PLOS ONE

RESEARCH ARTICLE

DNA metabarcoding reveals diverse diet of the three-spined stickleback in a coastal ecosystem

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Abstract

The three-spined stickleback (*Gasterosteus aculeatus* L., hereafter 'stickleback') is a common mesopredatory fish in marine, coastal and freshwater areas. In large parts of the Baltic Sea, stickleback densities have increased >10-fold during the last decades, and it is now one of the dominating fish species both in terms of biomass and effects on lower trophic levels. Still, relatively little is known about its diet—knowledge which is essential to understand the increasing role sticklebacks play in the ecosystem. Fish diet analyses typically rely on visual identification of stomach contents, a labour-intensive method that is made difficult by prey digestion and requires expert taxonomic knowledge. However, advances in DNA-based metabarcoding methods promise a simultaneous identification of most prey items, even from semi-digested tissue. Here, we studied the diet of stickleback from the western Baltic Sea coast using both DNA metabarcoding and visual analysis of stomach contents. Using the cytochrome oxidase (CO1) marker we identified 120 prey taxa in the diet, belonging to 15 phyla, 83 genera and 84 species. Compared to previous studies, this is an unusually high prey diversity. Chironomids, cladocerans and harpacticoids were dominating prey items. Large sticklebacks were found to feed more on benthic prey, such as amphipods, gastropods and isopods. DNA metabarcoding gave much higher taxonomic resolution (median rank genus) than visual analysis (median rank order), and many taxa identified using barcoding could not have been identified visually. However, a few taxa identified by visual inspection were not revealed by barcoding. In summary, our results suggest that the three-spined stickleback feeds on a wide variety of both pelagic and benthic organisms, indicating that the strong increase in stickleback populations may affect many parts of the Baltic Sea coastal ecosystem.

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Introduction

The three–spined stickleback (*Gasterosteus aculeatus* L., hereafter 'stickleback') is a common mesopredatory fish of high ecological interest, widespread all over the northern hemisphere in various habitats including coastal seas, estuaries, freshwater lakes and streams [1]. The stickleback is also an eco-genomic model organism, well-studied in terms of behavioural and evolutionary ecology [2–4]. Knowledge on the role of sticklebacks in aquatic food webs is, however, rather limited, especially in coastal and marine areas. To better understand the ecological role of sticklebacks, their feeding patterns and diet preferences need to be described, as feeding behaviour may affect community composition and food web functions.

In the brackish Baltic Sea, stickleback abundance has increased more than 10-fold during the last decade [5]. Currently, it constitutes up to 10% of the planktivorous biomass in offshore areas [5,6], and dominates fish assemblages in some coastal areas during summer, when adults immigrate from the open sea to spawn [6–8]. Experiments and field surveys indicate that sticklebacks may alter coastal food webs by feeding on and influencing lower trophic levels (e.g. grazers) [9], and worsening the effects of nutrient enrichment through cascading effects that increase the biomass of filamentous algae [6,7,10,11]. Moreover, sticklebacks may suppress populations of large predatory fish, such as northern pike and Eurasian perch, by predation on eggs and larvae, and the intraguild predation between sticklebacks and these large predatory fish may contribute to destabilizing food webs [5,12,13]. Thus, the increasing abundances of sticklebacks, in combination with their central role in ecosystem functioning, points to the need for more detailed knowledge on stickleback diets.

However, fish diet studies are challenging, with different methods having their own set of limitations. For the last century, the standard has been to visually identify prey from stomach contents, based on prey morphology [14]. This time-consuming method relies heavily on taxonomic expertise and can only be done when prey organisms are not too digested. Because most prey organisms rapidly degrade in stomachs, a high taxonomic resolution is often not possible, and a significant share of the prey tissue in the guts is often unidentifiable (e.g., [15]). A highly promising alternative to visual prey identification is metabarcoding methods, which combine DNA-based identification and high-throughput DNA sequencing, using taxonomically broad PCR primers to mass-amplify DNA barcodes from bulk samples (such as environmental samples or gut contents) [16]. Metabarcoding enables the identification of most prey items, even when diets are broad and diverse [17], and the simultaneous analysis of many samples. The aim of this study was to investigate the diet of the three-spined stickleback in coastal areas of the western central Baltic Sea, using a combination of classic (visual) and emerging (DNA metabarcoding) methods. Specifically, we addressed three questions: 1) what do sticklebacks eat in coastal areas, 2) how does stickleback diet depend on its body size, and 3) how do visual and DNA-based methods compare in terms of prey identification from stomach content. Accurate diet determination will provide more comprehensive information on coastal food webs, knowledge which is highly relevant in the context of ecosystem-based management to assess and potentially counteract the undesirable effects of massive increases of sticklebacks on the ecosystem [10,18].

Material and methods

This study was made in accordance with the ethical regulation laid down in the Swedish ordinance SJVFS 2012:26, which is the Swedish implementation of the Directive 2010/63/EU of the European Parliament and of the Council on the protection of animals used for scientific purposes. The fish died in the process of lifting the nets; after sticklebacks were removed from the nets they were immediately put in 95% ethanol. The fish sampling procedures applied in

the project were also judged and approved by the Ethical Board on Animal Experiments of the County Court of Uppsala, Sweden, permit C 139/13.

Study sites and sample collection

Sampling was performed in May 2014, after adult sticklebacks had migrated from their offshore winter areas into the coastal zone to spawn. Sampling was conducted in 16 bays situated along a 350 km stretch of the central Swedish Baltic Sea coast (Fig 1). Shallow bays are important reproduction areas for many coastal fish species, including sticklebacks [19]. They are characterized by a diverse and highly productive community of aquatic vegetation and macroinvertebrates, many of which constitute potential prey for sticklebacks [20]. The 16 bays were selected to represent a mix of shallow bay habitats along an archipelago gradient from the mainland to the outer archipelago, including sheltered shallow lagoons with narrow inlets, to more open and exposed bays.

Stickleback sampling. Sampling of stickleback stomachs was performed as part of a larger survey targeting the whole food webs of shallow vegetated bays (see [11]). Sticklebacks were caught using Nordic survey gillnets (European Union 112 standardized method EN 14757:2005). The nets were set at 0.5–3 m depth between 4–7 pm, and lifted between 7–9 am the following morning. The fish died in the process of lifting the nets; after sticklebacks were removed from the nets they were immediately put in 95% ethanol. In total, 196 individual fish were analysed (Fig 1). In bays where fewer than 15 sticklebacks were caught, all of the fish were analysed with respect to their diet composition. For bays where more individuals were caught, a subset representing the size distribution in the catch was selected for the diet analyses.

The total length (TL) of each fish was measured to the nearest 1 mm. The mean total length was 57.7 ±7.6 (SD), with a range of 35–72 mm (S1 Fig). Visual inspection of the resulting size frequency distribution indicates a left skew, i.e. an underrepresentation of large individuals (S1 Fig), which was not an effect of skewed subsampling. Only 2.5% (5 of the 196 individuals) were >70 mm; a much smaller proportion than that found in other, similar surveys in the Western Baltic Sea (unpublished; [5]).

Visual analysis of stomach content

Out of the 196 sticklebacks sampled, 192 were analysed using both visual methods and metabarcoding, and four were used in a pilot study for DNA metabarcoding. The stomachs were dissected and flushed with 80% EtOH to remove all stomach contents. To avoid cross-contamination, the dissection tools were rinsed with soap, bleach, and Milli-Q water before each individual dissection. Prey items visually distinguishable in the flushed stomach contents were identified to the highest taxonomic resolution possible, using a stereo microscope (magnification 20-80x). Frequency of occurrence for each prey item was estimated ($\%F_{vis}$, the percentage of stomachs in which a prey was present). Thereafter, all stomach contents were stored at -20˚C in 80% EtOH for subsequent DNA extraction. The level of digestion for each stomach was classified on a 1–5 scale, where 1 = intact prey, 2 = partially digested, 3 = extensively digested, 4 = very few prey parts discernible, and 5 = fully digested/ empty stomach.

DNA metabarcoding analysis

Sample processing. DNA was extracted from the 196 sticklebacks' gut contents using the UltraClean® Tissue and Cells DNA Isolation Kit (MO BIO Laboratories), according to the manufacturer's instructions. The dual PCR amplification method was used for Illumina MiSeq library preparation [21]. The cytochrome oxidase 1 (CO1) marker was first amplified using

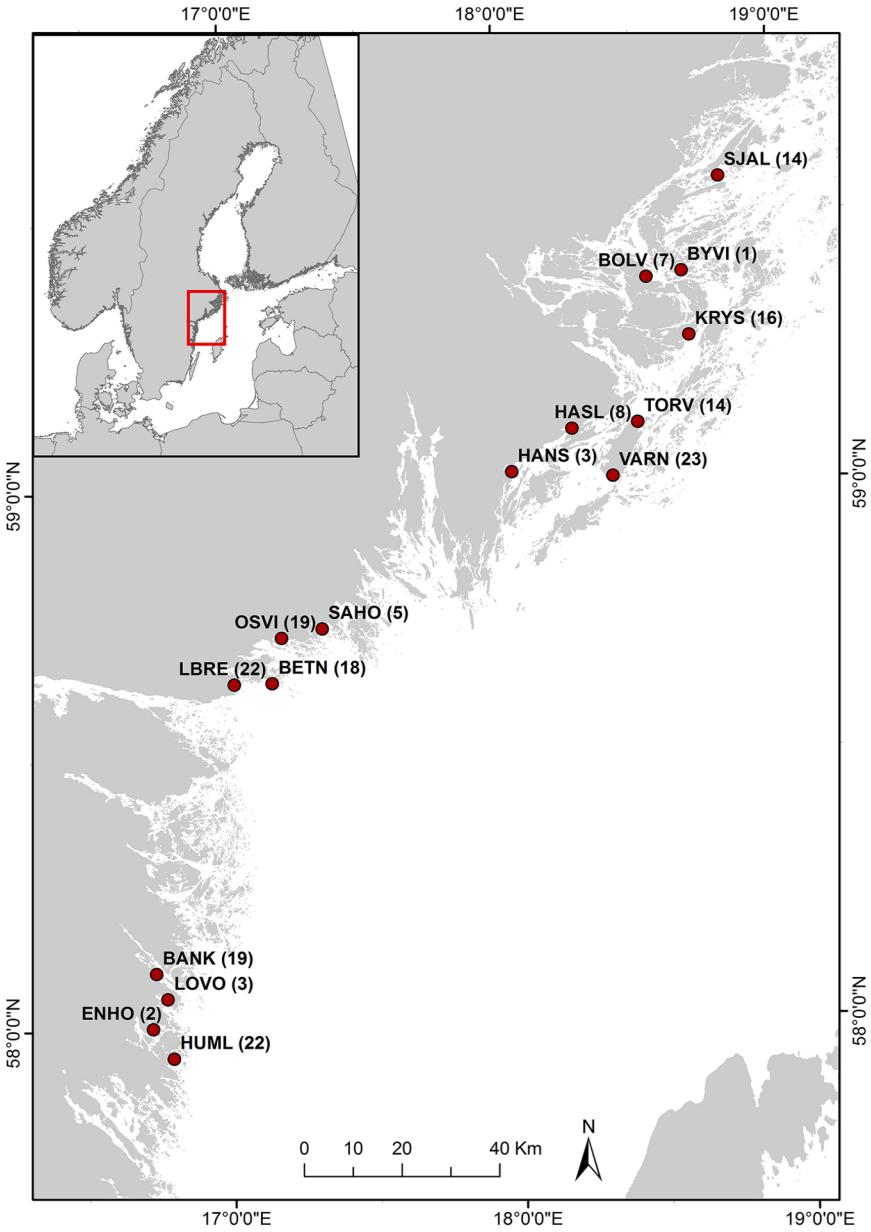

Fig 1. Sampling sites. Numbers in brackets indicate number of sticklebacks analysed from each bay.

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locus specific primers including an Illumina adapter overhang (amplicon PCR). The primers were based on Leray et al.'s (2013) [22] 'mini-barcode' yielding a 313 bp fragment (CO1mini_mI COIintF_MiSeq: TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGGWACWGGWTGAACWGTWT AYCCYCC, CO1_dgHCO2198_MiSeq: GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGTA AACTTCAGGGTGACCAAARAAYCA, CO1 specific sequence is shown in bold, and illumina adapter in regular font). A blocking primer was used in the amplicon PCR, to prevent amplification from

Table 1. Number of reads after each bioinformatic data processing step.

Paired-end joining	Primer trimming	Quality filtering
15 706 724	10 982 728	10 586 546

https://doi.org/10.1371/journal.pone.0186929.t001

G. aculeatus, following [23]. A Spacer C3 CPG was added to the 3' end of the blocking primer to prevent elongation without affecting annealing properties, minimizing predator DNA amplification (G_aculeatus_block_Hco_2198: CAAAGAATCAAAATAAGTGTTGGTAAAGA-C3). For each sample, two independent PCR reactions were performed and later pooled, ensuring greater coverage of prey items amplified. In a second PCR step, Illumina dual index adapters were incorporated to the amplicons using a limited number of cycles (Index PCR).

Amplicon PCRs were performed as 30 μl reactions with 20pm of each primer and 100pm of blocking primer and using Pfu proofreading DNA polymerase (Promega). Cycling conditions were as follows: 2 min at 95˚C (1x); 1 min at 95˚C, 45s at 55˚C, 1 min at 72˚C (40x); 5 min at 72˚C (1x); hold at 4˚C. Amplicons were checked on a 2% agarose gel. Agencourt® AMPure® XP paramagnetic beads (Beckman Coulter) were then used to purify the PCR products [21]. For index PCR, the Illumina Nextera XT kit (96 indices, 384 samples) was used according to manufacturer's instructions. Index PCR was performed as 50 μl reactions using 5 μl of cleaned up amplicons. Cycling conditions were as follows: 3 min at 95˚C (1x); 30s at 95˚C, 30s at 55˚C, 30s at 72˚C (8x); 5 min at 72˚C (1x); hold at 4˚C. Agencourt® AMPure® XP paramagnetic beads (Beckman Coulter) were then used to purify the PCR products, using a ratio of 0.8 that allows the selection of fragments larger than 200 bp. DNA quantification was carried out using a Qubit Fluorometer (Invitrogen) and the average fragment size was verified using Tapestation (Agilent Technologies). Pooled libraries were then sequenced as paired-ends using Illumina MiSeq Reagent v3, producing 30 103 790 paired-end reads of 300 bp in length.

Bioinformatic data processing and analysis. The processing steps were performed using Qiime (Quantitative Insights into Microbial Ecology) version 1.9.1 [24] and custom python scripts. Paired-end joining was done using the Qiime fastq-join tool. A 48% sequence loss was observed after the paired-end joining step due to poor sequence quality at read ends (the raw data are available from the NCBI sequence read archive under accession number SRP101702, BioProject number PRJNA378633). Dual indexes and Illumina overhangs were removed by the sequencing platform. Primer sequences were removed using a custom python script (https://github.com/Quiterie90/Primer_Removal), corresponding to a 30% loss (Table 1). Due to its stringency, the script quality filters sequences by removing incomplete reads or chimeras. Additional quality filtering with Qiime removed 3% of the reads. Finally, remaining chimeric reads were excluded using UCHIME [25], producing a final dataset (0.5% loss).

The Bayesian clustering algorithm CROP was used to cluster the sequences into operational taxonomic units (OTUs) based on the natural distribution of the data, using a Gaussian model [26]. According to a benchmarking study by Leray et al. [22], the best lower and upper bound values to cluster metazoan CO1 sequences are 3 and 4, corresponding to sequence dissimilarities between 6% and 8% (CROP -i <input.fasta> -b 211731 -z 470 -l 3 -u 4 -o <output>).

For taxonomic assignment of CO1 sequences, a custom database was created, consisting in a taxonomy file associated with a reference sequence file, of Metazoan sequences retrieved from BOLD (http://www.boldsystems.org/ downloaded in March 2016), combined with own reference databases of Chironomidae, Nemertea, Xenacoelomorpha and Oligochaeta and barcodes of Swedish Echinodermata, Mollusca, Cnidaria and Arthropoda from the Swedish Barcode of Life database (SweBol).

Taxonomic assignment was done using a 97% similarity threshold using the Uclust software implemented in Qiime with the default parameters [27]. In order to obtain matches for non-Metazoan taxa, we also did a Megablast search with a 97% similarity threshold, a minimum query coverage of 70% and an e-value inferior to 1E-100 against the Genbank nt (nucleotide) database (ftp://ftp.ncbi.nlm.nih.gov/blast/db/) with Geneious [28].

Data analysis. After sequencing, we obtained an OTU table showing the number of reads per taxon found in the stomach of each fish. For diet derived from this barcoding identification, frequency of occurrence was estimated ($\%F_{bar}$)—the percentage of stomachs in which a prey (OTU) was present.

To investigate the effect of fish body size (mm TL) on their diet and account for the hierarchical data structure, we performed permutational multivariate analysis of variance (PERMANOVA, *adonis* function in the vegan package for R [29] on the Bray–Curtis distance matrix with 'bay' (16 levels) as strata, fish size group as fixed predictor, and diet composition (counts of stomach with a certain prey present) as a response. Fish were divided into two size groups (TL): $\leq$6.5 cm (S), and >6.5 cm (L).

Comparison of visual vs DNA-based methods

The results from the visual analysis and the metabarcoding analysis were compared with respect to both number of taxa identified and to the taxonomic resolution of the data. The number of taxa was the mean number of taxa identified per stickleback in the two methods applied. To compare the methods with respect to their taxonomic resolution, ranks were given to each prey item in each stomach and then mean taxonomic rank of the stomach was used [30]. Taxonomic resolution was ranked as follows: species = 1, genus = 2, family = 3, infra-order = 4, order = 5, infra-class = 6, class = 7, phylum = 8. Infra-class and infra-order represent taxonomic rankings between class and order and between family and order, respectively. Paired t-tests were used to compare the resolution between the methods.

Results

Diet composition based on DNA barcoding

Using metabarcoding, 120 taxa were identified in the stomachs of sticklebacks: 15 phyla, 27 classes, 52 orders, 66 families, 83 genera, and 84 species (S1 Table). A broad range of phyla were found, but Arthropoda dominated by far (Fig 2). Given that this is the first barcoding-based study of Baltic Sea stickleback diet, we provide the whole list of taxa found (S1 Table). We only omit records from primates and birds, which were obviously contamination. Taxa likely to be accidental or secondary prey were also excluded from further analyses. Specifically, we excluded Fungi, Macroalgae and Chromista (as these are not targeted as food by sticklebacks), and kept only Metazoa in the primary prey list. A few OTUs of Metazoa were also excluded as they were either unlikely to be prey, or due to possible contamination (see S1 Table). In total, 103 taxa were considered primary prey and were used in the subsequent analyses.

Sticklebacks had a broad spectrum of prey items, of which Insecta (mainly chironomids), Maxillipoda (harpacticoid copepods) and Branchiopoda (cladocerans) were the dominating food items, found in more than 90% of the samples (S1 Table). At the species level, the main prey were the chironomid *Tanytarsus usmaensis*, the harpacticoid *Tachidius discipes*, and the cladoceran *Pleopis polyphemoides* (S1 Table).

Although the range of stickleback body lengths was too small to detect ontogenetic diet shifts, significant differences in stomach content depending on fish size were found (PERMANOVA, $F = 3.7$, $p = 0.01$). The diet of the large fish (>6.5 cm) differed from the group of

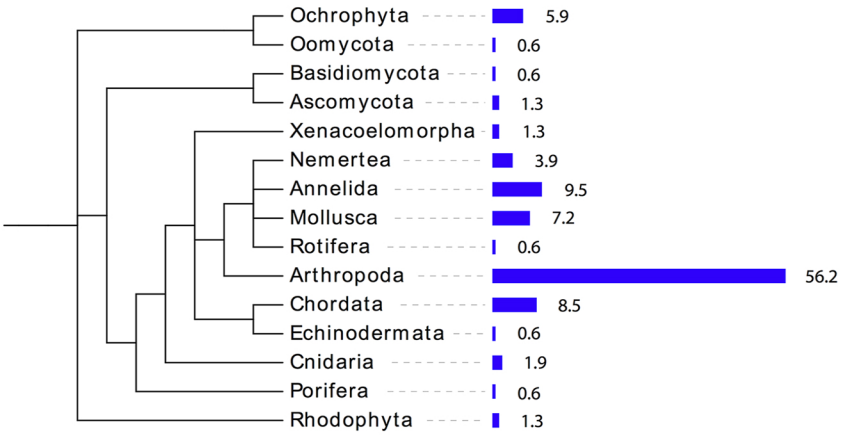

Fig 2. Frequencies of phyla identified in three-spined stickleback gut contents. Bar length corresponds to the frequency of OTU assigned to a specific phylum.

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smaller fish (≤6.5 cm). Specifically, amphipods, isopods and gastropods appeared to be more common in the diet of the larger fish, as well as insects like hemipterans and coleopterans (Fig 3).

Methods comparison: Visual identification vs DNA barcoding

The taxonomic resolution of the prey identified differed substantially between the two methods. DNA barcoding gave a much higher resolution (with median rank of genus, $p<0.0001$).

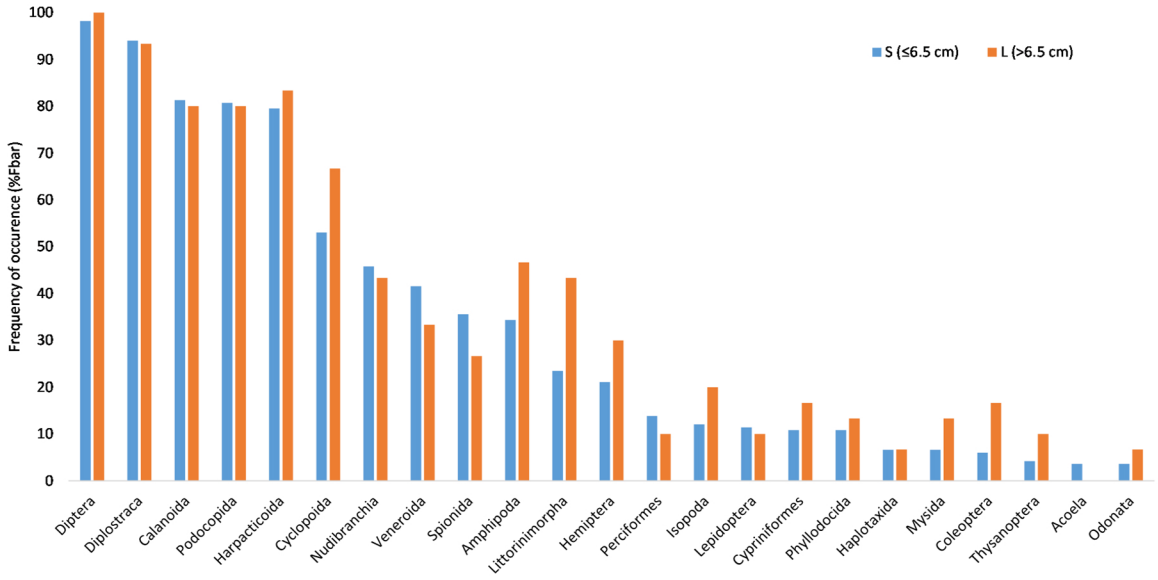

Fig 3. Diet composition of different size groups of stickleback (at order level). Only orders with >5% of frequency of occurrence are shown.

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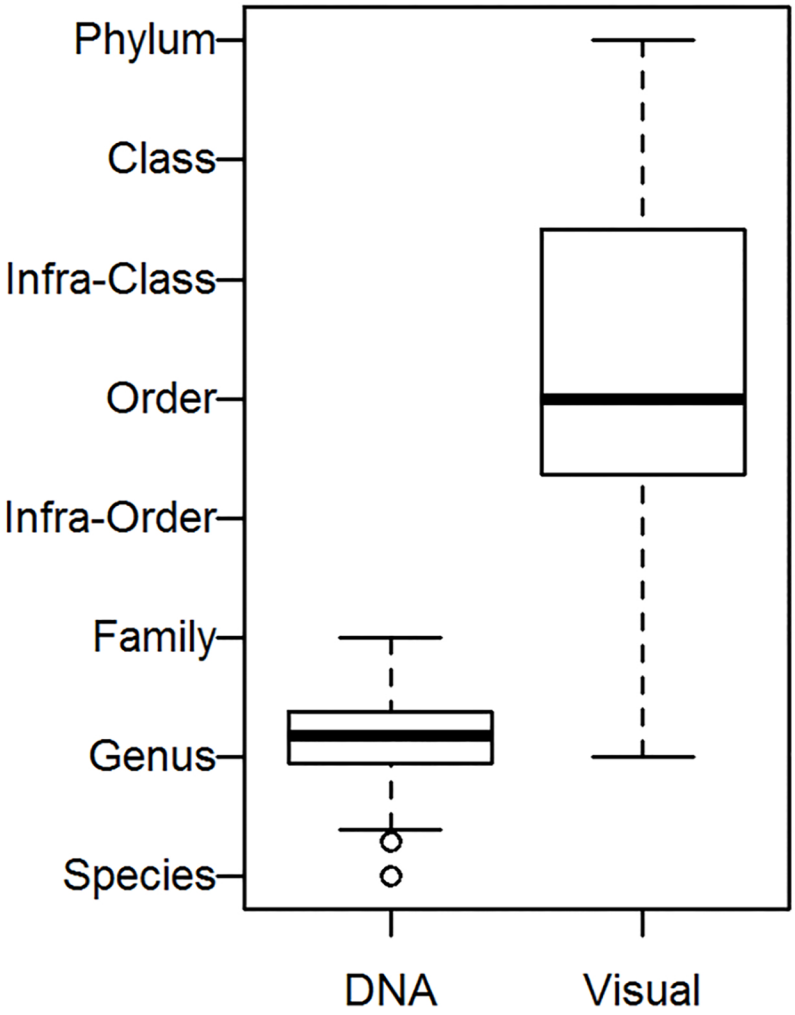

Fig 4. Mean taxonomic rank assigned to items within individual stomachs. DNA–assigned by barcoding, Visual–visual identification disregarding non-identified items. Midline represents median, boxes first and third quartiles, whiskers either maximum values or 1.5 times interquartile range (whichever is smaller) and circles outliers.

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Disregarding stomachs for which no visual identification could be done, the median taxonomic rank for visual inspection was order (Fig 4).

DNA barcoding also resulted in a much higher number of prey taxa identified per stomach than visual analysis ($p<0.0001$): 21.7±8.8 vs 1.96±1 (mean±SD). Also the total number of taxa identified using DNA barcodes was much larger than the number of taxa identified using visual quantification (120 vs 21; see S1 and S2 Tables). The average level of digestion was 3.6, meaning that gut contents were extensively digested and/or with very few prey items present. Not surprisingly, many taxa identified using DNA barcodes could not have been identified visually (e.g. due to their small size). However, some taxa identified by visual inspection were not revealed by barcoding (*Temora longicornis*, *Bosminidae*, *Hydracarina*).

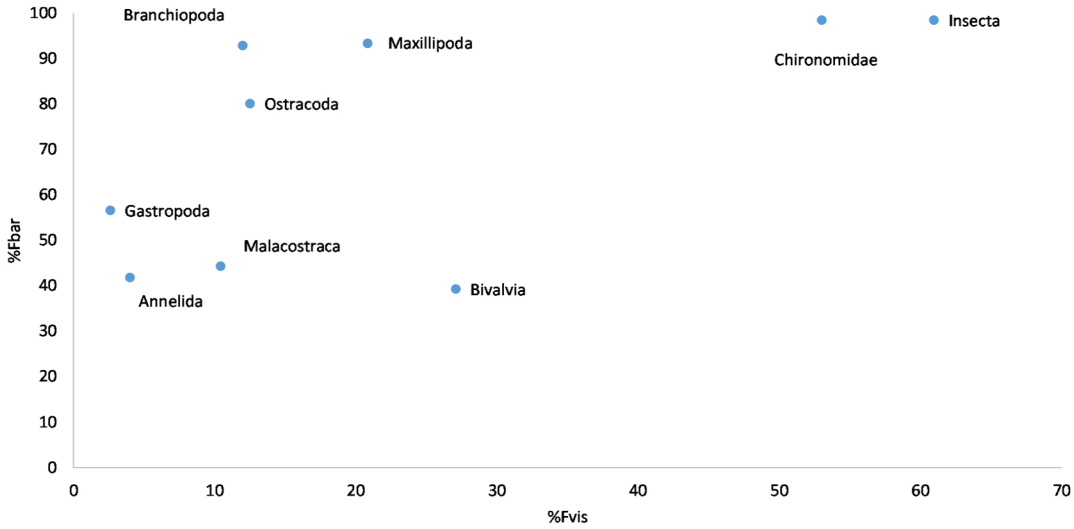

Fig 5. Diet of three-spined stickleback. Relationship between the results of two methods used: frequency of occurrence determined by metabarcoding ($\%F_{bar}$) and by visual analysis ($\%F_{vis}$).

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Irrespective of these minor differences, the two methods showed consistent patterns: at the population level, frequency of occurrence determined by visual analysis ($\%F_{vis}$) corresponded well with the frequencies based on OTU reads ($\%F_{bar}$; Fig 5), although the relationship was not linear. Instead, a curvilinear relationship was seen, where the frequencies of occurrence in the metabarcoding analyses were higher than in the visual identification for all taxa. Such a relationship is to be expected, since barcoding is capable of detecting even very little amounts of the prey, which could not be detected visually. Only Bivalvia appeared to have very similar frequencies detected by both methods.

Discussion

The aim of this study was to assess the diet of the three-spined stickleback in a coastal ecosystem, and to compare classic visual analysis and novel DNA metabarcoding for identifying fish prey in stomach contents. The main prey items found were chironomids, cladocerans and harpacticoid copepods. Large (>6.5 cm) sticklebacks had higher proportions of benthic herbivores, like amphipods, gastropods and isopods, in their diet. The results of the DNA barcoding revealed a highly diverse stickleback diet (more than 100 taxa in total, and >20 per individual) and provided a much higher taxonomic resolution than the conventional visual stomach content analysis.

Diet composition

While stickleback diets are well studied from other parts of the world, no previous studies have revealed such a high diversity of prey items, most likely because of limitations in the methods used (for some examples of previous studies, see S3 Table). Sticklebacks, however, inhabit many different habitats and ecosystems, so their diet varies accordingly. In pelagic areas of the Baltic Sea, where sticklebacks spend a large part of their life, they feed primarily on cladocerans and calanoid copepods [31–33], but at the coast the main prey items are insect larvae, harpacticoids and amphipods [34,35]. In freshwater systems, they are known to prey on both planktonic and

benthic prey. We found chironomids and harpacticoids to be a very important part of the stickleback diet, similar to the diet in the coastal zone of the Bothnian Bay, in the northern Baltic Sea [35].

From a food-web perspective, the high abundance of cladocerans found in the diet (Fig 3, S1 Table) might indicate competition with juvenile stages of other fish, especially when preference for cladocerans is evident [33,36,37]. Ljunggren et al. (2010) [6] suggested that recruitment of coastal predatory fish in the Baltic Sea (pike and perch) was impaired by limited food availability (zooplankton) for their larvae, due to competition with sticklebacks. The three-spined stickleback has indeed been shown to deplete zooplankton communities in brackish water lagoons with similar densities as in the current study area [38]. On the other hand, sticklebacks have also been shown to feed on small pike and perch larvae, which would constitute a more direct effect on populations of large predators, than competition [12]. We detected *Perciformes* in the stomachs of six fish (see S1 Table), potentially indicating sticklebacks may have been feeding on perch egg or larvae.

Concerning benthic prey, the most significant part of the diet consisted of chironomid larvae, which were one of the most common epibenthic organism groups in the 16 bays. Sticklebacks are well-known to feed on chironomids in freshwater areas ([35,36,39,40], S3 Table). Chironomids are a broad taxonomic group, with a diverse diet spanning between phytoplankton, epiphytic algae, detritus, macrophytes, and crustacean zooplankton [41]. More knowledge on the role of chironomids in the food webs and the interactions with sticklebacks is needed, since possible cascading effects from sticklebacks via chironomids to lower trophic levels may be present (e.g., [42]).

Sticklebacks seemed to have fed less on the gammarid amphipods than expected from previous experimental studies, where they have been shown to strongly reduce gammarid densities in the lab and in the field [7,9]. In our study, larger sticklebacks appear to feed more on amphipods (Fig 3). It is well known that stickleback mouth width and gape size influence the size of prey that can be eaten [43,44], and that jaw morphology (gape size, gill raker spacing) can change food handling efficiency [45]. Therefore, the optimum diet might differ between stickleback populations and/or habitats depending on their morphology. Hart and Ison (1991) found the size threshold of prey rejection to be at 6–7 mm [44], Byström et al. (2015) suggests the upper limit to be around 5 mm [12]. Given that fish above 5 cm eat amphipods [34,46], there were plenty of gammarids of appropriate size for sticklebacks to eat (see S2 Fig), showing that mouth morphology does not explain rejection of amphipods in small stickleback.

The most likely explanation for the relatively low proportion of gammarids in the overall diet is an underrepresentation of large individuals sampled in this study (S1 Fig) compared to several previous studies ([5]; and unpublished). These large individuals appear to feed the most on gammarids (Fig 3). The underrepresentation in the nets, which were placed at $>$ 1m depth, may indicate that the largest sticklebacks occupy the most beneficial habitats in the bays, i.e. the shallowest vegetated parts, where we could not fish using gillnets. These shallow areas are also the habitats with the highest abundances of gammarids, which may have led to the low frequency of stickleback predation upon gammarids apparent in the analysis. Large sticklebacks (>6.5 cm) also tend to have a higher frequency of occurrences of cyclopoid copepods than smaller ones (Fig 3), mainly driven by *Eucyclops macruroides*. This species is known to inhabit vegetation in the littoral zone, which again supports a possible small-scale differences in foraging habitats between stickleback size classes.

In many of the 16 bays there were relatively few stickleback individuals sampled, resulting in the inability to assess individual specialisation. To assess the link between sticklebacks and large, benthic crustaceans (e.g., gammarids), more detailed and intense sampling should be conducted, and the potential for individual specialisation should be investigated.

Visual inspection vs DNA barcoding

In general, the two methods gave consistent results with the same prey taxa dominating (Fig 5). However, as the stomach content was extensively digested and/or with very few prey items present, the visual prey species identification was in many cases obscured. Fish may have been caught in the nets up to 12 hours before they were preserved, making the visual analysis particularly difficult. On the other hand, this may also have had an effect on DNA degradation. In diet studies, a high proportion of unidentifiable material in the guts, which cannot be visually assigned to any prey category, is common [15]. Even though both methods are time-consuming and expensive, and despite the fact that some prey species were missed (*Temora longicornis*, *Bosminidae*, Hydracarina), barcoding provided a much higher taxonomic resolution and therefore produced a more accurate and detailed analysis of gut contents In terms of the resolution provided by the two methods, our results are similar to a previous fish feeding ecology study [30].Thus, we consider these discrepancies as minor, since barcoding still enabled the disclosure of unexpectedly high diversity in the stickleback diet.

Methodological shortcomings

The results of DNA metabarcoding and the visual analysis did not match fully.—Some prey taxa (*Temora longicornis*, *Bosminidae*, Hydracarina) were detected by visual inspection only, while Bivalvia had very similar frequency values estimated by both methods (see Fig 5). As we could visually identify these prey organisms, their DNA is unlikely to have been too degraded for barcoding to identify them. A more likely explanation is that even though the CO1 primers are designed to be taxonomically broad they may not bind equally well to all prey species, and maybe not at all to some. It is known that even minor primer–template mismatches can lead to substantial under-representation of the prey in the diet [47]. These biases are then accumulated through DNA amplifications during the PCR reaction [48,49]. Bosminidae was identified during visual inspection of stomach contents, but when barcoded only a higher corresponding taxon was detected (Diplostraca). Thus, only species or group specific primers would guarantee the most accurate identification.

Blocking primers are used to avoid 'predator sequences' (i.e. lots of non-informative reads), which can reduce potential of prey detection [50], but could also block prey DNA [51], which may bring in bias when analysing mixtures of DNA. We used a blocking primer to avoid stickleback sequences, but since predator and prey missed are not phylogenetically close, and the blocking primer used is specific to *G. aculeatus*, this should not have impaired the results.

There can be other biases introduced during the bioinformatic analysis steps, such as during the clustering of sequences, where the number of OTUs or 'species' found depends on the sequence similarity cut-off used, and during taxonomic assignment, which uses a sequence identity threshold of 97%. Also, it is obvious that if some species are not represented in the DNA reference library, no matches for these will be found.

Secondary consumption, i.e., prey of the prey, parasites or accidental material consumed during feeding, may confound the results in DNA-based studies [52–54]. The magnitude of potential error due to secondary predation depends on digestion rates [54]. We acknowledge that even though a few unlikely prey taxa were removed from the analysis, some secondary prey may still have been included in the analysis as primary prey items. However, DNA of secondary prey might be expected to represent only a minor part of total OTU reads compared to primary prey, due to a much lower total biomass and to a higher level of degradation.

When visually inspecting the often highly degraded stomach content, prey items such as fish eggs and larvae may be substantially underestimated (e.g., [55]). Although metabarcoding has the power to catch such prey species, the life stages of prey items remain unknown.

Moreover, prey analyses based on stomach content only represent a snapshot in time. To obtain more comprehensive knowledge on stickleback diets, future studies should be complemented with analyses of stable isotopes/fatty acids, which integrate the signal from different prey organisms over longer time.

Despite these shortcomings, DNA metabarcoding seems to be a viable method to assess stickleback diets. From a data quality perspective, we therefore, at least until metabarcoding methods are further developed, suggest to combine high-throughput DNA sequencing and traditional visual stomach content analysis, to achieve the best resolution of diet composition and diversity.

Implications

Using a powerful combination of visual and metabarcoding-based analyses of stomach contents, we show that the three-spined stickleback feeds on a wide variety of organisms in coastal areas of the Baltic Sea, including pelagic zooplankton and benthic epifaunal invertebrates. As a consequence, the major increase in stickleback abundance [5] could affect many parts of both pelagic and benthic food webs, resulting in competition with other fish species, and cascading effects down to primary producers [7,11]. Given that the expected increase in the Baltic Sea surface water temperatures [56] may be beneficial for stickleback population growth [57], studies such as this one could provide important information about the current and future impacts of three-spined sticklebacks on the Baltic Sea ecosystem.

Supporting information

S1 Table. Taxa found in three-spined stickleback stomachs as revealed by DNA metabarcoding (Primates and Aves excluded). Items in italics were considered as secondary/ accidental prey $\%F_{bar}$- frequency of occurrence (percentage of stomachs in which a prey was present).
(DOCX)

S2 Table. Diet of three-spined stickleback as revealed by visual stomach content analysis. $\%F_{vis}$—the percentage of stomachs in which a prey was present.
(DOCX)

S3 Table. Summary of some studies on three-spined stickleback diet.
(DOCX)

S1 Fig. Stickleback size (total length, mm) distribution in a sample.
(TIF)

S2 Fig. Gammaridae size distribution in the bays studied.
(TIF)

S1 Appendix. Comparison of quantification from OTU reads and results of visual stomach content analysis.
(DOCX)

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DNA metabarcoding reveals diverse diet of the three-spined stickleback in a coastal ecosystem

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S1 Table. Taxa found in three-spined stickleback stomachs as revealed by DNA metabarcoding (*Primates* and *Aves* excluded). Items in italics were considered as secondary/ accidental prey. $\%F_{bar}$- frequency of occurrence (percentage of stomachs in which a prey was present).

Phylum	**Class**	**Order**	**Family**	**Genus**	**Species**	**$\%F_{bar}$**
Annelida	Clitellata	Haplotaxida	Tubificidae	Paranais	Paranais frici	1,02
Annelida	Clitellata	Haplotaxida	Tubificidae	Tubificoides	Tubificoides benedii	0,51
Annelida	Clitellata	Haplotaxida				5,10
Annelida	Polychaeta	Phyllodocida	Nereididae	Hediste	Hediste diversicolor	8,67
Annelida	Polychaeta	Phyllodocida	Polynoidae	Polynoidae sp.		1,02
Annelida	Polychaeta	Phyllodocida	Sigalionidae	Pisione	Pisione remota	0,51
Annelida	Polychaeta	Phyllodocida				0,51
Annelida	Polychaeta	Spionida	Spionidae	Marenzelleria	Marenzelleria arctia	3,57
Annelida	Polychaeta	Spionida	Spionidae	Marenzelleria	Marenzelleria viridis	32,65
Annelida	Polychaeta	Spionida				2,55
Annelida	Polychaeta	Terebellida	Ampharetidae	Mugga	Mugga wahrbergi	0,51
Annelida	Polychaeta	Terebellida	Cirratulidae	Chaetozone	Chaetozone setosa	0,51
Annelida total						41,84
Arthropoda	Branchiopoda	Diplostraca	Daphniidae	Daphnia	Daphnia cucullata	0,51
Arthropoda	Branchiopoda	Diplostraca	Daphniidae	Daphnia		2,04
Arthropoda	Branchiopoda	Diplostraca	Leptodoridae	Leptodora	Leptodora kindtii	0,51
Arthropoda	Branchiopoda	Diplostraca	Macrothricidae	Macrothrix		0,51
Arthropoda	Branchiopoda	Diplostraca	Podonidae	Evadne	Evadne nordmanni	59,69
Arthropoda	Branchiopoda	Diplostraca	Podonidae	Pleopis	Pleopis polyphemoides	87,76
Arthropoda	Branchiopoda	Diplostraca	Sididae	Sida	Sida crystallina	14,80
Arthropoda	Branchiopoda	Diplostraca				34,18
Arthropoda	**Branchiopoda total**					92,86

S1 Table *Continued from previous page*

Phylum	Class	Order	Family	Genus	Species	%F_{bar}
Arthropoda	Collembola	Entomobryomorpha	Isotomidae	Isotoma	Isotoma anglicana	0,51
Arthropoda	Collembola	Symphypleona	Sminthuridae	Allacma	Allacma fusca	0,51
Arthropoda	*Insecta*	*Coleoptera*	*Curculionidae*	*Polydrusus*	*Polydrusus cervinus*	*1,02*
Arthropoda	Insecta	Coleoptera	Scirtidae	Cyphon	Cyphon padi	7,65
Arthropoda	Insecta	Diptera	Chironomidae	Chironomus	Chironomus aprilinus	78,06
Arthropoda	Insecta	Diptera	Chironomidae	Chironomus	Chironomus plumosus	73,47
Arthropoda	Insecta	Diptera	Chironomidae	Cladotanytarsus	Cladotanytarsus pallidus	58,67
Arthropoda	Insecta	Diptera	Chironomidae	Cricotopus	Cricotopus bicinctus	63,27
Arthropoda	Insecta	Diptera	Chironomidae	Cricotopus	Cricotopus patens	30,61
Arthropoda	Insecta	Diptera	Chironomidae	Cryptochironomus	Cryptochironomus supplicans	3,57
Arthropoda	Insecta	Diptera	Chironomidae	Dicrotendipes	Dicrotendipes modestus	66,33
Arthropoda	Insecta	Diptera	Chironomidae	Dicrotendipes	Dicrotendipes nervosus	5,61
Arthropoda	Insecta	Diptera	Chironomidae	Dicrotendipes	Dicrotendipes tritomus	7,65
Arthropoda	Insecta	Diptera	Chironomidae	Orthocladius	Orthocladius oblidens	77,04
Arthropoda	Insecta	Diptera	Chironomidae	Procladius	Procladius culiciformis	35,20
Arthropoda	Insecta	Diptera	Chironomidae	Psectrocladius		34,69
Arthropoda	Insecta	Diptera	Chironomidae	Tanytarsus	Tanytarsus cf. longitarsis	43,88
Arthropoda	Insecta	Diptera	Chironomidae	Tanytarsus	Tanytarsus mendax	3,06
Arthropoda	Insecta	Diptera	Chironomidae	Tanytarsus	Tanytarsus usmaensis	96,94
Arthropoda	Insecta	Diptera	**Chironomidae total**			98,47
Arthropoda	Insecta	Diptera				79,59
Arthropoda	Insecta	Hemiptera				21,94
Arthropoda	Insecta	Lepidoptera				11,22
Arthropoda	Insecta	Odonata	Libellulidae	Sympetrum		0,51
Arthropoda	Insecta	Odonata				3,57
Arthropoda	Insecta	Thysanoptera				5,10
Arthropoda	Insecta	Trichoptera				1,53

S1 Table *Continued from previous page*

Phylum	Class	Order	Family	Genus	Species	%F_{bar}
Arthropoda	Insecta					3,06
Arthropoda	**Insecta total**					98,47
Arthropoda	Malacostraca	Amphipoda	Gammaridae	Gammarus	Gammarus tigrinus	16,84
Arthropoda	Malacostraca	Amphipoda	Pontoporeiidae	Monoporeia	Monoporeia affinis	0,51
Arthropoda	Malacostraca	Amphipoda				32,14
Arthropoda	Malacostraca	Isopoda	Asellidae	Asellus	Asellus aquaticus	12,24
Arthropoda	Malacostraca	Isopoda	Janiridae	Jaera	Jaera albifrons	1,53
Arthropoda	Malacostraca	Mysida	Mysidae	Neomysis	Neomysis integer	5,61
Arthropoda	Malacostraca	Mysida	Mysidae	Praunus	Praunus inermis	3,06
Arthropoda	**Malacostraca total**					44,39
Arthropoda	Maxillopoda	Calanoida	Acartiidae	Acartia	Acartia bifilosa	36,73
Arthropoda	Maxillopoda	Calanoida	Acartiidae	Acartia	Acartia tonsa	33,16
Arthropoda	Maxillopoda	Calanoida	Centropagidae	Limnocalanus	Limnocalanus macrurus	0,51
Arthropoda	Maxillopoda	Calanoida	Diaptomidae	Eudiaptomus	Eudiaptomus graciloides	4,08
Arthropoda	Maxillopoda	Calanoida	Temoridae	Eurytemora	Eurytemora affinis	75,51
Arthropoda	Maxillopoda	Calanoida				59,18
Arthropoda	Maxillopoda	Cyclopoida	Cyclopidae	Cyclops	Cyclops abyssorum	4,08
Arthropoda	Maxillopoda	Cyclopoida	Cyclopidae	Eucyclops	Eucyclops cf. serrulatus	13,27
Arthropoda	Maxillopoda	Cyclopoida	Cyclopidae	Eucyclops	Eucyclops macruroides	13,27
Arthropoda	Maxillopoda	Cyclopoida	Cyclopidae	Macrocyclops	Macrocyclops distinctus	3,57
Arthropoda	Maxillopoda	Cyclopoida	Cyclopidae			47,96
Arthropoda	Maxillopoda	Harpacticoida	Tachidiidae	Tachidius	Tachidius discipes	79,59
Arthropoda	Maxillopoda	Sessilia	Balanidae	Amphibalanus	Amphibalanus improvisus	1,53
Arthropoda	**Maxillopoda total**					93,37
Arthropoda	Ostracoda	Podocopida	Cytherideidae	Cyprideis	Cyprideis torosa	16,84
Arthropoda	Ostracoda	Podocopida				78,06
Arthropoda	**Ostracoda total**					80,10

S1 Table *Continued from previous page*

Phylum	Class	Order	Family	Genus	Species	%F_{bar}
Ascomycota	*Eurotiomycetes*	*Eurotiales*	*Trichocomaceae*	*Penicillium*	*Penicillium digitatum*	*0,51*
Ascomycota	*Eurotiomycetes*	*Eurotiales*	*Trichocomaceae*	*Penicillium*	*Penicillium sclerotiorum*	*4,08*
Basidiomycota	*Microbotryomycetidae*	*Sporidiobolales*	*Sporidiobolaceae*	*Rhodotorula*	*Rhodotorula taiwanensis*	*0,51*
Chordata	Actinopterygii	Cypriniformes	Cyprinidae	Abramis	Abramis brama	6,12
Chordata	Actinopterygii	Cypriniformes	Cyprinidae	Phoxinus	Phoxinus phoxinus	8,67
Chordata	Actinopterygii	Cypriniformes	Cyprinidae	Tinca	Tinca tinca	0,51
Chordata	Actinopterygii	Cypriniformes				3,06
Chordata	Actinopterygii	Clupeiformes				1,02
Chordata	Actinopterygii	Gasterosteiformes	Gasterosteidae	Pungitius	Pungitius pungitius	79,08
Chordata	Actinopterygii	Perciformes	Gobiidae	Gobius	Gobius niger	0,51
Chordata	Actinopterygii	Perciformes	Gobiidae	Pomatoschistus	Pomatoschistus microps	4,59
Chordata	Actinopterygii	Perciformes	Gobiidae	Pomatoschistus	Pomatoschistus minutus	7,14
Chordata	Actinopterygii	Perciformes	Percidae	Gymnocephalus	Gymnocephalus cernua	2,55
Chordata	Actinopterygii	Perciformes				3,06
Chordata	*Actinopterygii*	*Salmoniformes*	*Salmonidae*	*Salmo*	*Salmo trutta*	*0,51*
Chordata	Leptocardii	Amphioxiformes	Branchiostomidae	Branchiostoma	Branchiostoma lanceolatum	0,51
Cnidaria	Hydrozoa	Anthoathecata	Hydridae	Hydra	Hydra oligactis	1,02
Cnidaria	Hydrozoa	Leptothecata	Melicertidae	Melicertum	Melicertum octocostatum	0,51
Cnidaria	Scyphozoa	Semaeostomeae	Ulmaridae	Aurelia	Aurelia aurita	0,51
Echinodermata	Ophiuroidea	Ophiurida				2,04
Mollusca	Bivalvia	Veneroida	Cardiidae	Cerastoderma	Cerastoderma glaucum	10,71
Mollusca	Bivalvia	Veneroida	Semelidae	Abra	Abra nitida	0,51
Mollusca	Bivalvia	Veneroida	Tellinidae	Macoma	Macoma balthica	31,63
Mollusca	**Bivalvia total**					39,29
Mollusca	Gastropoda	Hygrophila	Lymnaeidae	Radix		0,51
Mollusca	Gastropoda	Littorinimorpha	Hydrobiidae	Hydrobia	Hydrobia ulvae	26,02
Mollusca	Gastropoda	Littorinimorpha	Hydrobiidae	Potamopyrgus	Potamopyrgus antipodarum	0,51

S1 Table *Continued from previous page*

Phylum	Class	Order	Family	Genus	Species	%F_{bar}
Mollusca	Gastropoda	Mesogastropoda	Eulimidae	Haliella	Haliella stenostoma	0,51
Mollusca	Gastropoda	Nudibranchia	Calmidae	Calma	Calma glaucoides	43,88
Mollusca	Gastropoda	Nudibranchia				3,06
Mollusca	*Gastropoda*	*Stylommatophora*	*Agriolimacidae*	*Deroceras*	*Deroceras reticulatum*	*0,51*
Mollusca	**Gastropoda total**					56,63
Nemertea	Anopla		Lineidae	Lineus		1,53
Nemertea	Anopla					1,53
Nemertea	Enopla	Monostilifera	Emplectonematidae	Nemertopsis		0,51
Nemertea	Enopla	Monostilifera	Tetrastemmatidae	Tetrastemma		1,53
Nemertea	Enopla	Monostilifera				0,51
Nemertea	Enopla					2,04
Ochrophyta	*Bacillariophyceae*	*Cymbellales*	*Gomphonemataceae*	*Gomphonema*	*Gomphonema parvulum*	*0,51*
Ochrophyta	*Bacillariophyceae*	*Melosirales*	*Melosiraceae*	*Melosira*	*Melosira ambiqua*	*1,02*
Ochrophyta	*Bacillariophyceae*	*Melosirales*	*Melosiraceae*	*Melosira*	*Melosira nummuloides*	*16,84*
Ochrophyta	*Bacillariophyceae*	*Thalassiosirales*	*Skeletonemaceae*	*Skeletonema*	*Skeletonema marinoi*	*0,51*
Ochrophyta	*Eustigmatophyceae*	*Eustigmatales*	*Monodopsidaceae*	*Nannochloropsis*	*Nannochloropsis limnetica*	*9,18*
Ochrophyta	*Phaeophyceae*	*Ectocarpales*	*Acinetosporaceae*	*Pylaiella*	*Pylaiella washingtoniensis*	*19,39*
Ochrophyta	*Phaeophyceae*	*Ectocarpales*	*Chordariaceae*	*Leathesia*	*Leathesia difformis*	*1,02*
Ochrophyta	*Phaeophyceae*	*Ectocarpales*	*Ectocarpaceae*	*Ectocarpus*		*1,53*
Ochrophyta	*Phaeophyceae*	*Laminariales*	*Chordaceae*	*Chorda*	*Chorda filum*	*1,02*
Oomycota	*Peronosporea*	*Pythiales*	*Pythiaceae*	*Pythium*		*11,22*
Porifera	Demospongiae	Halichondrida				0,51
Rhodophyta	*Florideophyceae*	*Ceramiales*	*Callithamniaceae*	*Aglaothamnion*	*Aglaothamnion roseum*	*2,55*
Rotifera	Monogononta	Ploima	Brachionidae	Brachionus	Brachionus calyciflorus	0,51
Xenacoelomorpha		Acoela	Actinoposthiidae	Philactinoposthia	Philactinoposthia saliens	3,06
Xenacoelomorpha		Acoela	Isodiametridae	Aphanostoma		0,51
Total number of taxa identified						**120**
Total number of diet items (secondary/accidental items excluded)						**103**

S2 Table. Diet of three-spined stickleback as revealed by visual stomach content analysis. $\%F_{vis}$ - the percentage of stomachs in which a prey was present.

Phylum	Class	Order	Family	Genus + species	Identification name	$\%F_{vis}$
Algae					Algae	0.52
Mollusca	Bivalvia				Bivalvia	27.08
	Gastropoda				Gastropoda	2.60
Arthropoda	Arachnida	Hydracarina			Acari	1.56
	Branchiopoda	Diplostraca	Bosminidae	Bosmina spp.	Bosmina	7.29
			Chydoridae		Chydoridae	1.56
			Podonidae	Podon sp.	Podon	2.08
	Branchiopoda	Diplostraca			Cladocera	3.13
	Branchiopoda total					12
	Maxillopoda	Calanoida	Temoridae	Eurytemora affinis	Eurytemora affinis	2.08
			Temoridae	Temora longiremis	Temora longiremis	2.08
		Harpacticoida			Harpacticoida	2.60
					Copepoda	16.67
	Maxillopoda total					20.83
	Insecta		Chironomidae		Chironomidae	52.60
	Insecta				Insecta	8.85
	Insecta total					60.93
	Malacostraca	Amphipoda			Amphipoda	6.25
		Mysida	Mysidae		Mysidae	0.52
	Malacostraca				Malacostraca	3.65
	Malacostraca total					10.42
	Ostracoda				Ostracoda	12.50
Arthropoda					Eggs (Copepoda/Cladocera)	20.31
Annelida					Segmented worms	3.65
Nematoda/Nemertea					Other worms	2.60
Total number of diet items						**21**

S3 Table. Summary of some studies on three-spined stickleback diet.

Stickleback size (TL, mm)	Prey item (in descending order of abundance)	Season	Method for identification	Location	Longitude and latitude	Salinity Marine, brackish, freshwater	Habitat Pelagic Benthic	Reference
20 – 70	Bosmina longispina, Eurytemora affinis, Cercopagis pengoi	Early autumn (September 2- 6)	Visual	Baltic Sea, Gulf of Finland	Many locations in Gulf of Finland	brackish	pelagic	Peltonen H, Vinni M, Lappalainen A, Ponni J. Spatial feeding patterns of herring (L.), sprat (L.), and the three-spined stickleback (L.) in the Gulf of Finland, Baltic Sea. ICES J Mar Sci. 2004;61: 966–971. doi:10.1016/j.icesjms.2004.06.008
~45 – 75	Bosmina coregoni, Eurytemora affinis, Podon polyphemoides, Acartia bifilosa	Summer (August)	Visual	Baltic Sea, Gulf of Bothnia	Whole Gulf of Bothnia (many locations)	brackish	pelagic	Leinikki J. The diet of three-spined stickleback in the Gulf of Bothnia during its open water phase. Aqua Fenn. 1995;25: 71–75.
NA	Diptera (Chironomidae, Culicidae), Crustacea (Harpacticoida) Amphipoda (Gammarus) Mysidacea, Ostracoda	Early summer (May-June)	Visual	Baltic Sea, Bay of Bothnia	63°30’N 22°20’E	brackish	coastal, exposed sandy beach, shallower<50 cm	Frande C, Kjellman J, Leskela A, Hudd R. The food of three-spined stickleback (Gasterosteus aculeatus) on a whitefish (Coregonus lavaretus) nursery area in the bay of Bothnia. Aqua Fenn. 1993; 85–87.

Stickleback size (TL, mm)	Prey item (in descending order of abundance)	Season	Method for identification	Location	Longitude and latitude	Salinity Marine, brackish, freshwater	Habitat Pelagic Benthic	Reference
NA	Bosmina longispina, E. affinis, C. pengoi Podon spp. Acartia spp.	Summer (July)	Visual	Baltic Sea, Gulf of Riga	Many locations	brackish	pelagic	Lankov A, Ojaveer H, Simm M, Põllupüü M, Möllmann C. Feeding ecology of pelagic fish species in the Gulf of Riga (Baltic Sea): the importance of changes in the zooplankton community. J Fish Biol. 2010;77: 2268–84. doi:10.1111/j.1095-8649.2010.02805.x
33 - 70	Eurytemora affinis, Temora longicornis, Acartia spp. Eurytemora affinis, Bosmina spp. Acartia spp. Bosmina spp. Acartia spp.	Spring Summer Autumn	Visual	Southwest Baltic Sea		brackish	pelagic	Jakubavičiūtė E, Casini M, Ložys L, Olsson J. Seasonal dynamics in the diet of pelagic fish species in the southwest Baltic Proper. ICES J Mar Sci J du Cons. 2017;74: 750–758. doi:10.1093/icesjms/fsw224
Juvenile 9 – 27	Copepods (Temora longicornis, Microsetella norvegica) Ciliophoran (Helicostomella subulata) Oligochaetae Orthocladiinae	August - September	Visual	White Sea, Seldianaya Inlet of Kandalaksha Bay	66°20′14.5″N 33°37′27.8″E 66°20′N 33°37′E	marine	coastal Zostera seagrass beds	Demchuk A, Ivanov M, Ivanova T, Polyakova N, Mas-Martí E, Lajus D. Feeding patterns in seagrass beds of three-spined stickleback Gasterosteus aculeatus juveniles at different growth stages. J Mar Biol Assoc United Kingdom. 2015; 1–9. doi:10.1017/S0025315415000569

S3 Table *Continued from previous page*

Stickleback size (TL, mm)	Prey item (in descending order of abundance)	Season	Method for identification	Location	Longitude and latitude	Salinity Marine, brackish, freshwater	Habitat Pelagic Benthic	Reference
46-66	Main: Daphnia sp. Simuliidae (mostly in June), Chironomidae (mostly in July), Cladocera (mostly in May), Complementary: Copepoda, Ephemeroptera, Lepidoptera, Heteroptera, Sporadically - fish eggs and fry.	May-August	Visual	Warta River, Poland		freshwater	impounding river, submersed pond-weeds	Dukowska M, Grzybkowska M, Marszał L, Zięba G. The food preferences of three-spined stickleback, Gasterosteus aculeatus L., downstream from a dam reservoir. Oceanol Hydrobiol Stud. 2009;38: 39–50. doi:10.2478/v10009-009-0020-x
29-76	Cladocera pelagic microcrustaceans, littoral cladocerns, amphipods, chironomids, molluscs, ostracods, Trichoptera larvae	Autumn Spring Summer	Visual	Newfoundland lakes, Canada		freshwater	shallow oligotrophic lake	Campbell CE. Prey Selectivities of Threespine Sticklebacks (Gasterosteus aculeatus) and Phantom Midge Larvae (Chaoborus Spp) in Newfoundland Lakes. Freshw Biol. 1991;25: 155–167. doi:10.1111/j.1365-2427.1991.tb00481.x

S3 Table *Continued from previous page*

Stickleback size (TL, mm)	Prey item (in descending order of abundance)	Season	Method for identification	Location	Longitude and latitude	Salinity Marine, brackish, freshwater	Habitat Pelagic Benthic	Reference
31 – 75	Chironomids, Copepods, Cladocera, Ostracods, Rotifers, Clams (Bivalvia), Stickleback eggs	June-September	Visual	Karluk and Bare lake, Alaska		freshwater	lake	Greenbank J, Nelson P. Life history of the threespine stickleback Gasterosteus aculeatus Linnaeus in Karluk Lake and Bare Lake, Kodiak Island, Alaska. Fish Bull. 1959;59: 537–559
40 – 73	Higher Crustacea, Chironomids, Copepoda, Oligochaeta, Cladocera,	monthly	Visual	Birket, UK		freshwater	stream	Hynes H. The food of freshwater sticklebacks (Gasterosteus aculeatus and Pygosteus pungitius), with a review of methods used in studies of the food of fishes. J Anim Ecol. 1950;19: 36–58. doi:10.2307/1570
6 – 30	Ostracoda Cladocera, Copepoda, Chironomids Ostracoda, Rotifera, Diatoms							
38 – 96	Higher Crustacea, Copepoda, Diptera, Sticklebacks eggs and larvae, Annelida	August, June		Easdale Quarry, Argyll, UK		brackish 3.6 psu		

S3 Table *Continued from previous page*

Stickleback size (TL, mm)	Prey item (in descending order of abundance)	Season	Method for identification	Location	Longitude and latitude	Salinity Marine, brackish, freshwater	Habitat Pelagic Benthic	Reference
2 – 110	Higher Crustacea, Copepoda, Cladocera, Fish eggs and larvae, Insects, Ostracoda, Polychaeta Gastropoda		Visual	Denmark	Various Danish waters	brackish	*Zostera* regions	Blegvad, H. On the food of fish in the Danish waters within the Skaw. Rep. Danish Biol. 1917. Sta. 24: 19-72.
Adult	Copepoda, Hemiptera, Oligochaeta Chironomidae, Amphipoda, Nematoda, Fish eggs, Bivalvia	Spring Summer Autumn	Visual	St. Andrews, New Brunswick, Canada	45°5′N 67°5′W	brackish 0 – 28 psu	tidal saltmarshes	Delbeek, J.C.; Williams DD. Food resource partitioning between sympatric populations of brackishwater sticklebacks. Journal of Animal Ecology. 1987. pp. 949–967. doi:10.2307/4959
Juvenile	Harpacticoida, Calanoida Cyclopoida, Diatoms, Rotifera, Nematoda, Oligochaeta, Ostracoda, Amphipoda							

Stickleback size (TL, mm)	Prey item (in descending order of abundance)	Season	Method for identification	Location	Longitude and latitude	Salinity Marine, brackish, freshwater	Habitat Pelagic Benthic	Reference
average length 12 ± 3	Amphipods, Zooplankton (copepods, ostracods)	Summer July-August	Experiment	inner archipelago of the Askö area western Baltic Proper	58°48'N, 17°40' E	brackish 6.3 – 6.5 psu	sheltered bay, shallow (1.2 m deep)	Reiss K, Herriot MB, Eriksson BK. Multiple fish predators: Effects of identity, density, and nutrients on lower trophic levels. Mar Ecol Prog Ser. 2014;497: 1–12. doi:10.3354/meps10622
Larvae 9.1±0.8	Copepoda (Acartia spp., Eurytemora affinis) Cladocera (Bosmina longispina) Rotifera	July	Experiment	SW coast of Finland, the northern Baltic Sea		brackish 6 psu		Lehtiniemi M, Hakala T, Saesmaa S, Viitasalo M. Prey selection by the larvae of three species of littoral fishes on natural zooplankton assemblages. Aquat Ecol. 2007;41: 85–94. doi:10.1007/s10452-006-9042-6

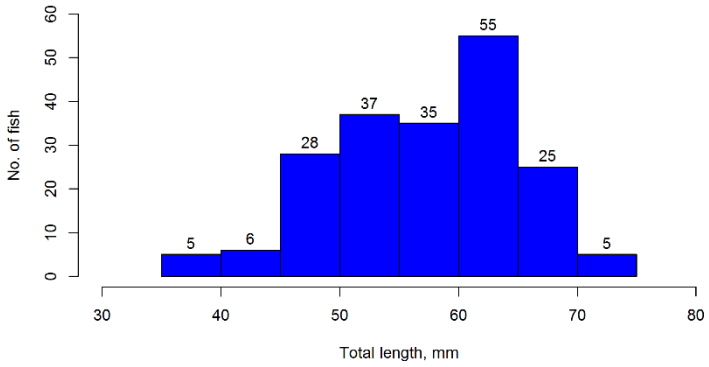

Figure S1 Stickleback size (total length, mm) distribution in a sample.

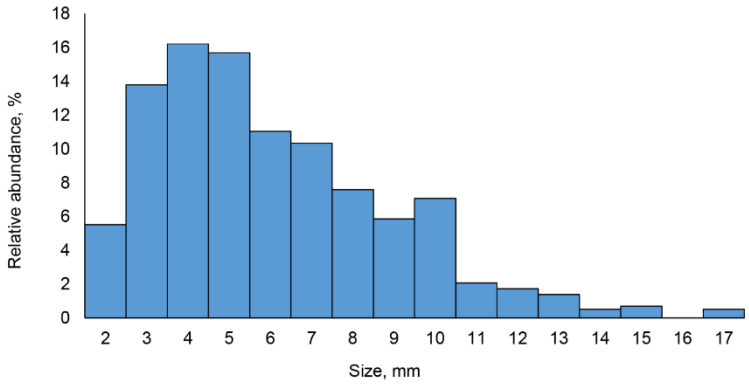

Figure S2 Gammaridae size distribution in the bays studied.

S1 APPENDIX

Comparison of quantification from OTU reads and results of visual stomach content analysis

METHODS

After sequencing, we obtained an OTU table showing the number of reads per taxon found in the stomach of each fish. To estimate the relative abundance of a certain prey in the stomach, and to make data from different fish individuals comparable, numbers of reads were normalized to the total number of reads in each sample (individual), and proportions of different taxa in each stomach were estimated (hereafter termed as "$\%N_{bar}$"). Thus, proportions of prey in the stomachs are based on the number of OTU reads per taxon ($\%N_{bar}$) (e.g., [1]). Frequency of occurrence was also estimated - $\%F_{bar}$, the percentage of stomachs in which a prey (OTU) was present.

RESULTS

As revealed by barcoding, Insecta (chironomids), Maxillipoda (harpacticoid copepods) and Branchiopoda (cladocerans) were the dominating food items, comprising 48%, 19% and 15% of all prey respectively (Fig A1). At the species level, the main prey were the chironomid *Tanytarsus usmaensis*, the harpacticoid *Tachidius discipes*, and the cladoceran *Pleopis polyphemoides* (Fig A2).

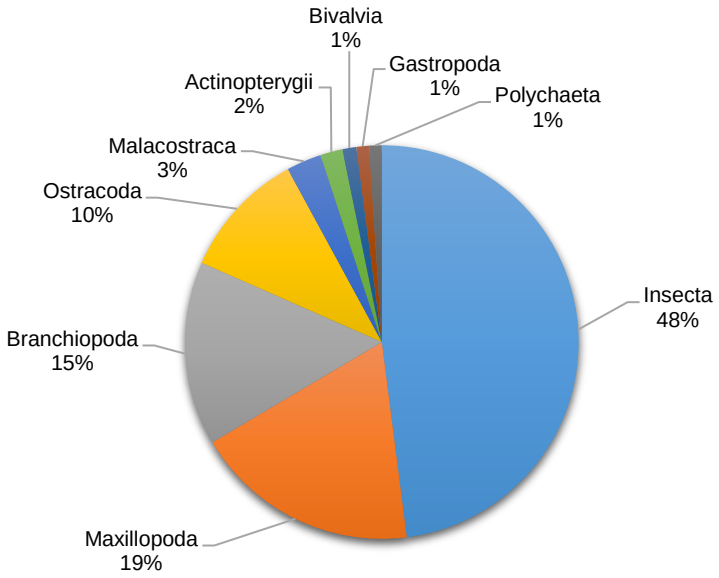

Fig A1. Proportion of different classes in stomachs based on number of OTU reads ($\%N_{bar}$). Only classes with >1% of OTU reads are shown.

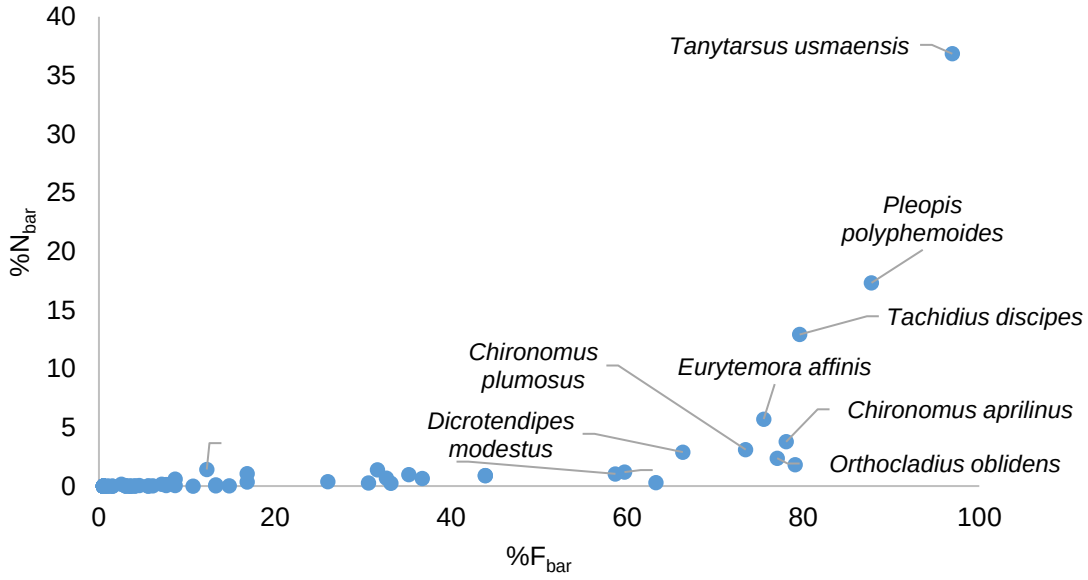

Fig A2. Main prey species (written) as indicated by relationship between relative abundance ($\%N_{bar}$, prey proportion in stomach based on number of OTU reads) and frequency of occurrence ($\%F_{bar}$).

In general, the two methods used – barcoding and visual stomach content analysis - showed consistent patterns: at the population level, frequency of occurrence determined by visual analysis ($\%F_{vis}$) correlated well with the proportions of prey in

the stomachs based on the number of OTU reads (%N_{bar}; Fig A3), except for Bivalvia, which may have been underrepresented in the barcoding analysis.

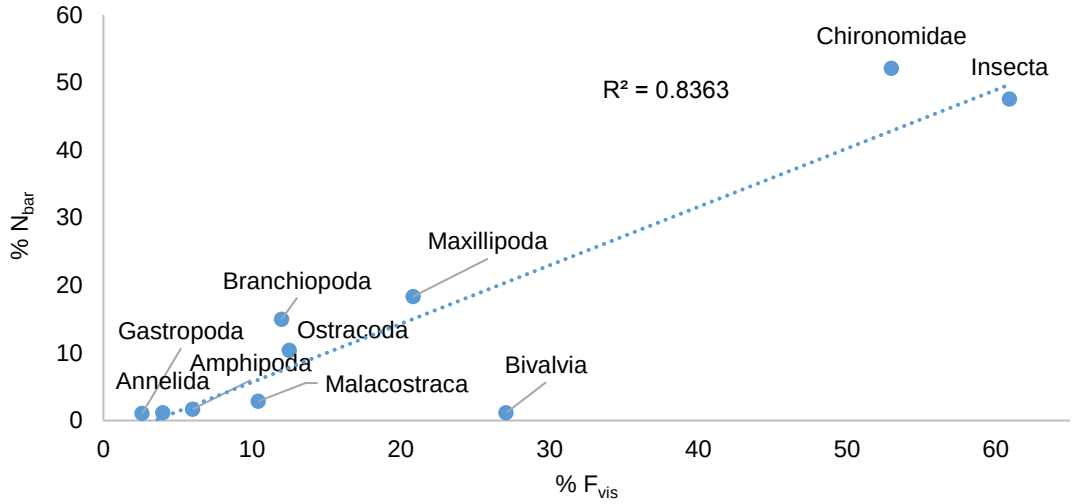

Fig A3. Diet of three-spined stickleback. Relationship between the results of two methods used: proportions of prey in the stomachs based on the number of OTU reads (%N_{bar}) and frequency of occurrence determined by visual analysis (%F_{vis}).

DISCUSSION

Although read counts can be used as a semi-quantitative proxy for diet composition [1–4], several well-known issues still impede the use of DNA metabarcoding for quantification. Quantitative estimates of certain prey in the stomach may be influenced by prey size, level of digestion, DNA preservation, as well as experimentally introduced biases from DNA extraction, primer-template mismatches, PCR amplification bias, OTU clustering, reference library quality and taxonomic assignment process [5–12]. One way to reduce such biases is to introduce correction factors, by creating a library of mixed prey standards and then using them to correct counts from unknown composition [13]. This was not done in our study, but the application of alternative methods (visual analysis) enabled us, at least to some extent, to validate the DNA metabarcoding results (Fig A3).

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PAPER III

Morphological divergence of three-spined stickleback in the Baltic Sea – implications for stock identification

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Abstract

Morphometrics is a beneficial, straightforward and relatively simple tool for fish stock delineation and identification. Analysis of spatial divergence, and morphological traits in relation to other factors, are of high interest both for management and in ecological studies. Morphology can help to delineate stocks, inform about local adaptations as well as ecological role the species play in the ecosystem. Morphological variation and differentiation of three-spined stickleback (*Gasterosteus aculeatus*) has been thoroughly studied in small freshwater systems, but less is known about differentiation of the species in an open marine system. The Baltic Sea inherits substantial environmental gradients in salinity, temperature, as well as in the nature and intensity of predation, gradients that all can foster local morphological adaptation and potential population differentiation. Previous studies have revealed some morphological differentiation of the species along the coast, but studies from the offshore are lacking. In this study we used traditional as well as geometric morphometrics to analyse divergence among sticklebacks from western and eastern Baltic Sea in morphological traits like body plate numbers, body- and otolith shape. Our results show that fish from the eastern Baltic (Curonian Lagoon) had significantly higher number of body plates, potentially indicating a response to higher predation pressure compared to the other areas studied. Body shape also significantly differed among locations with deeper bodied fish in the eastern Baltic. The most conservative and least plastic trait studied, otolith shape, did, however, not show any divergence among the areas studied. Our results suggest morphological divergence in plastic traits like body plates and body shape in response to local environmental conditions. The lack of divergence in otolith shape further suggests that the degree of population differentiation is weak or rather recent, as also highlighted in earlier studies using molecular markers or synchrony in population abundances. In all, our study shows that at least the number of body plates can serve as an effective stock delineator among genetic clusters of three-spined sticklebacks in the Baltic Sea.

Introduction

In order to understand fish population dynamics, it is crucial to consider the spatial structure of populations, and identification of spatial sub-division is essential to management (Cadrin et al., 2005). Many approaches exist to address the spatial structure of a population, including molecular markers (DNA, RNA, proteins), demographic characteristics (e.g., age and size distribution), or phenotypic traits (e.g., parasites, body shape, size at maturation; Begg and Waldman, 1999; Östman et al., 2017). Morphological analysis includes meristics (discrete counts of e.g., fin rays, gill rakers), traditional morphometrics (usually linear measurements), and geometric morphometrics (shape analysis using landmarks or outline methods; Begg and Waldman, 1999; Adams et al., 2004; Dean et al., 2013). The morphology of fish has been proven to be a beneficial tool for fish population discrimination since it can be used even if there is no or weak genetic divergence among sub-populations (Begg and Waldman, 1999; Cadrin and Friedland, 1999). Morphological traits are especially useful to study short-term environmentally induced differentiation (Kinsey et al., 1994), and morphological differences among sub-populations may hence reflect differences in environmental conditions, fishing pressure or the nature of predation (Liebhold et al., 2004).

Morphological variation of three-spined stickleback (*Gasterosteus aculeatus*, hereafter stickleback) in small freshwater systems has been studied extensively (Barrett, 2010; Bell and Foster, 1994). It is well known, that after isolation in freshwater environments, sticklebacks have undergone considerable diversification, and various aspects of the morphology, like body size (e.g., Snyder, 1991), body shape (e.g., Walker, 1997), or body armoring (e.g., Reimchen, 2000) have diversified and evolved within and among populations. It is, however, less known, whether or not a similar diversification has occurred in more open systems as oceans or large lakes.

Over the past decades, the abundance of sticklebacks has increased considerably in the western Baltic Sea (Bergström et al., 2015) as have their potential role in

the ecosystem. Sticklebacks migrate from wintering areas in the offshore Baltic to coastal areas during spring for spawning (Bergström et al., 2015; Ljunggren et al., 2010). Recent studies suggest that increased abundances of sticklebacks have a substantial impact on coastal ecosystem structure and functioning in the Baltic Sea (Ljunggren et al., 2010; Eriksson et al., 2011; Sieben et al., 2011; Byström et al., 2015; Östman et al., 2016) and potentially also so on the system in the offshore Baltic (Jakubavičiūtė et al., 2017). Understanding the spatial population structure and morphological divergence of sticklebacks in the Baltic Sea is hence warranted for our understanding of the future role of the species in the system as well as for potential future management of the species.

The Baltic Sea is a brackish water sea with pronounced gradients in salinity and biodiversity (HELCOM, 1996; Ojaveer et al., 2010; Voipio, 1981). It is also a highly impacted ecosystem with area-specific anthropogenic pressures like the level and nature of fishing pressure as well as nutrient concentration and loads (HELCOM, 2010). All these conditions in turn impact the species inhabiting the Baltic Sea, and favors genetic or plastic adaptation to environmental gradients and population differentiation (e.g., DeFaveri et al., 2013; Larsen et al., 2012; Wennerström et al., 2013).

So far, studies investigating stickleback population differentiation in the Baltic Sea have focused on genetic sub-division (DeFaveri et al., 2013; DeFaveri and Merilä, 2013) or spatial synchrony in demography (Östman et al., 2017). Differences in phenotypic traits like the number of lateral body plates or body shape can also inform about the spatial sub-division of sticklebacks (e.g., Zanella *et al.*, 2015), but has been less studied in the Baltic Sea. Individuals with higher number of body plates have, however, been found to be more common along the southern Baltic Sea coast (DeFaveri and Merilä, 2013), and relatively modest body shape divergence among populations inhabiting the Northeast Baltic Sea have been documented (Leinonen et al., 2006). To date, however, comprehensive studies analysing several traits in concert covering a larger geographic area and different habitat types are still scarce. In addition,

information on the environmental conditions favoring phenotypic differentiation of sticklebacks in the Baltic Sea is still lacking.

The aim of this study was to address possible morphological differentiation and potential population delineation of three-spined stickleback in the Baltic Sea. More specifically, we analyse morphological traits of different degree plasticity: number of lateral body plates, body shape, and otolith shape of sticklebacks from eastern (Curonian Lagoon) and western (Kalmar Sound, Baltic Proper and Bothnian Sea) parts of the Baltic Sea. We also discuss the link between spatial variation in morphology and environmental gradients.

Material and Methods

Sampling

There are two major genetic clusters of three-spined stickleback in the Baltic Sea, one eastern and one western (DeFaveri et al., 2013). Whereas the eastern cluster covers the Latvian, Lithuanian and Polish coasts, the western cluster is much more spatially extensive covering all major basins of the Baltic Sea and substantial environmental gradients. Samples used in this study was collected to represent both the eastern and western genetic cluster (hereafter termed as 'east' and 'west' divisions), and the west division was further divided into the smaller spatial units of Kalmar Sound, Baltic Proper and Bothnian Sea (hereafter termed as 'locations', Fig. 1, Table 1).

Fish were collected by trawling during the Baltic International Acoustic survey (Bothnian Sea and Baltic Proper), and during the Planfish project (Kalmar Sound) in 2010-2014 (Table 1). The trawl codend was 6-mm bar length. In the Curonian Lagoon, sticklebacks were caught with a trap-net or a beach seine (Table 1). From each survey, a randomly taken sub-sample was used for morphological analysis. Once caught, fish were frozen and later, slowly thawed in the laboratory. Fish from all sites were treated equally.

The number of body plates were analysed for in total 397 fish (N east division=130, N west division=267), the body shape for in total 270 fish (N east division=123, N west division=147), and the otolith shape in 71 fish (N east division=32, N west division=39) (Table 1). Fish were the same, but not for all fish all aspects (body shape, body plates, and otoliths) were analysed. We did not find an effect on the morphological traits analysed resulting from year (data for comparisons only available for Kalmar Sound) or gear type (data for comparisons only available for Curonian Lagoon), see below.

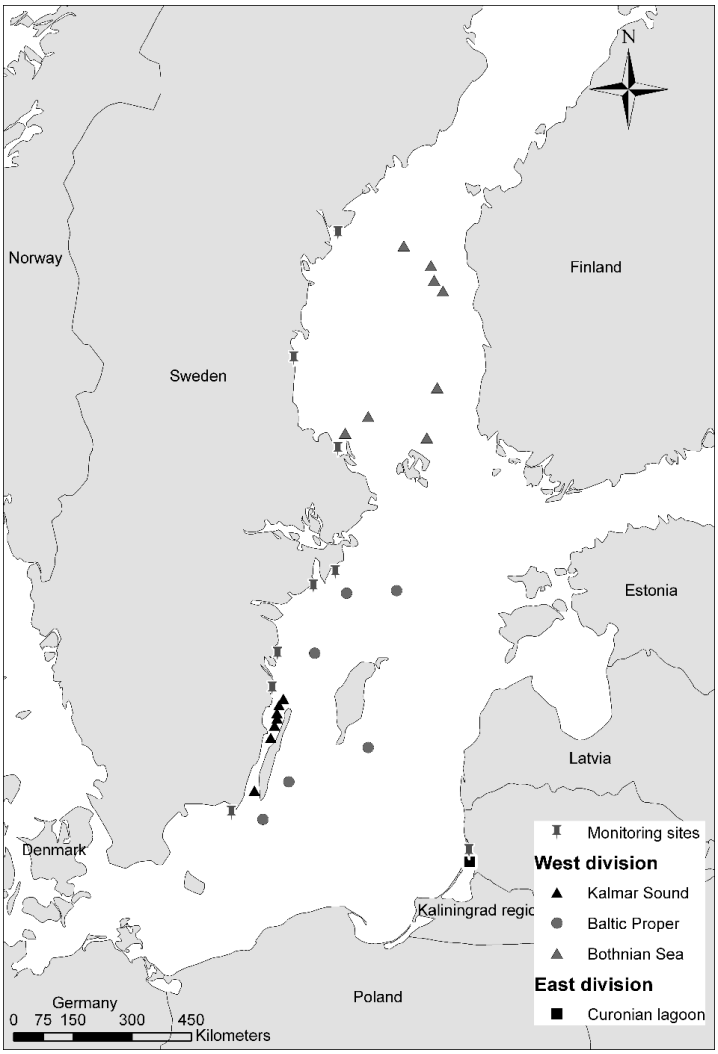

Fig. 1 Sampling locations of the samples from the east and west division. The west division was further divided into four locations, Kalmar Sound, Baltic Proper and Bothnian Sea. The coastal monitoring sites corresponds locations from where coastal predation estimates was derived.

Table 1. Sample sizes of three-spined stickleback for morphometric analysis.TL = total length, cm. N = number of fish analysed. In brackets are the number of fish used for a balanced subsample from the post-spawning period.

Division	Location	ICES Rectangle	Sampling date	Gear	N for body plates (TL≥4)	N for body shape (TL 5-7)	N for otolith shape
East	**Curonian Lagoon**	39 H1	2014-04-12	Beach seine	19	16	
		39 H1	2014-04-15	Beach seine	31	29	12
		39 H1	2014-10-13	Beach seine	30	30 (30)	11
		39 H1	2014-11-06	Trapnet	12	11	
		39 H1	2014-11-10	Trapnet	38	37	9
					130	**123 (30)**	**32**
West	**Kalmar Sound**	43 G6	2010-04-06	Trawl	42	12	4
		43 G6	2010-07-06	Trawl	14	11 (11)	
		43 G6	2010-10-13	Trawl	12	9 (9)	
		43 G6	2011-04-07	Trawl	37	27	11
		43 G6	2011-06-13	Trawl	22	15	3
		43 G6	2011-09-21	Trawl	22	11 (11)	
		43 G6	2012-07-03	Trawl	10	2	
					159	**87 (31)**	**18**
	Baltic Proper	46 G8	2014.10.06	Trawl	6		
		41 G6	2014.10.03	Trawl	10	4 (4)	
		45 G7	2014.10.06	Trawl	8	4 (4)	
		46 G9	2014.10.07	Trawl	10	8 (8)	
		43 G8	2014.10.10	Trawl	10	6 (6)	
		41 G7	2014.10.14	Trawl	10	8 (8)	
					54	**30 (30)**	
	Bothnian Sea	54 H0	2014.10.01	Trawl	6	3 (3)	4
		50 G8	2014.09.27	Trawl	10	7 (7)	9
		54 H0	2014.10.02	Trawl	6	1 (1)	3
		50 G7	2014.09.27	Trawl	10	6 (6)	
		50 H0	2014.10.05	Trawl	7	4 (4)	5
		54 G9	2014.10.02	Trawl	5	2 (2)	
		51 H0	2014.10.05	Trawl	5	2 (2)	
		53 H0	2014.10.01	Trawl	5	5 (5)	
					54	**30 (30)**	**21**
Total					**397**	**270 (121)**	**71**

Morphometric analysis

Number of body plates

The left side of each individual was positioned for observation of the number of lateral body plates. Individuals were stained with Alizarin red to emphasize the bony structures of each specimen. Fish of a total length (TL) of <40 mm were excluded from analysis to ensure that bone plate development was completed (Wootton 1976; Bell, 1981). Significantly larger sticklebacks were found in the

sample from the Bothnian Sea location (t-test; p=0.0003), and fish length (TL, from hereon denoted as fish size) was hence taken as a covariate in the later analysis (see below). For fish size distribution see Supplementary material, Fig. S1.

An ANCOVA (Analysis of Covariance) was used to test for differences in body plate numbers between locations and divisions, using fish length as a covariate to control for fish size. Since sample sizes from the divisions were highly uneven (Table 1), a balanced randomized subsample from each division were additionally analysed (60 fish from the west and east division respectively). This analyses showed the same patterns as when including all fish from both divisions (results not presented here). Season (Kalmar Sound and Curonian Lagoon) had no effect on the number of body plates, i.e. no differences in the number of body plates between the seasons were detected (Kalmar Sound: ANOVA, $p>0.05$; Curonian Lagoon: t-test, $p>0.05$, see also Supplementary material, Fig. S2). The number of body plates of fish across years (2010, 2011 and 2012, in Kalmar Sound) did not differ (ANOVA, $p>0.05$); neither did it differ between gears (trapnet and a beach seine) in the Curonian Lagoon (t-test, $p>0.05$).

The number of body plates in sticklebacks may be related to the predation pressure (Barber and Nattleship, 2010; Bell and Foster, 1994). To relate the number of body plates of the sampled sticklebacks to the predation pressure in the area, we used an abundance index of piscivorous fish in the area where samples were collected (Fig. 1). The index was estimated using publicly available coastal fish monitoring data (HELCOM, 2017) as estimated as the catch per unit effort of piscivores fish in each monitoring area (Supplementary material, Table S1). The index includes estimates of perch (*Perca fluviatilis*), pike (*Esox lucius*) and pikeperch (*Sander lucioperca*) in northern and eastern areas, while in more southern and western areas of the Baltic Sea, cod (*Gadus morhua*) and turbot (*Psetta maxima*) are the dominating piscivorous species (HELCOM, 2015). Although it is not clear if all these piscivorous fish predate equally on sticklebacks, all of them are potential predators on the species

(Almqvist et al., 2010; Jacobson, 2015; Mustamäki et al., 2014; Stankus, 2003). During the last two decades, there was no significant change in coastal piscivores fish abundance in the East (Curonian Lagoon) and Baltic Proper (except for one site with decreasing trend, Kvädöfjärden), whereas a decrease in abundance has been observed in the Kalmar Sound and an increase in one of the monitoring areas in the Bothnian Sea (Supplementary material, Table S1; HELCOM 2017, Bergström et al., 2016). The rationale for why only including a predation pressure at the coast is that no comprehensive predation index was available for offshore areas and that sticklebacks are exposed to predation when they migrate to the coast to spawn in the spring.

Analysis of body shape

Landmark based geometric morphometrics (Bookstein, 1991; Zelditch et al., 2004) was used for comparison of body shape of fish across divisions and locations. First, fish were positioned within a groove in a polystyrene block to prevent deformation of the body and images of the left side of each specimen were captured with a Canon EOS 700D. Then 22 landmarks were digitized for each fish (Fig. 2) using the tpsDig v 2.3 software (Rohlf, 2017). The landmarks used (see Supplementary material, Table S2) are similar to those used in previous studies of stickleback body shape (Albert et al., 2007; McGuigan et al., 2010; Walker, 1997; Webster et al., 2011).

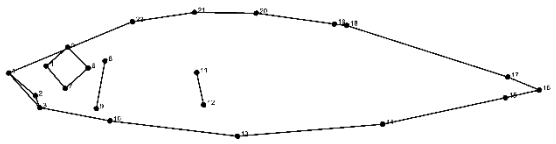

Fig. 2 Position of the 22 landmarks (for description see Supplementary Material, Table S2).

Using tpsRelw v 1.54 software (Rohlf, 2017) landmarks were superimposed to remove the non-shape part of variation (General Procrustes analysis or GPA, Rohlf and Slice, 1990). Finally, shape variation as obtained from the Procrustes shape coordinates (Mitteroecker and Bookstein, 2011) across divisions and locations was analysed using Principal Components Analysis (PCA) and Canonical variates analysis (CVA).

A subset of fish (only adult specimens of 5 – 7 cm) was chosen in this analysis to mitigate allometric effects on the body shape (Walker, 1993). There was, however, a season effect on body shape: samples collected during spring departed from all other samples. As a result, we divided fish into two groups, pre-spawning (spring) and post-spawning (summer and fall samples combined), in body shape analysis. In total, 270 fish were digitized (Table 1), but here, for illustrative purposes, we only present results of a balanced sub-sample per location of a post-spawning season (30-31 fish per location, Table 1, in brackets). Both seasonal groups (pre-spawning and post-spawning) showed consistent results (Supplementary material, Figs. S3-S5). In Kalmar Sound, the body shape did not differ across years (visual inspection with PCA, MANCOVA, $p>0.05$), neither it did between gears in the Curonian Lagoon (visual inspection with PCA, MANCOVA, $p>0.05$).

A permutational MANCOVA (adonis package in R) on the shape coordinates (partial warps) was used to test the effect of location on differences in body shape using size (log-transformed centroid size of each fish) as a covariate. Discriminant Functions analysis was used to compare body shapes of fish from the east and west divisions, and the degree of correct classification into the divisions was evaluated using jackknife cross-validation.

Analysis of otolith shape

Both left and right sagittal otoliths (Sagittae) were removed from the fish for analysis of differences in otolith shape between divisions and locations. Once removed, the otoliths were rinsed with distilled water, and, in case of remaining tissue material, cleaned using a Bandelin Sonorex ultrasonic bath. Subsequently,

digital captures of each otolith were taken under Olympus BX41 transmitted light microscope using an Olympus MicroPublisher 3.3 RTV camera. The otolith outline was analysed with Fourier and Wavelet analysis, using the open source software package ShapeR (Libungan and Pálsson, 2015). The two methods were chosen as they may provide different information. Unlike the Fourier analysis, the Wavelet analysis is capable to detect differences in shape in specific regions of the otolith. The Fourier analysis as a contrast is more powerful to distinguish differences in the overall shape of otoliths (Libungan and Pálsson, 2015).

To reduce the ontogenetic effects on otolith shape, the analysis was performed on adult fish of a size range of 5.5 – 7.6 cm total length (for fish size distribution see Supplementary material, Fig. S6). Otolith shape differences between the three locations were compared using an ANOVA-like permutation test (1000 permutations) in the vegan package of R (Oksanen et al., 2016). In total, 71 pair of otoliths was analysed from the three most geographically distant regions: Bothnian Sea, Kalmar Sound and Curonian Lagoon (Fig. 1; Table 1). Neither year (in Kalmar Sound) nor gear (in Curonian Lagoon) had an effect for otolith shape (ANOVA-like permutation test, $p>0.05$).

Results

Number of body plates

The number of body plates differed considerably between the east and west divisions ($F=244.16$, $p<0.0001$). In the east division, sticklebacks had on average 22.7 ± 0.2 (SE) body plates, while in the west division - 13.5 ± 0.4 (Fig. 3). Within the west division, there was a slight, but not significant differences in the number of body plates across locations ($F= 1.83$, $p= 0.16$). The average number of body plates was 15.4 ± 0.9, 14.3 ± 0.85 and 12.5 ± 0.5 in the Bothnian Sea, Baltic Proper and Kalmar Sound respectively (Fig. 3). Although the number of body plates was significantly associated with fish size ($F=33.7$, $p<0.0001$), the interaction between fish size and location on body plates was not ($F=0.17$,

p=0.92), indicating similar patterns in plate development over ontogeny across locations and divisions studied.

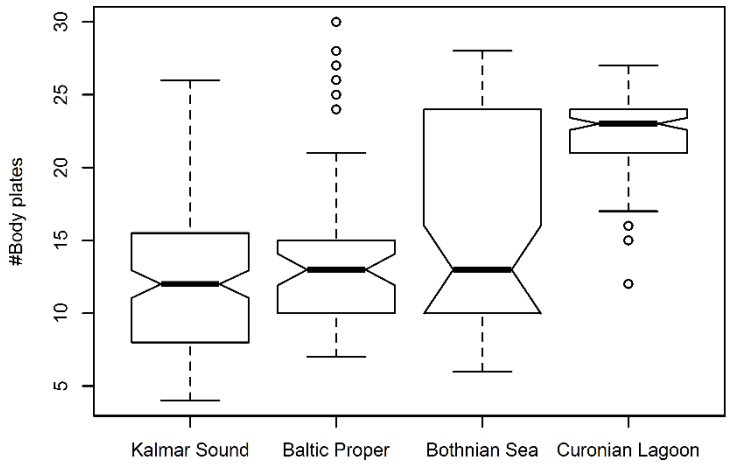

Fig. 3 Number of stickleback body plates in the locations studied.

The abundance index of piscivorous fish was highest in the Curonian Lagoon (Fig. 4), where the number of body plates of sticklebacks was also the highest. Within the west division, the abundance index of piscivorous fish was slightly higher in the Bothnian Sea, where there also was a slightly higher number of body plates of sticklebacks compared to the other locations within this division (Figs. 3 and 4).

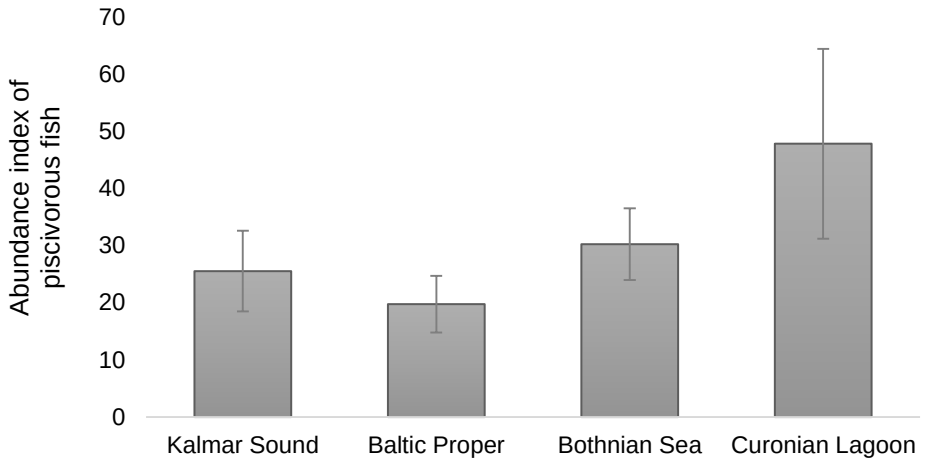

Fig. 4 Abundance index of coastal predatory fish in the areas studied (data of 2010-2014). Error bars are standard deviations (+/- 1 SD) of the annual means.

Body shape

Differences in body shape were observed both between divisions and among locations (Table 2). The main shape differences occurred along the second PC-axis where sticklebacks from the east division (Curonian Lagoon) had deeper bodies compared to fish from the west division (Fig. 5). The differentiation in body shape between the west and east division was confirmed by a high percentage of correctly classified fish (93 %) in the jackknife cross-validation analysis (see Supplementary material, Fig. S7).

Table 2. Results of permutational MANCOVA. Log-transformed fish centroid size (LCS) was treated as a covariate, division (west vs east, A) or location (Curonian Lagoon, Kalmar Sound, Bothnian Sea and Baltic Proper, B) was treated as a factor.

	df	Sum of squares	Mean Squares	F model	R^2	Pr (>F)
A						
LCS	1	9.22e+25	9.22e+25	4.79	0.03	0.0006
Division	1	1.90e+26	1.90e+26	9.89	0.05	0.0001
LCS*Division	1	2.97e+25	2.97e+25	1.55	0.01	0.14
B						
LCS	1	9.22e+25	9.21e+25	4.91	0.03	0.0006
Location	3	3.05e+26	1.02e+26	5.43	0.09	0.0001
LCS*Location	3	6.89e+25	2.29e+25	1.22	0.02	0.22

There was also significant variation in the body shape within the west division (Table 2). The Kalmar Sound fish were more distinct, while the Bothnian Sea and Baltic Proper fish were found to be most similar in terms of body shape (Table 3, Fig. 6). Fish from Kalmar Sound were more dorsally convex, while the fish in Bothnian Sea and Baltic Proper were more ventrally convex. The Kalmar Sound fish also had longer snouts (Fig. 6).

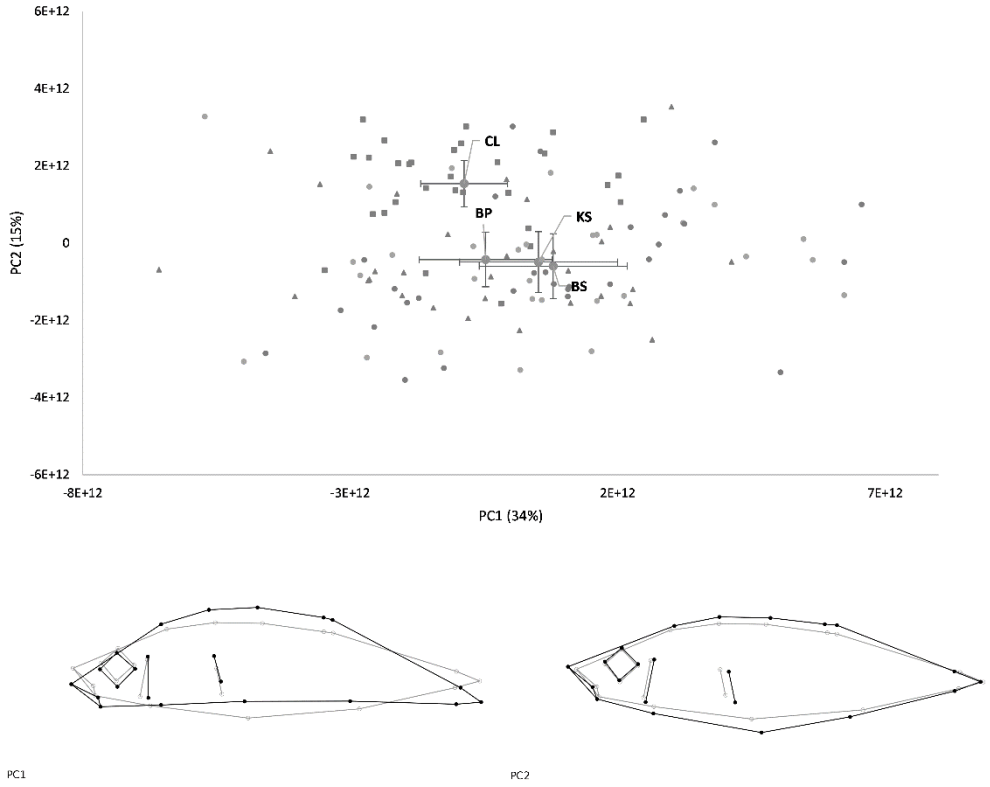

Fig. 5 Principal component analysis of body shape of sticklebacks from the different locations in the study (mean ± SD). CL = Curonian Lagoon (squares) BP = Baltic Proper (triangles), KS = Kalmar Sound (circles), BS = Bothnian Sea (rhombus). Bottom: Wireframe graphs, illustrating changes in body shape (black) in relation to mean shape (grey) along the PC1 (left), and along PC2 (right). Deformations presented correspond to the range of PC axes.

Although fish size was significant for explaining differences in body shape across divisions and locations (Table 2), the interaction between division/location and size was not significant suggesting that fish from all

samples share similar trajectories with respect to ontogenetic development of body shape.

Table 3. Differences in body shape of sticklebacks from different locations as addressed using Mahalanobis distances among locations. A higher value denotes a larger body-shape difference between sites. P-values of the distance estimates from permutation tests (999 permutations) are all <0.0001.

	Baltic Proper	**Bothnian Sea**	**Curonian Lagoon**
Bothnian Sea	3.89		
Curonian Lagoon	6.50	7.06	
Kalmar Sound	4.86	5.08	5.76

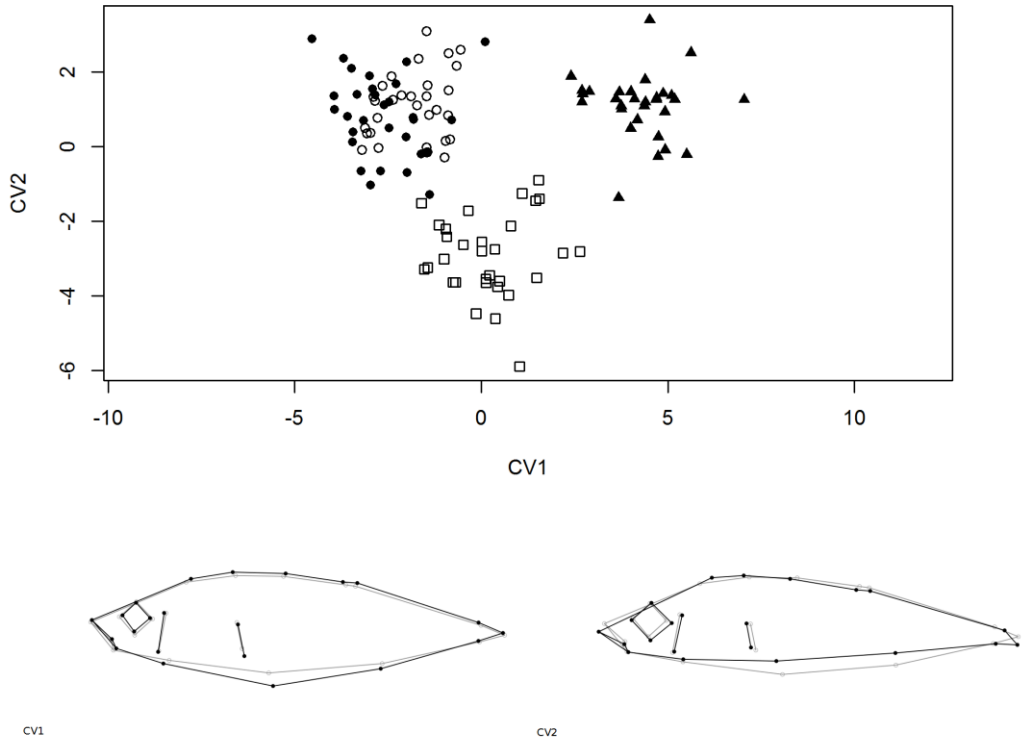

Fig. 6 CVA ordination showing the main axis of body shape variation among the locations studied. Triangles = Curonian Lagoon, squares = Kalmar Sound, filled circles = Bothnian Sea, open dots = Baltic Proper. Bottom: Wireframe graphs, illustrating changes in body shape (black) in relation to mean shape (grey) along the CV1 (left), and along CV2 (right). To ease illustration, deformations for CV2 are 2 x magnified.

Otolith shape

Both analyses used to address differences among divisions and locations in otolith shape showed similar results in terms of overall shape. No significant differences among locations and divisions were detected (Wavelet: F=1.56, p=0.14; Fourier: F= 0.7, p=0.7) for neither the right nor the left otolith. The Wavelet analysis revealed some minor differences in the *excisura major* region of the otolith (Fig. 7, see also Supplementary material, Fig. S8), but there were no overall strong differences across samples.

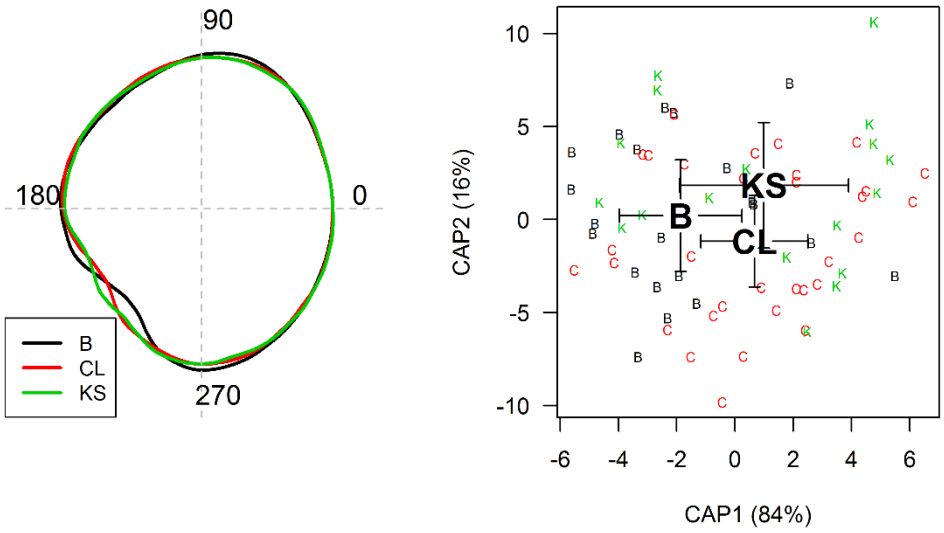

Fig. 7 Left: Average otolith shape, based on Wavelet reconstruction, of three-spined stickleback from the Bothnian Sea (B), Curonian Lagoon (CL), and Kalmar Sound (KS). Right: Otolith shape of three-spined stickleback from Bothnian Sea (B), Kalmar Sound (KS) and Curonian Lagoon (CL) using Canonical analysis of Principal Coordinates with Wavelet coefficients. Black letters represent the mean canonical value for each population, and smaller letters represent individual fish showing the first letter of each population. The error bars of the mean canonical values represent the standard error (mean +/- 1SE).

Discussion

In this study we show that stickleback morphology varies in space in spite of the Baltic Sea being a marine environment with potential for high gene flow between geographic regions. Our findings are in accordance with other studies in the Baltic Sea showing that sticklebacks exhibit divergence between regions (DeFaveri et al., 2013; DeFaveri and Merilä, 2013). In particular, the morphological characters analysed in our study reflect clear-cut differences between eastern and western Baltic Sea with respect to body plate numbers and body shape. This pattern might in turn be a response to the level of local predation pressure. The most conservative trait studied, otolith shape, did however not differ among samples in turn suggesting that population differentiation in sticklebacks in the Baltic Sea is either rather recent or weak as also highlighted by earlier studies using molecular markers or synchrony in population abundances (Östman et al., 2017).

Number of body plates

Sticklebacks are generally very diverse in morphology as a result of adaptation to various habitats and environments (Gow et al., 2007; Walker, 1997). It is well known that lateral body plate development, a part of the defense system in sticklebacks, can alter quite dramatically as a response to predation, especially in freshwater habitats (Barber and Nattleship, 2010; Bell and Foster, 1994; Cano et al., 2008; Reimchen, 2000). Relaxed predation pressure is presumed to be the main cause for a reduction in body plate number (Bell and Foster, 1994; Leinonen et al., 2011). However, different types of predators are likely to mediate armor development differently. Selection towards a higher number of body plates may be triggered by piscivores fish (Gross, 1978; Reimchen, 1983), while the response to macroinvertebrate or bird predation may be the opposite (Bergstrom, 2002; Reimchen, 1983; Zanella et al., 2015). The logic behind this is that body plates are suggested to be effective against gape-limited piscivorous fish, while such armor against birds and invertebrates may be less valuable

(Barber and Nattleship, 2010). Moreover, in a vegetated freshwater environment body plates may be lost as to increase maneuverability and improve escape performance (Barber and Nattleship, 2010). To that end, abiotic factors like concentration of calcium ions in the water (Spence et al., 2012), salinity (Barrett, 2010) and temperature (Reimchen, 2000) may also be associated with body armor development. Higher latitudes with lower temperatures in winter may be expected to correlate with a higher body plate number (Reimchen, 2000), and a positive selection towards a low-plate morphology in freshwaters has been observed and is believed to be related to increased growth rate in a low salinity conditions (see review and references therein Barrett, 2010). In a study of sticklebacks in the Baltic Sea, however, salinity did not influence the differentiation across regions in the number of body plates (DeFaveri and Merilä, 2013). To that end, variation in defensive structures, like the number of bony lateral plates, can thus illustrate population differentiation in sticklebacks (e.g., Hermida *et al.*, 2005), and the rate of divergence in and evolution of these traits have been shown to occur very rapidly (Kristjansson et al., 2002).

In our study, the observed divergence in body plate numbers among divisions and locations appears to be associated with different levels of predation pressure. Sticklebacks from the Curonian Lagoon had the highest number of body plates even despite being a freshwater (low salinity), relatively warm and highly vegetated area. The predation index was highest in the Curonian Lagoon suggesting that coastal piscivorous fish may foster the development of an elevated number of body plates of sticklebacks in the area. Within the west division, although not significant, there was a tendency for a higher body plate number in the sticklebacks where the salinity and temperature is the lowest (Bothnian Sea). There are likely differences between the studied divisions and locations originating from other sources of predation besides coastal fish such as birds (for example cormorants) and offshore fish species like cod. Due to a lack of comprehensive data, these sources of predation pressure are not considered in this study. As such, the results presented in here should be viewed

as a first attempt to link divergence in body armor of sticklebacks to natural environmental gradients in the Baltic Sea.

There is evidence for a genetic basis for lateral body plate differentiation along the Baltic Sea coast (DeFaveri and Merilä, 2013), and it is thus likely that the differences observed in our study might be a result of adaptive differentiation among sub-populations of sticklebacks in different regions.

Body shape

Numerous studies in freshwater have investigated the divergence in three-spined stickleback body shape in relation to adaptation to diverse environments (e.g., Aguirre and Bell, 2012). Differences in body shape have for example been demonstrated between anadromous and freshwater stickleback populations (Walker and Bell, 2000), between stream and lake populations (Berner et al., 2008), and also within lakes (McPhail, 1984; Walker, 1997; Willacker et al., 2010). This phenomenon has to date, however, received less attention in more open marine systems. In our study we found evidence for body shape differentiation in that sticklebacks from the Curonian Lagoon (east division) had deeper bodies compared to the other samples (west division). As for body plate number, fish predation can also influence the evasive morphology of sticklebacks (Walker, 1997) a feature that can exhibit quite drastic changes and develop very rapidly (Kristjansson et al., 2002; Mazzarella et al., 2015). Slender bodies in freshwater sticklebacks has been suggested to be a result of low predation (Walker and Bell, 2000).

Other features of the environment can also influence the body shape of sticklebacks. Higher salinity can for example induce shallower bodies in sticklebacks (Mazzarella et al., 2015) as a means to improve swimming performance in an open habitat (Blake et al., 2005). A deeper body does, however, not necessarily mean poorer swimming performance (Seebacher et al., 2016), and since sticklebacks from the Curonian Lagoon are anadromous in migrating to the Baltic Sea (Gaigalas, 2001; Jakubavičiūtė, unpublished), there is likely a trade-off between predation defense (deep and fully plated bodies) and

abilities to swim in a brackish more open system. In addition, a deeper body profile with a distended abdominal area has been shown to be associated with a low plate morphology (Bjærke et al., 2010). In our study we found a contrasting pattern in that sticklebacks from the Curonian Lagoon had the deepest bodies and the highest number of body plates. This again suggests that predation might be the most important driver of stickleback morphology divergence in the Curonian Lagoon.

Also within the west division there was a slight divergence among locations in the body shape of sticklebacks. Fish from the Kalmar Sound location were more dorsally convex and had longer snouts. These are both morphological features that is typically associated with a planktivorous diet (Bjærke et al., 2010; Willacker et al., 2010). Given that all fish within the west division were caught in the offshore pelagic area and hence were presumably planktivorous, and that the number of gill-rakers of the fish did not differ between the eastern and western divisions (Jakubavičiūtė unpublished data), we believe that differences in feeding behavior is not the key driver behind the morphological divergence of sticklebacks in the Baltic Sea.

Deeper bodies may also be a response to better conditions for sticklebacks in the Curonian Lagoon since it is a highly eutrophic water body with high resource densities (Gasiūnaitė et al., 2008). Fulton indexes of sticklebacks from Curonian Lagoon have indeed been found to be significantly higher than in the other locations investigated (see Supplementary material, Table S3). However, deeper bodied fish would naturally have a higher weight-length relationship, thus it is not clear whether the differences in morphology is caused by predation, habitat or food availability. Indications from numbers of gill rakers and predation pressures do not point at food availability, although interplay between all these factors cannot be rejected.

Otolith shape

Otolith shape analysis has been proven to be a valuable tool for separating even closely related fish species (e.g., Karahan et al., 2014), and also for discrimination between populations of the same species (e.g., Libungan et al., 2015). The shape of otoliths is usually a result of genetic separation but may also be affected by prevailing environmental conditions as temperature and diet (Cardinale et al., 2004; Vignon, 2012; Vignon and Morat, 2010). In our study, we did not find any significant differences in the otolith shape among locations or divisions. This in turn might suggest that stickleback population subdivision is not substantial as this trait likely is the least plastic among those investigated in our study.

It is possible that there is further subdivision with respect to morphological differentiation within the eastern and western divisions, especially so within the eastern one since at the time of this study only one replicate (Curonian Lagoon) was available. Despite this, we believe that the results are valid for the eastern and western delineation in morphology since the fish in the Curonian Lagoon migrate from the area to the Baltic Sea and back to spawn (Gaigalas, 2001; Jakubavičiūtė, unpublished). Although fish were sampled with different gears (see Table 1) implying different stickleback morphs (planktivorous vs benthic) may have been caught, no differences in the number of gill-rakers between eastern and western divisions were found. This again implies that all sticklebacks investigated were of the same planktivorous anadromic morph which spend a part of their life-cycle in the offshore Baltic Sea. Future studies, however, should include additional samples from foremost the eastern division to address further spatial sub-division.

Conclusions

Like variation in demography across areas (Östman et al., 2017), phenotypic plasticity in response to heterogeneous environments has been proven to have profound implications for stock identification (Swain et al., 2005). Our study suggests plastic traits can provide information on stock separation in open environments despite ample possibilities for gene flow between sub-populations. Morphological characteristics are still among the simplest, cost-effective and widely used tools to identify a stock (e.g., Bacha et al., 2014; Cadrin and Silva, 2005; Sadighzadeh et al., 2014). Phenotypic stocks are essential to determine in population modeling and fisheries management, since stocks in a population may differ, for instance, in ontogenetic rates or responses to exploitation (Cadrin and Silva, 2005). Ideally, for effective stock management, complementary genetic and phenotypic information should be available (Begg and Waldman, 1999); and this study brings morphological analysis to contribute the previous genetic investigations on the stickleback population structure in the Baltic Sea (DeFaveri et al., 2013; DeFaveri and Merila, 2011).

Our study shows that morphometrics may be a useful tool for detection of stickleback population differentiation and for stock identification in the Baltic Sea. The differentiation between locations and divisions in highly plastic traits as body plate number and body shape but not in the less plastic trait otolith shape suggest that population subdivision of sticklebacks in the Baltic Sea is rather recent or weak, something that has also been indicated by earlier studies on both the spatial synchrony in demography and molecular markers from (Östman *et al.*, 2017). Different traits may vary in their degree of plasticity in sticklebacks (Day et al., 1994). Number of body plates is a highly heritable trait in sticklebacks (Colosimo et al., 2004), and the profound differences in this trait as found in our study hence suggest adaptive phenotypic plasticity and genetic differentiation among regions in the Baltic Sea. Furthermore, since the morphological divergence as observed in our study corresponds to earlier studies

of genetic divergence of sticklebacks in the Baltic Sea, we further conclude that the population development of the species and its interaction with other parts of the Baltic ecosystem might as suggested in earlier studies differ between regions and areas in the Baltic Sea (Bergström et al., 2015; Olsson et al., 2015). We hence suggest that potential future management of sticklebacks in the Baltic Sea should consider the existence of weak but significant spatial population sub-division.

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Table S1. Data used for coastal fish predation estimation (abundance of functional group of piscivores fish, HELCOM, 2017).

Location	Year	Assessment unit	Monitoring site	Trend since 1995
Kalmar Sound	2010-2012	Western Gotland Basin Swedish Coastal waters	Vinö	Decreasing
Baltic Proper	2010-2014	Western Gotland Basin Swedish Coastal waters	Kvädöfjärden	Decreasing
	2010-2014	Bornholm Basin Swedish Coastal waters	Torhamn	No change
	2010-2014	Northern Baltic Proper Swedish Coastal waters	Askö	No change
			Muskö	No change
Bothnian Sea	2010-2014	Bothnian Sea Swedish Coastal waters	Gaviksfjärden	No change
			Långvindsfjärden	No change
			Forsmark	Increasing
Curonian Lagoon	2010-2012	Eastern Gotland Basin Lithuanian Coastal waters	Atmata/ Dreverna	No change
				No change

Table S2. Landmark positions used for body shape analysis.

Landmark number	Landmark position
1	anterior tip of the upper lip
2	the axis of the jaws
3	posterior edge of angular
4-7	the anterior-most, uppermost, posterior-most and lowermost point of the orbital circumference
8	dorsal extent of preopercular
9	posterioventral extent of preopercular
10	anterior tip of the ectocoracoid;
11-12	the lower and uppermost points of the pectoral fin base.
13	the posterior most edge of the left pelvic spine
14	anterior insertion of anal fin
15	origin of caudal fin membrane on ventral midline
16	posterior extent of caudal peduncle
17	origin of caudal fin membrane on the dorsal midline (DML)
18	base of the first dorsal fin ray at the DML
19-21	the posterior most edge of the first, second and third dorsal spines, at the points where they emerge from the dorsal surface
22	supraoccipital notch immediately lateral to DML

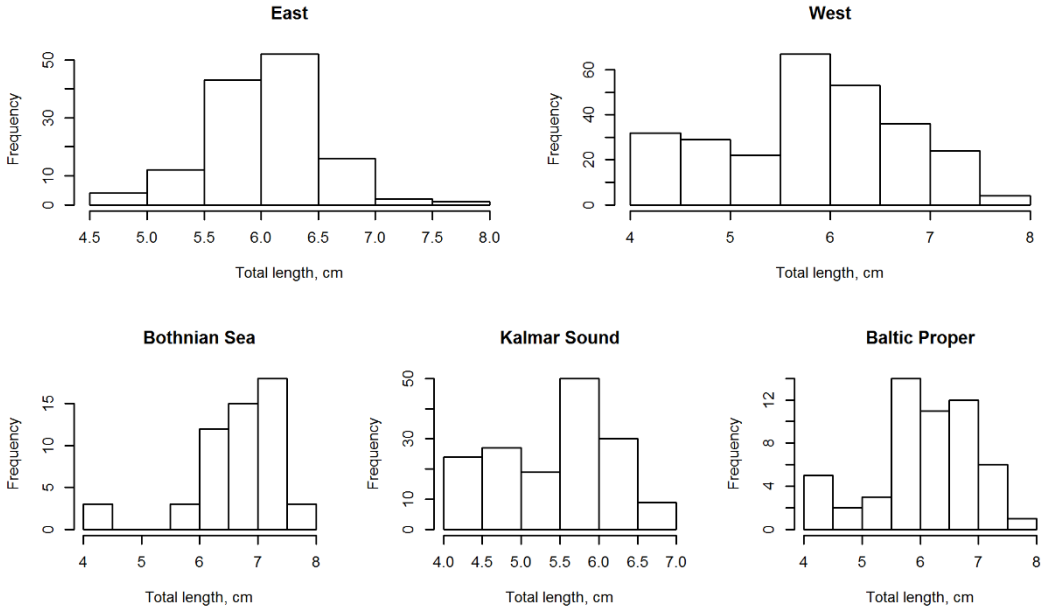

Fig. S1 Stickleback size (TL) distribution in a sample for body plates estimation: in divisions (at the top) and within West division (at the bottom).

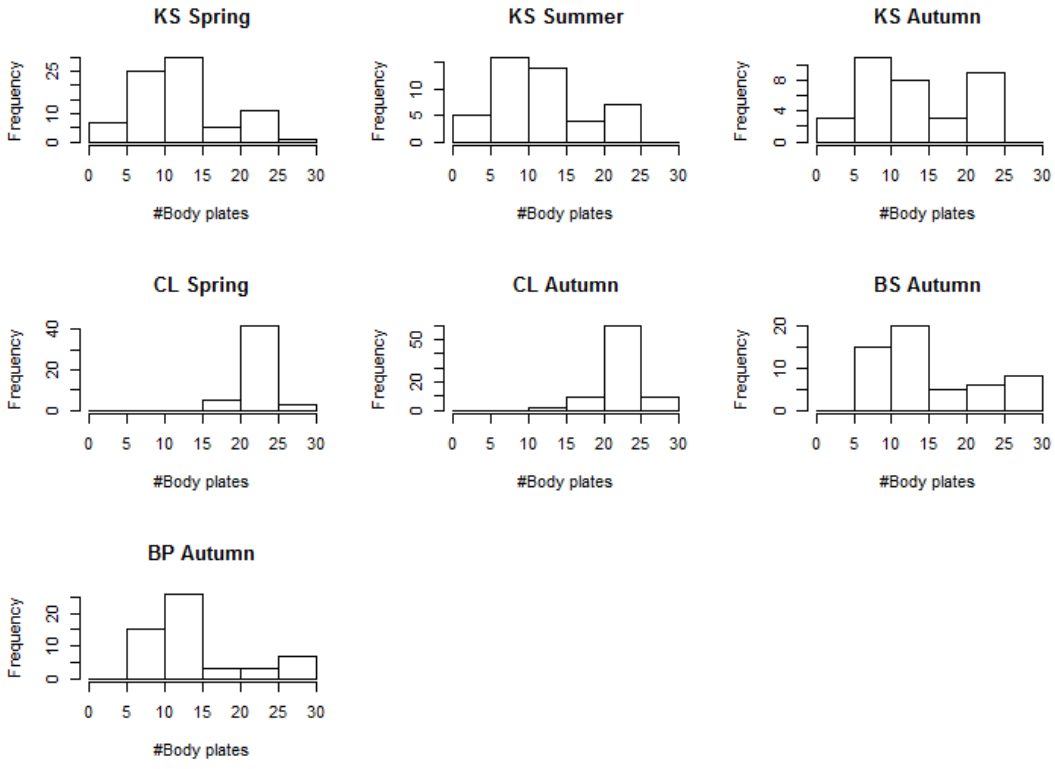

Fig. S2 Number of body plates in different seasons. KS – Kalmar Sound, CL – Curonian Lagoon, BS – Bothnian Sea, BP – Baltic Proper.

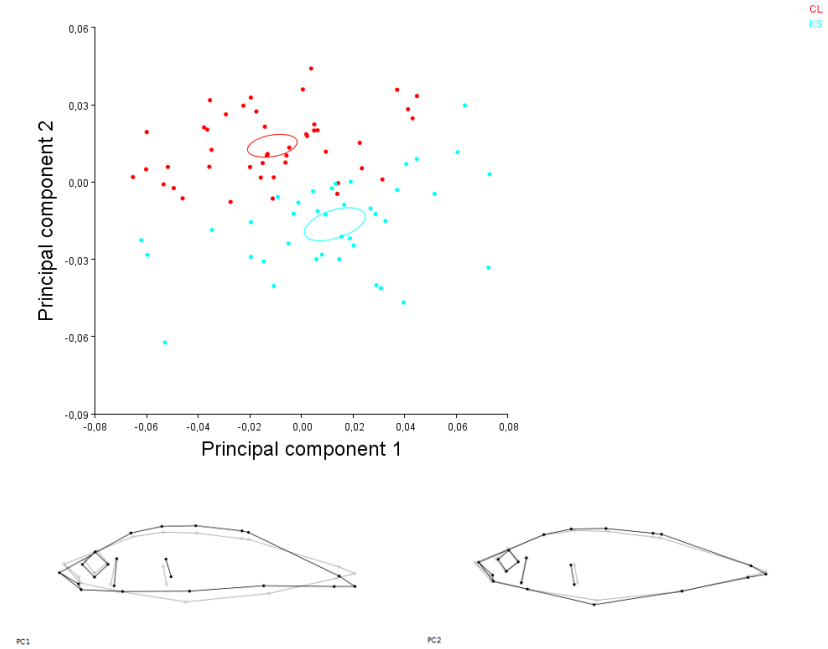

Fig. S3 PCA of body shape for samples from pre-spawning period (spring). Curonian Lagoon, N=45, and Kalmar Sound N=39. Circles indicate confidence ellipses (90%) for mean. Bottom: Wireframe graphs, illustrating changes in body shape (black) in relation to mean shape (grey) along the PC1 (left), and along PC2 (right). Deformations presented correspond to the range of PC axes.

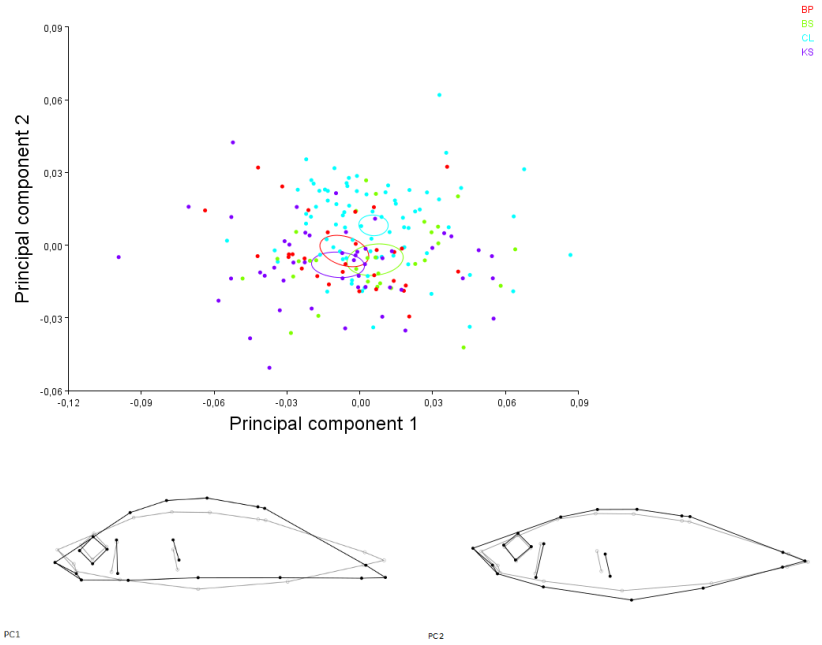

Fig. S4 PCA of body shape for samples from post-spawning period (summer and fall combined), unbalanced sample sizes. Curonian Lagoon N=78, Kalmar Sound N=49, Bothnian Sea N=30, Baltic Proper N=30. Circles indicate confidence ellipses (90%) for mean. Bottom: Wireframe graphs, illustrating changes in body shape (black) in relation to mean shape (grey) along the PC1 (left), and along PC2 (right). Deformations presented correspond to the range of PC axes.

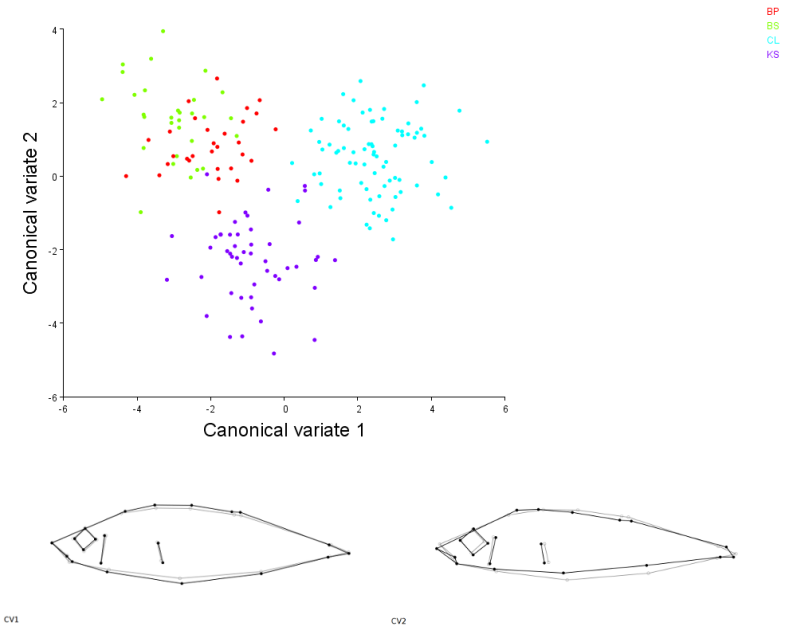

Fig. S5 CVA ordination of samples from post-spawning period (fall and summer combined) Curonian Lagoon N=78, Kalmar Sound N=49, Bothnian Sea N=30, Baltic Proper N=30.Wireframe graphs, illustrating changes in body shape (black) in relation to mean shape (grey) along the CV1 (left), and along CV2 (right). To ease illustration, deformations for CV2 are 2 x magnified and changes indicated as the score on CV2 decreases.

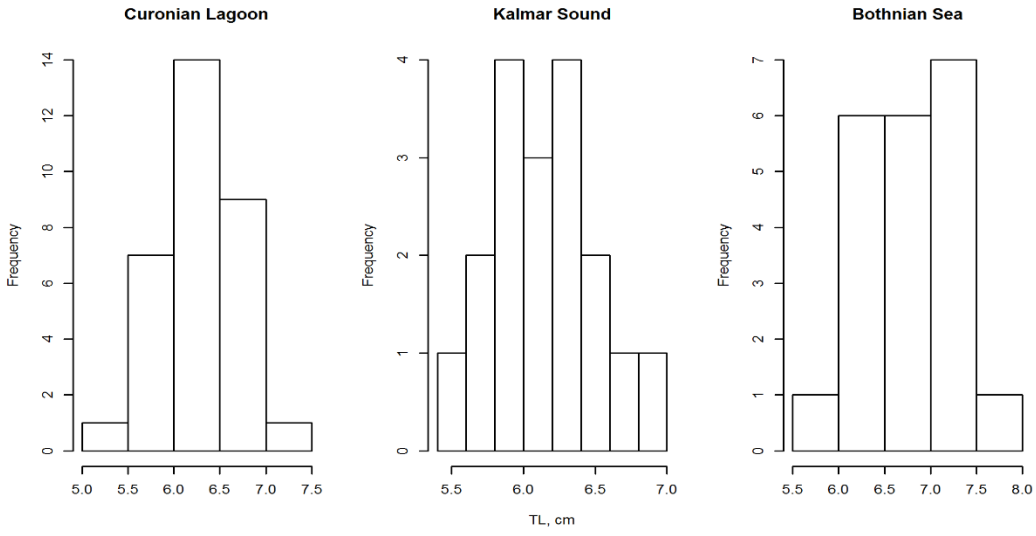

Fig. S6 Stickleback size (TL, cm) distribution used for otolith shape analysis.

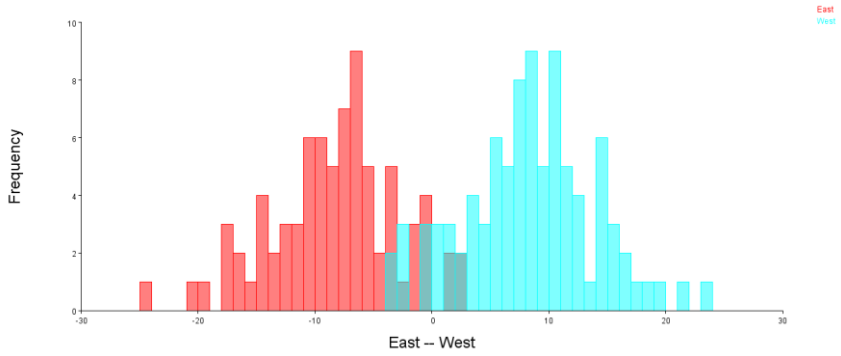

Fig. S7 Cross-validation discriminant function analysis of sticklebacks' body shape from the East (left) and West (right). Rate of correct classification was 93% (Jacknife).

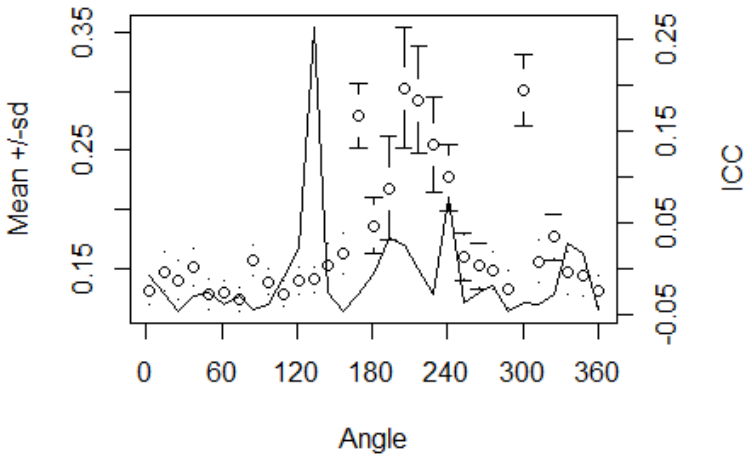

Fig. S8 Mean and standard deviation (sd) of the Wavelet coefficients for all combined otoliths and the proportion of variance among groups or the intraclass correlation (ICC, black solid line). The horizontal axis shows angle in degrees (°) based on polar coordinates where the centroid of the otolith is the center point of the polar coordinates.

Table S3. Mean (±1 SD) Fulton condition factors of three-spined sticklebacks in the post-spawning season. Means having the same letter are not significantly different ($p>0.05$, Tukey's test).

	Kalmar Sound	**Baltic Proper**	**Bothnian Sea**	**Curonian Lagoon**
Fulton condition factor	0.67 ± 0.15^{a}	0.83 ± 0.11^{b}	0.84 ± 0.11^{b}	1 ± 0.12^{c}

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Work experience

Since 2018	Junior researcher
	Nature Research Centre, Laboratory of Marine Ecology
	Akademijos str. 2, LT–08412, Vilnius
2011-2016	Biologist
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2008-2011	Laboratory assistant
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Education

2013-2017	PhD student in Ecology and Environmental Research
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2011-2013	MSc in Ecology, *Magna Cum Laude*
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	Degree thesis: Modelling perch population in the Curonian Lagoon: possibilities and difficulties
2007-2011	BSc in Ecology and Environmental Sciences
	Vilnius University, State University, Universiteto st. 3, LT-01513, Vilnius, Lithuania
	Degree thesis: Roach (*Rutilus rutilus*) behavioural and growth response to water salinity

Training and internships

May 2017 – June 2017	Swedish University of Agricultural Sciences, Sweden
	Three-spined stickleback stock assessment in the Baltic Sea
September 2015 – February 2016	University of Gothenburg, Sweden
	DNA metabarcoding for three-spined stickleback diet studies
2014 November	Institute of Coastal Research, Öregrund, Sweden
	Ageing and morphological analysis of three-spined sticklebacks
2014 November	Swedish University of Agricultural Sciences, Uppsala

	Ecosystem functioning course
2014 February	Swedish University of Agricultural Sciences, Uppsala
	R programming course
February 2013 – May 2013	Finnish Game and Fisheries Research Institute, Joensuu, Finland
	Fish population modelling and related analysis of catch and individual data on Lake Oulujärvi whitefish
January 2012 – June 2012	Technical University of Denmark, Copenhagen (Erasmus student exchange program)

International conferences

1. **Jakubavičiūtė E.,** Casini M., Ložys L., Olsson J., 2016. Are three-spined sticklebacks important food competitors for sprat and herring? Seasonal dynamics in the diet of pelagic fish species in the western Baltic Sea. International Council for the Exploration of the Sea (ICES) Annual Science Congress. Riga, Latvia.
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9. **Jakubavičiūtė, E.**, Ložys, L., 2010. Evaluation of perch (*Perca fluviatilis*) stock exploitation using 40-45 mm gillnets in the Curonian Lagoon. 5th

International Student Conference “Biodiversity and functioning of aquatic ecosystems in the Baltic Sea region”. Klaipėda, Lithuania.

10. **Jakubavičiūtė, E**., Ložys, L., 2009. Evaluation of perch (*Perca fluviatilis*) population age structure in the Curonian Lagoon using fish otolith processing and statistical analysis methods. 4th International Student Conference “Biodiversity and functioning of aquatic ecosystems in the Baltic Sea region”. Dubingiai, Lithuania.

National conferences

1. **Jakubavičiūtė, E**., 2017. Trispyglių dyglių ekologinė reikšmė Baltijos jūroje: mityba bei išteklių grupių identifikavimas. Jaunųjų mokslininkų konferencija „Bioateitis 10: gamtos ir gyvybės mokslų perspektyvos“. Lietuvos mokslų akademija, Vilnius.
2. **Jakubavičiūtė, E**., Olsson, J., Dainys, J., Ložys, L., 2016. Planktofagių žuvų mityba Baltijos jūros pelagialėje: trispyglės dyglės – mažos ir mažareikšmės? 9-oji mokslinė-praktinė konferencija „Jūros ir krantų tyrimai 2016“. Klaipėda.
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3. **Jakubavičiūtė, E**., 2015. Small fish – big challenges: three-spined stickleback in the Baltic Sea. HELCOM FISH-PRO, Copenhagen, Denmark. 2015-02.

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1. Vainikka, A., **Jakubavičiūtė, E**., Hyvarinen, P., 2017. Synchronous decline of three morphologically distinct whitefish (*Coregonus lavaretus*) stocks in Lake Oulujarvi with concurrent changes in the fish community. Fisheries Research, 196: 34-46. doi: 10.1016/j.fishres.2017.08.013
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Awards

- Lithuanian Academy of Sciences Award for the best Master thesis 2013 (Biology, Medicine and Geology section).