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Dietary resource analysis of two coexisting stickleback species (*Gasterosteus aculeatus* and *Pungitius pungitius*).

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Preface

I would like to thank the many people who helped me to realise this thesis, without whom it would not have been possible.

First and foremost, I would like to thank Joost Raeymaekers, who always inspired me with his scientific enthusiasm and who introduced me to the world of sticklebacks. His reliable qualities as a supervisor led me to a much better understanding of statistical and scientific analysis.

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Finally, I would like to thank Olivia Jecel for guiding me through the English language. I love you from the benthic of my heart!

Summary

This study explores the stomach content of two sympatric stickleback species (the three-spined stickleback (*Gasterosteus aculeatus* Linnaeus, 1758) and the nine-spined stickleback (*Pungitius pungitius* Linnaeus, 1758)) across ten sites in Belgium and the Netherlands and compares their niche breadth, niche overlap, and their proportion of limnetic prey (PLP). Additionally, classical multidimensional scaling was performed to find spatial signatures in the diet composition. Significant differences were found in the niche breadth and the PLP, prey items in three-spined stickleback were less diverse and more limnetic than prey items in nine-spined stickleback. The dietary niche overlapped at nine out of ten sites and a significant spatial signature in the diet composition was found. Additional approaches suggested for future studies include presence-absence methods of prey items (*e.g.*, kick sampling) and stomach content analyses covering a longer time period to cover potential seasonal variation.

Introduction

Community ecology

The amazing biodiversity of our planet has inspired generations of scientists to explore its ecosystems. What has been discovered during these centuries is fascinating: organisms form communities and occupy distinct roles in an ecosystem. The field of community ecology describes how populations of different species within a geographical area cope with many different conditions: these can be abiotic factors such as changes in temperature or the chemical composition of the environment – both of which can affect living organisms, as well as biotic factors including predator-prey interactions, competition for limited resources, diseases, pathogens, and human influence.

All in all, an ecosystem depends on its organisms to sustain its existence. The following example from Yellowstone National Park gives an understanding for how the food chain consists of a network of interacting, interdependent species which together sustain a functioning community. As top predators and keystone species in Yellowstone National Park, wolves played an important role as they controlled, by their presence or absence the population size of elk. Furthermore, they had a large effect on the overall composition and functioning of the ecosystem. From the 1800s to the middle of the 20th century local hunters overhunted the wolf, which led to a drastic reduction and eventually complete local extinction in 1926 when the last wolf pack in Yellowstone was killed. From then on, the complete absence of wolves led to an explosive increase in elks, which in turn induced overgrazing. Many other herbivores in the community were negatively affected by elks utilising too many resources. By observing this problem, the understanding for community ecology developed, and in the 1990s 31 grey wolves from western Canada were relocated to Yellowstone National Park. After the reintroduction, the elk population decreased, overgrazing was reduced, and other herbivores in the ecosystem started thriving again (Ripple & Beschta, 2004).

This example underlines on the one hand how strong keystone species drive ecological processes within a community, and on the other hand shows how anthropological influence can cause shifts in nature and a loss of biodiversity. On a global scale, the loss of biodiversity is one of the biggest issues and challenges of our time. Rainforests are being cut down to make space for the production of palm oil (Violita *et al.* 2016), industrialised agriculture can lead to soil degradation (Louwagie *et al.* 2011) and the loss of important pollinators (Gallai *et al.* 2009), and microplastics - with the chemicals and pesticides that adhere to them - affect aquatic life and precious marine habitats across the entire planet (Barrows, Cathey & Petersen, 2018). The extinction of species can in turn lead to a reduction of ecosystem processes (Griffin *et al.* 2011). Supported by our understanding of community ecology, applied conservation projects try to restore disturbed ecosystems. For example, rewilding projects focused on regaining precious biodiversity and ecosystems which were diminished or substantially reduced by humans. The solutions used are diverse: natural grazing allows the antagonistic relationship for forests and herbivores (Lord eat al. 2020), and sturgeon reintroduction projects helped to restore the biodiversity in the Danube (Friedrich, Reinartz, and Gessner, 2019). The rewilding of forests and rivers thus gives back more space for the resilience of ecosystems to function. In total, there are several ambitious projects which can help to restore what we as humans have often neglected to preserve.

Scientists want to understand the composition of ecological communities and how this composition is shaped by species interactions such as competition, parasitism, predation, herbivory, and mutualism. Within the field of community ecology, there are many questions regarding the distribution and abundance of species (Nielsen, 2014). Keystone species play an important role in communities and affect many other species by their presence or absence. Furthermore, feeding relationships of species and the influence which parasites have on the feeding behaviour of their hosts are examples of detailed information which can help answer questions about competition for finite resources within a community.

Data collection in the field forms the basis of many community ecology projects. Stomach content analyses serve as snapshots of the organism of interest its diet and gives insight of interactions with other organisms in the system (Baker *et al.* 2014). A more detailed and long-term approach popular in the field of community ecology is stable isotope analysis. Here, samples from for instance muscle or liver tissue are used to retrieve information about the trophic position of the species of interest in the food web. The measured isotopes ratios for carbon and nitrogen provide information on the source of primary producers in the system and the relative trophic levels of individuals, respectively (Dawson *et al.* 2007).

Food webs, as introduced by community ecologist Charles Elton in 1927, are scientific tools used by community ecologists, that visualise the transfer of food energy between trophic levels (Hui, 2012). For example, aquatic macroinvertebrates feed on aquatic plants, smaller fish prey on macroinvertebrates, bigger fish prey on small fish, and birds prey on bigger fish. As this shows, food webs can be complex, and a high number of species can be involved in a particular ecological community. A food web usually begins with autotroph organisms such as plants, which use light energy to metabolise carbohydrates via photosynthesis. They are classified as primary producers. Herbivorous animals including some groups of insects, rodents, and bigger ruminants live of plant materials and might serve as prey for secondary and tertiary carnivorous consumers. Some carnivorous consumers in a system could be apex predators, such as wolves, eagles, or pike, because they play a large role in the dynamics of the community. Within the food web, there are two important principles: the ecological niche and the competitive exclusion principle. The ecological niche describes that every organism in this web of interactions has its own function and interacts with the abiotic environment as well as all the other species (Peterson, 2011). The competitive exclusion principle implies that two species occupying the same ecological niche cannot coexist in a stable community. In other words: sooner or later, one of the species will drive the other to extinction (Hardin, 1960). However, as detailed by the example of ecological character displacement below, this type of competition is not inevitable, as organisms can evolve to reduce or avoid competition.

Ecological character displacement is an important term to understand niche partitioning. Coined by William L. Brown Jr. and E. O. Wilson in 1956, it states that sympatric species which overlap in the same area, differ in one or more characteristics (morphological, ecological, behavioural, or physiological) (Brown *et al.* 1956). When two closely related species live in similar niches, they are expected to compete for limited resources. However, those competitive situations can instead lead to niche partitioning, when both species start consuming slightly different forms of a resource, for example hunting on a different prey. Species might also share the same food resource, but browse for it at different times or in different locations (Griffin *et al.* 2011).

Just two years after the development of the term ecological character displacement, Robert H. MacArthur wrote a doctoral dissertation titled “Population Ecology of Some Warblers of Northeastern Coniferous Forests”. In this dissertation, Mac Arthur was one of the first scientists who described the coexistence of species in the same niche. He made interesting observations about five sibling warbler species who occupied the same trees to chase for insects during their breeding season. At first MacArthur wondered why one species did not outcompete the other, but during his field trips into the woods he observed unique behaviours: one species preferred the top of the tree, while the others spent their time in the dense branches in the middle (Figure 1.). After this discovery, he was one of the first scientists to define the term niche partitioning (MacArthur *et al.* 1958).

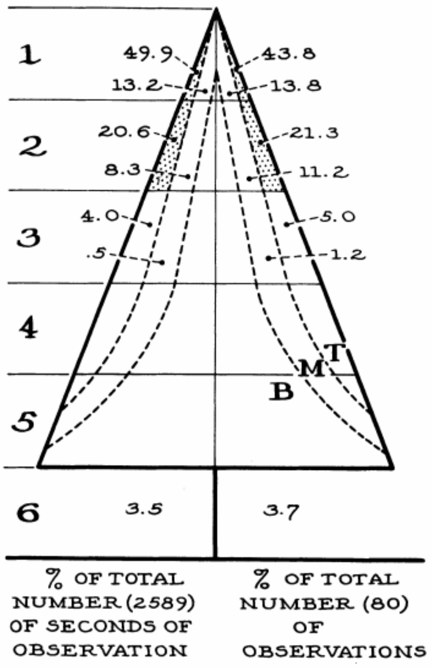


FIG. 2. Cape May warbler feeding position. The zones of most concentrated activity are shaded until at least 50% of the activity is in the stippled zones.

Figure 1. Schematic representation of the feeding positions of five warbler species in a tree. Sibling warbler species occupy different parts of the same tree to chase for insects (MacArthur et al. 1958).

Niche partitioning can also give an insight into the food web when sympatric species develop different diet preferences in distinct micro-habitats. Microhabitats are usually described as small areas that differ from the surrounding larger habitat by unique conditions which are suitable for the specific needs of certain species (O'Dwyer *et al.* 2014). To name an example that is related to this thesis, sympatric ecotypes of the three-spined stickleback in the lakes of British Columbia occupy different habitats in the water column (the benthic and the limnetic habitat), which gives each of them the advantage of feeding on different prey. The limnetic stickleback is described as a small and slender fish, which chases for zoo-plankton in the open water zone; the larger, deep bodied benthic stickleback feeds on larger invertebrates from the sediments and plants in the bottom of the water body (Gow *et al.* 2008).

There are other interesting examples of the insights to be gained through the concept of niche partitioning. One last example can be found within the class of insects. A study of bumblebees that was conducted in the mountains of Colorado shows how a species can adapt morphologically to its habitat. This study describes the morphological differentiation among coexisting bumblebees with different species specialising on specific plant species. To reach the precious nectar of the flowers, bumblebees with a short proboscis visit flowers with a short corolla, while species with a longer proboscis visit flowers with a longer corolla. As with the other examples, this study underlines how competitors coexist and partition resources in an ecosystem (Pyke, 1982).

As one can see, we can increase our understanding of the functioning of ecological communities by looking through the lens of niche partitioning. Not only can one better understand how species adapt morphologically to their habitat (Pyke, 1982), but we can also gain a better understanding of the roles that coexisting sympatric species occupy in an ecosystem (Gow *et al.* 2008; MacArthur, 1958). In this master thesis, niche partitioning plays an important role as the concept provides the main framework for understanding findings of the ecology of coexisting stickleback species.

Stickleback as a model organism

The current work consists of a study into the communities of two coexisting stickleback species, the three-spined stickleback (*Gasterosteus aculeatus* Linnaeus, 1758) and the nine-spined stickleback (*Pungitius pungitius* Linnaeus, 1758). The three-spined stickleback is a popular model organism for studying evolutionary and ecological concepts. Its wide distribution in lakes, coastal areas, ponds, rivers, and ditches leads for some populations to the evolution of reproductive isolation, which makes the three-spined stickleback interesting for the study of local adaptation and ecological speciation (McKinnon & Rundle 2002). Additionally, their occurrence in systems consisting of a dense network of ditches and rivers further enable to study gene flow and genetic drift (Raeymaekers *et al.* 2009; Raeymaekers *et al.* 2017; Bal *et al.* 2021). Due to the easy maintenance and reproduction of three-spined sticklebacks in laboratories, it is possible to conduct breeding and common garden experiments for the study of the adaptive significance of various phenotypic traits, including morphology (Colosimo *et al*. 2005; Cresko *et al.* 2004), behaviour (Kroken *et al.* 2021;

Boughman 2001), physiology (Raeymaekers *et al.* 2010) and parasite resistance (Raeymaekers *et al.* 2011; Konijnendijk *et al.* 2013).

There are also reasons why both three-spined and nine-spined sticklebacks are ideal model organisms: because of their relatively small body size of some centimetres, it is easier to use them in experiments and manipulate them during fieldwork. Costs can be held low because of their weight and size. Furthermore, a large sample size can be processed in the laboratory within a short time frame. A large scientific stickleback community all around the globe allows to share findings and new knowledge concerning the understanding of evolutionary and ecological processes.

Another good reason to use both species as model organisms is the chance to study their ecological communities and adaption to diverse habitats. Their habitat use seems to be different (Delbeek & Williams, 1987). Nine-spined sticklebacks occupy more often weedy areas and three-spined sticklebacks prefer the open water (Hart, 2003). As one can see in the figure below (Figure 2), nine-spined sticklebacks are more slender (Maitland & Campbell, 1992), while three-spined sticklebacks have a deeper body, which enables them to rotate faster around their vertical axis to chase for prey items on the bottom of the water body (Hart, 2003).

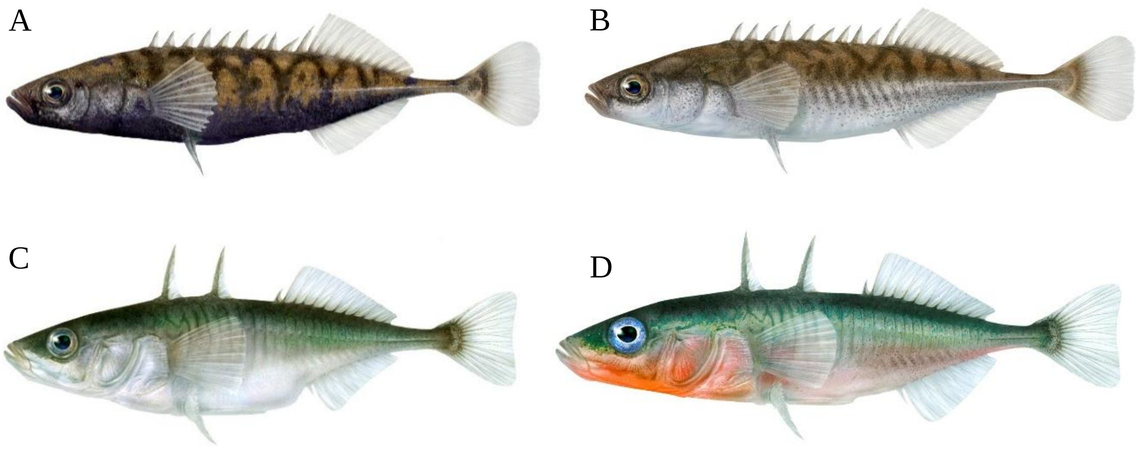
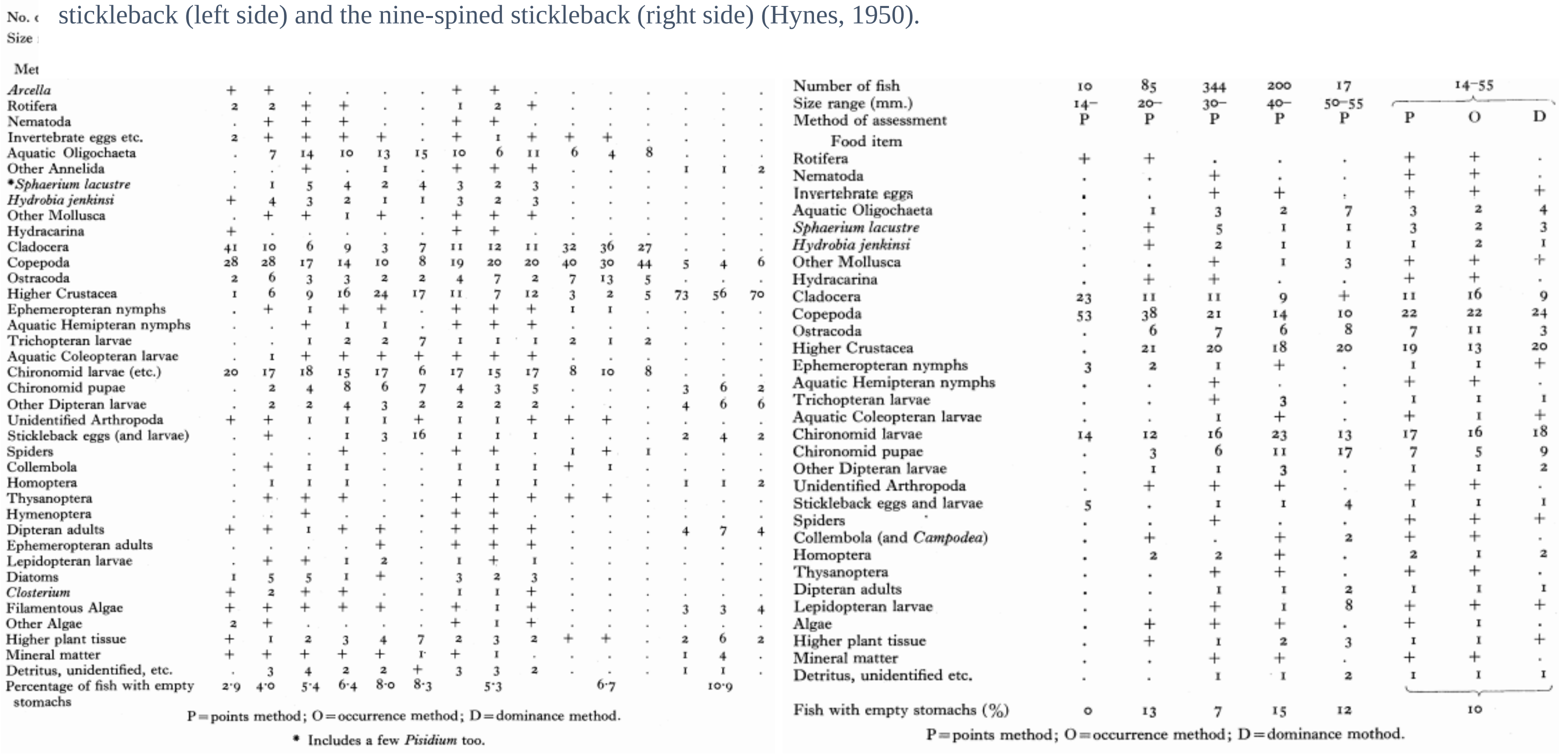


Figure 2. A: male nine-spined stickleback. B: female nine-spined stickleback. C: female three-spined stickleback. D: male three-spined stickleback (the proportion of all four images does not correspond to the natural size of the fish) (Jilg, 2021).

The two stickleback species feature in several studies on ecological divergence between the species, as well as local adaptation within each species. For instance, Hart (2003) analysed morphological traits linked to the habitat use of the two stickleback species. Through his experimental analysis he was able to conclude that morphological traits of both species gave them advantages using different specific habitats. Additionally, two further studies over the period of two decades (Schluter & McPhail, 1992; Gow *et al.* 2008) conducted in Little Quarry Lake in British Columbia show high levels of intraspecific morphological variation in three-spined stickleback. Specifically, Schluter & McPhail (1992) found morphological differences between a limnetic and a benthic type. The limnetic type was shown to have a smaller and slimmer body and smaller mouths compared to the benthic type, enabling it to chase for planktonic prey, while the benthic was shown to be more efficient in hunting on large benthic prey. Gow *et al.* (2008) were able to find an additional benthic-limnetic species pair in Little Quarry Lake. Both studies together provide strong evidence towards the ability of three-spined sticklebacks to quickly adapt to differing environments.

To investigate the ecological dynamics of coexistence of both species, this work is concerned with the analysis of stomach content. One study which has taken a very close look at the stomach content, *i.e.*, prey items, of sticklebacks is Hynes (1950). His detailed work includes some key information concerning prey categorisation which is also relevant for this study (Table 1.). All in all, this study shows that the two stickleback species chase for a similar range of food. Hynes´ findings were based on three different sampling sites.

Table 1. the percentage composition of the diet, as well as the change in composition of the food with increase in size of the three-spined stickleback (left side) and the nine-spined stickleback (right side) (Hynes, 1950).



No. c															
Size															
Met															
Arcella	+	+	.	.	.	.	+	+	.	.	.	.	.	.	.
Rotifera	2	2	+	+	.	.	1	2	+	.	.	.	.	.	.
Nematoda	.	+	+	+	.	.	+	+	.	.	.	.	.	.	.
Invertebrate eggs etc.	2	+	+	+	+	.	+	1	+	+	+	.	.	.	.
Aquatic Oligochaeta	.	7	14	10	13	15	10	6	11	6	4	8	.	.	.
Other Annelida	.	.	+	.	1	.	+	+	+	.	.	.	1	1	2
**Sphaerium lacustre*	.	1	5	4	2	4	3	2	3	.	.	.	.	.	.
Hydrobia jenkinsi	+	4	3	2	1	1	3	2	3	.	.	.	.	.	.
Other Mollusca	.	+	+	1	+	.	+	+	+	.	.	.	.	.	.
Hydracarina	+	.	.	.	.	.	+	+	.	.	.	.	.	.	.
Cladocera	41	10	6	9	3	7	11	12	11	32	36	27	.	.	.
Copepoda	28	28	17	14	10	8	19	20	20	40	30	44	5	4	6
Ostracoda	2	6	3	3	2	2	4	7	2	7	13	5	.	.	.
Higher Crustacea	1	6	9	16	24	17	11	7	12	3	2	5	73	56	70
Ephemeropteran nymphs	.	+	1	+	+	.	+	+	+	1	1	.	.	.	.
Aquatic Hemipteran nymphs	.	.	+	1	1	.	+	+	+	.	.	.	.	.	.
Trichopteran larvae	.	.	1	2	2	7	1	1	1	2	1	2	.	.	.
Aquatic Coleopteran larvae	.	1	+	+	+	+	+	+	+	.	.	.	.	.	.
Chironomid larvae (etc.)	20	17	18	15	17	6	17	15	17	8	10	8	.	.	.
Chironomid pupae	.	2	4	8	6	7	4	3	5	.	.	.	3	6	2
Other Dipteran larvae	.	2	2	4	3	2	2	2	2	.	.	.	4	6	6
Unidentified Arthropoda	+	+	1	1	1	+	1	1	+	+	+	.	.	.	.
Stickleback eggs (and larvae)	.	+	.	1	3	16	1	1	1	.	.	.	2	4	2
Spiders	.	.	.	+	.	.	+	+	.	1	+	1	.	.	.
Collembola	.	+	1	1	.	.	1	1	1	+	1	.	.	.	.
Homoptera	.	1	1	1	.	.	1	1	1	.	.	.	1	1	2
Thysanoptera	.	+	+	+	.	.	+	+	+	+	+	.	.	.	.
Hymenoptera	.	.	+	.	.	.	+	+	.	.	.	.	.	.	.
Dipteran adults	+	+	1	+	+	.	+	+	+	.	.	.	4	7	4
Ephemeropteran adults	.	.	.	.	+	.	+	+	+	.	.	.	.	.	.
Lepidopteran larvae	.	+	+	1	2	.	1	+	1	.	.	.	.	.	.
Diatoms	1	5	5	1	+	.	3	2	3	.	.	.	.	.	.
Closterium	+	2	+	+	.	.	1	1	+	.	.	.	.	.	.
Filamentous Algae	+	+	+	+	+	.	+	1	+	.	.	.	3	3	4
Other Algae	2	+	.	.	.	.	+	1	+	.	.	.	.	.	.
Higher plant tissue	+	1	2	3	4	7	2	3	2	+	+	.	2	6	2
Mineral matter	+	+	+	+	+	1	+	1	.	.	.	.	1	4	.
Detritus, unidentified, etc.	.	3	4	2	2	+	3	3	2	.	.	.	1	1	.
Percentage of fish with empty stomachs	2·9	4·0	5·4	6·4	8·0	8·3		5·3			6·7			10·9	

P = points method; O = occurrence method; D = dominance method.

* Includes a few *Pisidium* too.

Number of fish	10	85	344	200	17		14–55	
Size range (mm.)	14–	20–	30–	40–	50–55			
Method of assessment	P	P	P	P	P	P	O	D
Food item								
Rotifera	+	+	.	.	.	+	+	.
Nematoda	.	.	+	.	.	+	+	.
Invertebrate eggs	.	.	+	+	.	+	+	+
Aquatic Oligochaeta	.	1	3	2	7	3	2	4
Sphaerium lacustre	.	+	5	1	1	3	2	3
Hydrobia jenkinsi	.	+	2	1	1	1	2	1
Other Mollusca	.	.	+	1	3	+	+	+
Hydracarina	.	+	+	.	.	+	+	.
Cladocera	23	11	11	9	+	11	16	9
Copepoda	53	38	21	14	10	22	22	24
Ostracoda	.	6	7	6	8	7	11	3
Higher Crustacea	.	21	20	18	20	19	13	20
Ephemeropteran nymphs	3	2	1	+	.	1	1	+
Aquatic Hemipteran nymphs	.	.	+	.	.	+	+	.
Trichopteran larvae	.	.	+	3	.	1	1	1
Aquatic Coleopteran larvae	.	.	1	+	.	+	1	+
Chironomid larvae	14	12	16	23	13	17	16	18
Chironomid pupae	.	3	6	11	17	7	5	9
Other Dipteran larvae	.	1	1	3	.	1	1	2
Unidentified Arthropoda	.	+	+	+	.	+	+	.
Stickleback eggs and larvae	5	.	1	1	4	1	1	1
Spiders	.	.	+	.	.	+	+	+
Collembola (and *Campodea*)	.	+	.	+	2	+	+	.
Homoptera	.	2	2	+	.	2	1	2
Thysanoptera	.	.	+	+	.	+	+	.
Dipteran adults	.	.	1	1	2	1	1	1
Lepidopteran larvae	.	.	+	1	8	+	+	+
Algae	.	+	+	+	.	+	1	.
Higher plant tissue	.	+	1	2	3	1	1	+
Mineral matter	.	.	+	+	.	+	+	.
Detritus, unidentified etc.	.	.	1	1	2	1	1	1
Fish with empty stomachs (%)	0	13	7	15	12		10	

P = points method; O = occurrence method; D = dominance mothod.

While Hynes´ study focuses mainly on the stomach content and therefore gives more information on the coexistence of the stickleback species, there are other studies which have focused on the connection between prey items and niche partitioning. For instance, Dukowska & Grzybkowska (2014) analysed the gut contents of small-sized percids, cyprinids, and three-spined sticklebacks to assess how the fish coexist in their habitat. They concluded that their diets only partly overlapped, which supported the niche partitioning concept (Dukowska & Grzybkowska, 2014). Seeing as their results show that ruffe consumed most of the benthic *Chironomidae* (nonbiting midges), perch consumed epiphytic chrionomids and zooplankton, dace consumed prey in fast flowing waters, the generalist roach showed high intraspecific competition and low gut fullness coefficient values, which made them less competitive, and the three-spined stickleback consumed epiphytic back flies (*Simuliidae*) and fish eggs. Except of the roach, all of the fish species are in line with the niche overlap hypothesis, which states that maximal tolerable niche overlap can be higher under less intense competition.

Study system

The three-spined stickleback has a wide distribution. The geographical range of its habitat is along the northern hemisphere (Figure 3). This includes countries where it is native (for example Canada, United states, Scandinavia, United Kingdom, Belgium, The Netherlands, Germany). The nine-spined stickleback, on the other hand, has a wider and more inland distribution along the northern hemisphere, but also shows an overlap with the geographical range of the three-spined stickleback (Figure 4).

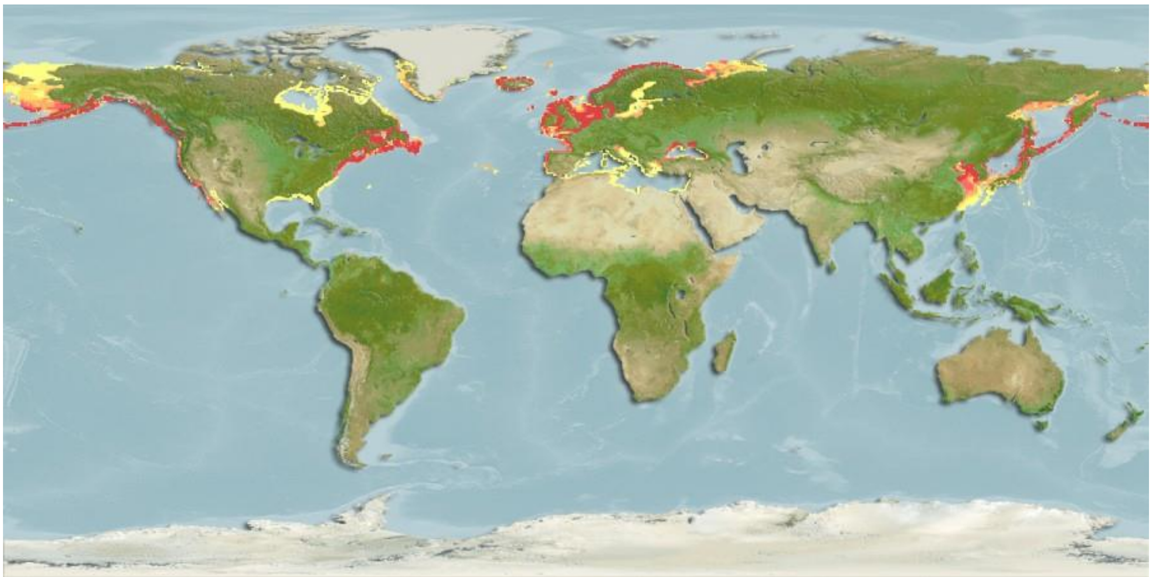


Figure 3. Geographical distribution of the three-spined stickleback. Distribution range colours (red: high degree, yellow: low degrees) indicate degree of suitability of habitat which can be interpreted as probabilities of occurrence (AquaMaps, 2019).

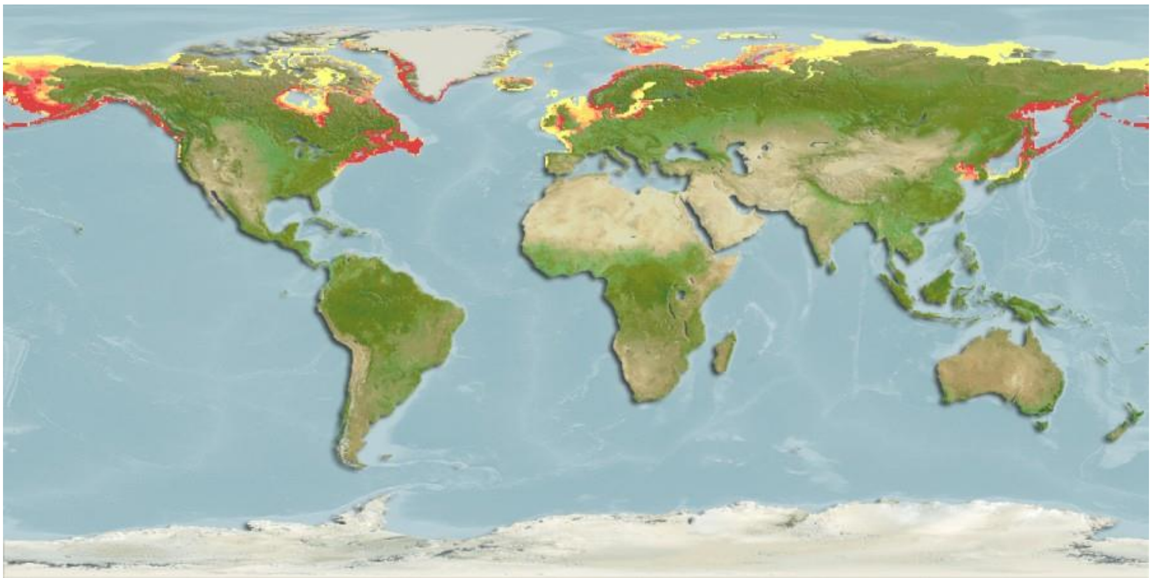


Figure 4. Geographical distribution of the nine-spined stickleback. Distribution range colours (red: high degree, yellow: low degrees) indicate degree of suitability of habitat which can be interpreted as probabilities of occurrence (AquaMaps, 2019).

In general, three-spined sticklebacks colonise coastal and freshwater habitats all over the northern hemisphere. In Europe, their distribution ranges from 35 to 70°N. Despite the fact that the nine-spined stickleback mainly evolved in freshwater, it is euryhaline and frequently shares brackish and freshwater habitats with the three-spined stickleback (Raeymaekers *et al.* 2017). In this comparative study, the three-spined stickleback and the nine-spined stickleback were sampled in Belgium and the Netherlands along a salinity gradient (brackish water to freshwater). The site characteristics in the typically dense network of water bodies in both countries range from manmade drainage systems, ditches in semi-natural grassland, and city canals, to natural streams (Raeymaekers *et al.* 2017). Figure 5 gives an impression of the study localities.

Figure 5. A typical landscape with ditches in Belgium and the Netherlands (Team Stickleback Nord, 2020).

Raeymaekers *et al.* (2014, 2017) investigated many details about stickleback species in this study system. Brackish and freshwater habitats of Belgium and the Netherlands have a Holocene origin. The historical expansion of the three-spined stickleback goes back to postglacial Eurasia, marine populations started to recolonise freshwater habitats, while nine-spined sticklebacks have been mainly freshwater-fish. More recently, both species of stickleback were, and still are, influenced by a shift of the coastline after the last glacial period as well as by the construction of the drainage systems across both countries (Raeymaekers *et al.* 2017).

Raeymaekers *et al.* (2017) concluded that the population divergence for phenotypic traits in nine-spined sticklebacks are partly explained by variation in habitat characteristics including salinity, turbidity and the density of macro-invertebrate predators such as backswimmers (*Notonecta glauca*), dragonfly larvae (*Anax sp. and Aeschna sp.*), and great diving beetles (*Dytiscus marginalis*). Likewise, trait divergence in three-spined stickleback could partly be explained by environmental variation. However, in three-spined sticklebacks a considerable larger part of the variation can also be partly explained by the interaction between environmental variation and spatial factors (Raeymaekers *et al.* 2017).

Aims and hypotheses

In this thesis, niche partitioning of three-spined sticklebacks and nine-spined sticklebacks plays an important role, as the stomach content analysis provides the main framework for understanding findings about coexisting species. The aim of this thesis is to examine the dietary intake and the dietary overlap of both stickleback species – the result of which might show how they manage to coexist, and how the diet in both species is influenced by habitat characteristics such as turbidity, and salinity. Therefore, we collected prey item counts of the stomach contents of nine-spined and three-spined sticklebacks collected in ten different sites along a salinity gradient across Belgium and the Netherlands.

The niche partitioning theory provides an insight to better understand the biodiversity of our planet’s ecosystems. It refers to natural selection, which drives competing species into different resource use and/or different niches (MacArthur, 1958). This leads to the main hypothesis of the current master´s thesis: that niche partitioning can be observed between coexisting three-spined and nine-spined stickleback.

Materials and Methods

Study area

Three-spined and nine-spined sticklebacks were sampled at ten different sites across Belgium and the Netherlands (Figure 6) in October 2020. Some of the localities, including small streams, ditches, city canals, and creeks were landlocked or anadromous. The sites were shallow (<150cm) and had a low current (Raeymaekers *et al.* 2014). Furthermore, the sites differ in environmental conditions, ranging from brackish to freshwater habitats.

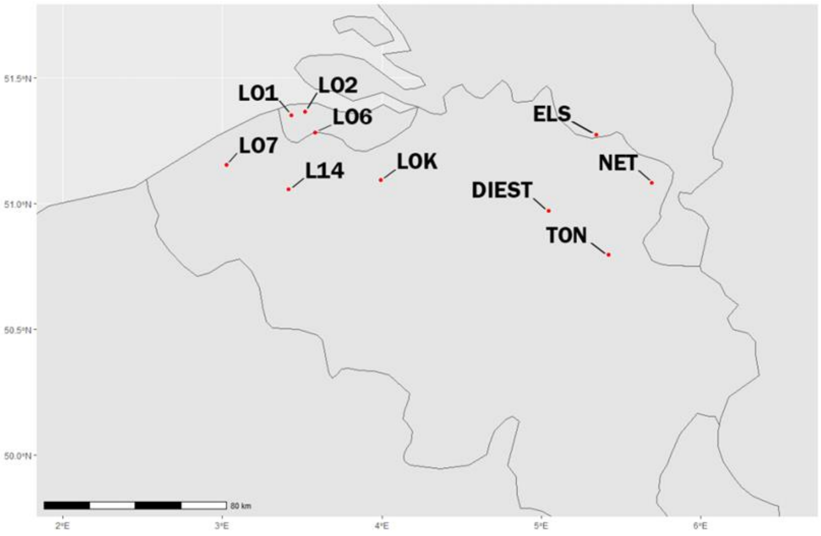


Figure 6. The fieldwork was performed in October 2020 at 10 different sites across Belgium and the Netherlands (Kohl and Malmsten 2021).

Field sampling

We selected seven freshwater sites and three brackish water sites for this study. In October 2020, we visited each site with the aim to capture twenty individuals per species (three-spined and nine-spined stickleback) per site (Diest, Elsenloop, L14, L01, L02, L06, L07, LOK, Net, TON). Field work at each location started by collecting environmental data, including the water temperature (°C), pH, conductivity (µS/cm), and oxygen (mg/L) using a Hach field monitoring devise (Hach, Loveland, Co, USA). Turbidity was measured with a Sneller tube. Sneller depth is the deepest point under water (in cm) where a mini Secchi disk, lowered in a grey PVC tube filled with the water of the specific site, can be seen (Van de Meutter *et al.* 2005). Turbidity was measured by two observers for seven of the sites (L01, L02, L06, L07, L14, LOK, ELS) and average numbers were taken for further statistical analyses. Diest, Net, and TON were measured by a single observer. A 100-metre stretch was sampled by a single person per site in order to estimate the density of nine-spined and three-spined sticklebacks as the number of individuals per metre (Raeymaekers *et al*. 2014). To standardise the density measurements to the highest possible extent, the same net was used for each stretch, with the responsible person using the same dip netting technique of scooping approximately once per metre. Water depth was measured at five points along 100-metres of the specific site in order to acquire an estimated depth range.

Sites with a conductivity >1000 µS/cm were classified as brackish water sites, except for TON (1022 µS/cm), which was categorised as a freshwater site because of its far inland position.

All resulting measurements, site names, depth, turbidity, coordinates, conductivity, the number of individuals sampled for each locality (N3s, N9s), and the classification as “upland/lowland” are shown in Table 2.

Table 2. Overview of site names, depth, turbidity, coordinates, conductivity, sampling size per site (N3s and N9s), and classification as upland/lowland. Note that the single values of the turbidity are an average (N=3).

Site	Depth (cm)	Turbidity	Coordinates (Lat, long)	Conductivity (µS/cm)	N3s	N9s	Upland/Lowland
L01	20 - 50	33	51.35296, 3.43384	3470	20	20	Lowland
L02	10 - 45	27	51.36564, 3.51974	2001	19	21	Lowland
L07	10 - 50	43	51.15488, 3.02463	821	28	19	Lowland
L14	5 - 55	9	51.05868, 3.41307	683	19	18	Lowland
L06	20 - 130	32	51.2828, 3.58352	1576	21	21	Lowland
LOK	20 - 150	27	51.09531, 3.99193	583	21	21	Lowland
ELS	20 - 60	53	51.27573, 5.34615	462	25	23	Upland
TON	20-60	36.5	50.796217, 5.419395	1022	18	20	Upland
Diest	40-50	37	50.9729, 5.0453	466	20	20	Upland
Net	40-110	35	51.08464, 5.69276	205,6	19	20	Upland

After the fish were captured, they were immediately anaesthetised with an overdose of MS-222 and frozen in dry ice.

Lab work

All samples were stored temporarily at KU Leuven University in Belgium until they were transferred to the Mørkvedbukta Research Station at Nord University in Bodø, Norway. In the lab, the sticklebacks were transferred from the -20°C freezer to a styrofoam box with ice for thawing. Three observers selected fish at random to avoid observation bias. Photographs with an individual label for each fish, standard length (excluding the caudal fin), total length (including the caudal fin), as well as fin clips were taken. Stomach content was preserved in 95% EtOH for later analysis. Stomach content was examined with an Olympus SZX16 stereomicroscope. Overall, the stomach contents turned out to be little digested and the visibility of prey items was high. The stomach fullness was estimated by eye and categorised as “full”, “half full”, and “empty”.

Prey items were identified according to Thorp & Covich (2001) and categorised into the following groups: cyclopoid and harpacticoid copepods (*Cyclopoida and Harpacticoida*), the class *Ostracoda*, three genera of the *Chydoridae* family (*Alona* sp., *Leydigia* sp., *Chydorus* sp.), water fleas (*Cladocera*) including the genus *Daphnia* sp., the family of nonbiting midges (*Chironomidae* including pupae and larvae), the family of biting midges (*Ceratopogonidae* including larvae), caddisfly larvae (*Trichoptera*), springtails (*Colembola*), the larvae of beetles (order: *Coleoptera*), may fly larvae (*Ephemenoptera*), the order *Amphipoda*, the order *Isopoda*, nematodes (phylum: *Nematoda*), different genera of aquatic snails, larval copepods (*Naupilus*), earthworms (phylum: *Annelida*), dragonflies and damselflies (order: *Odonata*), and water mites (*Acari* a subclass of arachnids). For each prey category, the number of items was counted.

Statistical analyses

All statistical analyses were performed using the statistics software R (R Core Team, 2020).

Diet proportions

To get a general overview of the prey categories consumed by three-spined and nine-spined sticklebacks, we visualised diet composition for each population by using stacked proportion barplots. We selected the nine prey categories that happened to have the higher overall mean to visualise the proportions of them for each site. Next, proportion tests were made to investigate the significance of the proportions between sites and species for the selection of the nine most abundant prey items (*Amphipoda, Ceratopogonidae, Chironomidae, Chydorus sp., Cyclopoida, Harpacticoida, Daphnia sp., Isopoda, Ostracoda*).

Stomach fullness

The stomach fullness was analysed with a Pearson's Chi-squared test to verify if there was an association between species and stomach fullness among sites.

Diet overlap

The numerical method was conducted to verify the counting of all prey items present in the sampled stomachs (Hynes, 1950). Therefore, diet overlap was quantified using the Morisita´s index (Mohamed, 2004). All prey categories were used for this analysis. The Morisita´s index is a similarity index, where 1 indicates complete overlap, and 0 indicates no overlap.

Morisita´s index is calculated using the equation,

$$\mathrm{M} ? \frac{2? \; p_{ij} p_{ik}}{? \; ^{n} p_{ij} ?(n_{ij} ? 1)/(N_j ? 1)?? \; ^{n} p_{ik} ?(n_{ik} ? 1)/(N_k ? 1)?}$$

Where, M =Morista's index of niche overlap between species *j* and *k*

p_{ij} =Proportion resource *i* is of the total resources used by species *j*

p_{ik} =Proportion resource *i* is of the total resources used by species *k*

n_{ij} =Number of individuals of species *j* that use resource category *i*

n_{ik} =Number of individuals of species *k* that use resource category *i*

N_j, N_k =Total number of individuals of each species in sample

Diversity

The Simpson´s diversity index was calculated as a proxy for the niche breadth of three-spined and nine-spined sticklebacks among sites. Therefore, the diet gives insight into which species tends to be a generalist or a specialist. Values of the Simpson´s diversity index range between zero (specialist) and one (generalist). A paired t-test was performed to investigate the differences of the mean Simpson´s indices in three- and nine-spined sticklebacks among sites.

To test the correlation between the Simpson´s diversity index for three- and nine-spined sticklebacks and the niche overlap, a Pearson correlation test was performed.

Proportion of limnetic prey

Benthic (in or on the substrate of the water body) and limnetic (open water) prey was classified based on Schluter & McPhail (1992). Harpacticoid copepods (*Harpacticoida*), ostracods (*Ostracoda*), isopods (*Isopoda*), biting midges larvae (*Ceratopogonidae*), larvae of beetles (*Coleoptera*), and nematodes (*Nematoda*) were classified as benthic prey. *Alona* sp., *Leydigia* sp., *Chydorus* sp, water fleas (*Cladocera*) including the genus *Daphnia* sp., springtails (*Colembola*), cyclopoid copepods (*Cyclopoida*), and larval copepods (*Naupilus*) were classified as limnetic prey. According to Berner *et al.* (2008), the statistical analyses are based on the number of limnetic prey relative to the number of limnetic and benthic prey combined. Furthermore, the proportion of limnetic prey (PLP) was calculated to see differences among the ten sites. We first visualised the PLP with boxplots and subsequently analysed the variances (ANOVA) to test for significant difference in three-spined and nine-spined stickleback diet among sites. Subsequently, we created a scatterplot by plotting the PLP data of all nine-spined stickleback against the PLP data of the corresponding three-spined stickleback population. Finally, we tested if there was an overall mean difference in PLP between the two species across the ten sites by using a paired t-test.

Classical multidimensional scaling

Bray-Curtis dissimilarities between the prey communities of each stickleback population were calculated using the mean of all limnetic and benthic prey data using the R package "vegan" (Oksanen *et al*. 2015). Classical multidimensional scaling (MDS) based on the Bray-Curtis dissimilarities was used to visualise these prey communities across the ten study sites and the two stickleback species. MDS was also conducted based on Bray-Curtis dissimilarities between all individuals of each stickleback species, again using all limnetic and benthic prey data. In order to visualise the niche breadth of each population, MDS plots were superimposed with 95 % confidence ellipses, based on the two first dimensions.

Results

Overall, the diet of three-spined and nine-spined sticklebacks was similar. Figure 7 gives a comparison of the diets of both species including all sites. The nine most abundant prey items across all sites and both fish species were cyclopoids, harpacticoids, ostracods, *Daphnia*, Chironomidae (including pupae and larvae), ceratopogonid larvae, amphipods, isopods, and *Chydorus* sp. (Table S1).

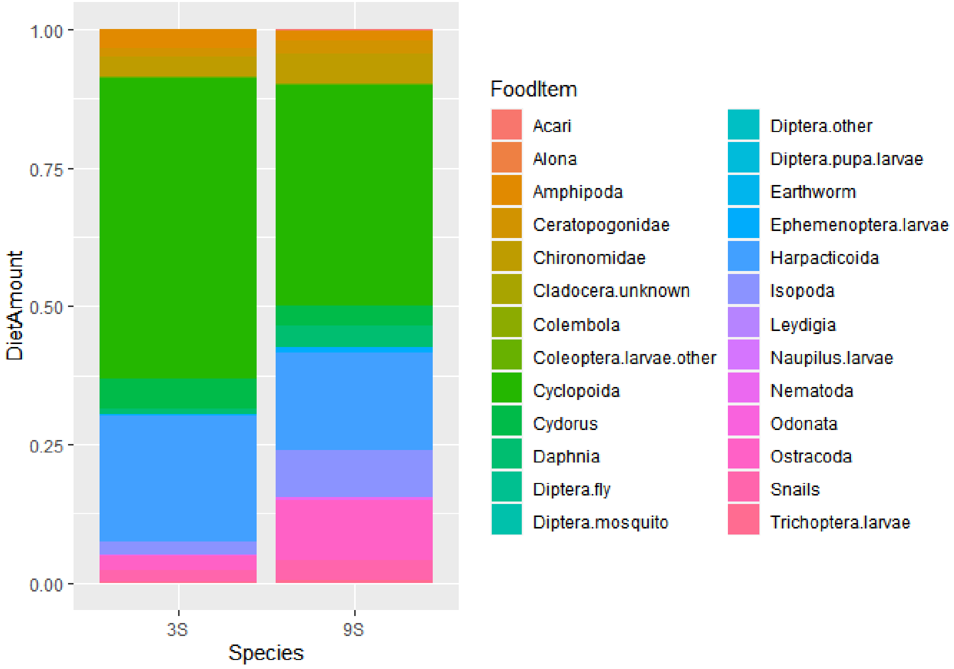


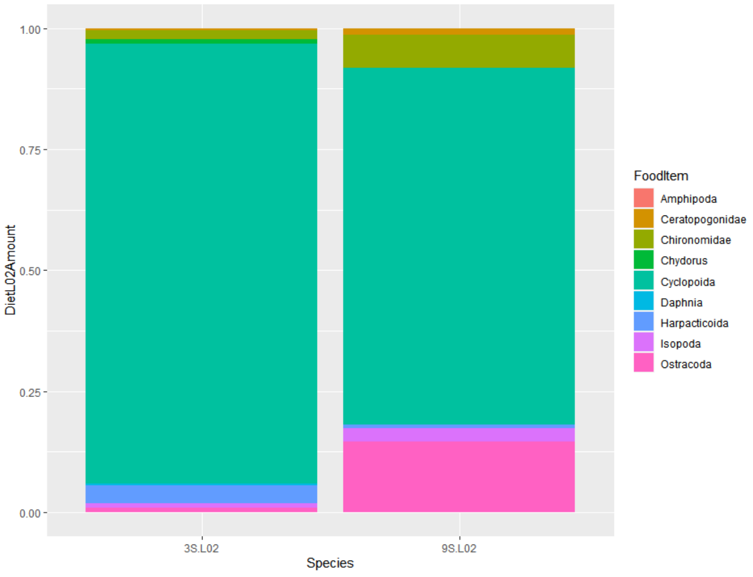
Figure 7. Proportions of all collected prey items consumed by the three-spined stickleback (3S) and the nine-spined stickleback (9S). All sites are included (L01, L02, L06, L07, L14, LOK, Diest, Net, Elsenloop, TON). Cyclopoid and harpacticoid were the most abundant prey items.

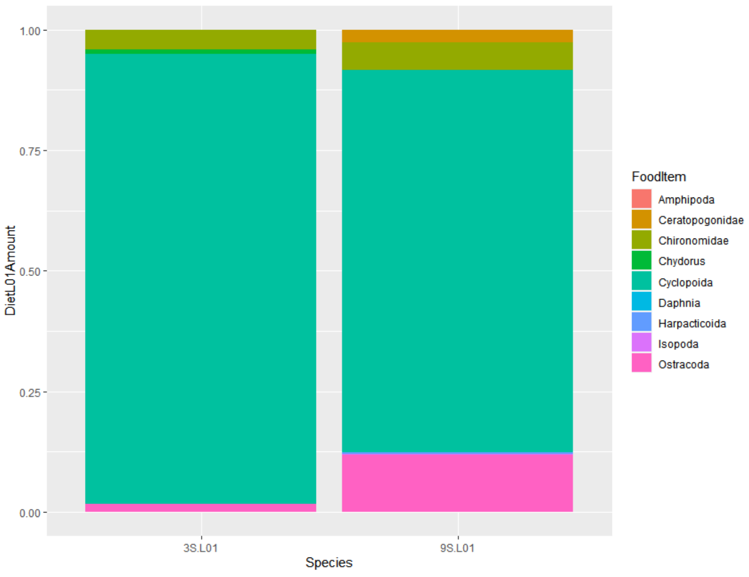
Diet proportions

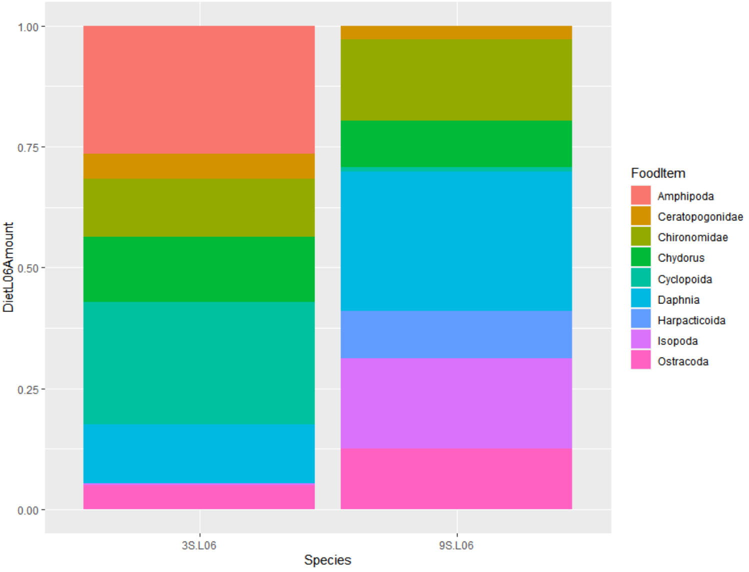
Overall, the consumed prey items seem to be similar between three-spined and nine-spined sticklebacks (Figure 8), but the visualisation of individual sites shows site-specific differences (Figure 8). For this analysis we compared the previous selection of the most abundant prey items. Except of site Diest, all proportion tests between sites and species revealed significant differences (Table 3).

Table 3. Results of the proportions between sites and species for the selection of the nine most abundant prey items

Site	X-squared	df	p-value of the proportion test
Diest	9.362	6	0.1542
Elsenloop	184.75	7	< 2.2e-16
L01	54.981	6	4.677e-10
L02	61.542	7	7.423e-11
L06	245.57	8	< 2.2e-16
L07	17.823	4	0.001336
L14	23.809	5	0.0002362
LOK	168.52	8	< 2.2e-16
Net	9.8519	4	0.043
TON	22.613	7	0.00199



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9S.L06
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Chironomidae
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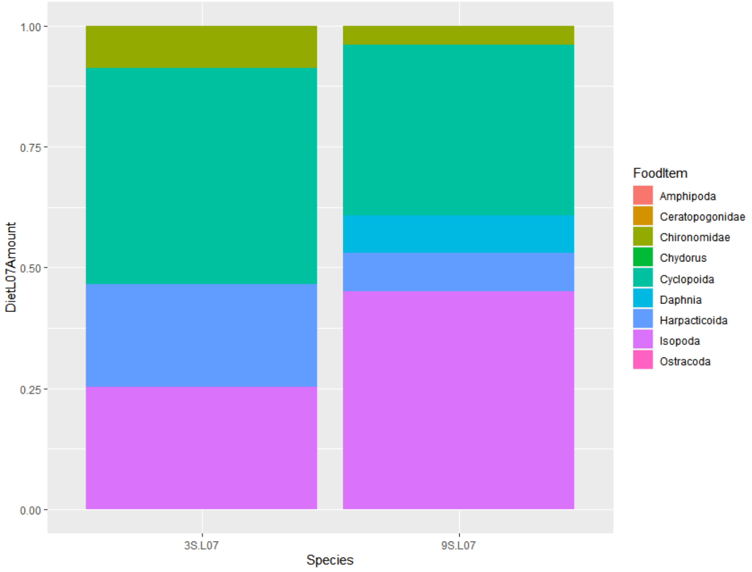
Daphnia
Harpacticoida
Isopoda
Ostracoda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Chironomidae
Chydorus
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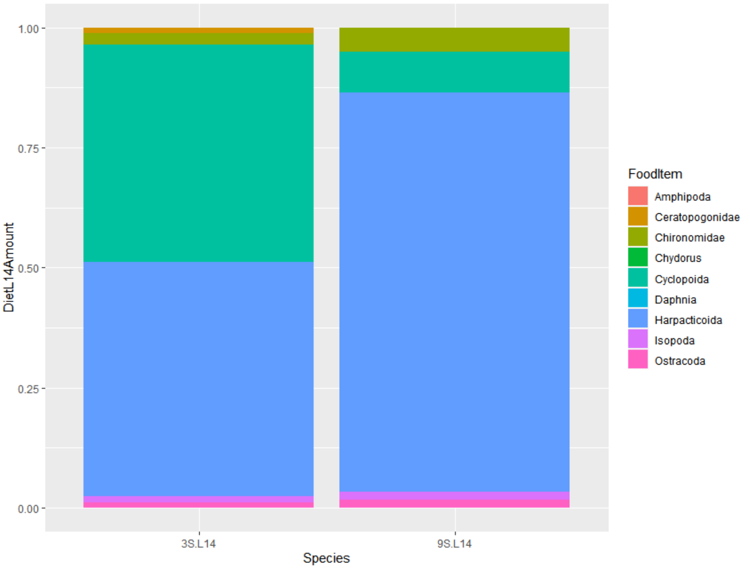
Daphnia
Harpacticoida
Isopoda
Ostracoda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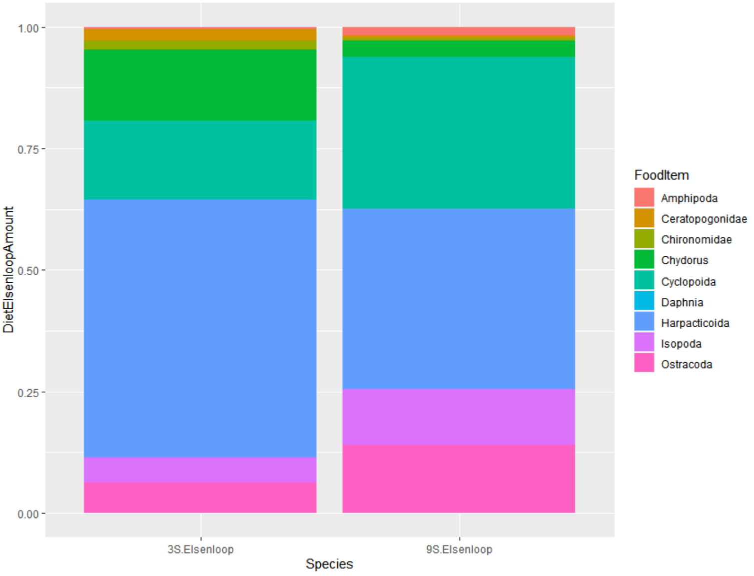
Harpacticoida
Isopoda
Ostracoda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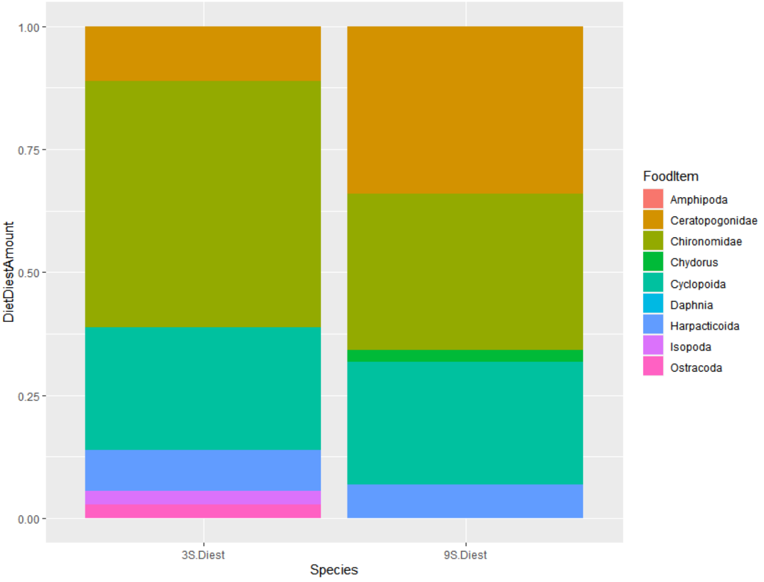
Ostracoda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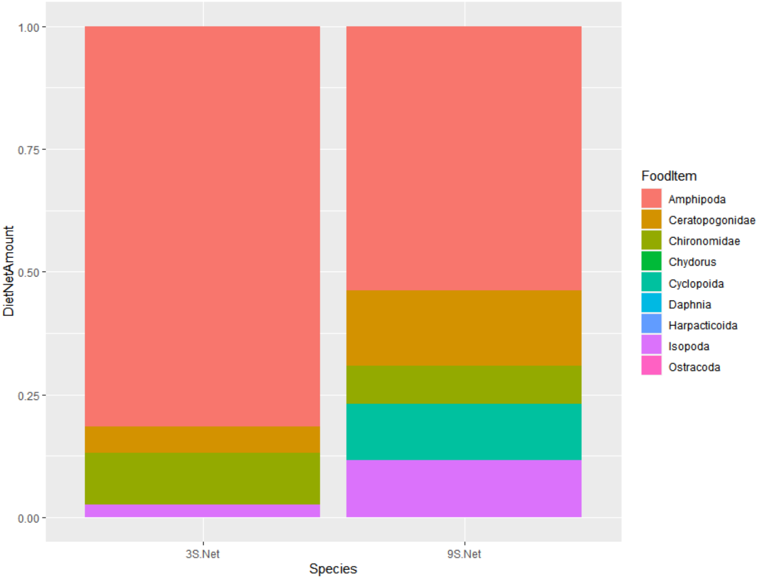
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Daphnia
Harpacticoida
Isopoda
Ostracoda

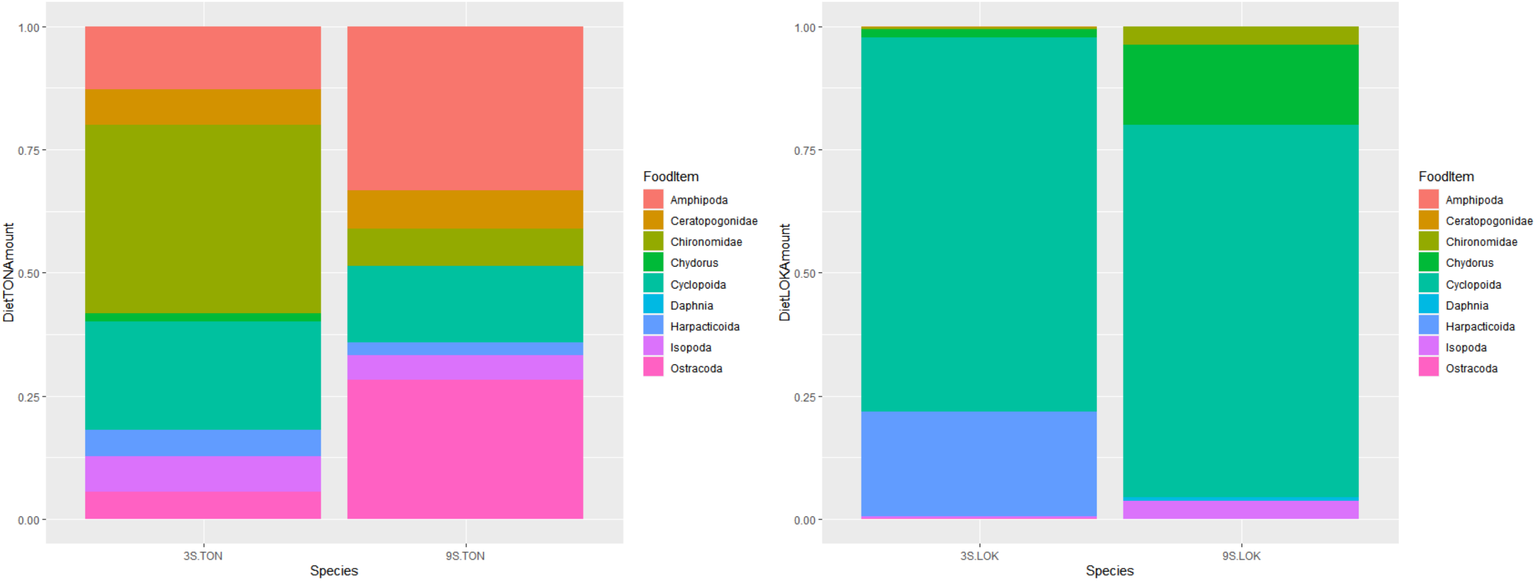

Figure 8. Overview of stomach content per species for each site: 10 graphs which visualise the selection of the most abundant prey items consumed by the three-spined stickleback and the nine-spined stickleback.

Stomach fullness

The results of the Pearson's Chi-squared test (Table S3) revealed that the distribution of stomach fullness over the three categories (“full”, “half full”, “empty”) did not differ between the two species among sites.

Niche overlap

With values frequently above 0.5, the Morisita index (Table 4), indicated that three-spined and nine-spined sticklebacks have high levels of niche overlap at most sites. One exception is L06, where the Morisita´s index of 0.43 indicates a lower overlap in the diet of both species.

Diversity

The mean Simpson diversity index of all sites was 0.43 for three-spined sticklebacks and 0.56 for nine-spined sticklebacks. The paired t-test revealed a significant difference in prey diversity between three-spined and nine-spined sticklebacks among sites ($P < 0.05$; t-value = -3.5072, df = 9). Overall, the three-spined stickleback thus seems to be more specialist than the nine-spined stickleback (see Table 4, and Figure 9).

The niche breadth of three-spined and nine-spined sticklebacks at site Diest, L02, and L07 was very similar. Three-spined and nine-spined sticklebacks at Diest and L07 tend to be generalists, while at L02 they tend to be specialists. At site LOK three-spined sticklebacks were clear specialists (Simpson´s index of 0.20), while nine-spined sticklebacks tended to be more generalists (Simpson´s index of 0.47). Similar results can be seen at site Net, TON, L06, and L01.

Table 4. Overview of the Simpson´s diversity (values 0 for specialists and values 1 for generalists.) for three-spined and nine-spined sticklebacks as well as the values of the Morisita´s index (values around 0 mean no overlap and values around 1 mean complete overlap) among sites.

Site	Simpson´s diversity index three-spined stickleback	Simpson´s diversity index nine-spined stickleback	Morisita´s diet overlap index
Diest	0.6081827	0.6777152	0.91580127
L06	0.3585484	0.5525527	0.43109778
L01	0.3982097	0.4631631	0.97766083
L02	0.4398963	0.4409982	0.95788699
L07	0.6041421	0.6113626	0.91346965
L14	0.5907007	0.7518421	0.78418926
LOK	0.195208	0.4705034	0.94320864
Elsenloop	0.4646369	0.559891	0.8867052
Net	0.2361111	0.5784439	0.91267549
TON	0.3965374	0.4524943	0.60900771

The Simpson´s diversity index for three-spined and nine-spined sticklebacks was correlated across sites (Pearson correlation r = 0.62; P = 0.05). No significant correlation was found between the Morisita´s index and the Simpson diversity index for three-spined (r = 0.06; P = 0.87) and the nine-spined (r = -0.03; P = 0.93) sticklebacks.

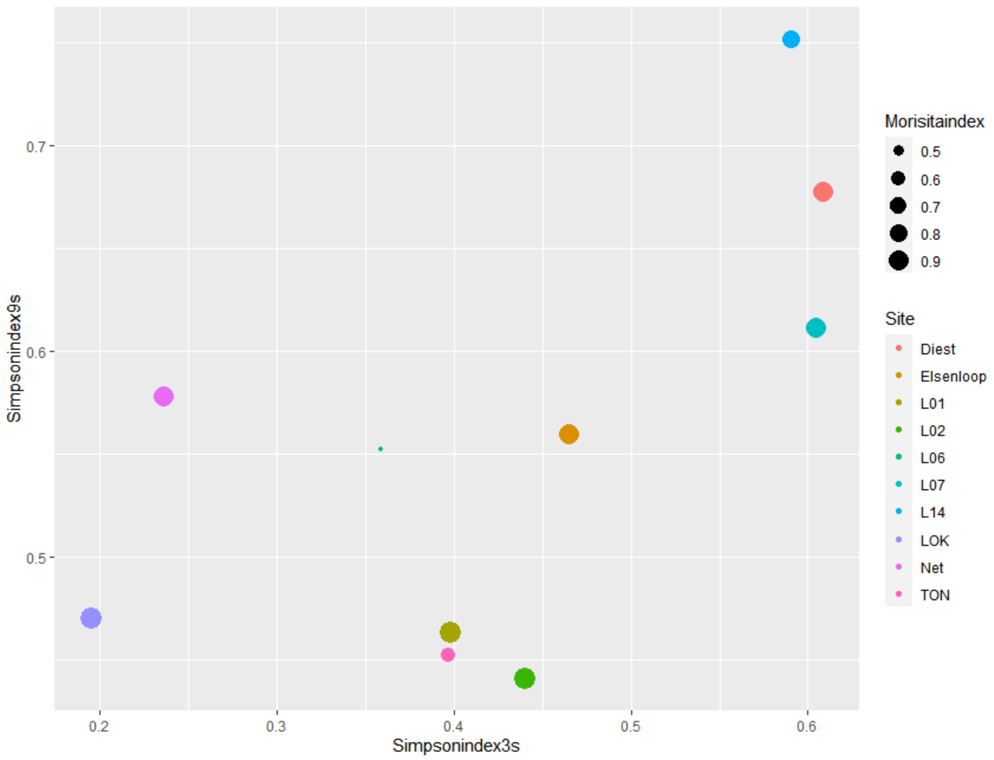

Figure 9. Bubble plot with the bubble size relative to the niche overlap of the three-spined and the nine-spined stickleback. As this analysis uses the Morisita´s index, values around 0 mean no overlap and values around 1 mean complete overlap. The Simpson´s index indicates the niche breadth of the three-spined and nine-spined stickleback, with values 0 for specialists and values 1 for generalists.

Proportion of limnetic prey

The analysis of the variances (ANOVA) revealed a significant difference of the proportion of limnetic prey (PLP) in three-spined (F-value = 13.42; df = 9; $P < 0.05$) and nine-spined stickleback diet among sites (F-value = 10.81; df = 9; $P < 0,05$). Three-spined sticklebacks tend to chase of more limnetic prey than the nine-spined stickleback (Figure 10 and11). The paired t-test of the mean PLP revealed that this difference was significant (t = -2.3311; df = 9; P = 0.04467). For instance, at L06 benthic amphipods and ostracods were proportionally more consumed by nine-spined sticklebacks. Similar patterns were observed for other sites including TON and L14. However, at site LOK three-spined sticklebacks proportionally consumed more benthic prey than nine-spined sticklebacks, where at Net both species consumed prey from both the benthic and the limnetic zone.

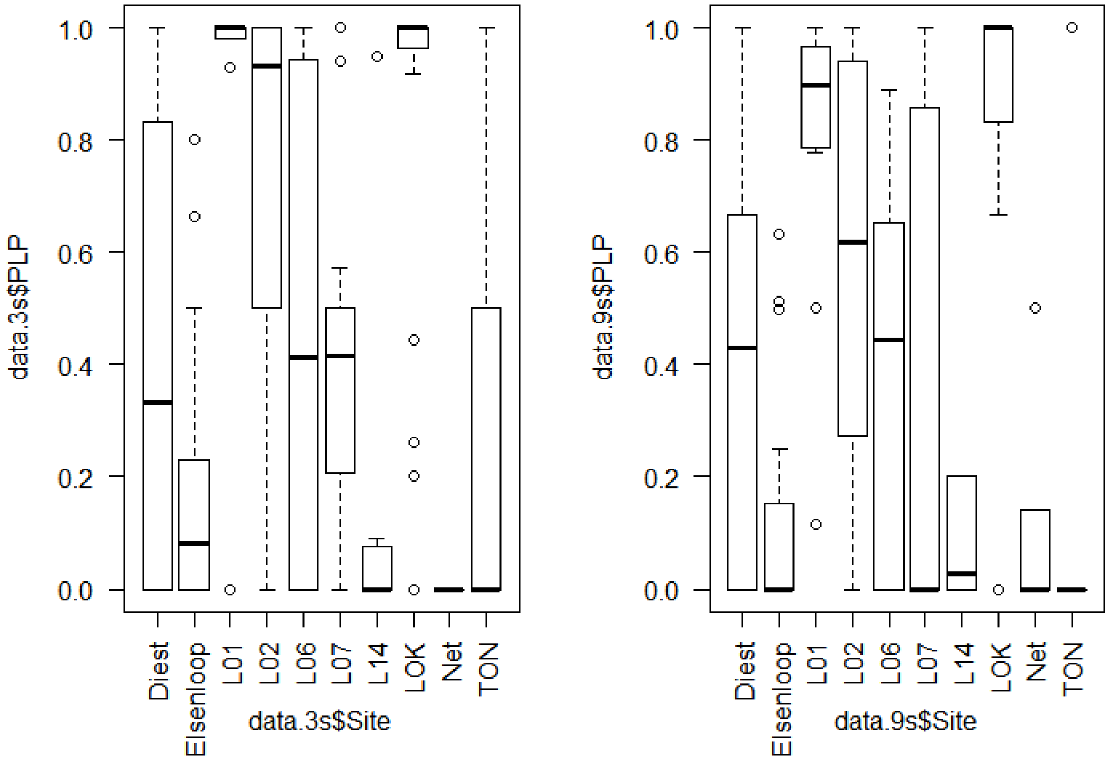

Figure 10. The median proportions of limnetic prey (PLP) show limnetic (close to one) and benthic (close to zero) patterns among sites. Three-spined sticklebacks generally show a slightly higher PLP than nine-spined stickleback.

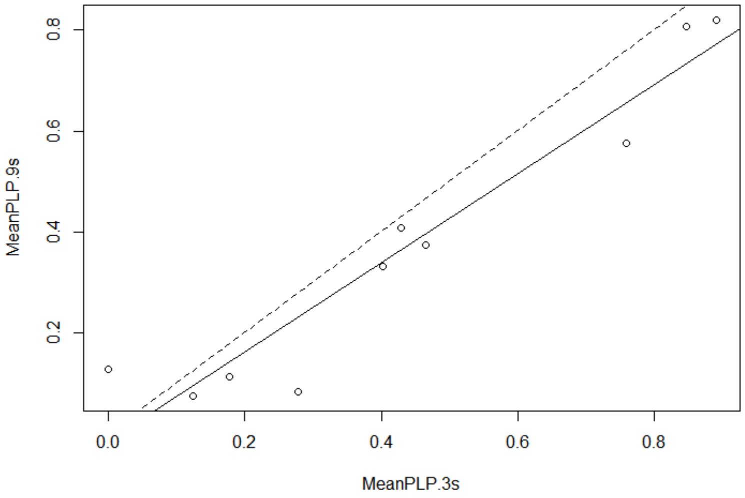

Figure 11. The 1:1 line (complete) and the regression line (dashed) show the relationship of the mean PLP of three-spined and nine-spined stickleback among all sites (dots in the graph). Seeing that more sites are under the 1:1 line, three-spined sticklebacks tend to chase more limnetic prey than nine-spined sticklebacks.

Classical multidimensional scaling (MDS)
MDS plots indicated grouping of the site´s prey community by comparing the benthic and limnetic diet of three-spined and nine-spined sticklebacks between sites (Figure 12.). Many sites, including Elsenloop, L07, Diest, TON, L02, L01, Net, L14, and LOK indicate grouping, while L06 seems to be far apart. This visualisation corresponds with the results of the niche overlap of this study, where three-spined and nine-spined sticklebacks at L06 seems to have less dietary overlap and all the other sites have high degrees of dietary overlap.

The MDS at the individual level revealed differences in niche breadth between the sites. For instance, the ellipse for three-spined sticklebacks at LOK appears to be very small, indicating a small niche breadth by comparing it to the generalist nine-spined sticklebacks at LOK where the ellipse appears to be bigger (Figure 13.). This corresponds with the Simpson´s diversity indices of 0,20 (small niche breadth) and 0,47 (bigger niche breadth) for three-spined and nine-spined sticklebacks, respectively.

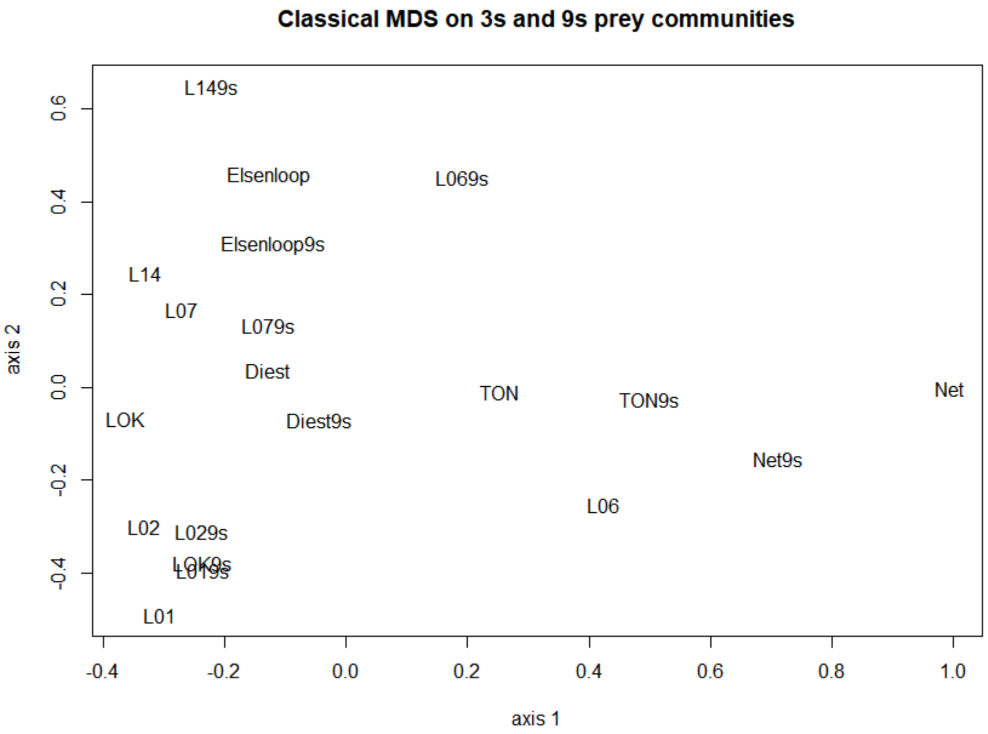

Figure 12. shows MDS plotting of the benthic and limnetic prey of three- and nine-spined stickleback populations at each site. Site labels indicate how dissimilar (or similar) the prey communities at each site are; sites close to each other have similar prey communities and sites far apart have dissimilar prey communities. Note that site labels without the term “9s” represent the prey community of three-spined sticklebacks (e.g., L02 versus L029s).

The mantel test revealed significant correlations between the prey communities of three- and nine-spined sticklebacks between sites (r = 0.49; P = 0.003).

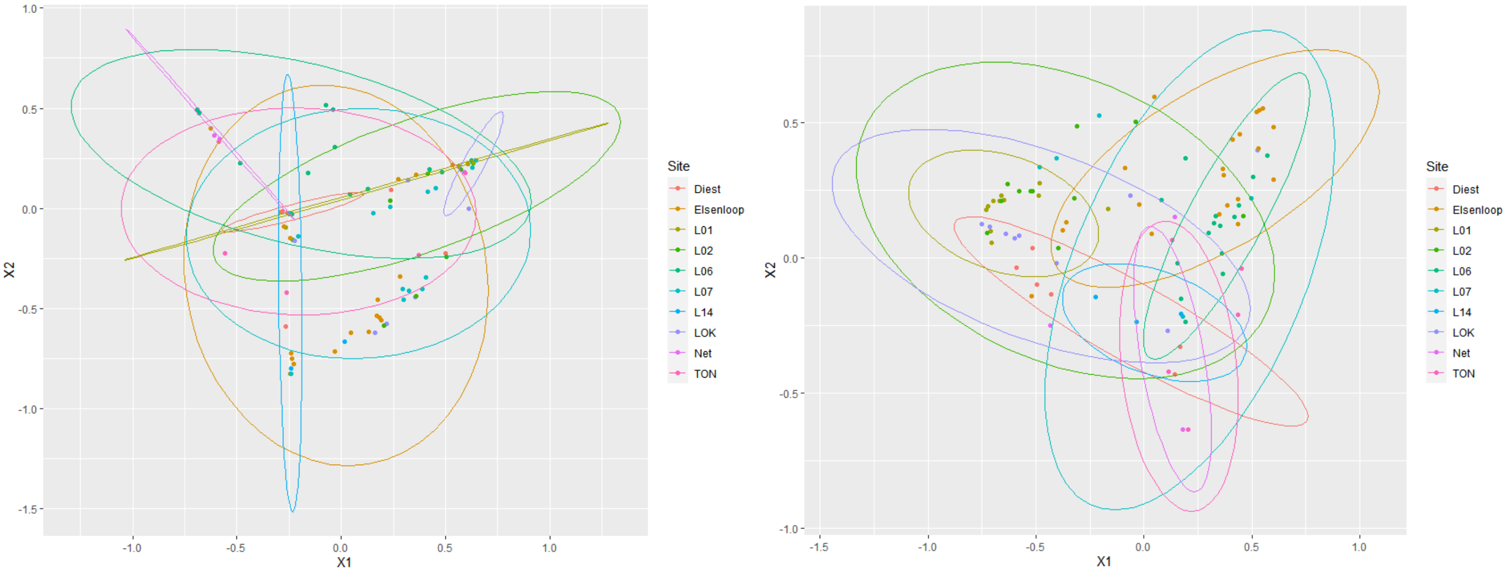

Figure 13. shows MDS plotting of the benthic and limnetic prey of three (left side)- and nine-spined (right side) stickleback populations at each site. Points indicate how dissimilar (or similar) the prey communities at the individual level are; points close to each other have similar stomach contents and points far apart have dissimilar stomach contents. The ellipses can be interpreted as the niche breadths of the stickleback populations, a small ellipse indicates specialists and a bigger ellipse indicates generalists.

Discussion

Overall, the diet of three-and nine-spined stickleback was similar. Nevertheless, by looking closer at the dietary results for each site, differences in composition and abundance can be observed. In particular, prey diversity was higher and more limnetic in nine-spined stickleback than in three-spined stickleback.

Sanchez-Hernandez *et al.* (2017) showed that food niche partitioning (assessed as dietary overlap) between Atlantic salmon (*Salmo salar*) and alpine bullhead (*Cottus poecilopus*) increases significantly with increasing prey diversity, our study revealed that high dietary overlaps correlate with generally low prey diversities (Simpson´s diversity index for three-spined sticklebacks 42,9% and for nine-spined sticklebacks 55,6%). Although the variables in the two studies vary, they both provide evidence for a correlation between prey diversity and dietary overlap.

Sympatric populations of three-spined and nine-spined sticklebacks occupying different microhabitats have been observed in many studies (Delbeek and Williams, 1987; Berner *et al*. 2008; Gow *et al*. 2008). Microhabitat segregation was found in this study too. Differences in the dietary composition can give insight into microhabitat preferences. We identified three- and nine-spined stickleback as being both benthic and limnetic feeders; however, nine-spined sticklebacks fed more from the benthos while three-spined sticklebacks preferred the limnetic zone to chase for prey.

Generalist species are characterised by having a wide dietary spectrum in their ecological community. Nevertheless, they can be outcompeted by other species which are more specialised to certain prey items that overlap with the diet of the generalists (Dukowska & Grzybkowska, 2014). For instance, Dukowska & Grzybkowska (2014) show that specialist percids consumed most of the chironomids (nonbiting midges), and that specialist three-spined stickleback consumed epiphytic back flies (*Simuliidae*) and fish eggs. In contrast, the generalist roach showed high intraspecific competition and low gut fullness coefficient values, which made them less competitive. However, in our findings this does not seem to be the case: The generalist nine-spined stickleback had a wide niche breadth in comparison with the specialist three-spined stickleback and did not seem to be outcompeted. We did not find interspecific competition between three-spined and nine-spined sticklebacks by comparing their stomach fullness. According to Hynes (1950), the reason for this could be the autumn season, which provides a relatively high abundance of potential prey availability, which again allows higher degrees of niche overlap.

Another study (De León *et al*. 2014) analysed the diet niches of sympatric ground finches coexisting on a single Galapagos Island. They revealed that morphological adaptation also depends on food limitations. Ground finches in their study were generalists that use overlapping diet resources in times of high food abundance, but when it comes to periods of food limitations, they retreated to resources to which they are best adapted. They are therefore "imperfect generalists". In our study similarities may be seen: At some sites (for instance Net, TON, Elsenloop, and L06) either one of the stickleback species consumed prey from both benthic and limnetic zones, which may indicate the previously mentioned potentially high food abundance in the autumn season (Hynes, 1950). Maitland & Campbell, 1992 described the morphological adaptation of three-spined and nine-spined sticklebacks to microhabitats, which could enable them to specialise for certain food items of the benthic or limnetic zone in times of food limitations. The hypothesis of species coexistence with substantial diet overlap may rest on temporal variation in niches (De León *et al*. 2014). Further studies over a long time period including seasonal data are of importance to better understand the coexistence of the two stickleback species.

Scientists developed several approaches to conduct stomach content analysis that best suits their research questions. However, there is no standardised methodology for stomach content analysis (Amundsen & Sanchez-Hernandez, 2019). A recommendation for a standardisation of methods is given by Amundsen & Sanchez-Hernandez (2019), where they suggest a combination of relative-fullness and presence-absence methods.

Kick sampling (presence absence method) is a frequent method to gain a better understanding of the prey abundance at a certain aquatic location. In order to do this, the observer uses a fine mashed hand net to take samples of the bottom of the waterbody. The resulting mud, including potential prey items, is stored in 4% formalin for later analysis. This method could give an additional insight into the potential prey and therefore into the niche breadth of the species of interest. Kick samples are available for our study but could not yet be analysed due the covid-19 pandemic.

Delbeek & Williams’ (1987) study about the food resource partitioning between sympatric stickleback populations used the Petraitis (1979) likelihood measurement. It measures the likelihood that the observed proportional resource usage (by the fish species of interest) is the same as the proportions of the prey in the environment. This method would have allowed us to better distinguish between resource availability and resource use, and thus provide better insight into how much the two species avoid niche overlap.

Additionally, Amundsen & Sanchez-Hernandez (2019) suggest the gravimetric method for the quantification of food consumption rates, and the numerical method for prey selection studies. In the current study, only the numerical method was conducted to verify the counting of all prey items present in the sampled stomachs (Hynes, 1950). Relatively easily identifiable body parts of the prey, such as insect heads, insect legs, shells, and carapaces (e.g., amphipods were barely digested) made the numerical method sufficiently feasible for this study. Nevertheless, one disadvantage of the method can be that it produces meaningless outcomes when the prey types show highly different body sizes (zooplankton versus large invertebrates), as such situations overemphasise small prey items taken in large numbers (Hyslop, 1980). However, a combination of three or more methods can increase the accuracy of the study (Amundsen & Sanchez-Hernandez, 2019; Berg, 1979).

According to other studies (Hynes, 1950; Dukowska & Grzybkowska, 2014, Delbeek & Williams, 1987), three-spined and nine-spined sticklebacks preferably feed on copepods, chironomids, and Cladocera, as also confirmed in this study. However, Dukowska & Grzybkowska (2014) analysed the food niche partitioning between small-sized percids, cyprinids, and three-spined sticklebacks in a large lowland river. They revealed that the three-spined stickleback mainly consumed epiphytic simuliids and other prey such as fish eggs, which resulted in food niche segregation with the other fish species. This could be an indicator for the dietary adaption of the three-spined stickleback to certain fish communities: Percids dominated most of the chironomids as prey, which could have been prey for the three-spined stickleback. Instead, the three-spined stickleback specialised on simuliids and fish eggs. Nevertheless, in this study, three-and nine-spined sticklebacks had a similar dietary spectrum, which could indicate on the one hand the seasonal variation of potential prey availability (including copepods and chironomids) (Hynes, 1950), and on the other hand no major food competitors (Dukowska & Grzybkowska, 2014).

One limitation of this study was the lack of categorisation of the chironomid prey into pupae and larvae. Usually, chironomid larvae inhabit the benthic zone, and chironomid pupae the limnetic zone. Larvae are extremely important prey for many species in aquatic food chains, which is also reflected in this study (chironomids were abundant in all sites) (Thorp & Covich, 2001). Unfortunately, the classification into limnetic pupae or benthic larvae was overlooked for this study, while it could have provided better resolution to compare the foraging space of both stickleback species.

Another limitation is the stomach content analysis itself. It represents the food in a particular stomach, which can be useful for research objectives related to dietary composition and prey analysis (Amundsen & Sanchez-Hernandez, 2019). However, if the food web of an ecosystem is the topic of interest, a combination of the dietary analysis and the stable isotope analysis could be useful, as demonstrated in Andrade *et al*. (2018), where they were able to identify the trophic position in the food web of three herbivorous serrasalmid species by comparing it with the dietary composition. The stable isotope analysis of the muscle tissue reflects an assimilation of the dietary intake over a timescale of several weeks to months, whereas the diet analysis represents only a snapshot of food items consumed by the fish in the hours before killing them (Vander Zanden *et al*. 2015). Nevertheless, combining those two analyses could reveal more information about the trophic relations among fish communities over a longer period.

A sample size of approximately twenty stomachs per species (three-spined and nine-spined sticklebacks) was a sufficient representation of the diet (Delbeek & Williams, 1987). This means that we sampled approximately 40 fish per site in total (twenty three-spined and twenty nine-spined sticklebacks), which resulted in a total sample size of approximately 400 fish for ten sites.

Conclusion

Prey items in three-spined stickleback were less diverse and more limnetic than prey items in nine-spined stickleback, but overall, the diet of both species were similar. High niche overlaps are not associated with the niche partitioning hypothesis in this study, but there are other factors which could allow the coexistence of three-spined and nine-spined sticklebacks, including prey abundance and its seasonal availability. Additionally, both stickleback species tended to occupy different microhabitats (three-spined sticklebacks consumed more limnetic prey than nine-spined sticklebacks).

To conclude, three-spined and nine-spined sticklebacks have the capacity to adapt to changing environments, which could allow the coexistence and reduce the interspecific competition. Evidence for the dietary composition of sticklebacks has solidified our understanding of the coexistence of these aquatic species, as well as for the fact that natural selection can drive adaption to alternative environments (Gow *et al.* 2008).

References

Amundsen, P. A., & Sanchez-Hernandez, J. (2019, Dec). Feeding studies take guts - critical review and recommendations of methods for stomach contents analysis in fish. J Fish Biol, 95(6), 1364-1373. https://doi.org/10.1111/jfb.14151

Andrade, M. C., Fitzgerald, D. B., Winemiller, K. O., Barbosa, P. S., & Giarrizzo, T. (2018). Trophic niche segregation among herbivorous serrasalmids from rapids of the lower Xingu River, Brazilian Amazon. Hydrobiologia, 829(1), 265-280. https://doi.org/10.1007/s10750-018-3838-y

Animal Ecology. (2001). SciTech Book News, 25(3), 67. Ringgold, Inc.

AquaMaps (2019, October). Computer generated distribution maps for *Pungitius pungitius* (Ninespine stickleback), with modelled year 2050 native range map based on IPCC RCP8.5 emissions scenario. Retrieved from https://www.aquamaps.org.

AquaMaps (2019, October). Computer generated distribution maps for *Gasterosteus aculeatus* (Three-spined stickleback), with modelled year 2050 native range map based on IPCC RCP8.5 emissions scenario. Retrieved from https://www.aquamaps.org.

Baker, Ronald, Buckland, Amanda, & Sheaves, Marcus. (2014). Fish gut content analysis: robust measures of diet composition. Fish and Fisheries (Oxford, England), 15(1), 170–177. https://doi.org/10.1111/faf.12026

Bal TMP, Llanos-Garrido A, Chaturvedi A, Verdonck I, Hellemans B & Raeymaekers JAM (2021). Adaptive divergence under gene flow along an environmental gradient in two coexisting stickleback species. Genes 2021, 12, 435. https://doi.org/10.3390/genes12030435

Barrows, A. P. W., Cathey, S. E., & Petersen, C. W. (2018). Marine environment microfiber contamination: Global patterns and the diversity of microparticle origins. Environmental Pollution, 237, 275-284. https://doi.org/10.1016/j.envpol.2018.02.062

Berg, J. (1979). Discussion of methods of investigating the food of fishes, with reference to a preliminary study of the prey of Gobiusculus flavescens (Gobiidae). Marine biology, 50(3), 263-273. https://doi.org/10.1007/BF00394208

Berner, D., Adams, D. C., Grandchamp, A. C., & Hendry, A. P. (2008, Nov). Natural selection drives patterns of lake-stream divergence in stickleback foraging morphology. J Evol Biol, 21(6), 1653-1665. https://doi.org/10.1111/j.1420-9101.2008.01583.x

Boughman, J.W. (2001). Divergent sexual selection enhances reproductive isolation in sticklebacks. Nature 411: 944–948.

Brown, W. L, & Wilson, E. O. (1956). Character Displacement. Systematic Zoology, 5(2), 49–64. https://doi.org/10.2307/2411924

Colosimo PF, Hosemann KE, Balabhadra S *et al.* (2005) Widespread parallel evolution in sticklebacks by repeated fixation of Ectodysplasin alleles. Science, 307, 1928–1933.

Cresko WA, Amores A, Wilson C *et al.* (2004) Parallel genetic basis for repeated evolution of armor loss in Alaskan threespine stickleback populations. Proceedings of the National Academy of Sciences, USA, 101, 6050–6055.

Dawson, T. E., & Siegwolf, R. T. W. (2007). Stable isotopes as indicators of ecological change (1st ed.). Academic Press.

De León, L. F., Podos, J., Gardezi, T., Herrel, A., & Hendry, A. P. (2014). Darwin's finches and their diet niches: the sympatric coexistence of imperfect generalists. Journal of evolutionary biology, 27(6), 1093–1104. https://doi.org/10.1111/jeb.12383

Delbeek, J. C., & Williams, D. D. (1987). Food Resource Partitioning Between Sympatric Populations of Brackishwater Sticklebacks. The Journal of animal ecology, 56(3), 949-967. https://doi.org/10.2307/4959

Dukowska, M., & Grzybkowska, M. (2014). Coexistence of fish species in a large lowland river: food niche partitioning between small-sized percids, cyprinids and sticklebacks in submersed macrophytes. PLoS One, 9(11), e109927. https://doi.org/10.1371/journal.pone.0109927

Friedrich, T., Reinartz, R., & Gessner, J. (2019). Sturgeon re-introduction in the Upper and Middle Danube River Basin. Journal of Applied Ichthyology, 35(5), 1059-1068. https://doi.org/10.1111/jai.13966

Gallai, N., Salles, J.-M., Settele, J., & Vaissière, B. E. (2009). Economic valuation of the vulnerability of world agriculture confronted with pollinator decline. Ecological economics, 68(3), 810-821. https://doi.org/10.1016/j.ecolecon.2008.06.014 (Ecological Economics)

Gow, J. L., Rogers, S. M., Jackson, M., & Schluter, D. (2008). Ecological predictions lead to the discovery of a benthic–limnetic sympatric species pair of threespine stickleback in Little Quarry Lake, British Columbia. Canadian Journal of Zoology, 86(6), 564-571. https://doi.org/10.1139/z08-032

Griffin, J. N., Silliman, B. R. (2011). Resource Partitioning and Why It Matters. Nature Education. https://www.nature.com/scitable/knowledge/library/resource-partitioning-and-why-it-matters-17362658/

Hardin, G. (1960, April 29). The Competitive Exclusion Principle

Hart, P. J. B. (2003). Habitat Use and Feeding Behaviour in Two Closely Related Fish Species, the Three-Spined and Nine-Spined Stickleback: An Experimental Analysis. The Journal of animal ecology, 72(5), 777-783. https://doi.org/10.1046/j.1365-2656.2003.00747.x

Hui, D. (2012). Food Web: Concept and Applications. Nature Education https://www.nature.com/scitable/knowledge/library/food-web-concept-and-applications-84077181/

Hynes, H. B. N. (1950). The Food of Fresh-Water Sticklebacks (Gasterosteus aculeatus and Pygosteus pungitius), with a Review of Methods Used in Studies of the Food of Fishes. The Journal of animal ecology, 19(1), 36-58. https://doi.org/10.2307/1570

Hyslop, E. (1980). Stomach contents analysis- a review of methods and their application. Journal of Fish Biology, 17, 411-429.

Jari Oksanen, F. Guillaume Blanchet, Michael Friendly, Roeland Kindt, Pierre Legendre, Dan McGlinn, Peter R. Minchin, R. B. O'Hara, Gavin L. Simpson, Peter Solymos, M. Henry H. Stevens, Eduard Szoecs and Helene Wagner (2020). vegan: Community Ecology Package. R package version 2.5-7. https://CRAN.R-project.org/package=vegan

Jilg, K. (2021). Artfakta, SLU Artdatabanken. SLU. https://artfakta.se/artbestamning/taxon/gasterosteoidei-6000062/galleri?categories=helbild&categories=helbild%5Ck%C3%B6nen%20lika&categories=helbild%5Chane&categories=helbild%5Chona&src=1&class=11

Keast, A. (1977). Diet overlaps and feeding relationships between the year classes in the yellow perch (Perca flavescens). Environmental biology of fishes, 2(1), 53-70. https://doi.org/10.1007/BF00001416

Kohl, N., Malmsten., I. (2021). The parasites of two stickleback species (*Gasterosteus aculeatus* and *Pungitius pungitius*) and their relationship to salinity and other environmental variables. [Bachelor´s Thesis Nord University]. Norway.

Konijnendijk N, Raeymaekers JAM, Vandeuren S, Jacquemin L & Volckaert FAM (2013). Testing for local adaptation in the *Gasterosteus–Gyrodactylus* host-parasite system. Evolutionary Ecology Research 15, 489–502.

Kroken, K. K., Saethre, A. A., Nicolaisen, O., Egeland, T. B., & Nordeide, J. T. (2021, Aug). Carotenoids-based reddish pelvic spines in nonreproducing female and male sticklebacks (Gasterosteus aculeatus) - Signalling social dominance? Ecol Evol, 11(16), 11038-11050. https://doi.org/10.1002/ece3.7892

Lord, C. M., Wirebach, K. P., Tompkins, J., Bradshaw-Wilson, C., & Shaffer, C. L. (2020). Reintroduction of the European bison (*Bison bonasus*) in central-eastern Europe: a case study. International journal of geographical information science: IJGIS, 34(8), 1628-1647. https://doi.org/10.1080/13658816.2019.1672876

Louwagie, G., Gay, S. H., Sammeth, F., & Ratinger, T. (2011). The potential of European Union policies to address soil degradation in agriculture. Land Degrad. Dev, 22(1), 5-17. https://doi.org/10.1002/ldr.1028

MacArthur, Robert H. (1958). Population Ecology of Some Warblers of Northeastern Coniferous Forests. Ecology (Durham), 39(4), 599–619. https://doi.org/10.2307/1931600

Maitland, P. S., Campbell, R.N. (1992). Freshwater Fishes. Harper Collins.

Marine Fisheries – Building Mass Balance Trophic and Simulation Models (pp. 148-158). Winter School on Ecosystem Based Management of Marine Fisheries.

McKinnon, J. S., & Rundle, H. D. (2002). Speciation in nature: the threespine stickleback model systems. Trends in Ecology & Evolution, 17(10), 480-488. https://doi.org/10.1016/S0169-5347(02)02579-X

Nielsen, U. N. (2014). Community Ecology. Nature Education https://www.nature.com/scitable/knowledge/community-ecology-13228209/

O'Dwyer, K., Kamiya, T., & Poulin, R. (2014). Altered microhabitat use and movement of littorinid gastropods: the effects of parasites. Marine biology, 161(2), 437-445. https://doi.org/10.1007/s00227-013-2349-7

Peterson, A. T. (2011). Ecological niches and geographic distributions: Vol. no. 49 (Course Book.). Princeton University Press.

Pyke, G. H. (1982). Local geographic distributions of bumblebees near Crested Butte, Colorado: competition and community structure. Ecology 63, 555–573.

R Core Team (2020). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL https://www.R-project.org/.

Raeymaekers JAM, Boisjoly M, Delaire L, Berner D, Räsänen K & Hendry AP (2010). Testing for mating isolation between ecotypes: laboratory experiments with lake, stream and hybrid stickleback. Journal of Evolutionary Biology 23, 2694-2708.

Raeymaekers JAM, Raeymaekers D, Koizumi I, Geldof S & Volckaert FAM (2009). Guidelines for restoring connectivity around water mills: a population genetic approach to the management of riverine fish. Journal of Applied Ecology 46, 562-571.

Raeymaekers JAM, Wegner KM, Huyse T & Volckaert FAM (2011). Infection dynamics of the monogenean parasite *Gyrodactylus gasterostei* on sympatric and allopatric populations of the three-spined stickleback *Gasterosteus aculeatus*. Folia Parasitologica 58, 27-34.

Raeymaekers, JAM., Chaturvedi, A., Hablutzel, P. I., Verdonck, I., Hellemans, B., Maes, G. E., De Meester, L., & Volckaert, F. A. M. (2017, Aug 16). Adaptive and non-adaptive divergence in a common landscape. Nat Commun, 8(1), 267. https://doi.org/10.1038/s41467-017-00256-6

Raeymaekers, JAM., Konijnendijk, N., Larmuseau, M. H., Hellemans, B., De Meester, L., & Volckaert, F. A. (2014, Jan). A gene with major phenotypic effects as a target for selection vs. homogenizing gene flow. Mol Ecol, 23(1), 162-181. https://doi.org/10.1111/mec.12582

Ripple, W. J., & Beschta, R. L. (2004). Wolves and the Ecology of Fear: Can Predation Risk Structure Ecosystems? BioScience, 54(8), 755-766. https://doi.org/10.1641/0006-3568(2004)054[0755:WATEOF]2.0.CO

Sanchez-Hernandez, J., Gabler, H. M., & Amundsen, P. A. (2017, Apr). Prey diversity as a driver of resource partitioning between river-dwelling fish species. Ecology and Evolution, 7(7), 2058-2068. https://doi.org/10.1002/ece3.2793

Schluter, D., & McPhail, J. D. (1992). Ecological Character Displacement and Speciation in Sticklebacks. Am Nat, 140(1), 85-108. https://doi.org/10.1086/285404

Thorp, J. H., Covich, A. P., & Thorpe, J. H. (2001). Ecology and Classification of North American Freshwater Invertebrates. San Diego: Elsevier Science & Technology.

Van de Meutter, F., Stoks, R., & De Meester, L. (2005). The effect of turbidity state and microhabitat on macroinvertebrate assemblages: a pilot study of six shallow lakes. In Aquatic Biodiversity II (pp. 379-390). https://doi.org/10.1007/1-4020-4111-x_32

Vander Zanden, M. J., Clayton, M. K., Moody, E. K., Solomon, C. T., & Weidel, B. C. (2015). Stable isotope turnover and half-life in animal tissues: A literature synthesis. PLoS One, 10(1), e0116182-e0116182. https://doi.org/10.1371/journal.pone.0116182

Violita, V., Triadiati, T., Anas, I., & Miftahudin, M. (2016). Fine Root Production and Decomposition in Lowland Rainforest and Oil Palm Plantations in Sumatra, Indonesia. Hayati: journal of biosciences, 23(1), 7-12. https://doi.org/10.1016/j.hjb.2015.10.008

Zacharia, P. U., & Abdurahiman, K. P. (2004). METHODS OF STOMACH CONTENT ANALYSIS OF FISHES. In K. S. Mohamed (Ed.), Towards Ecosystem Based Management of Marine Fisheries – Building Mass Balance Trophic and Simulation Models (pp. 148-158). Winter School on Ecosystem Based Management of Marine Fisheries.

Appendix

Diet proportions

Table S1. shows the total numbers of consumed prey items by three-spined and nine-spined sticklebacks.

Species	Cyclopoida	Harpacticoida	Ostracoda
3s	2010	844	91
9s	816	362	221

	Cladocera.unknown	Daphnia	Chironomidae.pupa.larvae
3s	1	38	137
9s	0	79	112

	Diptera.pupa.larvae	Diptera.fly	Diptera.mosquito
3s	0	0	0
9s	0	0	0

	Amphipoda	Isopoda	Nematoda
3s	122	88	6
9s	40	174	10

	Leydigia	Chydorus	Odonata
3s	0	204	5
9s	0	73	0

	Trichoptera.larvae	Colembola	Acari
3s	2	1	3
9s	7	0	3

	Coleoptera.larvae.other	Ephemenoptera.larvae	Diptera.other
3s	5	8	0
9s	8	23	0

	Nauplius.larvae	Earthworm	Snails
3s	0	0	84
9s	1	0	77

	Alona	Ceratopogonidae.larvae
3s	0	52
9s	1	44

Stomach fullness

Table S2. numbers of the stomach status of three-spined (3s) and nine-spined (9s) sticklebacks among sites. Stomach fullness is categorised as “full”, “half full”, and “empty”.

Site	Species	full	half full	emty
Diest	3s	3	15	2
	9s	4	11	5
L01	3s	3	16	1
	9s	4	12	4
L02	3s	1	17	2
	9s	5	13	2
L06	3s	11	10	0
	9s	11	9	1
L07	3s	1	23	4
	9s	4	13	3
L14	3s	2	14	4
	9s	0	9	10
LOK	3s	2	19	1
	9s	3	14	3
TON	3s	5	13	0
	9s	4	12	3
Net	3s	4	14	1
	9s	3	14	3
Elsenloop	3s	8	15	1
	9s	15	7	1

Table S3. shows the results of the Pearson's Chi-squared test. The distribution of stomachs over the 3 categories (“full”, “half full”, and “empty”) do not differ between the two species.

Site	X-squared	df	p-value
Diest	2.044	2	0.3599
L01	2.5143	2	0.2845
L02	3.2	2	0.2019
L06	1.0526	2	0.5908
L07	3.4841	2	0.1752
L14	5.6364	2	0.05971
LOK	1.8666	2	0.3933
TON	3.1264	2	0.2095
Net	1.118	2	0.5718
Elsenloop	5.0205	2	0.08125

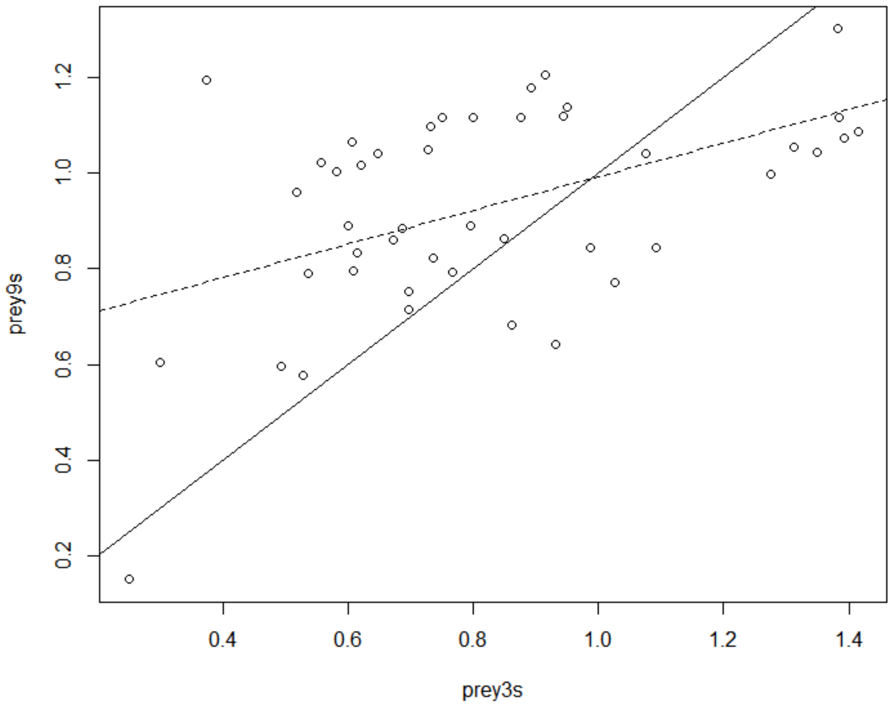

Figure S1. The scatter plot shows the mantel test which reveals significant correlations between the prey communities of three- and nine-spined sticklebacks between sites (r = 0.49; p = 0.003).