Trophic niche partitioning in communities of African annual fish: evidence from stable isotopes

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Abstract Annual killifish of the genus *Nothobranchius* often co-occur in temporary savannah pools. Their space- and time-limited environment does not allow for any substantial habitat or temporal segregation. Coexisting species are therefore predicted to have well separated trophic niches to avoid intense food competition. Although in a previous "snapshot" study using stomach content analysis (SCA), the trophic niches of three sympatric species (*N. furzeri*, *N. orthonotus*, and *N. pienaari*) were found to vary among species, the difference was relatively weak and inconsistent across different sites. Here, we used the time-integrative capacity of stable isotope analysis to test whether the trophic niches of sympatric Mozambican *Nothobranchius* are more distinct over a long-term period. Analysis of carbon and nitrogen stable isotopes separated the trophic niche and trophic position of *N. pienaari* but failed to find any difference between *N. furzeri/N. kadleci* and *N. orthonotus*. No segregation was found at the sites with low prey diversity. In contrast, SCA identified *N. orthonotus* as the species with the most distinct trophic niche. We discuss the effect of prey diversity and different sensitivities of stomach content and stable isotope analysis in general and conclude that the trophic niches of the three sympatric *Nothobranchius* species are well separated.

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Introduction

Annual fishes of the genus *Nothobranchius* are a fascinating group of fish inhabiting spatially limited, temporary savannah pools of east Africa. Although the genus is species rich, with ca. 70 species currently recognised, species typically show only minor morphological differences and their taxonomy is mostly based on extremely diverse male colouration (Wildekamp, 2004). *Nothobranchius* have a life cycle that is unusual in many ways: they survive the dry season as dormant eggs buried in the dry substratum, but the eggs simultaneously hatch following the onset of the annual rains. The larvae grow rapidly, mature and produce eggs that will populate the pool in the

following rainy season (Wildekamp, 2004, Polačik et al., 2011).

Several killifish species often coexist in a single pool. The coexistence of South American annual killifish is often characterised by mutual predator–prey relationships (Laufer et al., 2009). In contrast, sympatric African *Nothobranchius* are characterised by only marginal differences in size and shape (Wildekamp, 2004) and they do not use different microhabitats within the pool (Reichard et al., 2009). The competitive exclusion principle (Gause, 1934) predicts that such circumstances should lead to particularly well separated trophic niches of the sympatric species.

In a previous study (Polačik & Reichard, 2010) using stomach content analysis (SCA), extensive qualitative trophic niche overlap among three Mozambican *Nothobranchius* species (*Nothobranchius furzeri* Jubb, 1971; *Nothobranchius orthonotus* Peters, 1844; *Nothobranchius pienaari* Shidlovskiy, Watters & Wildekamp, 2010 under the name *N. rachovii*) was demonstrated. Quantitative analysis using relative proportions of ingested prey through SCA allowed for consistent statistical support of interspecific trophic niche separation across sites in one species (*Nothobranchius orthonotus* Peters, 1844). The trophic niches of the two remaining species showed clear separation, only when the diversity of available prey was high.

An important drawback of such "snapshot" studies is that they only reveal short-term patterns in prey consumption (Bearhop et al., 2004). The temporary pools inhabited by *Nothobranchius* are by definition naturally highly dynamic ecosystems, going through a series of ecological stages from their initial filling with water, through to their eventual desiccation. Each stage is normally characterised by stage-specific invertebrate fauna (Allan et al., 1995; Meintjes, 1996), giving rise to seasonal variation in prey abundance/occurrence. The level of trophic niche segregation between sympatric species may fluctuate considerably over time, reflecting seasonal abundance of particular prey. For example, Wilson et al. (2010) showed that although two sympatric snake species appeared on average to have very similar isotopic compositions, their trophic niches were actually distinct, due to seasonal differences in prey availability, as revealed by sampling in the respective seasons.

To follow up on our earlier snapshot study, which found only minor trophic niche segregation especially between the sympatric *N. furzeri* and *N. pienaari* (Polačik & Reichard, 2010), we applied analysis of stable isotope ratios (SIA) of carbon (δ^{13}C) and nitrogen (δ^{15}N) to test whether the potentially different trophic niches of three sympatric *Nothobranchius* species are reflected in their isotopic compositions. Consumer stable isotope values are closely associated with that of their long-term, time integrated diet, providing a longer-term indication of assimilated diet compared to SCA. We hypothesised that over a long-term period there will be clear statistical separation in the trophic niches of the sympatric species, here defined as the centroid of δ^{15}N–δ^{13}C isotope space.

Materials and methods

Samples for SIA were collected in Gaza and Inhambane provinces, southern Mozambique (Fig. 1). Three sites with sympatric occurrence of three *Nothobranchius* species were sampled (Table 1) to obtain samples for SIA in February and March 2011. Site 1 (22°33.28′S 32°43.64′E) and site 3 (21°52.41′S 32°48.04′E) were situated in the Chefu River basin and site 2 (21°14.71′S 34°42.65′E) was located in the Save River basin. Two species, *N. orthonotus* and *N. pienaari* occurred at all three sites (Table 2). The third species *N. furzeri* was replaced by its geographical vicariant *Nothobranchius kadleci* Reichard, 2010 at site 2. *Nothobranchius kadleci* was recently described as a new species (based on colouration and allopatric occurrence), but has a similar size, morphology and ecology to *N. furzeri* (Reichard, 2010; Polačik et al., 2011). Due to their general similarity, these two sister species (Reichard, 2010; Dorn et al., 2011) were regarded here as a single ecological unit and pooled in analyses (see below).

Fish were collected using a triangular dip-net and seine net. As all sampled sites were shallow and small (Table 1), kick sampling by a rectangular hand net (Frost, 1971) likely provided a representative sample of prey diversity in the pools. Within each pool, all available habitat types (e.g., open water, aquatic vegetation, shallows and relatively deeper parts) were sampled for 3 min each. Details on sampling sites for habitats and fish are shown in Tables 1 and 2, respectively. After sampling, both the collected fish and the entire contents of the invertebrate hand net were fixed in 70% ethanol (freezing was not possible due to logistic reasons).

Fig. 1 *Map* of southern Mozambique showing the river network and important settlements. Sample sites (1–3) are indicated by *triangles*. Note that many rivers are temporary and may not form flowing sections every rainy season. International borders are indicated (*dotted lines*). Named rivers are shown in *italics*
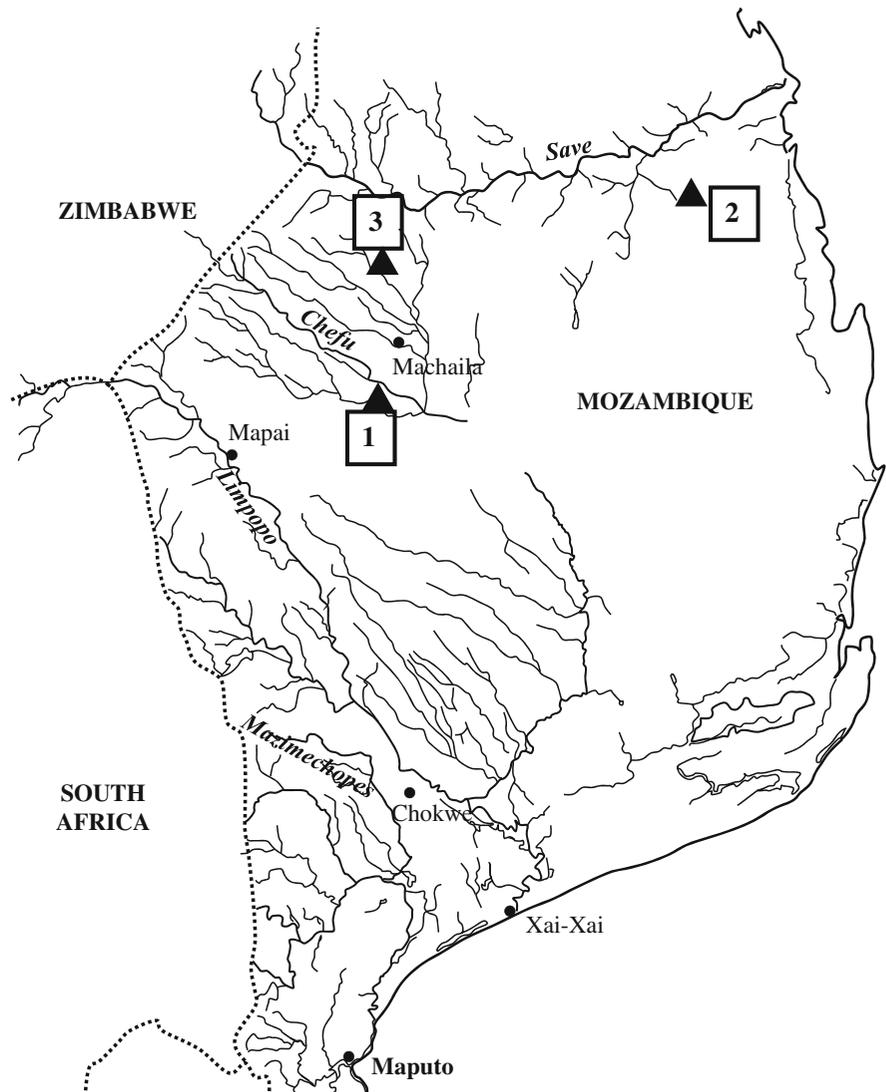

Table 1 Characteristics of the study sites and *Nothobranchius* community

Site	Habitat area (m^2)	Max. depth (cm)	Aquatic vegetation	*Nothobranchius* density (dip. fish^{-1})	Species ratio	N
1	600	30	None	0.2	70:23:8	88
2	480	40	Dense	0.5	17:53:30	129
3	400	60	Dense	0.9	68:16:16	68

Nothobranchius density was estimated as number of fish per dip of the survey net. Species ratio is expressed as the percentage of fish of each species encountered (*N. furzeri*–*N. kadleci*: *N. orthonotus*: *N. pienaari*), with sample size (all fish collected at site during quantitative sampling) shown (N)

In the laboratory, a piece of dorsal muscle tissue was dissected from each fish. Muscle samples were dried at 60°C for 48 h and ground into a fine powder.

Sub-samples (0.4–1 mg) were weighed into tin capsules for SIA. Sampled invertebrates were separated from the substratum and organic debris, sorted into

Table 2 Summary of samples dissected for SIA analysis

	Site	N	Sex ratio	Mean TL (SD)
N. furzeri	1	10	5:5	36.4 (4.4)
	3	10	5:5	34.9 (4.4)
N. kadleci	2	10	5:5	34.8 (3.3)
N. orthonotus	1	10	3:7	49.2 (5.1)
	2	10	5:5	38.7 (3.5)
	3	10	5:5	38.9 (3.3)
N. pienaari	1	7	2:5	29.0 (2.0)
	2	10	5:5	30.9 (2.8)
	3	10	3:7	29.3 (3.0)

The number of fish analysed (*N*), their sex ratio (male to female), mean total length (TL, with SD in parenthesis) is shown for each species

taxonomic categories (order or family level) and counted. The percentage of each taxonomic group was calculated based on the sum of all invertebrates in the sample. Depending on their size, a group of individuals (50–100 for zooplankton, 5–10 for insect larvae, three for molluscs) from all taxonomic categories were ground together (to obtain a mean amalgamated value for that category in the site) and placed in the tin capsules. Since the biomass of ecologically similar cladocerans and copepods, and conchostracans and ostracods found in the samples was not sufficient to treat them separately, pooled samples were used for analysis. Soft body tissues were prepared for isotopic analysis from gastropods and bivalves.

Analysis to provide stable isotope ratios ($\delta^{13}C$ and $\delta^{15}N$) and elemental concentrations (%C and %N) was performed at the [14]Chrono Centre, Queen's University in Belfast using a Thermo Scientific Delta V Advantage Isotope Ratio Mass Spectrometer. Repeated analysis ($N = 49$) of an internal standard of known isotopic value showed that sample precision was ≤0.1‰ for both $\delta^{13}C$ and $\delta^{15}N$.

As lipid stores are ^{13}C depleted (DeNiro & Epstein, 1977), $\delta^{13}C$ values of fish muscle tissue were mathematically lipid corrected to remove the influence of variable lipid concentrations prior to statistical analysis (Kiljunen et al., 2006). Although storage in ethanol can affect stable isotope ratios (e.g., Syväranta et al., 2008), all samples were treated similarly, and any preservation effect is likely to be constant across statistical treatments and is not considered to affect our conclusions. We used PERMANOVA (Anderson,

2001; Anderson et al., 2004) to test for differences in $\delta^{13}C$–$\delta^{15}N$ centroids (i.e., lack of trophic overlap). PERMANOVA is non-parametric, permutation-based analogue of Analysis of Variance between multiple groups based on a distance measure, in this case Euclidean distances. We used gastropod $\delta^{15}N$ (assumed trophic position = 2) values to provide isotopic baselines for estimates of fish trophic position following Cabana & Rasmussen (1996), but with a trophic enrichment factor ($\Delta^{15}N$) of 2.9‰, reflecting the mean value for fishes estimated by McCutchan et al. (2003). The formula for calculation of individual TP was: consumer trophic position = [{(consumer $\delta^{15}N$ − gastropod $\delta^{15}N$)/2.9} + 2] (Cabana & Rasmussen, 1996).

Results

Prey availability was highly variable across the sampled sites both in terms of species richness and proportions (Table 3). A total of 14 taxonomic categories were found and analysed to provide isotopic information for putative prey (Fig. 2). Crustaceans (including Cladocera, Copepoda, Ostracoda and Conchostraca) were abundant at all sites. Insect larvae were less abundant and only occurred in higher proportions at sites 2 and 3 (Table 3). There was considerable variation in the stable isotope values of the different putative prey taxa (Fig. 2), both between different taxa inhabiting individual sites and within individual taxa found across the three sites. Individual *Nothobranchius* had $\delta^{13}C$ values intermediate between the extreme values displayed by the putative prey, but were typically ^{15}N enriched relative to the invertebrates.

Within-site comparisons of $\delta^{15}N$-$\delta^{13}C$ centroids (PERMANOVA) revealed that the trophic niches of the three sympatric *Nothobranchius* species differed at the sites with relatively more diversified availability of potential prey (Fig. 2, sites 2 and 3, $P < 0.001$ both). However, at both sites only the trophic niche of *N. pienaari* was significantly different from the other two species ($P < 0.01$ in all cases). No difference was found between *N. furzeri/N. kadleci* and *N. orthonotus* at any site (Fig. 2, site 1: $P = 0.28$; site 2: $P = 0.18$; site 3: $P = 0.59$). All three coexisting species showed complete isotopic overlap at site 1 where prey diversity was low (Fig. 2, $P = 0.28$).

Table 3 Relative abundance (in %) of prey categories collected at respective sites sampled for stable isotope analysis

	Site 1 (N = 496)	Site 2 (N = 738)	Site 3 (N = 1,012)
Amphibia larvae	–	–	0.3
Cladocera and Copepoda	51.8	42.3	25.3
Chironomidae larvae	2.1	8.9	5.3
Corixidae	–	0.4	0.3
Culicidae larvae	–	1.3	–
Dytiscidae larvae	–	–	0.2
Ephemeroptera larvae	–	–	4.6
Hirudinea	–	–	0.6
Notonectidae	9.3	4.9	2.7
Odonata larvae	0.4	3.1	11.9
Oligochaeta	–	2.5	5.9
Ostracoda and Conchostraca	36.4	36.6	42.9

Analysis of estimated trophic position revealed identical pattern to the outcome of the analysis of centroid location. The trophic position of *N. pienaari* was lower at those sites (2 and 3) with more diverse invertebrate communities (Fig. 3). It indicated the tendency of this species to feed on organisms at lower positions in the food chain (primary consumers) (PERMANOVA $P < 0.01$). No difference was found between *N. pienaari* and the other two species at site 1, and between *N. furzeri*/*N. kadleci* and *N. orthonotus* at any of the sites (Fig. 3, $P > 0.1$ in all cases).

Discussion

We showed that sympatric annual *Nothobranchius* which are similar in size, morphology, and environmental requirements, and with limited opportunity for habitat segregation, partly separate their trophic niches over the period of assimilation reflected by analysis of muscle stable isotope ratios. Although there are no known studies of muscle turnover in the study taxa, work on other fishes indicates it typically varies between 2 months and 1 year (Tieszen et al., 1983; Perga & Gerdeaux, 2005). The timeframe for prey assimilation reflected in the isotopic values is limited by duration of *Nothobranchius* pools, typically lasting only several months (Wildekamp, 2004). The degree of segregation varied between respective sympatric species and appeared to depend on the diversity and abundance of available prey.

Our SIA analysis revealed that among the three *Nothobranchius* species living in sympatry, *N. pienaari* had the most distinct trophic niche. Its trophic position indicates that this species tends to feed on organisms close to the bottom of the food chain, e.g., primary consumers such as zooplankton. *Nothobranchius pienaari* (referred to as *N. rachovii* in publications prior to 2011) was also found to prefer small-bodied prey in our previous SCA-based study (Polačik & Reichard, 2010). However, in contrast to SIA, the SCA provided only weak support for the separation of the trophic niches of *N. pienaari* and *N. furzeri*. The combination of results of both SIA and SCA data suggests that the food preferences of *N. pienaari* and *N. furzeri* differ over the longer-time period reflected by stable isotope data but may overlap considerably, e.g., seasonally or when prey diversity is low.

The isotopic composition of *N. furzeri*/*kadleci* and *N. orthonotus* did not provide any support for the segregation of their trophic niche or that they assimilated energy at different trophic positions. Conversely, previous SCA clearly identified *N. orthonotus* as the species with the most distinct trophic niche among the three species living in sympatry. The distinctness of *N. orthonotus* was to a large extent based on its tendency to consume relatively large-bodied prey—small vertebrates such as tadpoles or juvenile lungfish, and large insect larvae such as dragonflies. The absence of interspecific niche segregation when using stable isotope analysis may be explained by two factors. First, the consumption of small vertebrates by *N. orthonotus* is strictly seasonal due to predator–prey size compatibility. In a temporary pool, both small vertebrates and large insect larvae soon attain sizes than make them
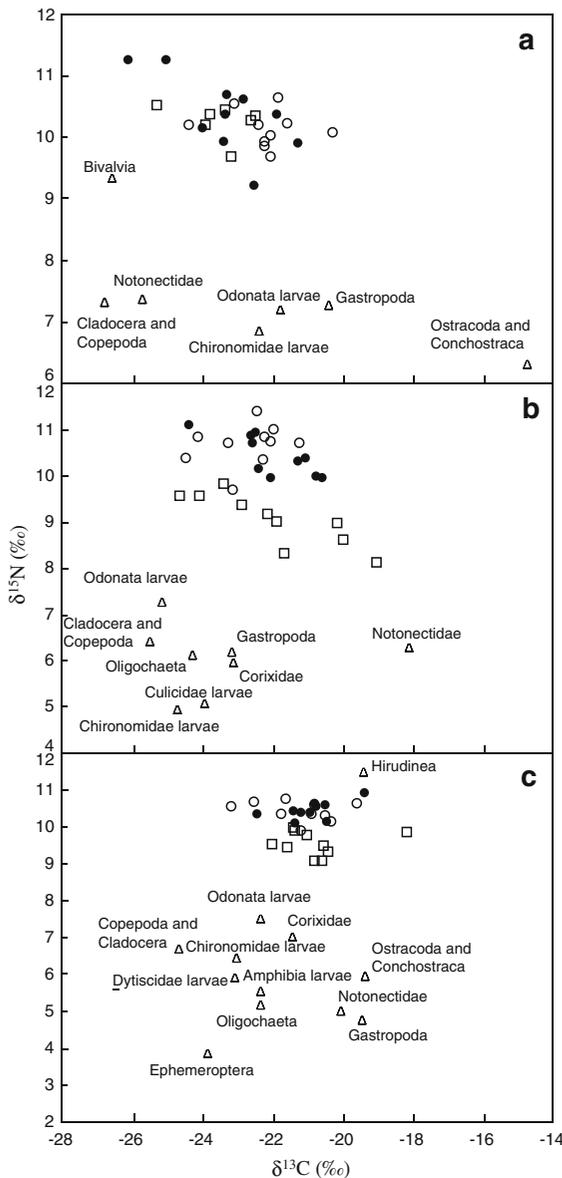
Fig. 2 $\delta^{13}C$–$\delta^{15}N$ bi-plot of *N. furzeri* (*filled circle*), *N. orthonotus* (*open circle*), *N. pienaari* (*open rectangle*) and their prey (*open triangle*). Site 1—(**a**), site 2—(**b**), site 3—(**c**)

Fig. 3 Variation in estimated individual trophic position of sympatric *Nothobranchius* species—*N. furzeri* (*N. kadleci* at site 2) in *black*, *N. orthonotus* in *grey* and *N. pienaari* in *white*—at the three study sites. The data identified by a similar *letter* are not statistically different. *Horizontal central lines* reflect median values, *boxes* represent the inter-quartile range, and *whiskers* reflect values within 1.5× the interquartile range

in the diet of *N. orthonotus*, Polačik & Reichard, 2010) had been previously regarded as relatively uniform in their feeding strategy, Schiesari et al. (2009) recently found that they represent a group with diversified feeding ecology and complex trophic relationships, including algae grazers, carnivores and detritivores. In general, SCA has greater potential to detect trophic niche differences based on prey morphology, but may link disparate food chains if morphologically similar prey occupy different trophic roles. Conversely, SIA can fail to identify substantial differences in a consumer's trophic niche when prey show little variation in isotopic composition, but may actually have distinct ecological roles (size, taxon specific habitat use).

Snapshot sampling of the prey available to consumers may complicate the relationship between the actual isotopic values of both the consumers and their prey. The highly variable nature of temporary pools means that important prey taxa may only be present for short periods. Assuming that trophic fractionation in the target fishes follows the patterns typical for other fishes (McCutchan et al., 2003), the data shown in Fig. 2a suggest that not all prey types consumed by the fishes were present in the sample. The variation in prey (except bivalves) $\delta^{15}N$ values was limited, and the fish consumers spanned a much broader range. Our sampling method provided a good representation of the occurrence of putative prey, especially with

inconsumable for the gape-limited fish predator and therefore can be preyed upon only within a narrow time window (Curry-Lindahl, 1956). As a consequence, their contribution to the assimilated diet of the consumers examined here may not be sufficient to affect their isotopic composition. Second, the isotopic values of the larger-bodied prey may not be specific enough to allow for niche discrimination between *N. furzeri* and *N. orthonotus*. For example, although anuran larvae (found
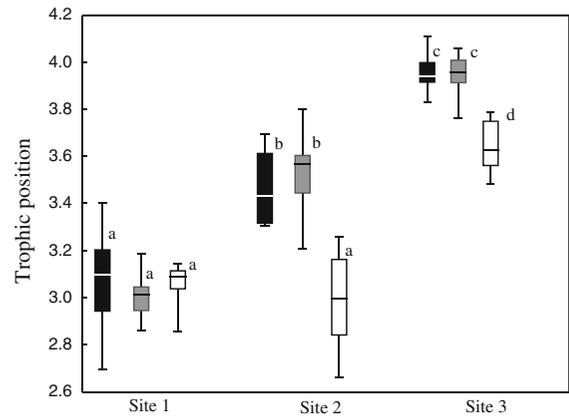
regards to the size and depth of the sampled sites (Table 1). However, it may have underestimated overall prey diversity, e.g., by not including ephemeral prey only present in the system for a brief time. Our approach, i.e., stable isotope analysis of single pooled samples of putative prey, meant that in this case, we were not able to identify the prey types driving the interspecific isotopic niche separation. Conversely, we believe that our data on relative differences in prey diversity between the sampled sites remain valid and we ranked the sampled sites correctly. Invertebrate diversity is known to correlate with habitat heterogeneity, mainly with the extent of aquatic vegetation (e.g., Ferreiro et al., 2011). Two of our sampled sites with high prey diversity were densely overgrown with aquatic vegetation whereas in the site with low prey diversity aquatic plants were absent (Table 1). As supported by our other studies, a strong positive relationship between the occurrence of various invertebrate taxa and aquatic plants is a general rule in our *Nothobranchius* study area in southern Mozambique.

The combination of both SCA and SIA methods has allowed us to identify the scale of trophic niche segregation in the three sympatric *Nothobranchius* species. The actual segregation of trophic resources may be subtle, manifested through the consumption of varying proportions of particular prey types rather than strong qualitative differences. Studies examining trophic niches show general agreement in advocating benefits of the combination of classical SCA and SIA (e.g., stream foodweb: Mantel et al., 2004; tropical fishes: Davis et al., 2012; sympatric gastropods: Sitnikova et al., 2012). For example Nagelkerken et al. (2009) reviewed the advantages and disadvantages of both methods using aquatic foodwebs and concluded that SIA offered only low taxonomic resolution of consumed prey, despite the fact that taxonomic status may actually play a role in niche segregation. Conversely, SCA may underestimate the proportion of poorly identifiable, but still effectively assimilated prey (soft or very small organisms). In the work described here and in our previous study, it is apparent that each of the two methods was only sensitive to particular characteristics of the data and clearly separated only one out of three species. SCA had a greater potential to differentiate respective diets based on the size and taxon of the consumed prey and therefore it allowed for the separation of *N. orthonotus* (Polačik & Reichard, 2010). Conversely, SIA was

capable of revealing long-term (and diminishing the influence of short-term) propensity in consumption of particular prey items, and consistent feeding of *N. pienaari* at a lower trophic level was reflected in its isotopic separation.

In general, at sites with low prey diversity trophic niche separation between species tended to be less clear, regardless of the method used (site 1 in Polačik & Reichard, 2011; site 1 in this study). Unstable environmental conditions that *Nothobranchius* spp. may encounter during their life (Reichard et al., 2009; Watters, 2009; Polačik et al., 2011) cannot lead to a strictly specialised trophic niche and *Nothobranchius* spp. are therefore generalists. When prey is scarce, sympatric species are likely to compete for food intensively, due to the limited choice in space-restricted habitats, which may potentially lead to competitive exclusion (Gause, 1934). Our field data show that the number of species occurring in sympatry at particular sites is subject to interannual fluctuations (unpublished data). Although other factors cannot be ruled out, competitive exclusion due to highly overlapping trophic niches is one of plausible explanations. Under this scenario, occasional flood events provide vectors for multiple colonisations, as individual pools temporarily merge together (Watters, 2009; Dorn et al., 2011; M. Reichard, pers. obs.). Resource availability is then one of the decisive factors of the long-term coexistence of *Nothobranchius* spp. in a given pool.

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