

Coexistence of surface and cave amphipods in an ecotone environment

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Abstract

Interspecific interactions between surface and subterranean species may be a key determinant for species distributions. Until now, the existence of competition (including predation) between these groups has not been tested. To assess the coexistence and potential role of interspecific interactions between surface *Gammarus fossarum* and subterranean *Niphargus timavi*, and to determine their micro distributions, we conducted a series of field and laboratory observations. We aimed to determine: (1) species substrate preference, (2) whether the presence of *G. fossarum* influences the habitat choice of *N. timavi*, and (3) possible predation effects on micro habitat choice of small juveniles. Throughout a small river in SW Slovenia, *N. timavi* was predominantly found in leaf litter and gravel, but rarely in sand. In the sand however, we exclusively found juveniles. In contrast, surface *G. fossarum* sheltered mainly in leaf litter. A similar, body size dependent, micro distribution was observed in *G. fossarum*, where small individuals were generally found in gravel and sand. The presence of *G. fossarum* affected the micro distribution of juvenile, but not adult, *N. timavi*. In the laboratory we observed predation and cannibalism in both species. *Niphargus timavi*, however, appeared to be a more efficient predator than *G. fossarum*. In particular, juvenile *N. timavi* were most vulnerable to preying by adults of both species. This probably affected the distribution of juvenile *N. timavi* that chose finer substrates when placed with adult individuals in an aquarium with granules of different size. To understand the distribution of subterranean species, the summed effect of intraspecific interactions, as well as surface – subterranean species interactions, in particular between individuals of different size, should be taken into account.

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Introduction

Environmental changes may result in range expansions of some species (e.g. Jażdżewski *et al.*, 2004). Invasions due to expanding species ranges inherently lead to additional interspecific relationships. These are often asymmetric and can eventually dramatically change the local fauna, the survival of which in turn depends exclusively on the number of appropriate refugia (Savage, 1981, 1982; Dick, 2008). Although poorly studied, caves are not an exception. Sket (1977) reported how mild pollution of sinking rivers enabled immigration and survival of surface species deep in the caves, which significantly reduced abundance of cave-specialized species, so-called troglobionts. It is assumed that surface species outcompete subterranean species if resources are abundant, but conversely, subterranean species outcompete surface species in food-limited environments (reviewed in Culver and Pipan, 2009). If these hypotheses were true, they might be of key importance for understanding distributions of subterranean species. First, surface fauna may be a major factor in preventing movement of subterranean species to the surface (Sket, 1981; Culver and Pipan, 2009). Second, troglobiotic fauna of small cave systems may be vulnerable to pollution mainly on account of changes in competitive strengths of surface *versus* subterranean species.

Despite the presumed importance of interspecific interactions between surface and subterranean species for their distributions, the existence of competition (including predation) between them has not been explicitly tested before. A hypothesis about competitive and predation interactions between surface and subterranean species could be tested in ecotones, such as springs (*sensu* Connell, 1980), which was the approach taken in this study.

Our research focused on two amphipod species. Amphipods represent a substantial portion of aquatic

fauna, both in biomass and in species numbers (e.g. Conlan, 2008). They are widely distributed in both surface and subterranean waters (Pinkster, 1978; Botosaneanu, 1986). The role of negative interactions such as competition or predation has been studied extensively across surface species (MacNeil et al., 1997) with particular emphasis on invasive species (Bollache et al., 2008; Dick, 2008). Moreover, there are also a few reports indicating negative interspecies interactions between subterranean species (e.g. Culver et al., 1991; summarized in Culver and Pipan, 2009). Thus, amphipods may serve as the appropriate model organisms for studying interspecific interactions between surface and subterranean species.

Recent observations of a spring in SW Slovenia identified an appropriate natural setting with two amphipod species for this study. *Gammarus fossarum* Koch, 1835 (Gammaridae) represents surface species (note that this name might cover a number of cryptic species). The species *Niphargus timavi* S. Karaman, 1954 (Niphargidae) can be found in surface (springs) and subterranean habitats (Fišer et al., 2006; it could be categorized as eutroglophile sensu Sket, 2008). Both species coexist along the studied stretch of brook. The spatio-temporal shares of both species vary significantly throughout the year, suggesting that both species may compete for space and food. We predicted that *G. fossarum*, either as a result of its higher reproductive potential or through enhanced predation, could constrain the distribution of *N. timavi* in surface habitats (Fišer et al., 2006, 2007). Indeed, the stomachs contents corroborate the hypothesis that both species overlap in feeding behaviour (Fišer et al., 2010).

To assess the coexistence and the potential role of interspecific interactions between surface and subterranean amphipod species, we conducted a series of field and laboratory observations. The setup was designed to determine: (1) whether both species show similar substrate preference, (2) whether the presence of *G. fossarum* affects habitat choice of *N. timavi*, and (3) whether predation has any effect on microhabitat choice of younger and weaker juveniles (MacNeil et al., 2008).

Material and methods

Field experiment and data analysis

We positioned 12 sampling stations along the Kolaški potok (brook) near Ilirska Bistrica in SW Slovenia.

About 250 m below the primary spring, the brook sinks and re-emerges as resurgence after about 150 m. *Niphargus timavi* is present along the entire brook, while *Gammarus fossarum* appears to be confined to the reaches below the point of resurgence (details in Fišer et al., 2007). Samples were collected in upstream (control) and downstream stretches (test).

In February and March 2008, we sampled each site for four times. We submerged six plastic cups, two for each substrate: decaying leaf litter (predominately beech tree leaves), sand (2-5 mm in diameter) and gravel (5-60 mm) based on unified classification (Buol et al., 2008). Cups were 12 cm in radius and 7 cm in height, with 33 drilled holes ($d = 10$ mm). To prevent accidental drift of the cups, we lodged them with rocks. Thereafter, we inspected each plastic cup weekly and washed out all animals. Sampled amphipods were preserved in 70% ethanol *in loci*, preventing any loss due to predation after sample collection. Samples were sorted according to species and size in the laboratory.

The following questions were proposed:

- 1 Does either species show a preference for a particular substrate? To answer this, we pooled individuals for each species by substrate categories. We tested for differences in numbers of individuals present in different substrate categories.
- 2 Does the presence of *G. fossarum* affect the habitat choice of *N. timavi*? To answer this question, we pooled individuals for each species by substrate type; however, the data for *N. timavi* from the upper part of the stream (above the resurgence, where only *N. timavi* occurs) were treated separately from the data collected in the lower stretch (below the resurgence, where both species are present). We first tested whether the substrate preferences of *N. timavi* (i.e. gravel vs. sand) in the upper and lower stretches of the stream differ. Afterwards, we tested whether the number of individuals of *N. timavi* within a selected substrate differed between the upper and lower stretches of the stream (i.e. gravel above versus gravel below the resurgence).
- 3 For each species we asked whether individuals of different size invaded the same substrate. We selected subsamples from various sampling sites in order to attain at least 50 individuals of each species from all substrate types. We estimated their body size by measuring body length (measured from the genal lobes, along the insertia of pereopod coxae – insertia of pleopods – insertia along uropods) (Fišer et al., 2009). We opted for non-parametric tests because

data were not normally distributed. Differences in substrate preferences of different sized individuals were analysed using Kruskal-Wallis test; pairwise comparisons were done by Mann-Whitney U test (using SPSS 14).

Laboratory work and data analysis

Given that arthropods present a part of both species' diet (see Fišer *et al.*, 2010), we tested whether either species showed predatory or cannibalistic behaviour.

Collected animals were separated by species, transferred to glass aquaria in a speleolaboratory (a dark, insulated room kept at approximately 10°C) and fed *ad libitum* with leaf litter from the study site for four days. Thereafter, we transferred the animals to Petri dishes. The number of animals in the dishes was counted every 24 hours for four days.

Prey size may affect the prey choice of predatory amphipods (MacNeil *et al.*, 2008), which implies that fully developed individuals would prey only on smaller individuals. To test this, we prepared con- and inter-specific pairs of large males (>12 mm) of *G. fossarum*, *N. timavi* and *N. timavi* + *G. fossarum*, each pair in a separate Petri dish. All combinations were run in five parallels for two weeks. All 30 individuals survived this testing period as expected, and so only small (<4 mm) and mid-sized (5-7 mm) individuals were used as the potential prey in the experiments described below.

Furthermore, we placed three individuals of one or a mix of both species into a Petri dish. The experimental set up was constrained by limited numbers of available small *N. timavi*. Each Petri dish contained either one large *G. fossarum* or large *N. timavi* (predator) and one out of nine possible pairs of small and/or mid-sized *G. fossarum* and/or *N. timavi* individuals. In doing so, we obtained four categories of prey (small- and mid-sized individuals of both species), the vulnerability of which was estimated by their survival. This experimental design allowed the estimation of prey vulnerability in a pair (*i.e.* which individual survives longer), however, generalizations on vulnerability of prey category were implicit, since individual predators could not select among all possible prey categories. Predation/cannibalism rates were estimated from survival rates, which were calculated from how many individuals of each prey category survived to the next day.

We asked whether *N. timavi* and *G. fossarum* differed in predatory behaviour. To answer this, we pooled the numbers of survivors per day for each predatory species. The data were log-transformed and the

predation rate was then estimated as the slope of the linear regression line. To test whether predation rates of the two species were the same, we compared the slopes of the two regression lines (coefficients) using the Snedecor and Cochran test (Snedecor and Cochran, 1976).

To determine whether prey categories differed in vulnerability, we pooled the data by the focal prey categories per predator species. For instance, if we were interested in small *N. timavi* preyed upon by *N. timavi*, we pooled the data from Petri dishes where prey of *N. timavi* were two small *N. timavi*, small *N. timavi* + small *G. fossarum*, small + mid-sized *N. timavi* and small *N. timavi* + mid-sized *G. fossarum*. To assess which category was most vulnerable, we pairwise tested the differences in slopes of regression lines for each prey category as described above (Snedecor and Cochran, 1976).

Furthermore we asked, whether small niphargids search shelter in fine sand due to cannibalistic pressure or due to seeking the contact with substrate. We filled four aquaria (100 mm × 50 mm × 200 mm) with differently sized glass pebbles, with each layer being 50 mm thick. Top, middle and bottom layers were constructed with pebbles of diameter 10, 5 and 2 mm, respectively. Well-aerated water from the site was used to fill the aquaria. Due to small number of subjects per aquarium and short experiment duration (circa one day), no additional water aeration was provided. We considered small juveniles to be less than 5 mm in length and adults larger than 15 mm. We observed the distributions of one juvenile when a) alone, b) in the presence of an adult male and c) in the presence of an adult female. Distribution of juveniles was examined every 20 minutes for five hours. Differences in distributions were tested using contingency tables and χ^2 .

Results

Field experiment results

Both, *Gammarus fossarum* and *Niphargus timavi* differently inhabited the substrate types (Kruskal-Wallis: $\chi^2 = 7.83$, d.f. = 2, $p < 0.05$ * and $\chi^2 = 21.150$, d.f. = 2, $p < 0.001$ ***, respectively). Gammarids more often sheltered in leaf litter (Fig. 1, Table 1) than in gravel and sand (Mann-Whitney U = 376.00, $p < 0.05$ *; U = 284.00, $p < 0.05$ *, respectively). However, they did not distinguish between gravel and sand (U = 407.50, $p > 0.1$). In contrast, *Niphargus timavi* showed no difference in

choosing between leaf litter and gravel (Figs 1-2, Table 2; $U = 525.00$, $p > 0.1$), but it preferred either of those substrates to sand (gravel – sand: $U = 142.00$, $p < 0.001$ ***; leaf litter- sand: $U = 185.00$, $p < 0.001$ ***).

In general, the substrate choice of *N. timavi* tended to be effected by the presence of *G. fossarum* (Kruskal-Wallis: $\chi^2 = 2.51$, d.f. = 1, $p > 0.1$; Figs 1- 2, Table 2). In the presence of *G. fossarum*, *N. timavi* showed similar preferences for substrates (Kruskal-Wallis: $\chi^2 = 22.668$, d.f. = 2, $p < 0.001$ ***). It showed no preferences when choosing gravel or leaf litter ($U = 82.00$, $p > 0.1$), but avoided sand (gravel – sand: $U = 3.00$, $p < 0.001$ ***; leaf litter – sand: $U = 15.50$, $p < 0.001$ ***). Furthermore, the numbers of individuals in leaf litter and gravel in the upper, compared to lower stretch of the brook, showed no difference ($U = 122.00$, $p > 0.1$ and $U = 105.00$, $p > 0.1$, respectively). However, in comparison to the upper stretch, more individuals invaded sand in the lower stretch of the brook ($U = 13.00$, $p < 0.001$ ***), on account of smaller individuals (see Table 4).

Both species showed similar patterns in size-dependent micro distribution - smaller individuals of both species invaded their adult stage non-preferential substrates significantly more often than larger individuals. Individuals of *N. timavi* found in sand were significantly smaller than individuals found in gravel

Table 1. Differences in abundances of *G. fossarum* in different substrates in the lower stretch of the brook. Mann-Whitney U-Test, statistically significant differences in boldface.

	Leaf litter	Gravel
Gravel	$U = 376.00$ $p < 0.05^*$	
Sand	$U = 284.00$ $p < 0.05^*$	$U = 407.50$ $p > 0.1$

Table 2. Differences in abundances of *N. timavi* in different substrates. Values above the diagonal refer to data in the upper stretch of the brook where *N. timavi* lives alone, the values below the diagonal refer to data in the lower stretch of the brook where *N. timavi* and *G. fossarum* coexist. Diagonal cells refer to comparison of abundances of *N. timavi* within the same substrate in the lower and upper stretch of the brook. Mann-Whitney U-Test, statistically significant differences in boldface.

	Leaf litter	Gravel	Sand
Leaf litter	$U = 122.00$ $p > 0.1$	$U = 525.00$ $p > 0.1$	$U = 185.00$ $p < 0.001$***
Gravel	$U = 82.00$ $p > 0.1$	$U = 105.00$ $p > 0.1$	$U = 142.00$ $p < 0.001$***
Sand	$U = 15.50$ $p < 0.001$***	$U = 3.00$ $p < 0.001$***	$U = 13.00$ $p < 0.001$***

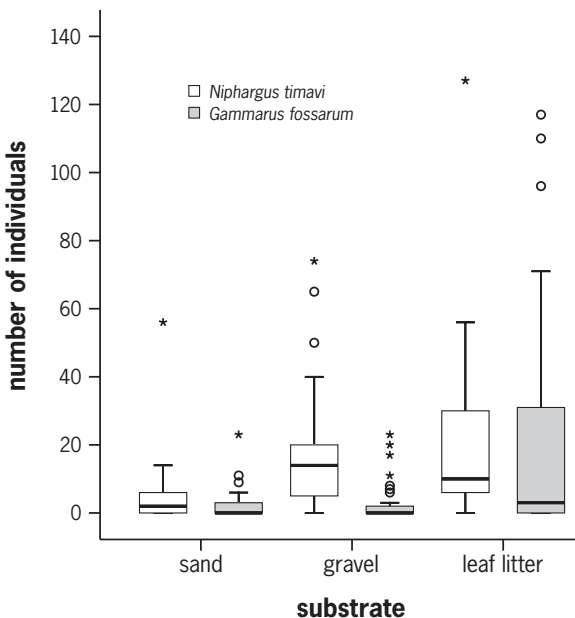


Fig. 1. Number of individuals of *G. fossarum* and *N. timavi* in three different substrates in the lower stretch of the brook (below the resurgence) where the two species coexist.

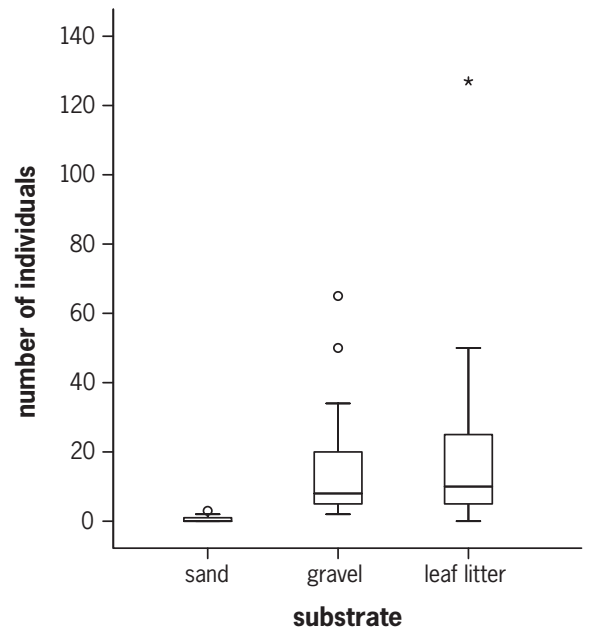


Fig. 2. Number of individuals of *N. timavi* in the upper stretch of the brook (above the resurgence) where the species appears alone.

or leaf litter (sand-gravel: $U = 757.00$, $p < 0.001$ ***; sand – leaf litter: $U = 1008.50$, $p < 0.001$ ***), while body lengths of individuals found in gravel and leaf litter did not differ ($U = 974.00$, $p > 0.1$, Tables 3-4). For *G. fossarum*, leaf litter was invaded by significantly larger individuals than gravel ($U = 829.50$, $p < 0.01$ **). Invaders of leaf litter showed a trend with marginal statistical significance to be larger than those invading sand ($U = 1015.50$, $p = 0.057$). For *G. fossarum*, no difference in sizes was detected between the two types of non-preferential substrates ($U = 1222.00$, $p > 0.1$; Tables 3-4).

Laboratory work results

Niphargus timavi appeared to be a superior predator over *G. fossarum*. Thirty adult *N. timavi* consumed about 60% of available prey items in 24 hours and 21% of prey survived the full five days test period. By contrast, *G. fossarum* consumed only 24% of available prey in the first 24 hours and consumed only 39% of available prey items in the five day test period. This pattern was consistent across prey categories. In all cases, *N. timavi* preyed faster and more efficiently than *G. fossarum*.

Niphargus timavi preyed more efficiently upon small- over mid-sized individuals of both species. By contrast, *G. fossarum* most efficiently hunted small *N. timavi*, while it preyed on the rest of the prey categories with roughly the same efficiency (Table 5, Fig. 3). Our data indicate that juvenile *N. timavi* present the most vulnerable size (age) class in this particular brook.

The presence of an adult changed the distribution of juvenile niphargids. When no adult was present, juveniles inhabited mostly the middle layer, whereas it was evenly distributed in the other two layers. In the presence of an adult, a juvenile was almost never found in the layer with large pebbles, being more often found in the layers with middle and small sized pebbles (large pebbles: $\chi^2 = 0.24$, $p < 0.01$ **; middle sized pebbles: $\chi^2 = 0.235$, $p < 0.01$ **; small pebbles: $\chi^2 = 0.202$, $p < 0.05$ **, Table 6).

Discussion

Our study shows that *G. fossarum* chose leaf litter more often than gravel and sand, implying only accidental and temporary use of the later two habitats. By contrast, *N. timavi* chose no single substrate, but was

Table 3. Differences in body lengths in different substrates for *N. timavi* (above diagonal) and *G. fossarum* (below diagonal). Mann-Whitney U-Test, statistically significant differences in boldface, trends in italics.

	Leaf litter	Gravel	Sand
Leaf litter		$U = 974.00$ $p > 0.1$	$U = 1008.50$ $p < 0.001$ ***
Gravel	$U = 829.50$ $p < 0.01$ **		$U = 757.00$ $p < 0.001$ ***
Sand	$U = 1015.50$ $p > 0.05$ (*)	$U = 1222.00$ $p > 0.1$	

Table 4. Body sizes of *Niphargus timavi* and *Gammarus fossarum* in three different substrates in the lower stretch of the brook (below the resurgence), where the two species coexist.

Substrate	Descriptive statistic	<i>Niphargus timavi</i>	<i>Gammarus fossarum</i>
Gravel	min	1.91	0.51
	1. quartile	3.57	4.9
	median	5.47	7.18
	mean \pm SD	6 ± 3.08	6.35 ± 2.62
	3. quartile	7.89	8.08
	max	13.63	11.15
Leaf litter	min	1.17	4.3
	1. quartile	3.13	5.58
	median	3.73	8.37
	mean \pm SD	5.12 ± 2.94	8.28 ± 2.81
	3. quartile	6.54	10.47
	max	11.4	13.25
Fine sand	min	1.1	0.81
	1. quartile	2.32	2.27
	median	2.52	6.56
	mean \pm SD	3.19 ± 2.18	6.76 ± 3.99
	3. quartile	2.93	10.3
	max	13.2	13.81

found in sufficiently large spaces among either particle grains or leaves. Moreover, the presence of *G. fossarum* had only little effect on *N. timavi* microdistribution where the two populations overlapped (in the lower stretch of the brook).

Differently sized individuals of both species distributed non-randomly among substrates. Body size differences in habitat choice were greater in *N. timavi* than in *G. fossarum*, but in both species smaller individuals sought shelter in substrates that were preferred less by adults (gravel, sand).

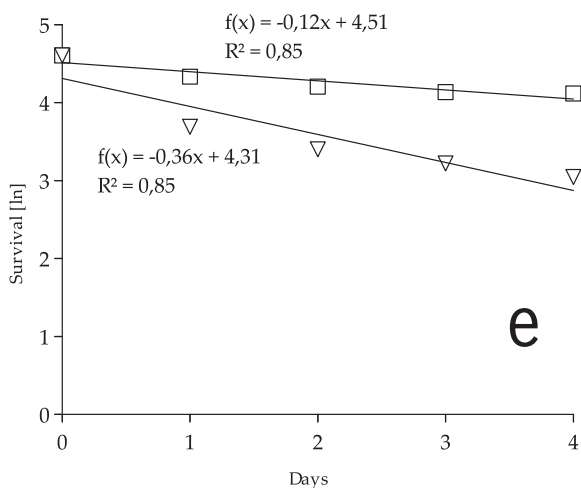
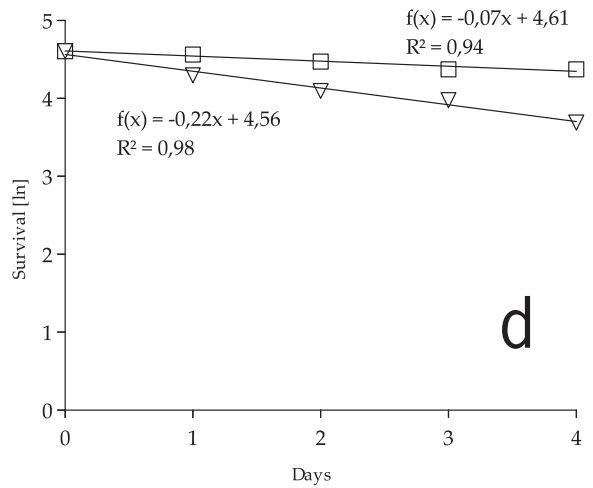
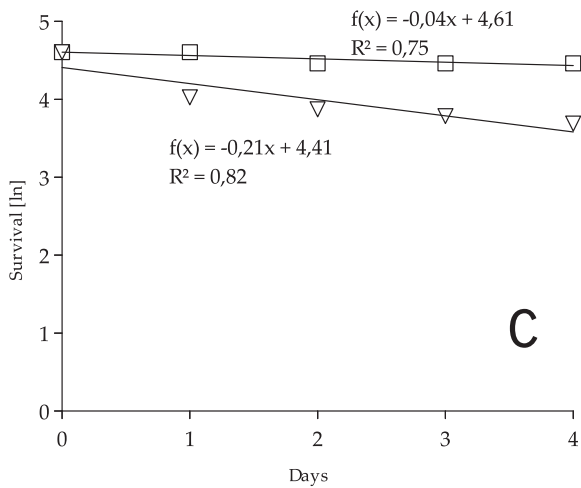
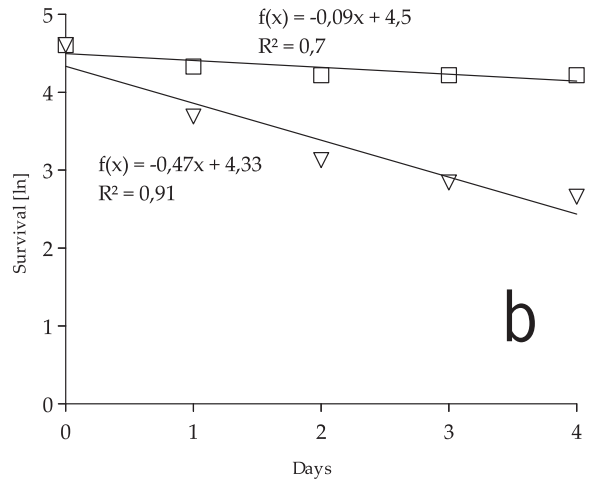
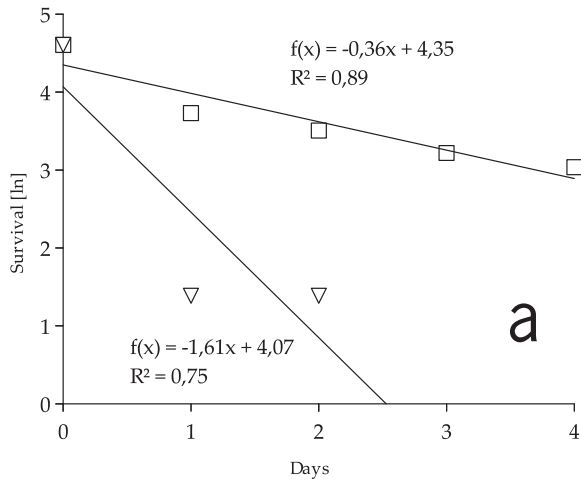


Fig. 3. Effectiveness of predation (cannibalism) by adult *G. fossarum* (□) and *N. timavi* (Δ) upon differently sized individuals of both species: a) small *N. timavi*, b) middle *N. timavi*, c) small *G. fossarum*, d) middle *G. fossarum* and e) total effectiveness for all aforementioned groups by adults of both species. See Materials and methods for class sizes. The slopes of all regression lines-pairs are statistically different ($p < 0.05^*$).

Niphargus timavi was a more efficient predator and cannibal than *G. fossarum*. This predatory behaviour may broaden available food sources, which might be advantageous in the food-limited subterranean environment. Juvenile *N. timavi*, the most vulnerable prey category, invaded sand seeking not only contact with substrate, but also in response to predatory pressure from large *N. timavi* whose movement through the sand was constrained. Interpreting the micro distribution of small *N. timavi* in this light matches with the field results,

Table 5. Differences in predation preferences by *N. timavi* (above diagonal), *G. fossarum* (below diagonal) and the differences between species. Each cell indicates the most vulnerable prey category and probability that coefficients of the two regression lines are the same. Statistically significant values are indicated in boldface, statistical trends are indicated in italics. Diagonal cells list the probability that both species equally prey the particular prey category.

	Small <i>N. timavi</i>	Middle <i>N. timavi</i>	Small <i>G. fossarum</i>	Middle <i>G. fossarum</i>
Small <i>N. timavi</i>	<i>N. timavi</i> <i>p > 0.05</i> (*)	small <i>N. timavi</i> p < 0.05 *	p > 0.1	small <i>N. timavi</i> p < 0.05 *
Middle <i>N. timavi</i>	small <i>N. timavi</i> p < 0.01 **	<i>N. timavi</i> p < 0.01 **	small <i>G. fossarum</i> p < 0.05 *	p > 0.1
Small <i>G. fossarum</i>	small niphargid p < 0.05 *	small <i>G. fossarum</i> <i>p > 0.05</i> (*)	<i>N. timavi</i> p < 0.01 **	small <i>G. fossarum</i> p < 0.05 *
Middle <i>G. fossarum</i>	small <i>N. timavi</i> p < 0.01 **	p > 0.1	p > 0.1	<i>N. timavi</i> p < 0.05 *

Table 6. Distribution of small sized niphargids in layers of artificial substrate of different grain size when alone and in the presence of adult conspecific under laboratory settings.

Substrate size	Alone	Adult present	
10 mm pebbles	12	2	4
5 mm pebbles	33	47	32
2 mm pebbles	15	11	24

which seem to indicate that hiding behaviour of small *N. timavi* was most pronounced in the lower stretch of the stream, where occasional *G. fossarum* predation accompanies cannibalistic acts by larger *N. timavi*. Similar segregations of juvenile individuals have also been observed at least in *Niphargus rhenorhodanensis* Schellenberg 1937 (Mathieu *et al.*, 1987), *Thermosphaeroma thermophilum* (Richardson, 1897) (Jormalainen and Shuster, 1997), *Gammarus pseudolimnaeus* Bousfield, 1958 (Williams and Moore, 1986), *Gammarus pulex* (McGrath *et al.*, 2007) and *Pseudoniphargus grandimanus* Stock, Holsinger, Sket and Iliffe, 1986 and *Pseudoniphargus carpalis* Stock, Holsinger, Sket and Iliffe, 1986 by Stock *et al.* (1986).

In contrast, *G. fossarum* showed less inter- and intraspecific interactions. The distribution of differently sized animals in different substrates may simply reflect rivalry for the preferred substrate, where the largest individuals outcompete smaller ones. Similar results were obtained in observations in aquaria (unpublished observations).

Micro distribution of *N. timavi* and *G. fossarum* apparently depends on a broad array of biotic and abiotic

factors. The role of the substrate, interspecific competition, intraguild predation or a combination of all of the above have been broadly acknowledged to structure various communities (Crowder and Cooper, 1982; Polis, 1984, 1989; Skadsheim, 1984; Adams *et al.*, 1987; Dick, 1996, 2008; MacNeil *et al.*, 1997, 2008; Beisel *et al.*, 1998; Dick and Platvoet, 2000; Dick *et al.*, 2002; van Riel *et al.*, 2006; McGrath *et al.*, 2007; Kley *et al.*, 2009; MacNeil and Briffa, 2009). Nevertheless, at least in certain cases, intraspecific relationships has been neglected or obscured on account of interspecific relationships (Sket, 1981; Culver and Pipan, 2009). In this particular study, the hypothesis of competition between surface and subterranean fauna remains generally unsupported. However, juvenile *N. timavi* were observed to take refuge in finer substrates (sand) more often in the presence of cannibalism and predation by their conspecifics and *G. fossarum*, respectively. Large-scale effects may arise due to additive effects of predation and cannibalism upon the smallest (the most vulnerable) individuals of *N. timavi*, thereby significantly decreasing the number of individuals in the next generation.

To conclude, the two species differed in their habitat choice. Smaller and weaker individuals of *N. timavi* and *G. fossarum* inhabited microhabitat less preferred by adults, probably due to cannibalism and competition, respectively. Despite the difference in habitat choice between the two species, the occasional predation of *G. fossarum* upon juvenile *N. timavi* in addition to cannibalism may accelerate the decrease of niphargid population along the brook (as observed by Fišer *et al.*, 2007). To understand distribution of subterranean species, the summed effect of intraspecific

interactions as well as surface – subterranean species interactions, in particular between differently sized individuals, should be taken into account.

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