

The interplay between habitat use, morphology and locomotion in subterranean crustaceans of the genus *Niphargus*

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ABSTRACT

Locomotion is an important, fitness-related functional trait. Environment selects for type of locomotion and shapes the morphology of locomotion-related traits such as body size and appendages. In subterranean aquatic arthropods, these traits are subjected to multiple, at times opposing selection pressures. Darkness selects for enhanced mechano- and chemosensory systems and hence elongation of appendages. Conversely, water currents have been shown to favor short appendages. However, no study has addressed the variation in locomotion of invertebrates inhabiting cave streams and cave lakes, or questioned the relationship between species' morphology and locomotion. To fill this knowledge gap, we studied the interplay between habitat use, morphology and locomotion in amphipods of the subterranean genus *Niphargus*. Previous studies showed that lake and stream species differ in morphology. Namely, lake species are large, stout and long-legged, whereas stream species are small, slender and short-legged. We here compared locomotion mode and speed between three lake and five stream species. In addition, we tested whether morphology predicts locomotion. We found that the stream species lie on their body sides and move using slow crawling or tail-flipping. The species inhabiting lakes move comparably faster, and use a variety of locomotion modes. Noteworthy, one of the lake species almost exclusively moves in an upright or semi-upright position that resembles walking. Body size and relative length of appendages predict locomotion mode and speed in all species. We propose that integrating locomotion in the studies of subterranean species might improve our understanding of their morphological evolution.

1. Introduction

Locomotion – any type of propulsion – is an essential trait for survival and reproduction as it is involved in foraging, escaping predators or approaching potential mates (Dickinson et al., 2000; Irschick and Higham, 2012; Foster et al., 2015). Similarly to most functional traits, locomotion patterns and performance have been shaped by ecological demands of the environment (Ribera and Foster, 1997; Harrison et al., 2015; Crumière et al., 2016; Winchell et al., 2018). On a species or population level, habitat use selects for optimal locomotion, and influences the morphology of structures involved in locomotion (Harrison et al., 2015; Pontzer, 2016). Morphological traits such as appendages and their accessory structures generate thrust by pressing against a

substratum. The properties of these structures, such as appendage length and physiology, determine performance and mode of locomotion (Collins et al., 2015; Harrison et al., 2015; Higham et al., 2015a,b; Aiello et al., 2017).

Arthropods specialized for life in groundwater are interesting and insightful model organisms for studying the interrelations between habitat use, morphology and locomotion. In these animals, the appendage length has been subjected to multiple, at times opposing selection pressures (Trontelj et al., 2012; Delić et al., 2016; Zakšek et al., 2019). Given that appendage length usually corresponds to the locomotion mode (Belanger, 2013), locomotion-independent selection forces (see below) acting on appendage morphology might indirectly affect locomotion. The relationships between morphology and locomotion of

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subterranean species, to our knowledge, remain unexplored (but see Christiansen, 1965).

The prime selection force affecting appendage morphology derives from the absence of light. Permanent darkness is an ubiquitous characteristic of all subterranean habitats (Culver and Pipan, 2009, 2014) that led to substantial modifications of sensory systems, where mechano- and chemoreception substituted for photoreception (Hüppop, 2000; Culver and Pipan, 2009). Many arthropods' extraoptic receptors are arranged along their appendages. In order to maximize the reception of environmental information, selection favored the elaboration of extraoptic sensory systems and elongation of appendages (Hüppop, 2000; Langecker, 2000; Konec et al., 2015). Indeed, darkness commonly selects for long appendages (Culver and Pipan, 2009, 2014). The second selection force derives from the properties of the microhabitat in which species live, in particular water currents. Subterranean aquatic environments are a heterogeneous habitat template comprising capillary water in crevices, waterfalls, streams and lakes (Sket, 2004; Trontelj et al., 2012; Culver and Pipan, 2014). Water velocity apparently acts as a potent selective force. Species from streams have short appendages, presumably resistant to catastrophic drift and physical injuries during high water levels (Pacioglu et al., 2019). As the appendages' length influences the locomotion mode (Belanger, 2013), we can expect that short-legged stream species move differently than their long-legged close relatives from lakes.

We explored the relationships between habitat use, morphological traits and locomotion in amphipod species from the genus *Niphargus* Schiodte, 1849. The genus comprises almost exclusively subterranean species, which colonized all subterranean aquatic microhabitats, including tiny crevices, streams and lakes (Sket, 1999; Trontelj et al., 2012). Previous studies showed an association between the species' morphology and their respective habitat use. Species from subterranean streams have slender bodies and shorter appendages, allowing them to shelter under stones and gravel, and making them less vulnerable to catastrophic ecological drifts caused by floods. By contrast, lake species are large and stout, with long appendages (Trontelj et al., 2012; Fišer et al., 2013; Delić et al., 2016). Similarly to most amphipods (Dahl, 1977; Gilbert et al., 2018), *Niphargus* commonly rest laterally, on either body side, and move by crawling or occasionally by swimming. However, some lake species have been consistently observed in an upright position in the field (unpublished field observation). Hence, the observed morphological and ecological diversity of *Niphargus*, jointly with field observations, suggest that these species might differ in locomotion as well.

In this study we recorded locomotor behaviors of specimens belonging to eight *Niphargus* species from two ecologically distinct microhabitats, i.e., streams and lakes; and measured several morphological traits that are involved in their locomotion. Using these data, we tested whether species from the two microhabitats differ in behaviors related to locomotion and explored the relationship between morphology and locomotion. We hypothesized i) that stout and long-legged lake species use a different mode of locomotion than slender and short-legged stream species, and ii) that morphology, despite subjected to more complex selection, can be used as a valid surrogate for the mode of locomotion and speed in future comparative analyses.

2. Methods

2.1. Animals, field collection and acclimation

We studied specimens of eight *Niphargus* species. Three species (*N. croaticus* Jurinac, 1887, *N. longiflagellum* Karaman, 1950 and *N. pachytelson* Sket, 1960) live predominantly in subterranean lakes, in permanently flooded zones with little or no water current. Five species (*N. brachytelson* Karaman 1952, *N. podpecanus* Karaman, 1952, *N. dalmatinus* Schäferna, 1932, *N. sphagnicolus* Rejic, 1956 and *N. spinulifemur* Karaman, 1954) live in subterranean streams; the last three regularly

occur also in springs.

Animals were collected between April 2016 and March 2017. All species were collected in caves, except for *N. sphagnicolus* and *N. spinulifemur*, which were collected in springs. *Niphargus croaticus* was collected by cave diving. We aimed to obtain at least 10 intact adult individuals per species, although this was not always feasible, and the sample size varied between 8 and 19 individuals. After collection, we transported the animals to a cave laboratory (Department of Biology, Biotechnical Faculty, University of Ljubljana), preliminarily identified them to the species level, and allocated each individual into its own Petri dish (90 × 15 mm) filled with dechlorinated tap water. Animals were acclimated for at least one week in complete darkness at 12 °C, which approximated the temperatures at the sampling sites (precision ± 2 °C). We fed the animals with commercial fish food (astaksantin, granules of 2 mm) ad libitum and changed water in Petri dishes once a week. For all necessary animal handling prior to observations we used exclusively weak red light to which *Niphargus* species are insensitive (Fišer et al., 2016). We started the behavioral observations in March 2017 and ended in May 2017. After observations, animals were killed and their species identities were verified to eliminate possible mistakes made earlier when identifying live and moving animals. Sex was determined whenever possible; however, given that studied morphological traits are sexually monomorphic, gender was not included into further analyses. Detailed information regarding locations and collection dates, as well as dates of observations, for each individual are summarized in Appendix, Table S1.

2.2. Behavioral observations and video-tracking

We recorded the animals' behavior using the modified experimental setup described in detail by Fišer et al. (2019). A red light (630 nm) emitting panel was used to provide background illumination for a rectangular plexiglass aquarium (20 cm long, 8 cm wide and 7 cm high) that was filled with dechlorinated tap water to the height of 3 cm. Animals were kept and observed in stagnant water. Low light intensities used (0.15–0.25 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$) did not warm up the water during the timespan of observations. A single individual was gently taken from its Petri dish and placed into the aquarium where its behavior was recorded for 20 minutes using a webcam (Logitech C930e FullHD). Videos were captured in VirtualDub 1.10.4 at 5 frames per second. The water in the aquarium was changed before every test.

Videos were used to extract locomotion-related behaviors and average speed for each individual. To construct ethograms, we measured the total durations of amphipod locomotion-related behaviors, including crawling, walking (movement in upright, or more commonly semi-upright position) and swimming, and counted occurrences of tail-flipping, side-flipping and direction changings. Descriptions of these behaviors are provided in Table 1. Videos were played back in VLC media player software and ethograms were constructed from five 1-minute intervals evenly spaced across the video duration, i.e., from 3–4 min, 7–8 min, 11–12 min, 15–16 min, and 19–20 min. Average speed was calculated from the trajectories obtained by video-tracking each individual using idTracker 2.1 (Pérez-Escudero et al., 2014) and custom functions in R 3.5.1 (R Core Team, 2016).

2.3. Morphological traits

We measured six morphological traits that are involved in amphipod locomotion: body length, lengths of pereopods V–VII and depths of coxal plates II–III. The latter form a channel along the ventral side of the body and is important in generation of a jet of water currents used for swimming (Dahl, 1977, Fig. 1). Intact animals were photographed, after which their appendages were dissected, mounted on glycerin slides, and photographed as well. Olympus ColorView III digital camera mounted on an Olympus SZX9 stereomicroscope was used for photographing. Traits were measured from photos to the precision of 0.01

Table 1
A list and description of behaviors used for ethogram construction.

Behavior	Description
Crawling	The animal is lying on its side. Propulsion is generated by the strokes of pleopods and pereopods V–VII, but only pereopods from the body side in contact with the substratum are involved.
Walking	The animal is in an upright or semi-upright position and both pairs of pereopods V–VII are in contact with substratum. Propulsion is generated by the action of pleopods and all pereopods V–VII.
Swimming	The animal detaches from the substratum into free water and propulsion is generated by the action of pleopods. We did not discriminate between swimming “back-up”, “back-down” and “on a side”.
Tail-flipping	The animal makes an explosive or “jumpy” move. Propulsion is generated with the entire body when “C” or “hook” shaped body is instantaneously stretched.
Side-flipping	The animal switches the side of the body that is oriented towards the substratum (from left to right body side or vice versa). This may proceed via upright position, swimming or tail-flipping.
Direction changing	The animal changes the direction of crawling, walking or swimming for more than 90 degrees.
Resting†	The animal is motionless for at least 2 seconds. Its body is on the side, in an upright or semi-upright position.
Cornering†	The animal moves into in the corner of the aquarium and usually tries to squeeze into it or explores it with its appendages. If it was motionless in the corner, we marked the behavior as resting.

† These behaviors were not included in the analyses, as they do not describe a mode of locomotion.

mm using Olympus Cell B 2.8. software and relying on landmarks described by (Fišer et al., 2009). In five animals we damaged the appendages during handling after observations and could not obtain reliable measurements. To keep our dataset as large as possible, we replaced these missing values using population means, corrected for the individual’s body size. Four specimens lacked more than two appendages and were discarded from the analyses. Behavioral and morphological data are available in Appendix, Table S2.

2.4. Phylogeny

To correct results for the phylogenetic relatedness between the focal species, we used the most recent ultrametric phylogeny of the genus *Niphargus*, calculated in a Bayesian framework (Copilaş-Ciocianu et al., 2018). This is the most complete phylogeny of the genus currently available and summarizes relationships for 157 *Niphargus* species using one mitochondrial (cytochrome oxidase 1) and two nuclear markers

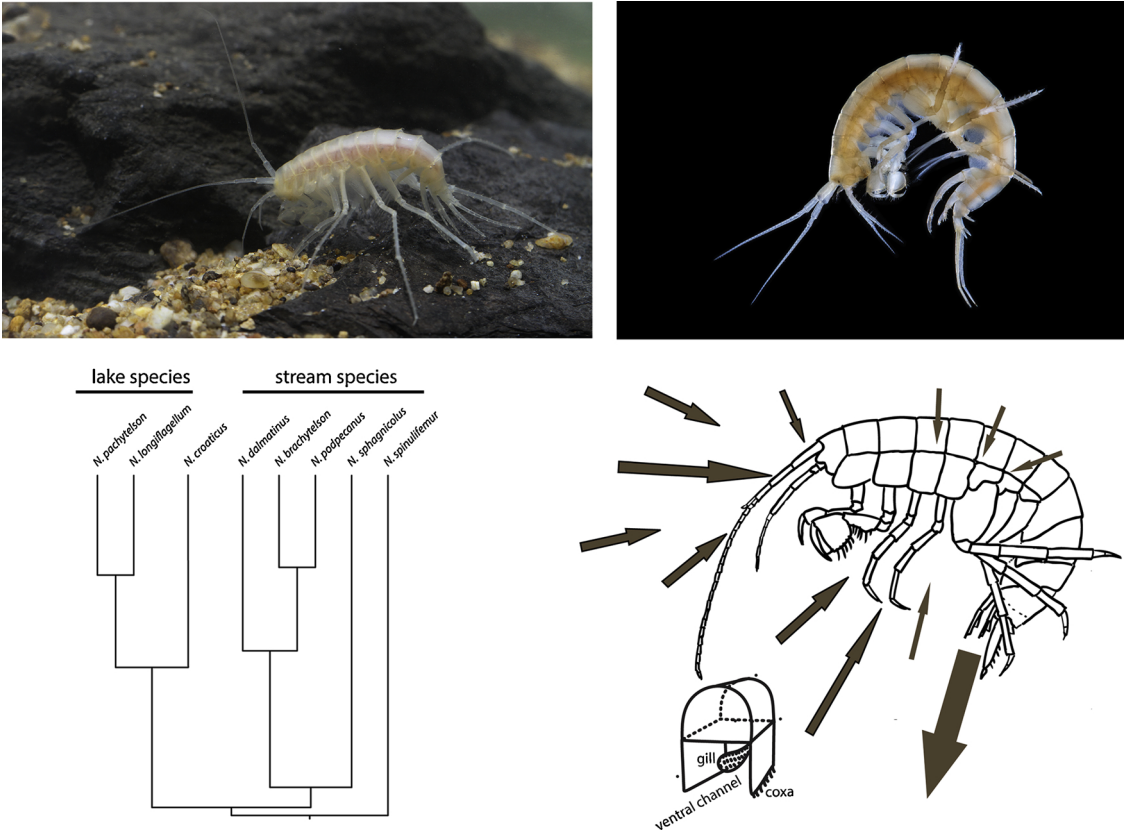


Fig. 1. Presentation of studied *Niphargus* ecomorphs: morphology, phylogenetic relationships and functional morphology. *Upper left:* lake species *Niphargus croaticus*, with a large and stout body and long legs, in a typical upright position. *Upper right:* stream species *Niphargus dalmatinus*, with a small and slender body with short appendages. *Bottom left:* Phylogenetic relationships between the studied species. The phylogenetic tree was taken from Copilaş-Ciocianu et al. (2018), and pruned such that only the focal species were retained. Habitat use of the studied species is labelled as “stream” and “lake”. *Bottom right:* Amphipod swimming relies on self-generated water currents. Coxal and epimeral plates create a channel along the ventral side of an animal. Broom-like appendages in the rear part of the body (pleopods, not illustrated) create water currents (arrows) that deliver oxygen to gills, and exit at the rear end of the animal (large arrow). When the animal is bent in a “C” or “hook” shaped posture, the sum of currents is zero, such that an animal can lie motionless even on a smooth surface. If stretched, the currents turn into a jet propulsion and an animal switches to swimming. Swimming is rare, the animals more commonly crawl on a side with pereopods, or use the whole body to tail-flip (redrawn from Fišer, 2012). Photos courtesy of Teo Delić.

(histone H3 and 28S rRNA). We used 1000 random trees that were integrated into the consensus phylogeny, and pruned them for the needs of our analyses using the *phytools* package (Revell, 2012) in R 3.5.1 (R Development Core Team, 2016). The pruned phylogenetic tree is shown on Fig. 1.

2.5. Statistical analyses

We tested for morphological and locomotion differences between lake and stream species. In the first step we reduced the number of morphological variables by running a principal component analysis (PCA) on a correlation matrix using the program IBM SPSS Statistics 20.0.0. To estimate the relevant number of factors to extract, we used Kaiser’s eigenvalue-one rule and the visual inspection of scree-plot. An individual’s scoring on the extracted factor was estimated by the regression method. Any correlation of 0.5 or above was regarded as relevant for the variable loading on each factor. The PC scores were used in further analyses. In the same way, we reduced the number of behavioral variables obtained from ethograms and video-tracks. First, we discarded all non-locomotor behaviors (resting, cornering). Each side-flipping and direction changing event was assigned the duration of one second. Then, we calculated the proportions of time that individuals spent in each individual behavior. The behavioral PCA was based on shares spent for crawling, walking, swimming, tail-flipping, direction changing, side-flipping and average speed. In order to account for large differences among behavioral variables, the behavioral PCA was calculated from correlation matrix.

In both PCA analyses, the PCA determinant of the correlation matrix exceeded 0.0001, confirming there was no multicollinearity or singularity in the data subjected to PCA. Measures of sampling adequacy indicated that the data matrix was appropriate for both PCAs (morphology (hereinafter as PCA_M): Kaiser–Meyer–Olkin measure of sampling adequacy = 0.77, Bartlett’s test of sphericity, $p < 0.001$; locomotion (hereinafter as PCA_L): Kaiser–Meyer–Olkin measure of sampling adequacy = 0.43, Bartlett’s test of sphericity $p < 0.001$). We extracted two principal components from the morphological data (PC_{M1}, PC_{M2}) and three principal components from the behavioral data (PC_{L1}, PC_{L2}, PC_{L3}) (see Results). Components with eigenvalues smaller than one were discarded from other analyses.

To test whether lake and stream species differ in morphology and locomotion, we ran generalized linear mixed models within a Bayesian framework using the package *MCMCglmm* (Hadfield, 2010) in R 3.5.1 (R Development Core Team, 2016). We used microhabitat (lake, stream) as a fixed predictor and species identity as a random effect. We ran separate models for each of the five response variables, i.e., PC_{M1}, PC_{M2}, PC_{L1}, PC_{L2}, PC_{L3}. We could not correct the model for phylogenetic non-independence, because the low number of species was not sufficient for the accurate estimation of phylogenetic effects (J. Hadfield, personal communication, Nov. 2018). We used non-informative priors and ran 100,000 Monte Carlo Markov Chain iterations, sampled every 10th iteration and discarded the first 10% of samples as burn-in.

Next, we tested whether morphology can be used as a surrogate for locomotion in future macroecological and comparative analyses. Morphology is easier to measure than locomotion but is subjected to a more complex selective regime. To test whether morphology predicts locomotion traits we performed several regression analyses using phylogenetically corrected generalized least squares (PGLS) in a Bayesian framework in Bayes Traits V3.0.1 (Pagel et al., 2004). The predictors were morphological traits, and response variables were locomotion traits, both expressed as PCA scores. To account for the within-species variation, all measured individuals were added to the tips of the phylogenetic tree (see above) using short, equally long branches (branch length = 10^{−20}). The relationship between individuals of the same species was arbitrary, however, the branches were more than ten-times

shorter than the branches between the most closely related *Niphargus* sister species (see also Witt et al. (2006) for a discussion on inter- and intraspecific distances in amphipods). To account for phylogenetic uncertainty, we used a sample of 1000 trees. Priors for intercept and regression coefficients were drawn from a uniform distribution on the interval between −100 and 100. Monte Carlo Markov Chain ran for 10 million iterations, which were sampled every 1000th iteration with a burn-in of 100,000. Mixing, the proportion of proposed changes to a chain that is accepted was auto-tuned with an acceptance rate between 25–40%. The significance of regressions was assessed by distribution of regression coefficients, i.e., whether or not they cross zero.

We applied a model selection procedure in order to assess whether complex models using two predictors (PC_{M1} and PC_{M2}) better explained the variation in a response variable than simple models using only one predictor (PC_{M1} or PC_{M2}). Thus, we ran one complex and two simple regression models for each of the three locomotion-related response variables (PC_{L1}, PC_{L2}, PC_{L3}). The preferred model was assessed using Bayes factors which were calculated from marginal likelihoods using the function “stepping stones” and default settings in Bayes Traits V3.0.1 (Pagel et al., 2004).

3. Results

3.1. Principal component analyses of morphological and locomotion traits

PCA of morphological traits identified two principal components with eigenvalues greater than one that together explained 98.9% of variance (PC_{M1}: 87.2%; PC_{M2}: 11.7%, Table 2). All traits loaded strongly positively on the first component, hence PC_{M1} can be considered as a surrogate of body size. PC_{M2} differentiated between animals with relatively short and long appendages, the latter having higher scores.

PCA of locomotion traits identified three principal components that together explained 78.8% of variance (PC_{L1}: 31.9%; PC_{L2}: 29.1%; and PC_{L3}: 17.8%; Table 3). The first principal component mainly distinguished between walking (positive values) and crawling (negative values), as well as between fast (positive values) and slow locomotion (negative values). We therefore defined it as a “fast walkers versus slow crawlers” variable in further analyses. The second principal component differentiated swimmers and tail-flippers (positive values) from walkers (negative values), and we named this variable as “swimmers and tail-flippers versus walkers”. The third principal component distinguished individuals that often changed direction or flipped from one body side to the other (positive values) and individuals that did not show these behaviors (negative values), and we termed it as a “direction-changers and flippers versus non-flippers”.

3.2. Differences in morphology and locomotion between lake and stream species

Niphargus species from lakes differed from species from streams in

Table 2
Results of principal component analysis for morphological traits. Loadings of individual traits on the first two principal components are shown. Variables with loadings higher than |0.5| are bolded.

Morphological trait	PC ₁ (87.2% of variance)	PC ₂ (11.7% of variance)
Body length	3.489	−1.52
Coxal plate III length	0.393	0.143
Coxal plate III width	0.243	−0.065
Pereopod V length	1.92	0.789
Pereopod VI length	2.68	0.821
Pereopod VII length	2.54	0.610

Table 3

Results of principal component analysis for locomotion traits. Loadings of individual traits on the first three principal components are shown. Variables with loadings higher than $|0.5|$ are bolded.

Behavioral trait	PC ₁ (31.9% of variance)	PC ₂ (29.1% of variance)	PC ₃ (17.8% of variance)
Crawling	-0.87	0.143	-0.258
Walking	0.795	-0.441	0.214
Swimming	0.383	0.917	0.072
Tail-flipping ¹	0.383	0.917	0.072
Changing direction ¹	-0.386	0.075	0.72
Side flipping ¹	-0.355	0.017	0.76
Average speed	0.526	-0.369	0.182

¹ Each event in these behaviors was treated as 1 second.

both morphology and locomotion. Lake animals were overall larger than animals from streams (PC_{M1}: posterior mean difference between groups (post. mean) = 1.089, 95% credible interval (CI) = [0.724, 1.475], $p < 0.001$; Fig. 2a). Stream animals had shorter legs than lake animals (PC_{M2}: post. mean = 1.461, 95% CI [1.159, 1.775], $p < 0.001$; Fig. 2b).

Lake animals were faster and showed a tendency to walk, while stream animals were slower and more often crawled and swam (PC_{L1}: post. mean = 0.982, 95% CI = [0.629, 1.318], $p < 0.001$, Fig. 2c; PC_{L2}: post. mean = 0.517, 95% CI = [0.348, 0.681], $p < 0.001$, Fig. 2d). Noteworthy, lake species also exhibited a higher between-species variation of the first two locomotion principal components than stream species (Fig. 2). Movement in an upright or semi-upright position that we herein refer as walking was clearly expressed by all *N. croaticus* individuals, whereas a tendency towards this mode of locomotion was displayed only by few individuals of the remaining two lake species. Animals from the two microhabitats did not significantly differ in direction changing or side-flipping frequency (PC_{L3}: post. mean = 0.232, 95% CI = [-0.222, 0.665], $p = 0.298$, plot not shown).

3.3. Relationships between morphology and locomotion

Using phylogenetically corrected regressions, we showed significant relationships between morphology and locomotion (Table 4). Scores on PC_{M1} and PC_{M2} positively related to scores on PC_{L1}. Namely, larger body size, deeper ventral channel and longer appendages (PC_{M1} and PC_{M2}) predicted fast movement, at times in upright or semi-upright position resembling walking (PC_{L1}). On the other hand, smaller body size, shallower ventral channel and shorter appendages predicted slow crawling. The complex model that included both PC_{M1} and PC_{M2} as predictors was substantially better than either of the two simple models that included only one of these predictors (a difference in Bayes factors between the simple and complex models were > 28 , meaning a very strong support for the complex model, see Pagel et al., 2004).

Scores on PC_{M1} and PC_{M2} negatively correlated to PC_{L2}. Thus, the tendency for swimming and tail-flipping was associated with smaller body size, shallower coxae and shorter appendages. The complex model had a higher marginal likelihood than either of the two simple ones (a difference in Bayes factors between the simple and complex models were > 19).

Finally, PC_{M1} scores significantly and negatively correlated to PC_{L3} scores (changing direction and side flipping). Hence, frequent changes of direction and side-flips were associated with smaller bodies, shallower coxae and shorter appendages. The PC_{M2} scores did not predict PC_{L3} scores. Accordingly, a simpler model with only PC_{M1} as a predictor was favored over the more complex model that included both morphological PCs as predictors. The latter model had a lower marginal likelihood and the differences in Bayes factors between more complex and more simple models were negative.

4. Discussion

We have shown that closely related *Niphargus* species differ in morphology and locomotion according to their habitat use and thereby provided support for our main hypothesis. Specifically, the small and

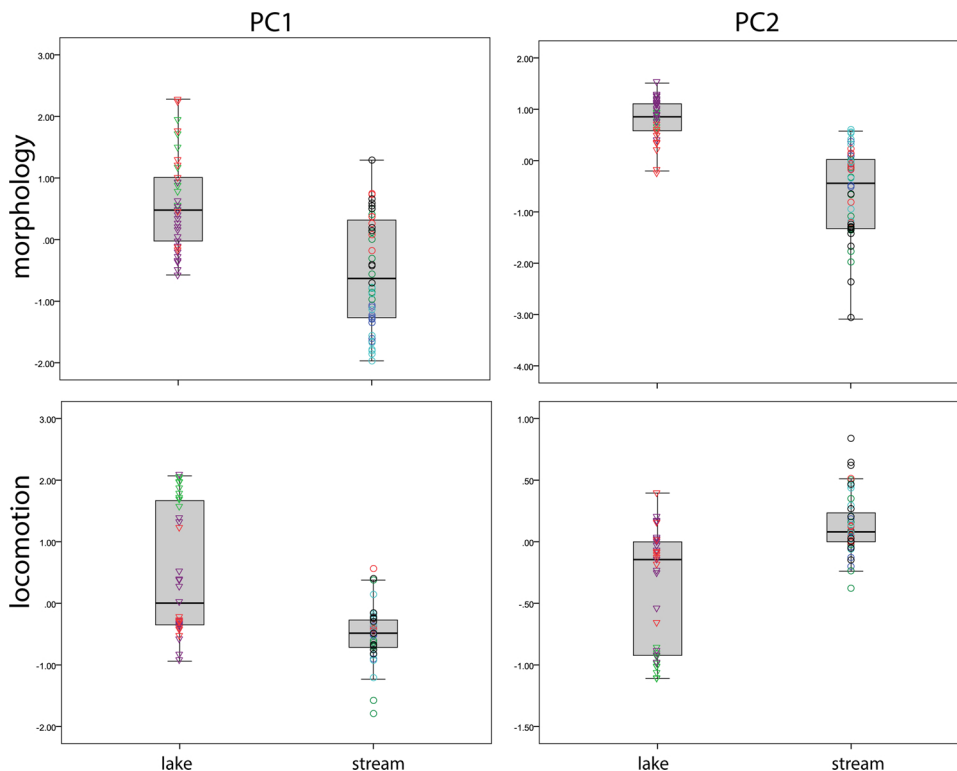


Fig. 2. Scores of principal components 1 (left) and 2 (right) for morphology (upper) and locomotion (bottom) in lake and stream animals. Lake and stream animals significantly differed in their respective scores. Triangles indicate lake species (green – *N. croaticus*, red – *N. pachytelson*, violet – *N. longiflagellum*) whereas circles indicate stream species (blue – *N. brachytelson*, green – *N. dalmatinus*, black – *N. spinulifemur* and red – *N. podpecanus*).

Table 4
A summary of the best regression models using PGLS. We report mean values.[†]

Response variable (locomotion)	Predictors (morphology) in the optimal model [†]	Log marginal likelihood	Intercept [‡]	Regression coefficient(s) ± SE [§]	Sum of squared error	R ²
Fast walkers vs. slow crawlers (PC _{1,1})	All traits (PC _{M1} , PC _{M2})	-53.14	-0.08 ± 0.03	0.44 ± 0.05 (PC _{M1})	8.15	0.80
Swimmers vs. walkers (PC _{1,2})	All traits (PC _{M1} , PC _{M2})	7.95	-0.09 ± 0.01	-0.17 ± 0.03 (PC _{M1})	1.75	0.83
Flippers vs non-flippers (PC _{1,3})	Size and appendage lengths (PC _{M1})	-30.16	-0.07 ± 0.02	-0.18 ± 0.04 (PC _{M1})	5.52	0.28

[†] Bayes Traits estimates all parameters in the model, including standard errors. We report mean values of all parameters.
[‡] We report the model that the best fits the data. Selection of the models was made by comparison of Bayes factors. We report only significant models, i.e., models where regression coefficients do not cross zero point.
[§] We report means of estimated values of coefficients and estimations of standard error.

short-legged species from streams mainly moved by crawling on the side, tail-flipping or swimming and were relatively slow. Large and long-legged lake species were faster than stream species and showed a greater variation in type of locomotion; one of the species (*N. croaticus*) almost exclusively walked in an upright or semi-upright position. Furthermore, we found significant relationships between morphology and locomotion, i.e., large and long-legged species move faster, possibly in upright position. Thus, body size and appendage length can be used as surrogates for locomotion in future evolutionary or macroecological studies. For example, studies that account for dispersal can use body sizes and appendage lengths as proxies for species' dispersal capacity, which can be explicitly incorporated into statistical models as predictors of the gene-flow or species range sizes.

It has been previously suggested, albeit not empirically tested, that the elongated appendages in cave animals evolved to enhance the extraoptic sensory capacity (Langecker, 2000; Culver and Papan, 2009). Results of the present study broaden this explanation, by hinting that evolution of the elongated appendages might have been intertwined with the modifications of the locomotion mode, i.e., from crawling and swimming to walking. The amphipod body is laterally flattened, with a high center of gravity and hence relatively unstable in an upright position. The most common types of locomotion are crawling and swimming, i.e., modes that minimally use pereopods (Dahl, 1977). Lake species evolved elongated and laterally extended appendages, in particular pereopods V-VII. These apparently act as side anchors, which stabilize the body in an upright position and enable a different type of locomotion, walking. Movement in semi-upright position in the absence of currents was on average a faster and likely more efficient mode of propulsion than crawling or tail-flipping for these species. Hence, we propose that selection for efficient movement in lakes has acted in parallel to selection for enhanced extraoptic sensory equipment. Noteworthy, such upright posture was observed in many other *Niphargus* lake species (e.g., *N. balcanicus* Absolon, 1927; *N. orcinus* Joseph, 1869). An analogous example to *Niphargus* may be the Mexican tetra (*Astyanax mexicanus* De Filippi, 1853), a fish species with surface and subterranean populations. In nature, surface populations with eyes feed on the water surface. When transferred to darkness, these populations switch to a rather ineffective way of bottom feeding. They swim with their body axes oriented almost perpendicularly to the ground, thus only few chemoreceptors at the tips of their lips can explore the lake bottom. This swimming posture makes swimming inefficient and at the same time the sensory apparatus is poorly exploited. By contrast, cave populations swim at an approximate 45° angle when feeding at the bottom. This posture makes swimming more energetically effective. In addition, it enhances the exploration of the lake bottom using the entire widened lower jaw that bears numerous chemoreceptors. Hence, a switch in the body posture during swimming maximizes both, the energetic efficiency in locomotion and chemical reception of the environment (Schemmel, 1980; Hüppop, 2000; Langecker, 2000; Protas and Jeffery, 2012).

In streams, on the other hand, water currents have selected against long appendages (Trontelj et al., 2012; Delić et al., 2016). Moreover, stream environment selects for slender body shape as this enables sheltering in crevices during the floods. Short appendages, especially in combination with a slender or worm-shaped body, might have precluded the shift into the upright posture. Hence, stream animals retained the typical amphipod locomotion, i.e., crawling, tail-flipping and occasional swimming (Dahl, 1977). We note, however, that the effects of water currents on locomotion as well as on appendage damage remains to be studied in an explicit experimental framework. Nevertheless, we hypothesize that evolution of appendage length is much more complex than previously proposed, and under at least two different selection forces (sensory and locomotion), the relative strengths of which vary in different microhabitats. The relative strengths and hierarchy among selection forces remain to be tested.

The present study is the first to empirically investigate locomotion

in cave invertebrates. Despite being theoretically well-founded (Boudrias, 1991), explicit studies on amphipod locomotion behavior are rare. The most relevant for comparison with the findings of our study are the in situ recordings of two *Princaxelia* Dahl, 1959 species from Pacific depths of 7700–9300 m (Jamieson et al., 2012). The comparison of *Niphargus* and *Princaxelia* is based on the ecological similarities between the deep sea and caves. Both habitats share the absence of light and patchy food distribution (Parzefall, 1996; Martens and Danielopol, 1999; Poulson, 2001). Both deep sea species have long legs and move by walking in an upright position (inferred from Fig. 2 in Jamieson et al. (2012)), and thus resemble posture and locomotion of the lake *Niphargus* species. In accordance to our results in *Niphargus* species, both marine amphipod species showed a positive correlation between body size and speed (Jamieson et al., 2012). Hence, striking similarities emerging between the cave lake and deep sea amphipods from different families may imply general evolutionary principles underlying amphipod locomotion, such as a switch to the upright posture likely accompanied with walking-like locomotion in stagnant water, as well as a positive relationship between body size and speed. Future comparisons of marine, deep lake and cave lake amphipods warrant an integrative research of biomechanics, functional ecology and evolutionary biology.

Declaration of Competing Interest

Authors declare no conflict of interest.

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