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Sexual dimorphism in subterranean amphipod crustaceans covaries with subterranean habitat type

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Abstract

Sexual dimorphism can evolve in response to sex-specific selection pressures that vary across habitats. We studied sexual differences in subterranean amphipods *Niphargus* living in shallow subterranean habitats (close to the surface), cave streams (intermediate), and cave lakes (deepest and most isolated). These three habitats differ because at greater depths there is lower food availability, reduced predation, and weaker seasonality. Additionally, species near the surface have a near-even adult sex ratio (ASR), whereas species from cave lakes have a female-biased ASR. We hypothesized (a) a decrease in sexual dimorphism from shallow subterranean habitats to cave lake species because of weaker sexual selection derived from changes in the ASR and (b) an increase in female body size in cave lakes because of stronger fecundity selection on account of oligotrophy, reduced predation, and weaker seasonality. We measured body size and two sexually dimorphic abdominal appendages for all 31 species and several behaviours related to male competition (activity, risk-taking, exploration) for 12 species. Species with an equal ASR that live close to the surface exhibited sexual dimorphism in all three morphological traits, but not in behaviour. The body size of females increased from the surface to cave lakes, but no such trend was observed in males. In cave lake species, males and females differed neither morphologically nor behaviourally. Our results are consistent with the possibility that sexual and fecundity selection covary across the three habitats, which indirectly and directly, respectively, shape the degree of sexual dimorphism in *Niphargus* species.

Keywords: adult sex ratio, Amphipoda, caves, environmental gradient, fecundity selection, sexual selection, sexual size dimorphism

Introduction

Sexual dimorphism is a common phenomenon in which females and males of the same species differ phenotypically due to divergent selection on traits that increase an individual's fitness (Hunt et al., 2009; Pincheira-Donoso & Hunt, 2017). In a broad sense, sexual dimorphism is attributed to natural selection (Endler, 1986) when its components, such as sexual selection, fecundity selection, and ecological selection, operate differently between the sexes. Pre-copulatory sexual selection arises in response to competition for mates and/or mate choice, and it is typically stronger in males (Darwin, 1859; Hunt et al., 2009; Janicke et al., 2016; but see Dale et al., 2015). Fecundity selection primarily acts on females and favours traits (e.g., body size) that directly enhance their lifetime reproductive output (e.g., larger brood sizes) (Pincheira-Donoso & Hunt, 2017). Additionally, both sexes may experience sex-specific, ecological selection due to competition for food, leading to specialization in different trophic

niches, and subsequent divergence of traits between the sexes (Butler et al., 2007; Hedrick & Temeles, 1989; Slatkin, 1984). Sexual, fecundity, and ecological selection can all occur within the same species and jointly determine the degree of sexual dimorphism (Beltrán et al., 2022; Miller & Svensson, 2014; Shine, 1989).

It is difficult to quantify the relative contributions of different selection components to the evolution of sexual dimorphism (Dugo-Cota et al., 2022; Krüger et al., 2014). In comparative studies, these contributions are sometimes estimated by combining two different methodological approaches. One method is to run an analysis of male traits mapped against female traits to infer whether selection seems to predominantly act on males or females and whether the studied traits evolved in response to sexual selection on males or fecundity selection on females (Fairbairn, 1997). Sexual dimorphism emerges when a trait in one sex has a different allometry to that in the other sex (Adams et al., 2020; Fairbairn, 2007). At the clade level,

the sex under stronger selection shows greater trait variation than the opposite sex (Fairbairn, 1997; Fairbairn & Preziosi, 1994). These relationships can be tentatively inferred from a regression slope, where log-transformed male traits are plotted against log-transformed female traits. Slopes steeper than one (>1) indicate higher male variation, which has been interpreted that sexual selection on males is stronger than fecundity selection on females (Fairbairn & Preziosi, 1994). Slopes shallower than one (<1) indicate higher variation in female traits, and this has been used to infer that fecundity selection on females is stronger than sexual selection on males (Fairbairn, 1997). The former pattern (slope > 1) is known as Rensch's rule, recently re-formulated as an empirical pattern where the ratio of female to male size increases with decreasing mean body size of the species (Meiri & Liang, 2021) (for further discussions, see Adams et al., 2020; Fairbairn, 1997; Fairbairn & Preziosi, 1994).

In addition to the analysis of allometry, a second method to infer the importance of different types of selection in driving sexual dimorphism is to compare taxa living in different habitats. It is then assumed that the degree of sexual dimorphism has evolved in response to environmental factors that may generate sex-specific selection. The empirical evidence for a link between sexual dimorphism and habitat type is mixed. For instance, Caribbean frogs of the genus *Eutheolerodactylus* showed no association between habitat type and sexual size dimorphism (Dugo-Cota et al., 2022), but many other studies show that environmental conditions do predict the magnitude of sex differences (Butler et al., 2007; Herczeg et al., 2010; Morbiato et al., 2023). A particularly complex case is the evolution of habitat-driven differences in hummingbird species, where sexual dimorphism varies with altitude, but it depends on the type of vegetation cover in the habitat, and trait type. With increasing altitude, morphological sexual dimorphism decreases in the understory, dichromatism increases in open habitats, and song complexity decreases in mixed habitats (Beltrán et al., 2022). These patterns are interpreted as reflecting changes in the strength of ecological and sexual selection due to habitat differences in key environmental factors. Relating analyses of allometry and sexual dimorphism differences among habitats to environmental factors can help researchers to identify likely variation in underlying selection pressures leading to sex differences.

Closely related species occupying habitats along surface–subterranean gradients are a suitable system to study the evolution of sexual dimorphism due to environmental variation. The transition from surface to subterranean environments is characterized by a substantial change in ecological conditions over short distances (Culver & Pipan, 2019). Subterranean environments are dark, oligotrophic, and buffered from environmental fluctuations (Culver & Pipan, 2019). Subterranean communities are simpler, have fewer species (Mammola, 2019), and have lower predation pressure than their adjacent areas above ground (Manenti et al., 2023). The shift in ecological conditions with depth may affect the strength of fecundity and sexual selection and presents an opportunity to investigate whether and how ecological factors shape sexual dimorphism. Weak seasonality and lower predation may strengthen fecundity selection. Animals inhabiting low-predation environments, such as deep caves, may be selected for prolonged development due to lower pre-maturation mortality, allowing them to become larger and thus more fecund (Herczeg et al., 2010, 2012; Kozłowski, 1992). In caves, there

is also no end-of-season penalty favouring earlier breeding, making larger body size at maturation more advantageous (Stearns, 1992). The strength of sexual selection may change along the surface–subterranean gradient in response to food shortage and changes in the adult sex ratio (ASR). Food shortage has profound effects on metabolic rates and reproductive strategies, which usually differ between males and females (Arnqvist et al., 2022). Subterranean animals have a lower metabolic rate (Hervant et al., 1999), and females in general exhibit a lower metabolic rate than males (Videlier et al., 2021). In low-food environments like caves, mating likely imposes higher costs on females due to the loss of foraging time. To offset this cost, females may bolster their resistance to mating, intensifying sexual selection on males for traits that can overcome female resistance (Ortigosa & Rowe, 2002). However, in deep caves, the ASR becomes more female biased (Premate et al., 2021; Vonk & Nijman, 2006), reducing male–male competition and potentially lowering sexual selection on males.

The handful of studies examining how sexually dimorphic traits differ between surface and subterranean species have yielded mixed results. While surface and subterranean populations of crayfish showed no difference in sexual dimorphism (Taylor et al., 2010), other studies have detected changes in the degree of sexual dimorphism. Examples include surface–subterranean isopod populations, where sexual dimorphism decreased in traits related to female-guarding but increased in activity (Balázs et al., 2021; Berisha et al., 2023; Herczeg et al., 2023), and in cave fish, where females had a more regressed *tectum opticum* in brains than their surface counterparts (Eifert et al., 2015). The mechanisms underlying these differences remain unclear (Herczeg et al., 2023).

Here, we study morphological (*n* = 31 species) and behavioural traits (*n* = 12 species) of groundwater-dwelling crustaceans of the genus *Niphargus*. These species are found at the surface boundary (hereafter abbreviated as shallow subterranean habitats, SSH; Culver & Pipan, 2019), in cave streams (at intermediate depth), and in cave lakes (the most isolated habitats at the greatest depth). These habitats lie along a gradient of increasing isolation from the surface, with associated decreases in seasonality, food availability, and predation (Culver & Pipan, 2019). In *Niphargus* species, ASR ranges from roughly balanced (1:1) in SSH to strongly female biased in deep cave lakes (Premate et al., 2021). We therefore predicted that (a) female body size will increase with distance from the surface due to direct effects of environmental factors, such as reduced predation and seasonality that select for delayed maturation to increase fecundity, and (b) males from SSH will be more active, more exploratory, larger, and have longer uropods (sexually dimorphic traits, see below) than males in cave lakes due to a less female-biased ASR that increases the strength of sexual selection. As a net result, we expected a decline in sexual dimorphism with increasing isolation from the surface.

We measured body size, which affects both female fecundity (Fišer et al., 2013; Sainte-Marie, 1991) and males competitiveness (Birkhead & Clarkson, 1980; Ward, 1983). In addition, we measured morphological and behavioural traits that provide a competitive advantage against rivals when competing for mates. These include abdominal appendages, called uropods (Figure 1), which not only function as posterior antennae (mechano- and chemoreceptors) but also anchor the animal to the substrate and aid propulsion (Dahl,
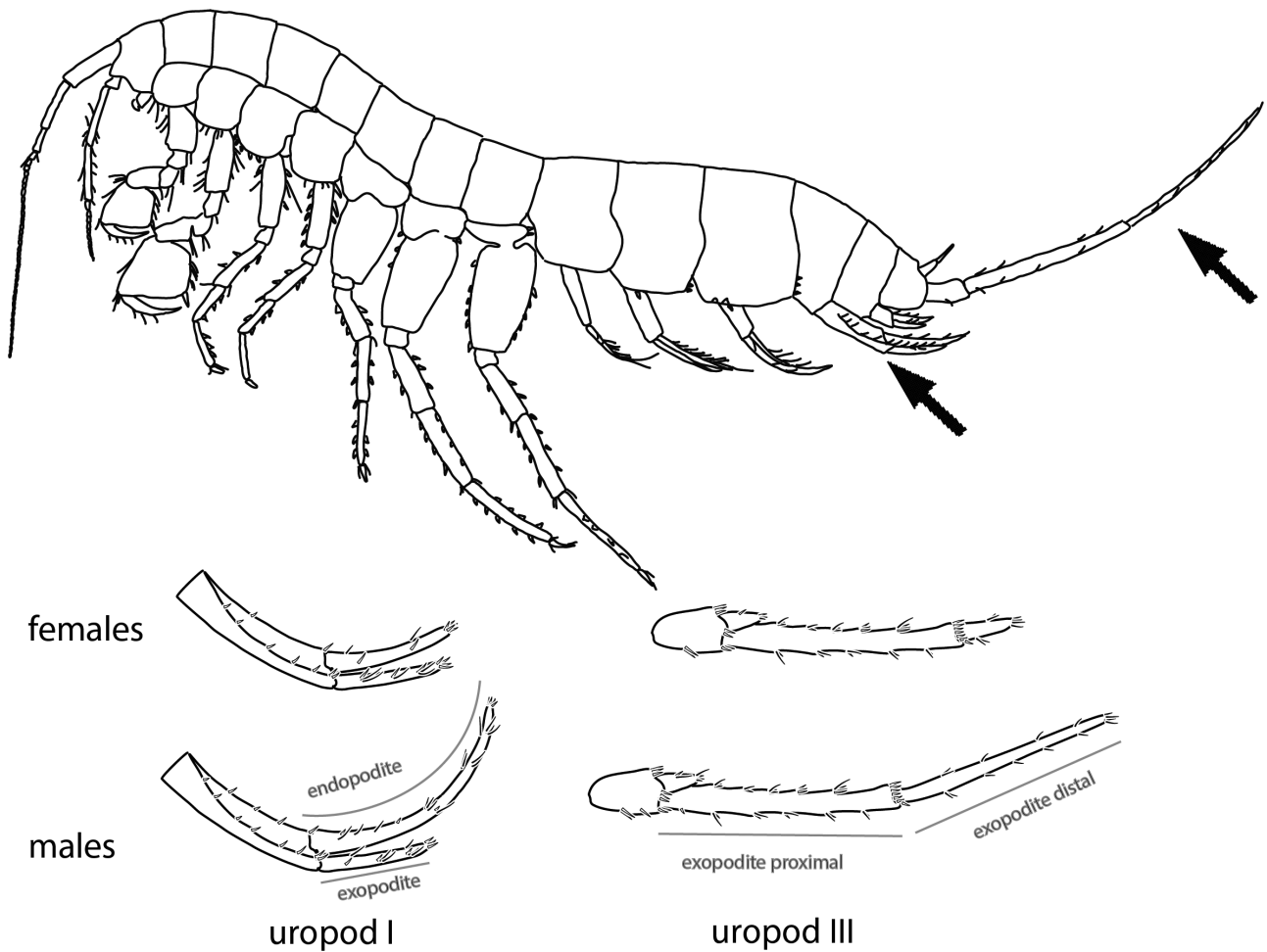
Figure 1. *Niphargus* morphology. (Top) General appearance. The arrows show the appendages that were measured. (Bottom) Cases of sexual dimorphism in uropod I (left) and uropod III (right).

1977; Kutschera et al., 2012). As such, uropods potentially contribute to more efficient movement and mate finding. We also measured several behaviours, namely activity, risk-taking, and exploration, which may help male *Niphargus* to find and monopolize females. To test whether these focal traits might have evolved in response to stronger selection on male or female traits, we explored the cross-species relationships between male and female traits. Subsequently, we used phylogenetic regression and phylogenetically corrected analysis of variance to test whether sexual dimorphism is predicted by the ASR (due to sexual selection on males) and habitat type (due to fecundity selection on females).

Materials and methods

Study system and data

We studied 31 *Niphargus* species, of which 13 were collected in SSH, 9 in cave streams, and 9 in cave lakes. Although the surface–subterranean gradient is a continuum, here we focus on three types of habitats for pragmatic reasons. Aside from depth below the surface, several other variables affect the ecological conditions we are interested in (e.g., absolute and relative amounts of surface water inflow, absolute and relative amounts of nutrient inflow, insulation from temperature fluctuations), and it is unclear how these variables should be weighted to quantify a site's precise position along the continuum. To circumvent this problem, we employ a categorical scale that, we believe, captures much of the relevant ecological variation.

We collected SSH species from springs or seepage springs, i.e., habitats with abundant food that are exposed to daily and seasonal temperature fluctuations, comprising communities with many surface predators, such as salamanders, leeches, and dragonfly larvae (Manenti et al., 2023). Cave streams represent an intermediate habitat. The streams from which we collected animals are sinking rivers that seasonally deliver organic material from the surface into cave systems (Simon et al., 2007). Cave lakes are the most isolated habitat with stagnant water, limited food availability, and buffered from environmental fluctuations.

We selected species for which ASR estimates were available, and that were as phylogenetically independent as possible, to reduce possible phylogenetic dependency in the statistics. Newly collected samples were supplemented with specimens from the collection in the Department of Biology at the Biotechnical Faculty (University of Ljubljana). In total, we measured 685 individuals, with at least five specimens per sex per species (Table 1).

To quantify activity, risk-taking, and exploratory behaviour, we studied a subset of 12 species, four per habitat type. The choice of species for our behavioural study was limited by availability. Some species are rare and could not be obtained

Table 1. Overview of species, sample sizes, means, and *SD* of measured morphological and behavioural traits.

H	Species	SR	Sex	Sample size		Morphological traits			Behavioural traits		
				Mor.	Beh.	BS	UPI	UPIII	ACT	RT	EXP
Cave lake	*N. arbiter*	**Yes**	M	9	9	15.70 ± 3.16	1.07 ± 0.06	0.12 ± 0.01	0.58 ± 0.32	0.34 ± 0.28	0.88 ± 0.15
			F	14	14	14.79 ± 2.87	1.07 ± 0.03	0.13 ± 0.02	0.78 ± 0.23	0.21 ± 0.19	0.90 ± 0.13
	N. balcanicus	**Yes**	M	6	*NA*	23.18 ± 3.49	1.16 ± 0.03	0.09 ± 0.02	*NA*	*NA*	*NA*
			F	8	*NA*	25.10 ± 3.13	1.18 ± 0.06	0.08 ± 0.02	*NA*	*NA*	*NA*
	N. croaticus	**Yes**	M	15	*NA*	19.70 ± 2.84	1.03 ± 0.03	0.09 ± 0.01	*NA*	*NA*	*NA*
			F	25	*NA*	21.25 ± 2.62	1.03 ± 0.03	0.10 ± 0.02	*NA*	*NA*	*NA*
	N. kolombatovici	No	M	8	*NA*	12.72 ± 3.58	1.13 ± 0.06	0.16 ± 0.02	*NA*	*NA*	*NA*
			F	13	*NA*	15.41 ± 3.90	1.11 ± 0.06	0.13 ± 0.02	*NA*	*NA*	*NA*
	N. longiflagellum	**Yes**	M	5	5	14.66 ± 2.05	1.09 ± 0.06	0.15 ± 0.01	0.32 ± 0.35	0.22 ± 0.34	0.65 ± 0.38
			F	26	26	16.40 ± 2.74	1.09 ± 0.06	0.13 ± 0.01	0.35 ± 0.23	0.32 ± 0.28	0.80 ± 0.21
	N. orcinus	**Yes**	M	1	*NA*	26.49	1.01	0.14	*NA*	*NA*	*NA*
			F	7	*NA*	26.04 ± 1.21	1.04 ± 0.02	0.14 ± 0.00	*NA*	*NA*	*NA*
	N. cf. pachytelson	**Yes**	M	3	3	18.65 ± 4.50	1.16 ± 0.08	0.12 ± 0.00	0.29 ± 0.26	0.23 ± 0.28	0.66 ± 0.47
			F	14	14	19.35 ± 3.87	1.11 ± 0.09	0.13 ± 0.01	0.37 ± 0.18	0.44 ± 0.19	0.85 ± 0.10
	N. subtypicus	**Yes**	M	7	7	13.85 ± 2.96	1.07 ± 0.07	0.13 ± 0.01	0.60 ± 0.33	0.10 ± 0.09	0.82 ± 0.19
			F	12	12	12.99 ± 1.49	1.05 ± 0.06	0.13 ± 0.02	0.4 0 ± 0.34	0.15 ± 0.17	0.77 ± 0.21
	N. vjetrenicensis	**Yes**	M	8	*NA*	24.81 ± 4.08	1.12 ± 0.05	0.11 ± 0.02	*NA*	*NA*	*NA*
			F	7	*NA*	25.42 ± 1.98	1.10 ± 0.05	0.11 ± 0.02	*NA*	*NA*	*NA*
Cave stream	*N. dalmatinus*	**Yes**	M	7	*NA*	17.96 ± 2.08	1.52 ± 0.11	0.37 ± 0.05	*NA*	*NA*	*NA*
			F	7	*NA*	15.41 ± 2.97	1.22 ± 0.07	0.27 ± 0.02	*NA*	*NA*	*NA*
	N. miljeticus	No	M	6	*NA*	23.83 ± 2.43	1.22 ± 0.05	0.72 ± 0.09	*NA*	*NA*	*NA*
			F	6	*NA*	21.19 ± 1.52	1.17 ± 0.07	0.17 ± 0.03	*NA*	*NA*	*NA*
	N. novomestanus	No	M	8	*NA*	14.34 ± 1.22	1.48 ± 0.12	0.61 ± 0.09	*NA*	*NA*	*NA*
			F	9	*NA*	11.79 ± 1.61	1.16 ± 0.04	0.28 ± 0.02	*NA*	*NA*	*NA*
	N. podpecanus	No	M	13	13	18.05 ± 2.31	1.57 ± 0.21	0.55 ± 0.15	0.46 ± 0.31	0.23 ± 0.22	0.81 ± 0.33
			F	17	17	16.23 ± 1.94	1.18 ± 0.10	0.32 ± 0.05	0.34 ± 0.28	0.22 ± 0.18	0.80 ± 0.25
	N. rhenorhodanensis	No	M	5	*NA*	18.65 ± 3.73	1.29 ± 0.13	0.70 ± 0.15	*NA*	*NA*	*NA*
			F	5	*NA*	16.10 ± 1.44	1.10 ± 0.03	0.34 ± 0.05	*NA*	*NA*	*NA*
	N. scopicauda	No	M	13	13	13.08 ± 2.39	1.00 ± 0.10	0.76 ± 0.19	0.88 ± 0.11	0.10 ± 0.12	0.94 ± 0.14
			F	17	17	11.47 ± 2.26	1.03 ± 0.03	0.48 ± 0.07	0.90 ± 0.16	0.08 ± 0.05	0.94 ± 0.14
	N. spoeckeri	No	M	19	11	19.61 ± 3.27	1.53 ± 0.23	0.44 ± 0.14	0.61 ± 0.21	0.18 ± 0.14	0.91 ± 0.07
			F	11	9	13.94 ± 1.48	1.08 ± 0.03	0.24 ± 0.02	0.73 ± 0.28	0.12 ± 0.13	0.87 ± 0.18
	N. stygius	No	M	27	22	16.87 ± 3.28	1.31 ± 0.14	0.72 ± 0.24	0.52 ± 0.30	0.10 ± 0.07	0.90 ± 0.09
			F	23	18	14.47 ± 2.24	1.13 ± 0.04	0.26 ± 0.05	0.59 ± 0.27	0.10 ± 0.05	0.95 ± 0.08
	N. zagrebensis	No	M	9	*NA*	25.79 ± 4.60	1.63 ± 0.21	0.65 ± 0.15	*NA*	*NA*	*NA*
			F	6	*NA*	17.27 ± 2.19	1.25 ± 0.09	0.32 ± 0.03	*NA*	*NA*	*NA*
SSH	*N. cf. dalmatinus*	No	M	7	*NA*	14.29 ± 3.07	1.77 ± 0.18	0.54 ± 0.14	*NA*	*NA*	*NA*
			F	7	*NA*	10.94 ± 1.07	1.20 ± 0.03	0.29 ± 0.04	*NA*	*NA*	*NA*
	N. hadzii	No	M	7	*NA*	23.45 ± 4.29	1.44 ± 0.17	0.28 ± 0.05	*NA*	*NA*	*NA*
			F	11	*NA*	18.86 ± 3.36	1.19 ± 0.11	0.18 ± 0.02	*NA*	*NA*	*NA*
	N. hrabei	No	M	15	*NA*	5.97 ± 1.18	1.14 ± 0.03	0.22 ± 0.04	*NA*	*NA*	*NA*
			F	15	*NA*	7.60 ± 1.70	1.11 ± 0.02	0.21 ± 0.02	*NA*	*NA*	*NA*
	N. hvarensis	No	M	4	*NA*	18.79 ± 2.54	1.12 ± 0.05	0.69 ± 0.10	*NA*	*NA*	*NA*
			F	4	*NA*	17.54 ± 3.03	1.08 ± 0.03	0.23 ± 0.03	*NA*	*NA*	*NA*
	N. illidzensis	**Yes**	M	5	*NA*	20.05 ± 5.46	1.62 ± 0.11	0.41 ± 0.11	*NA*	*NA*	*NA*
			F	7	*NA*	12.89 ± 2.05	1.26 ± 0.12	0.22 ± 0.04	*NA*	*NA*	*NA*
	N. krameri	No	M	24	24	20.67 ± 4.15	1.33 ± 0.25	0.39 ± 0.13	0.12 ± 0.06	0.27 ± 0.34	0.67 ± 0.30
			F	11	11	17.32 ± 3.07	1.18 ± 0.03	0.23 ± 0.04	0.13 ± 0.12	0.24 ± 0.37	0.59 ± 0.36
	N. cf. longicaudatus	No	M	7	*NA*	16.16 ± 2.74	1.22 ± 0.20	0.85 ± 0.09	*NA*	*NA*	*NA*
			F	7	*NA*	10.34 ± 0.98	1.08 ± 0.05	0.31 ± 0.10	*NA*	*NA*	*NA*

Table 1. Continued

H	Species	SR	Sex	Sample size		Morphological traits			Behavioural traits		
				Mor.	Beh.	BS	UPI	UPIII	ACT	RT	EXP
	N. sanctinaumi	Yes	M	5	*NA*	16.19 ± 3.45	1.08 ± 0.02	0.49 ± 0.15	*NA*	*NA*	*NA*
			F	7	*NA*	13.71 ± 1.56	1.16 ± 0.06	0.22 ± 0.03	*NA*	*NA*	*NA*
	N. slovenicus	No	M	8	*NA*	16.86 ± 2.89	1.71 ± 0.19	0.70 ± 0.12	*NA*	*NA*	*NA*
			F	9	*NA*	11.51 ± 0.97	1.20 ± 0.09	0.35 ± 0.03	*NA*	*NA*	*NA*
	N. sphagnicolus	No	M	10	10	12.61 ± 2.44	1.48 ± 0.21	0.92 ± 0.14	0.24 ± 0.29	0.22 ± 0.22	0.71 ± 0.25
			F	20	20	9.91 ± 1.27	1.12 ± 0.07	0.39 ± 0.07	0.24 ± 0.19	0.13 ± 0.14	0.71 ± 0.34
	N. spinulifemur	No	M	20	20	19.82 ± 4.33	1.63 ± 0.24	0.58 ± 0.16	0.09 ± 0.08	0.09 ± 0.12	0.49 ± 0.37
			F	10	10	13.64 ± 0.93	1.24 ± 0.05	0.25 ± 0.03	0.10 ± 0.09	0.11 ± 0.19	0.52 ± 0.39
	N. timavi	No	M	26	20	14.17 ± 2.63	1.81 ± 0.30	0.67 ± 0.16	0.29 ± 0.26	0.08 ± 0.16	0.73 ± 0.31
			F	17	10	10.16 ± 1.18	1.21 ± 0.06	0.33 ± 0.05	0.35 ± 0.27	0.15 ± 0.25	0.82 ± 0.18
	N. valachicus	No	M	17	*NA*	9.48 ± 1.16	1.18 ± 0.04	0.23 ± 0.04	*NA*	*NA*	*NA*
			F	17	*NA*	11.4 ± 2.23	1.21 ± 0.09	0.20 ± 0.03	*NA*	*NA*	*NA*

Note. ACT = activity; beh. = behavioural traits; BS = body size; EXP = exploration; F = females; H = habitat; M = males; mor. = morphological traits; RT = risk-taking; SR = female-biased sex ratio; UPI = uropod I ratio; UPIII = uropod III ratio. NA in species and traits we did not measure. Note that the sample size given is the maximum sample size and is not always the same across all traits (due to damage), and this is accounted for in calculations of means and *SD*. Complete dataset is available at Zenodo: https://zenodo.org/records/10006270.

in sufficient numbers. We video-recorded 335 individuals (Table 1). Sex ratio data are from Premate et al. (2021) and are expressed as the proportion of males.

To correct for phylogenetic dependence in our analyses, we used the most recent phylogeny of *Niphargus* (513 species and 7 loci subjected to Bayesian inference, calibrated using four calibration points, details in Borko et al., 2022). To account for phylogenetic uncertainty, we drew 100 random trees from the stationary phase of the Bayesian analysis, from which we pruned all non-studied species.

A dataset with the origin of species, morphological and behavioural data, as well as the reference phylogeny and the R script used for all analyses is deposited in Zenodo at https://zenodo.org/records/10801507.

Morphology

We used standard landmarks to quantify (a) body length, (b) relative length of the endopodite to exopodite of uropod I, and (c) relative length of the distal to proximal articles of the exopodite of uropod III (Figure 1, Fišer et al., 2009). Animals were photographed under an Olympus SZX9 stereomicroscope mounted with a ColorView III camera. We made measurements using the Olympus cellSens Entry programme.

Amphipods have indeterminate growth, so estimating the mean body size of a species is challenging. We identified the largest individuals in the samples. Individuals smaller than 1/3 of the largest individual of either sex were excluded from the analyses. Despite uncertainty about the maximum size of a given species, this approach reduces underestimation of mean body size due to the potential erroneous inclusion of subadults. We measured body length three times and used the mean length per individual.

Uropods are the three posterior-most pairs of appendages (Figure 1). Sexual dimorphism in uropod I (elongated endopodite vs. exopodite) and uropod III (elongated terminal vs. exopodite article) develops only in adult males: Juvenile and subadult males do not differ from females (Fišer et al., 2008). We expressed the development of uropods as two ratios. For

uropod I, we calculated the ratio of the endopodite to exopodite, and for uropod III, we calculated the ratio of the distal to proximal exopodite article.

Behaviour

We brought animals collected in the field into the cave laboratory of the University of Ljubljana (no light, 11–13 °C). We kept all individuals of the same species for 3–30 days in a common container in dechlorinated tap water, which we changed regularly, and fed them lab-raised potworms (Enchytraeidae) (details in Supplementary Table S1). Five days prior to recording their behaviour, we allocated each individual to its own Petri dish (90 × 15 mm) with dechlorinated tap water and food. We removed the food 1 day prior to recording.

We video-recorded animals for 1 hr in the dark. We placed up to 36 individuals (each in its own Petri dish) onto a custom-built infrared light (920-nm) emitting panel (Berisha et al., 2023), which allowed us to record undisturbed animals (Fišer et al., 2016). The light source did not increase the water temperature (data not shown). Petri dishes were randomly distributed across the panel with respect to an individual's sex and species identity. We made recordings using webcams (Logitech C920) and modified to improve the quality of videos recorded under infrared (Berisha et al., 2023; Horváth et al., 2023). We simultaneously employed three webcams; each captured 12 individuals at 5 frames/s at FullHD resolution (1920 × 1080 pixels). We used the open-source software Bonsai 2.6.3 (Lopes et al., 2015) to drive the webcams and save the videos.

We performed video-tracking in Bonsai 2.6.3 and extracted behavioural parameters using a custom script in R 4.1.1 (R Development Core Team, 2022). We used the proportion of time that the animal changed location with its whole body as a proxy for general movement activity (hereafter activity). We also calculated the proportion of time an individual spent near the wall of the Petri dish and in the central zone of the Petri dish, either active or resting. These two zones were of the same size (the diameter of the central zone was 6 cm). We

used the time spent in the central zone as a proxy for risk-taking behaviour (Kohler et al., 2018) (hereafter risk-taking). For our third measure, used as a proxy for exploratory behaviour, we quantified the proportion of the Petri dish that an animal visited within an hour (hereafter exploration). We projected a 10 × 10 square grid with cell size of 8.5 mm over the bottom of the Petri dish and counted the number of cells visited. Cells whose area was not entirely within the Petri dish were weighted by their reduced size in the final proportion calculation.

Data analyses

Relationship between male and female traits

To evaluate whether traits seem most likely to have evolved in response to sex-specific selection, we analysed the relationships between males and females for each trait separately. We regressed log-transformed male means onto log-transformed female means under the null expectation of an isometric relationship (slope coefficient = 1) (Fairbairn & Preziosi, 1994; Sztepanacz & Houle, 2021).

We used phylogenetically corrected reduced major axis (RMA) regression (Fairbairn, 2007; Fairbairn & Preziosi, 1994) using the phytools package (Revell, 2012). To check the robustness of conclusions obtained by RMA residuals (Meiri & Liang, 2021), we reran the analyses as a phylogenetic generalized least squares model (PGLS) using the estimated Pagel's λ to account for phylogenetic correlation using the nlme package (Pinheiro & Bates, 2022). As the results did not differ qualitatively, we report only RMA; results of PGLS are available in R script deposited in Zenodo at https://zenodo.org/records/10801507.

Phylogenetic regressions

To test whether sexual dimorphism in morphological and behavioural traits covaries with the ASR, we ran a series of PGLS. In all models, we used ASR as the predictor and the degree of sexual dimorphism in the focal morphological or behavioural trait as the response variable. ASR was expressed as the proportion of males (Premate et al., 2021). The degree of trait dimorphism was expressed as the sexual dimorphism index (hereafter SDI) calculated as [(mean trait value in males/mean trait value in females) – 1] (Fairbairn, 2007). We tested all three morphological traits (body size and the two uropod length ratios) and three behavioural traits (activity, risk-taking, exploration). Positive index values suggest that sexual dimorphism of a trait is biased in favour of males and negative values that it is biased in favour of females.

Phylogenetic ANOVA

To test whether habitat type accounts for variation in sexual dimorphism across species, we used phylogenetic ANOVA assuming trait evolution modelled by Brownian motion. We performed phylogenetic ANOVAs using the phylANOVA function from the phytools R package (Revell, 2012), with species' habitat (SSH, cave stream, cave lakes) as the predictor variable and morphological and behavioural traits as the response variables. Doing so, we simplified modelling of the behavioural traits by exclusion of random factors (date of collection, time of acclimation) and thus avoided model over-parametrization. For each trait, we ran three separate analyses: for males, females and for the SDI. While the evolution of a trait is not independent for males and females if there is a cross-sex genetic correlation, separate analyses for

each sex offer insights as to which sex showed greater trait evolution. In contrast, SDI is a composite measure derived from female and male measures that can be considered as a property of a species. SDI is a measure of the net sexual dimorphism in a trait, but it is not diagnostic of the underlying evolutionary processes. The phylANOVA for each response variable was run 100 times to account for phylogenetic uncertainty (see above). We extracted the mean F-statistic, mean p-value, and mean corrected p-value from 100 runs for each response variable. In post hoc pairwise comparison tests, we adjusted p-values via the method by Holm (1979).

Results

Relationship between male and female traits: test of Rensch's rule

Sex-specific distributions of morphological and behavioural traits for each species are available in Supplementary Material (Supplementary Figures S1 and S2). The analysis of log-transformed male traits plotted against log-transformed female traits showed that male variation in uropods I and III exceeds variation in females (regression slope > 1, $p < 0.05$), which is in agreement with Rensch's rule. In contrast, the regression slope in the male–female relationship did not deviate from 1 for body size or any of the three behavioural traits (Table 2, Figure 2).

Phylogenetic regressions: does ASR predict degree of sexual dimorphism?

ASR predicts sexual dimorphism in both uropods I ($p = 0.02$) and uropods III ($p = 0.03$) (Figure 3, Table 3). Species with a female-biased ASR tend to have sexually monomorphic uropods, whereas species with a more even ASR are sexually dimorphic for both uropods. In contrast, sexual dimorphism in body size or the three behavioural traits showed no significant relationship with the ASR.

Phylogenetic ANOVA: does sexual dimorphism covary with habitat?

The magnitude of sexual dimorphism in morphology, but less so in behaviour, can be explained by habitat type (Figure 4, Supplementary Figure S3, Table 4). For males, uropod I differed significantly among the three habitats, with the greatest elongation in males from SSH ($p = 0.036$). However, none of the pairwise differences between the three habitats reached statistical significance after corrections for phylogenetic dependence and

Table 2. Results of reduced major axis regressions of log-transformed male traits against log-transformed female traits (morphological and behavioural).

Trait	R^2	Slope	T-statistics	p-Value
Body size	0.70	1.04	0.41	0.69
Uropod I	0.35	2.26	5.46	**<0.001**
Uropod III	0.74	1.71	5.69	**<0.001**
Activity	0.86	0.99	0.03	0.98
Risk-taking	0.32	1.02	0.099	0.92
Exploration	0.65	1.09	0.44	0.67

Note. Slopes that deviate statistically significantly from 1 (bolded) are interpreted as indicating differential selection on males and females.
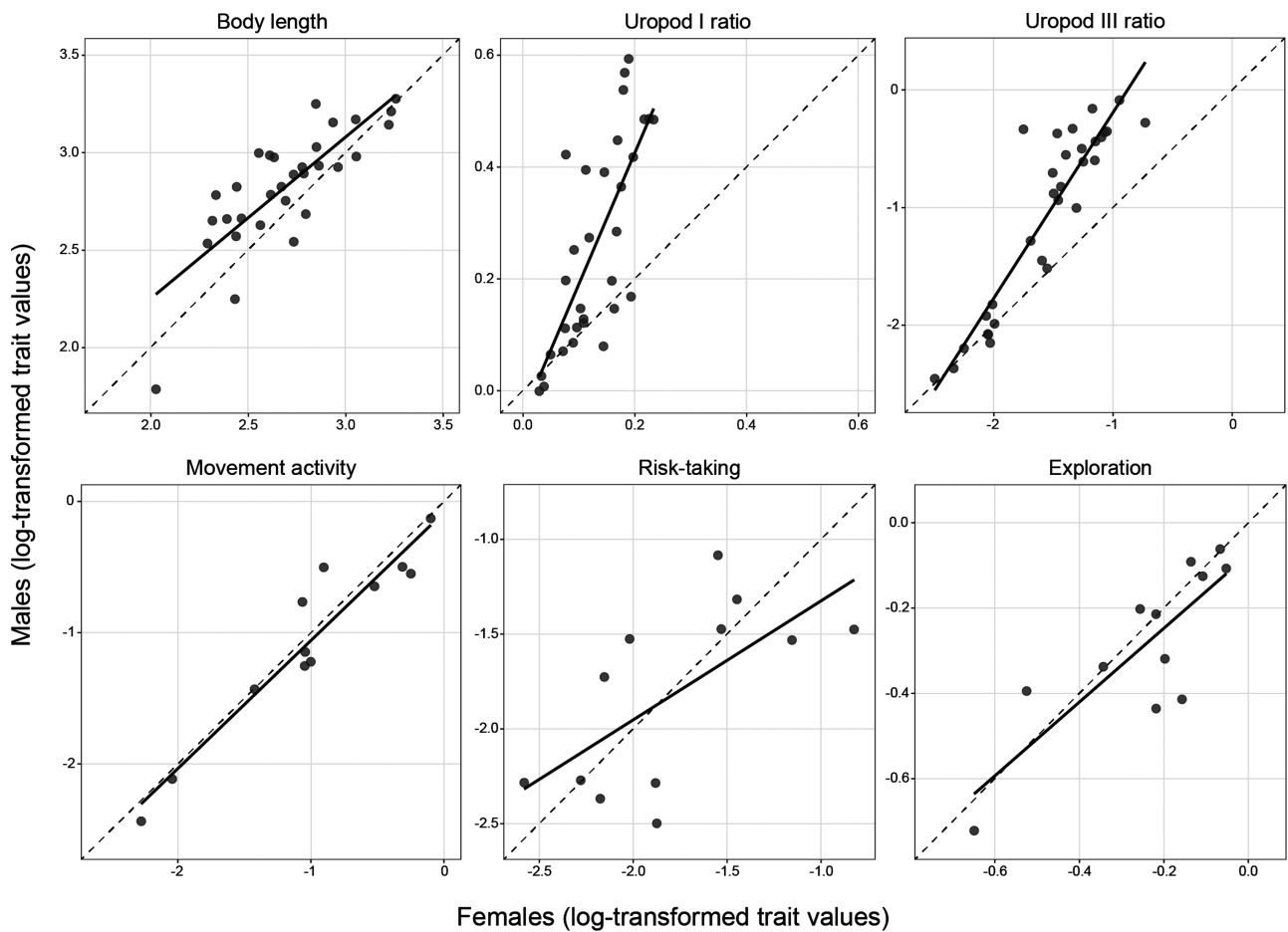
Figure 2. Log-transformed male traits regressed against log-transformed female traits. Each trait was modelled separately using reduced major axis regression, taking into account phylogenetic relationships. The slope was statistically significantly steeper than 1 only in uropod I and uropod III, matching Rensch's rule.

multiple comparisons. The greatest contrast was between males from cave lakes and SSH ($p = 0.066$). For females, uropod I did not differ significantly among the three habitats. The habitat effect on SDI in uropod I indicated a marginally non-significant trend of sexual dimorphism differing among the three habitats ($p = 0.057$), being greatest in SSH.

Uropod III differed significantly among habitats for both males ($p = 0.001$) and females ($p = 0.002$), as did SDI ($p = 0.015$). For both sexes, pairwise comparisons of habitats showed that species from the cave lakes had a significantly shorter terminal article of uropod III, whereas species from cave streams and SSH did not differ from each other (Table 4).

Female body size differed significantly among the three habitats ($p = 0.027$), but male body size did not. The largest females were in cave lakes and the smallest in SSH (difference; $p = 0.035$), whereas females from cave streams were of intermediate size and did not differ significantly in size from females from the other two habitats (Table 4). The effect of habitat on SDI in body size was significant ($p = 0.039$). However, none of the pairwise differences was statistically significant after phylogenetic correction and correction for multiple comparisons. The greatest difference was between species from cave lakes and SSH ($p = 0.068$).

The only statistically significant behavioural difference among habitats was in male activity ($p = 0.034$). Males

of species from SSH were less active than those from cave streams ($p = 0.022$). Males from SSH and cave lakes, or from cave lakes and cave streams, did not differ significantly in their behaviour.

Discussion

We found substantial variation in sexual dimorphism in morphological, but not behavioural traits in *Niphargus*. The degree of sexual dimorphism in morphological traits varied across the three habitats (SSH, cave streams, cave lakes). The most pronounced sexual dimorphism was found in species from habitats closer to the surface (SSH, cave streams), with larger males and more enlarged appendages compared to those of females. The results of different analyses were broadly congruent with the idea that sexual dimorphism evolved in response to both sexual selection on males and fecundity selection on females.

Variation in the two uropod appendages suggests that both these traits evolved in response to selection on males, as variation across species is higher in males than in females. Species living in habitats well connected to the surface (cave streams, SSH) were sexually dimorphic, but those from cave lakes were not. As sexual dimorphism in these traits covaries with the ASR, we suggest that one potential driver of evolutionary change in uropods is sexual
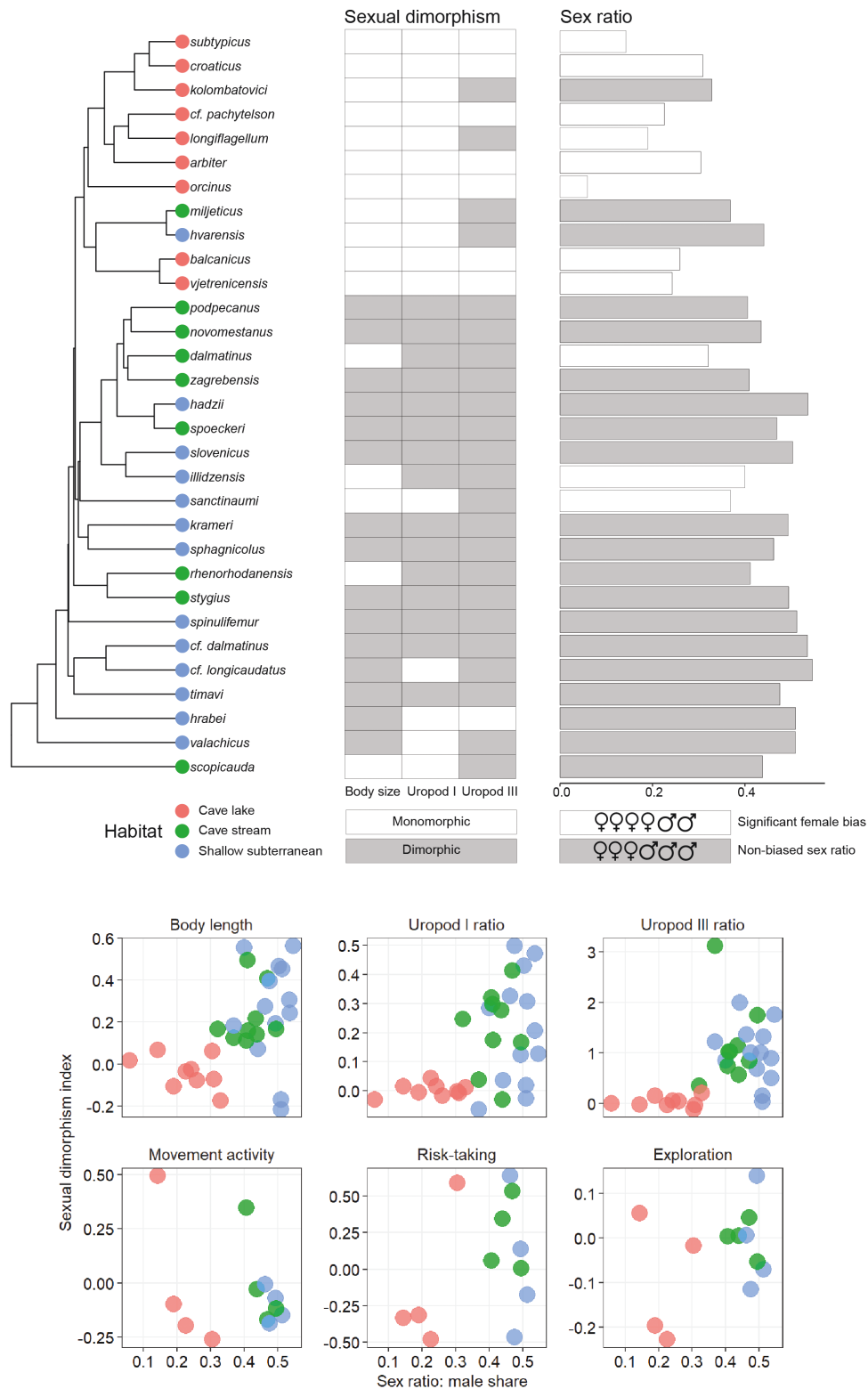
Figure 3. (Upper left) Phylogenetic relationships of the studied *Niphargus* species from three habitats (shallow subterranean habitats, cave stream, cave lake). (Upper right) Adult sex ratio (ASR) of the species expressed as the share of males. (Bottom) The relationship between ASR and degree of sexual dimorphism expressed as a sexual dimorphism index for morphological and behavioural traits.

selection on males. Additionally, water currents generally select for relatively short legs and antennae (Delić et al., 2016). Species with males with more elongated uropods than females live in places with stronger currents, implying a potential conflict between drag reduction and sexual

selection. Both sexes showed significant habitat-related variation in the length of the terminal article of uropod III, but this variation was significantly larger in males (Figure 4). This suggests that net selection is stronger on males than on females.

Table 3. Results of phylogenetic generalized least squares model, testing whether adult sex ratio predicts the degree of sexual dimorphism of a trait.

Trait	Pagel's λ	Slope	T	p-Value
Body size	1.05	0.20	0.66	0.51
Uropod I	1.10	0.48	2.39	**0.02**
Uropod III	0.81	2.41	2.27	**0.03**
Activity	−0.49	−0.66	−1.42	0.18
Risk-taking	−0.80	1.08	1.48	0.16
Exploration	−0.54	0.35	1.76	0.11

Note. ASR was expressed as the share of males, and the degree of sexual dimorphism was expressed as a sexual dimorphism index (SDI = (mean trait value in males/ mean trait value in females) − 1). Statistically significant *p*-values are bolded.

In contrast to uropod length, sexual size dimorphism might have evolved mainly as a response to stronger habitat-related selection on females, although the evidence for this claim is inconsistent. The regression of male body size on female body size showed no deviation from isometry. However, inspection of male and female body size differences among the three habitats showed that variation in male size is not associated with habitat type, while female size is: Females in cave lakes are significantly larger than those in SSH ([Figure 4](#)). The observed sex difference in the effect of habitat on body size suggests that the lower sexual size dimorphism in cave lake species can be attributed to increased female body size rather than reduced male size. We suggest that greater female size in cave lakes is probably selected because it increases lifetime fecundity, while greater male body size might be constrained by food limitations. It is tempting to suggest that there are also reduced benefits of greater male size in cave lakes due to weaker sexual selection ([Blanckenhorn, 2000](#)). In support of this, uropod dimorphism is reduced in cave lakes, and uropod length has been linked to sexual selection. However, the ASR—which is a widely used predictor of the strength of sexual selection ([Kappeler et al., 2022](#); [Schacht et al., 2022](#))—did not predict the magnitude of sexual size dimorphism. We therefore tentatively conclude that the most likely evolutionary driver of body size dimorphism is fecundity selection, which presumably covaries with habitat type.

Contrary to our predictions, we did not detect sex-specific evolution of behaviours deemed important in male–male competition for females. We expected stronger dimorphism in SSH species due to their more male-biased ASR. The only exception was slightly higher activity of males from cave streams than SSH, which was against our predictions. Apart from low sample sizes (four species per habitat), several factors could contribute to our findings. First, we might have collected samples at an inappropriate time. If reproduction is seasonal for species from the SSH, we might have underestimated sexual differences in behaviours associated with reproduction. Unfortunately, there are little data about general phenology for species in these habitats ([Copilaş-Ciocianu & Boroş, 2016](#); [Fišer et al., 2007](#)). Second, if metabolically costly behaviours are reduced under food limitation (e.g., aggressive behaviour in cave mollies; [Plath et al., 2004](#)), sexual dimorphism might disappear. However, the starvation capacity of *Niphargus* ([Hervant et al., 1999](#)) implies that reduction of male sexual behaviours due to food availability is unlikely.

Third, we cannot rule out that our experimental design or differences in time of acclimation (due to species availability) were responsible for our failure to detect behavioural variation detected in other studies ([Berisha et al., 2023](#); [Horváth et al., 2023](#)).

Our conclusions should be treated with caution. The evidence that sexual dimorphism evolved in response to sexual and fecundity selection acting predominantly on males and females, respectively, is indirect. This is due to the correlative nature of comparative analyses and the usage of indirect proxies for sexual and fecundity selection instead of measuring both. That said, the conclusions of our study could be strengthened by additional comparative analyses that test for sex differences in the rates of evolution of the studied traits. The analysis of evolutionary rates is, however, sensitive to incomplete taxon sampling. Incomplete taxon sampling is common in subterranean species and could potentially impact some aspects of the present study. Our current dataset is limited by the availability of samples that allow estimation of ASR and sexual dimorphism. Another issue is that sample sizes can influence estimates of adult morphological traits, particularly if subadults were inadvertently included in samples (see *Materials and methods*). However, sexual dimorphism in *Niphargus* is usually male biased and emerges due to exaggerated male traits (body size, uropods), so a potential bias could occur only upon systematic error, e.g., all males were subadult. This type of sampling error would underestimate sex differences in sexually dimorphic species and would affect conclusions if more prevalent when sampling certain habitats. There is, however, no obvious natural history or logistic reason to expect systematic bias across sex and ecological category.

The results of our analyses of the three morphological traits integrate into a consistent argument that sexual dimorphism in *Niphargus* has evolved in response to decreased selection on males and increased selection on females from surface to deep subterranean habitats. This conclusion should be treated with caution due to the correlative nature of our analyses, untested assumptions, and indirect inferences of the strength of selection. Given these caveats, we tentatively propose that our finding of less sexual dimorphism with greater isolation from the surface is one of the few expected patterns of variation of sexual dimorphism in groundwater animals. To generalize our results, below we reconsider the biology of groundwater organisms in relation to their ecological conditions. We place special emphasis on the role of metabolism in the evolution of life-history traits and mating economics ([Arnqvist et al., 2022](#); [Carazo, 2022](#)).

Increasing isolation from the surface is associated with lower food availability and smaller environmental fluctuations. Both factors presumably determine the strength of sexual ([García-Roa et al., 2020](#); [Morbiato et al., 2023](#)) and fecundity selection ([Pincheira-Donoso & Hunt, 2017](#)), as well as metabolic rate. Oligotrophy reduces metabolic rates in all subterranean organisms ([Hervant, 2012](#); [Hervant et al., 1999](#); [Simčič et al., 2005](#)). Reduced metabolism presumably slows down physiological, behavioural, and life-history processes, the so-called pace-of-life syndrome (POLS) ([Réale et al., 2010](#)). Indeed, groundwater organisms live longer ([Lunghi & Bilandžija, 2022](#)), reach sexual maturity later, grow larger, and shift towards iteroparity ([Poulson, 1963](#); [Venarsky et al., 2023](#)).
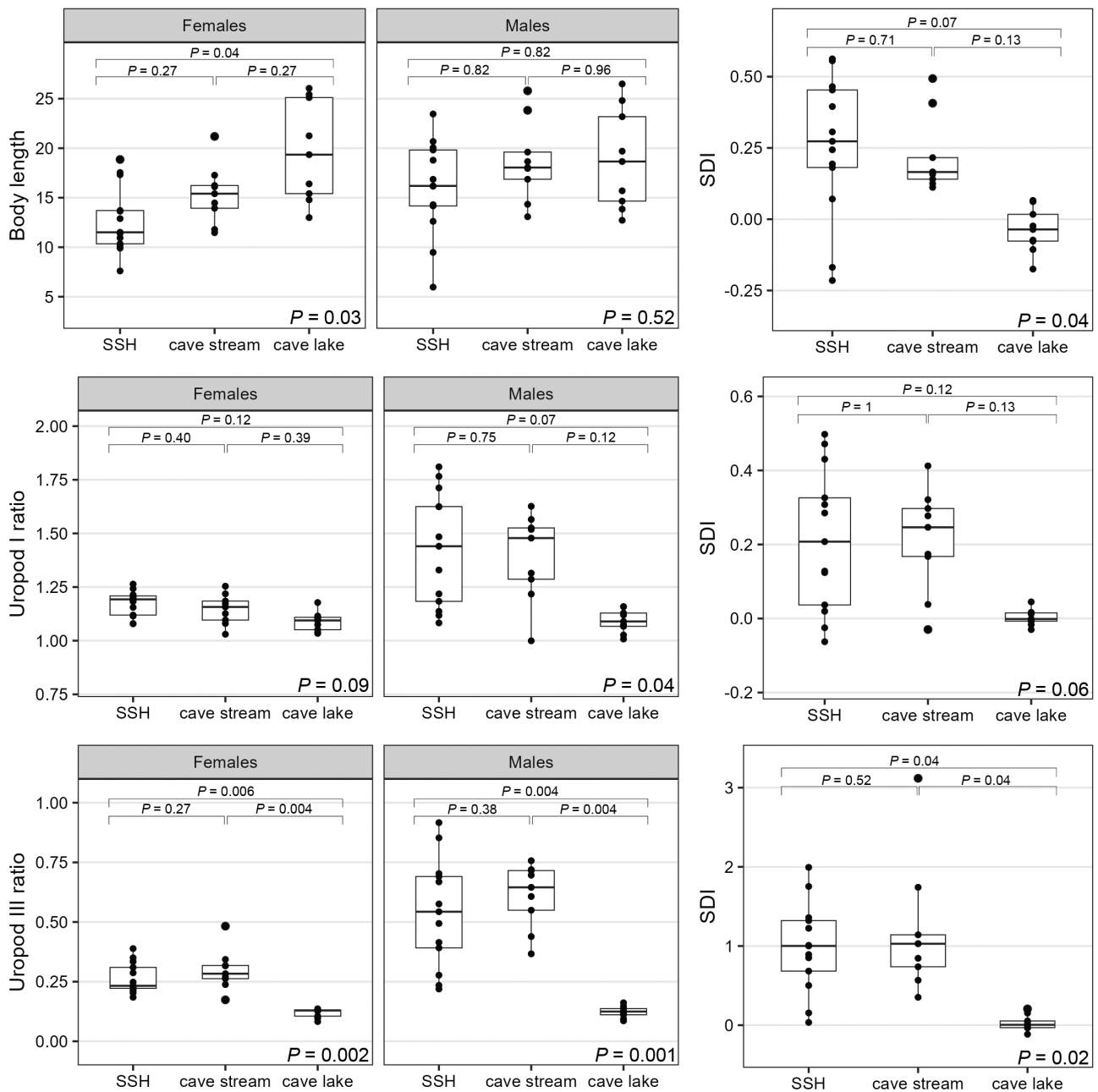
Figure 4. Species' morphological traits across the three habitats for males, females, and also expressed as a sexual dimorphism index (SDI). Statistical significance of the phylogenetic ANOVA is presented at the bottom right, while the statistical significance (after Holm's correction) of pairwise comparisons between habitats is shown above the boxplots. SSH = shallow subterranean habitat.

A slower pace of life may change the economics of female mating (Arnqvist et al., 2022). In groundwater species, this manifests as occasional (less predictable) reproductive events in low-density populations and reduced reproductive investment per event, mediated through the allometric relationship between body size and clutch size (Fišer et al., 2013; Venarsky et al., 2023). We tentatively propose that female body size in groundwater species in general increases, as increasing oligotrophy in conjunction with greater environmental stability and fewer predators selects for a slower metabolism and pace of life (but see Herczeg et al., 2023).

The evolution of males in groundwater is less clear than that of females. If females in deep subterranean compartments reproduce less often (see above), then male–male competition for receptive females intensifies and males should invest more into mating (as predicted by Ortigosa and Rowe, 2002 and Arnqvist et al., 2022). Consequently, in deep caves, we can expect greater sexual dimorphism in metabolism and POLS, due to selection on males (see Arnqvist et al., 2022). This might also manifest in greater sexual dimorphism in traits used to detect, approach, and defend females and in traits that allow males to assess female quality in darkness (Plath et al., 2006). These predictions are, however, in stark contrast to our current findings. One possible explanation is that a history of inbreeding in *Niphargus* selected for a female-biased offspring sex ratio in isolated cave lakes (Premate et al., 2021), resulting in more adult females per male, weaker sexual selection, and reduced sexual dimorphism. Therefore, we

Table 4. Results of phylogenetic ANOVA, testing whether habitat type (SSH, cave stream, cave lake) predicts trait values in males, females, and sexual dimorphism index (labelled as SDI).

Trait	Sex/SDI	*F*-value	*p*-Value	Cave lake—SSH (*p*-value)	Cave lake—cave stream (*p*-value)	Cave streams—SSH (*p*-value)
Body size	Female	8.636	**0.027**	**0.035**	0.272	0.272
	Male	1.290	0.523	0.821	0.960	0.820
	SDI	7.588	**0.039**	*0.068*	0.133	0.707
Uropod I	Female	5.492	0.085	0.120	0.392	0.401
	Male	7.775	**0.036**	*0.066*	0.117	0.752
	SDI	6.462	*0.057*	0.122	0.133	0.997
Uropod III	Female	21.349	**0.002**	**0.006**	**0.004**	0.273
	Male	23.732	**0.001**	**0.004**	**0.004**	0.383
	SDI	10.417	**0.015**	**0.043**	**0.038**	0.518
Activity	Female	5.303	*0.085*	0.314	0.386	*0.063*
	Male	8.308	**0.034**	0.179	0.273	**0.022**
	SDI	0.226	0.867	1.000	1.000	1.000
Risk-taking	Female	3.462	0.173	0.323	0.304	0.667
	Male	0.733	0.641	1.000	1.000	1.000
	SDI	0.788	0.623	1.000	1.000	1.000
Exploration	Female	6.901	*0.052*	0.140	0.520	**0.041**
	Male	5.917	0.068	0.360	0.360	0.047
	SDI	1.027	0.544	1.000	1.000	1.000

Note. SDI = sexual dimorphism index. The first two columns show *F*- and *p*-values of the overall model, and the remaining three columns report *p*-values of pairwise comparisons after Holm's correction. All analyses were repeated on 100 phylogenetic trees drawn from the stationary phase of a Bayesian analysis; *F*- and *p*-values are means across 100 replicates. Statistical significance ($p < 0.05$) is shown in boldface and marginal non-statistical significance ($0.05 < p < 0.1$) in italics.

tentatively propose that the evolution of males in groundwater species is more strongly determined by the evolution of the ASR. Unfortunately, the ASR is rarely studied in groundwater organisms (Nijman & Vonk, 2022; Premate et al., 2021; Wilson et al., 2021), so we cannot yet state how frequently it affected the evolution of their sexual dimorphism.

In sum, groundwater provides a simple environment characterized by simple but harsh environmental conditions. Subterranean organisms that inhabit groundwater are therefore intriguing, and potentially tractable, systems to study the evolution of sexual dimorphism. We tentatively propose that female evolution is often under natural selection imposed by a few key environmental factors (e.g., food availability, seasonality, predation risk), whereas male evolution is additionally affected by sexual selection that indirectly arises if environmental conditions change the social setting (e.g., the ASR). This leads to an expectation that convergent evolution should be more common in female traits than in male traits. Our empirical study is one of the first attempts to fill this knowledge gap.

Supplementary material

Supplementary material is available at *Journal of Evolutionary Biology* online.

Data availability

The raw data are deposited in public repository Zenodo at https://zenodo.org/records/10801507.

Author contributions

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Conflicts of interest

None declared.

Ethical statement

Old samples were obtained from the Zoological collection at the Department of Biology, Biotechnical Faculty, University of Ljubljana. New samples were collected under permits as follows.

Slovenia, Agencija RS za okolje, document no. 35602-46/2016-4 (issued March 16, 2017)

Slovenia, Agencija RS za okolje, document no. 35602-41/2021-5 (issued August 1st, 2022)

Bosnia and Herzegovina, Republika Srpska, Ministarstvo prosvjete i kulture, document no. 07/1/30/625-059/20 (issued February 5, 2020)

Bosnia and Herzegovina, Republika Srpska, Ministarstvo prosvjete i kulture, document no. 07/1/30/625-056/21 (issued February 15, 2021)

Bosnia and Herzegovina, Federacija Bosne I Hercgovine, Federalno ministarstvo okoliša i turizma, document 04-23-448/19 (issued July 4, 2019)

Species used in this study are not protected. Maintenance of live animals was compliant with Slovenian legislation.

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