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Female-biased adult sex ratio in *Niphargus*

NO ROOM FOR MALES IN CAVES: FEMALE-BIASED SEX RATIO IN SUBTERRANEAN AMPHIPODS OF THE GENUS *NIPHARGUS*

Abstract

Sex allocation theory predicts that the proportion of daughters to sons will evolve in response to ecological conditions that determine the costs and benefits of producing each sex. All else being equal, the adult sex ratio (ASR) should also vary with ecological conditions. Many studies of subterranean species reported female-biased ASR, but no systematic study has yet been conducted. We test the hypothesis that the ASR becomes more female-biased with increased isolation from the surface. We compiled a dataset of ASRs of 35 species in the subterranean amphipod *Niphargus*, each living in one of three distinct habitats (surface-subterranean boundary, cave streams, phreatic lakes) representing an environmental gradient of increased isolation underground. The ASR was female-biased in 27 of 35 species; the bias was statistically significant in 12 species. We found significant difference in the ASR among habitats after correction for phylogeny. It is

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most weakly female-biased at the surface-subterranean boundary and most strongly female-biased in phreatic lakes. Additional modelling suggests that the ASR has evolved towards a single value for both surface-subterranean boundary and cave stream dwelling species, and another value for 9 of 11 phreatic lake dwellers. We suggest that a history of inbreeding in subterranean populations might lower inbreeding depression such that kin selection favours mating with siblings. This could select for a female-biased offspring sex ratio due to local mate competition among brothers. The observed patterns in sex ratios in subterranean species make them a group worthy of more attention from those interested in sex allocation theory.

Keywords: sex allocation theory, Amphipoda, extreme habitats, caves

Introduction

Sexual reproduction involving males and females, or sometimes simultaneous hermaphrodites, is the most common mode of reproduction in the animal kingdom (Hartfield & Keightley, 2012). There is, however, considerable variation in the adult sex ratio across the tree of life (Futuyma, 2009). Some of this variation is due to a bias in the sex ratio at conception or birth. Sex allocation theory has had great success in explaining biases in the offspring sex ratio. The Düsing-Fisher theory predicts equal investment into each sex, which leads to an even sex ratio, when the production of each sex and the benefits they provide to parents are identical (West, 2009). The ratio is, however, biased towards one sex if it is cheaper to produce. In addition, selection favours biased sex allocation towards whichever sex offers a higher marginal fitness gain (i.e., the increment caused by a small additional investment) (Charnov, 1979). Sex-specific marginal fitness gains can be driven by ecological factors that differentially affect each sex (Taylor, 1981). For example, when there is limited dispersal, brothers are more likely to compete with each other for mates (local mate competition; Hamilton, 1967; Bulmer & Taylor, 1980). The fitness gains to mothers from investments into sons therefore saturate at a lower investment level, selecting for the production of daughters. Similarly, there is selection for greater production of the more widely dispersing sex when offspring compete with their mother and/or their siblings for resources (local resource competition; Clark, 1978; Bulmer & Taylor, 1980). The opposite patterns arise with selection for greater production of the less-dispersing sex when offspring of this sex increase the fitness of their mother or siblings (e.g. through sex-specific cooperative behaviour; Emlen *et al.*, 1986).

Accounting for variation in sex ratios is a challenging task, which requires a detailed understanding of natural history (Frank, 1990). One profitable approach has been to seek out patterns of variation in different sex ratios (e.g. at birth, maturation and adult) in taxa living in ecologically unusual habitats. For example, Hamilton's seminal work on extraordinary sex ratios was inspired by the observation that, "among small arthropods, wherever reproduction is quite regularly by brother-sister mating there seems to be extreme economy in the production of males" (Hamilton, 1967, p. 481; also see Hamilton, 1996). Additionally, when studying adult sex ratios – as in the present study – one must be aware that these can differ from offspring sex ratios due to sex-specific juvenile and adult mortality (Ancona *et al.*, 2017).

Caves and other subterranean habitats provide a model system to investigate the effect of an extreme environment (Mammola, 2019). Dispersal between different caves is low, making them natural replicates

(Trontelj *et al.*, 2009). These habitats are dark, food deprived, have strongly reduced daily and seasonal climatic fluctuations, and harbour fairly simple biotic communities (Culver & Pipan, 2009; Fišer *et al.*, 2014). In addition to morphological, physiological, and behavioural adaptations (Pipan & Culver, 2012), subterranean dwellers show markedly distinct life histories from their surface relatives. These include slower growth rates, longer lifespans (Voituron *et al.*, 2011; Venarsky *et al.*, 2012; Carpenter, 2021), fewer but larger eggs per clutch (Poulson, 1963; Fišer *et al.*, 2013), and higher levels of iteroparity than seen in related terrestrial species (Poulson, 1963; Carpenter, 2021). Interestingly, many authors report that subterranean species show a tendency to be facultatively or obligately parthenogenetic, leading to a very biased female sex ratio (Borgonie *et al.*, 2011; Tilquin & Kokko, 2016). In general, parthenogenesis is associated with low population density and/or high levels of inbreeding that lower genetic variation and reduce the benefits of sexual reproduction (Tilquin & Kokko, 2016). Intriguingly, a few studies suggest that subterranean species have an adult sex ratio that is skewed towards females (Culver & Holsinger, 1969; Vonk & Nijman, 2006; Carpenter, 2021), although the ubiquity of this pattern has not yet been tested. A female-biased adult sex ratio is consistent with low dispersal and higher rates of inbreeding that favour a bias towards the production of daughters, in line with Hamilton's quote, cited above.

Here we tested if species in subterranean environments have female-biased adult sex ratios, using amphipod crustaceans of the genus *Niphargus* as our model system. *Niphargus* is a species-rich genus distributed in the Western Palearctic (Fišer, 2012). Its members primarily inhabit subterranean waters, such as deep cave (phreatic) lakes and cave streams. Secondarily some species have also colonized habitats at the subterranean-surface boundary, such as springs, seeps, or even surface streams that arise from the upwelling of groundwater (Trontelj *et al.*, 2012; Copilaş-Ciocianu *et al.*, 2017; Copilaş-Ciocianu *et al.*, 2018; Fišer *et al.*, 2019). There is a clear surface-subterranean ecological gradient which is mirrored in the phenotype of *Niphargus* (Trontelj *et al.*, 2012; Fišer *et al.*, 2013, 2019; Esmaeili-Rineh *et al.*, 2020; Kralj-Fišer *et al.*, 2020).

To test whether there is an increasing bias towards females in deeper subterranean habitats, we compiled a dataset of adult sex ratios for 35 *Niphargus* species from three habitats representing distinct points along the surface-subterranean ecological gradients (phreatic, cave streams and shallow subterranean). First, we tested whether sex ratios in each species deviate from the standard 1 : 1 null expectation. Second, after taking into account phylogenetic relatedness, we tested whether the adult sex ratios differ among the inhabitants of the three habitats. Finally, we determined which dynamic model of evolution best accounts for the observed

adult sex ratio variation across the *Niphargus* phylogeny by asking whether ecologically different species had different evolutionary attractors (analogous to ‘adaptive peaks’) for the adult sex ratios.

Materials and methods

Samples, quantification of sex ratio and assumptions

Sampling of subterranean species is challenging, as sites are hard to access and many species occur at low densities or are rare. Efficient sampling often requires special gear, rope techniques and even scuba diving in caves. Large sample sizes of any given species are therefore the exception. We recorded the sex of all individuals from samples of *Niphargus* species collected over the last two decades from multiple underground sites and now housed at the Department of Biology at the University of Ljubljana, Slovenia. These samples were collected either as general surveys of fauna, or for experimental studies (Fišer *et al.*, 2015, 2016; Delić *et al.*, 2016). We present data on species for which a single sample provided >19 individuals, or for which multiple samples jointly provided >30 individuals and each sample minimally provided 10 individuals. We included data for an additional species (*N. cvetkovi*) from literature (Kenderov & Andreev, 2015).

Each species was assigned to one of three habitat categories: phreatic (lentic water bodies in permanently flooded zone, oligotrophic, highly isolated and stable habitat), cave streams (endogenous streams and sinking rivers, less oligotrophic, less isolated but stable habitats) and shallow subterranean habitat at the boundary with surface (springs, seepages or peat-bogs, non-limiting food, influenced by some seasonality). We used the European Groundwater Crustacean Dataset (Zagmajster *et al.*, 2014) to assign each species to the habitat in which it is most commonly found. Species from multiple sites were collected from the same habitat. For species known from only a single site (e.g. *N. cf. dalmatinus*), or where records indicated that the species inhabits multiple habitats (e.g. *N. dalmatinus*, *N. stygius*), we classified them according to the habitat at the collection site, based on the assumption that sex ratios vary among populations due to local conditions (Charnov *et al.*, 1981).

We compiled a dataset of 35 species: 11 from phreatic waters, 10 from cave streams and 14 from shallow subterranean habitats (Table 1, Supplementary Material S1). The total number of individuals sexed was 3173 (range: 22-428 per species).

All individuals were examined under a Leica S9E stereomicroscope (Leica, Germany). Males were identified by the presence of genital papillae between the seventh pereopods; females were identified by the presence of oostegites, i.e. leaf shaped outgrowths from appendages, forming a brooding pouch (Kaestner, 1967). Unlike some isopods where oostegites develop only during breeding, these structures are permanently present in *Niphargus* females, and are visible as tiny leaf-shaped structures next to the gills in smaller (i.e. younger) females. We found no intersexes. Individuals that were too young to develop observable sex-specific characters were excluded from the analysis. We then calculated the *adult sex ratio* (ASR) of each species, which will differ from birth or operational sex ratio (i.e., proportions of males and females in a breeding pool) if there is sex-biased mortality and/or a difference in the duration of sexual receptivity (Jennions *et al.*, 2017). We define the ASR as the proportion of males. We report the mean ASR for species with two or more samples (maximum time between sampling was five years, see Supplementary Material S1). If the samples were from the same site (i.e., the same population was sampled on different occasions), we report the sample-size weighted mean; if the samples were from different sites, we report non-weighted means. In treating the estimated ASR as matching the true ASR we make three assumptions. First, that there was no sampling bias. This assumption is plausible as sex cannot be accurately determined in the field and the collections were mainly obtained using a hand net to catch all available individuals (but see Ancona *et al.*, 2017). Second, that the ASR shows low seasonal variation because subterranean habitats have buffered seasonality. Third, that all individuals with observable secondary sexual characters are sexually mature.

Statistical analyses

We performed three analyses in R v. 3.6.0 (R Development Core Team, 2020). First, we ran separate χ^2 tests for each species to test if the ASRs deviate from a 1 : 1 ratio. Second, we tested whether the ASRs differed among species based on their habitat category. To account for phylogenetic dependence among species, we ran a phylogenetic ANOVA using the most recent multilocus calibrated phylogeny of *Niphargus* (Borko *et al.*, in preparation). Briefly, the phylogeny was reconstructed using 512 species sequenced for one mitochondrial and six nuclear markers, with a total concatenated alignment length of 7076 bp. This dataset was analysed in a Bayesian framework and calibrated using four calibration points (Borko *et al.*, 2021). For our study, all non-focal species were pruned from the tree. To account for phylogenetic uncertainty, we sampled 100 random trees from the stationary phase (Revell, 2012).

Third, we tested whether the ASRs in species from the three habitat categories converge towards different values (i.e., if habitat-specific attractor values exist). If this is the case, the ASRs modelled on the phylogeny should fit the Ornstein-Uhlenbeck model better than Brownian motion model. Brownian motion is the simplest model used for modelling quantitative traits. It assumes that the evolution of a trait is a purely neutral process that can change in any direction in any given time period. Thus, the variation of a given trait for a given clade increases over time, and the model generates only one parameter, the evolutionary rate. An elaborated model assumes two or more clade-specific rates. By contrast, the Ornstein-Uhlenbeck model explicitly assumes environment-specific selection, and that trait variation at the phylogeny tips corresponds to one or more environment-specific evolutionary attractors. This evolutionary model generates parameters that describe: i) the time-dependent rate of neutral evolution, ii) one or more evolutionary attractors (i.e., trait values that attract the dynamics of the system) and iii) the strength of attraction towards these trait values (i.e. strength of selection) (Butler & King, 2004). We explored which evolutionary model best explains the distribution of ASRs on the tips of the phylogeny. We ran models of neutral evolution with Brownian motion (models with a single or multiple rates) and Ornstein-Uhlenbeck models that explicitly included one or more evolutionary attractors. We compared the models using AIC values and AIC weights. In this analysis, we assigned different attractor values *a priori* according to each species' ecology using *mvMORPH v.1.1.3* (Clavel *et al.*, 2015). We then performed an additional analysis using *SURFACE v.0.5* (Ingram & Mahler, 2013), which in a two-step procedure using AIC searches for the optimal distribution of the number of evolutionary attractor values on the phylogeny free of any information on species ecology. Briefly, in the first step the algorithm assigns the optimal number of evolutionary attractor values to the phylogeny nodes in the direction from the root towards the tips, using the AIC criterion. In the second step, the algorithm tries to simplify the model from tips to root through lumping similar evolutionary attractors using the AIC criterion (Ingram & Mahler, 2013; Mahler *et al.*, 2013).

Results

The adult sex ratio (ASR) varied between 0.06 (*N. orcinus*, phreatic) and 0.55 (*N. cf. longicaudatus*, spring). In 27 of 35 species there were more females than males (Table 1). A significant female-biased ASR occurred in eight of 11 phreatic species, two of ten cave stream species and two of 14 shallow subterranean species (Table 1). In no species was the ASR significantly male-biased. The pattern of a more female-biased ASR in

species from phreatic sites was robust to correction for phylogeny. The ASRs differed significantly among the species from the three habitat categories (phylogenetic ANOVA $F = 27.78$, $p = 0.001$; p -values of pairwise comparisons after Holm correction: phreatic – cave stream $p = 0.012$; phreatic – shallow subterranean habitats $p = 0.030$; cave streams – shallow subterranean habitats $p = 0.036$; Fig. 1).

Table 1. Dataset summary for 35 species of *Niphargus*. The habitat type, sample size and adult sex ratio (ASR; proportion of males) are shown. Significant deviations from a 1 : 1 ratio were tested by χ^2 tests. Significant ($p < 0.05$) deviations from a 1:1 ASR are indicated in boldface. Detailed data on localities are available in the Supplementary material S1.

Species	Ecology	#locality /#samples	total number of individuals	ASR	p-value
<i>Niphargus arbiter</i>	phreatic	5 / 5	112	0.30	<0.01
<i>Niphargus balcanicus</i>	phreatic	1 / 1	27	0.26	0.01
<i>Niphargus croaticus</i>	phreatic	7 / 7	119	0.31	<0.01
<i>Niphargus gammariformis</i>	phreatic	1 / 1	84	0.44	0.28
<i>Niphargus hebereri</i>	phreatic	1 / 1	28	0.36	0.13
<i>Niphargus kolombatovici</i>	phreatic	2 / 2	24	0.33	0.25
<i>Niphargus longiflagellum</i>	phreatic	3 / 4	104	0.19	<0.01
<i>Niphargus orcinus</i>	phreatic	2 / 2	34	0.06	<0.01
<i>Niphargus cf. pachytelson</i>	phreatic	1 / 2	31	0.23	<0.01
<i>Niphargus subtypicus</i>	phreatic	1 / 2	28	0.14	<0.01
<i>Niphargus vjetrenicensis</i>	phreatic	3 / 3	61	0.24	<0.01
		(Kenderov & Andreev, 2015)	235	0.33	<0.01
<i>Niphargus cvetkovi</i>	cave stream				
<i>Niphargus dalmatinus</i>	cave stream	1 / 1	78	0.32	<0.01
<i>Niphargus miljeticus</i>	cave stream	1 / 1	19	0.37	0.25
<i>Niphargus novomestanus</i>	cave stream	1 / 1	23	0.43	0.53
<i>Niphargus podpecanus</i>	cave stream	1 / 1	101	0.41	0.06
<i>Niphargus rhenorhodanesis</i>	cave stream	1 / 1	102	0.41	0.07
<i>Niphargus scopicauda</i>	cave stream	1 / 1	89	0.44	0.24
<i>Niphargus spoeckeri</i>	cave stream	1 / 1	81	0.47	0.58
<i>Niphargus stygius</i>	cave stream	1 / 1	91	0.49	0.92
<i>Niphargus zagrebensis</i>	cave stream	1 / 2	44	0.41	0.23
<i>Niphargus cf. dalmatinus</i>	ssh ¹	2 / 2	62	0.53	0.61
<i>Niphargus elegans</i>	ssh ¹	1 / 1	75	0.44	0.30
<i>Niphargus hadzii</i>	ssh ¹	1 / 3	69	0.54	0.55
<i>Niphargus hrabei</i>	ssh ¹	4 / 4	37	0.51	0.87
<i>Niphargus hvarensis</i>	ssh ¹	1 / 2	68	0.44	0.33
<i>Niphargus illidzensis</i>	ssh ¹	1 / 1	95	0.40	0.05
<i>Niphargus krameri</i>	ssh ¹	1 / 1	150	0.49	0.87

<i>Niphargus cf.</i>		1 / 1	22	0.55	0.67
<i>longicaudatus</i>	ssh ¹				
<i>Niphargus sanctinaumi</i>	ssh ¹	1 / 1	57	0.37	0.05
<i>Niphargus slovenicus</i>	ssh ¹	2 / 2	49	0.50	0.89
<i>Niphargus sphagnicolus</i>	ssh ¹	2 / 4	247	0.46	0.07
<i>Niphargus spinulifemur</i>	ssh ¹	2 / 2	177	0.51	0.15
<i>Niphargus timavi</i>	ssh ¹	1 / 1	122	0.48	0.59
<i>Niphargus valachicus</i>	ssh ¹	1 / 11	428	0.51	0.70

¹ssh stands for Shallow Subterranean Habitat.

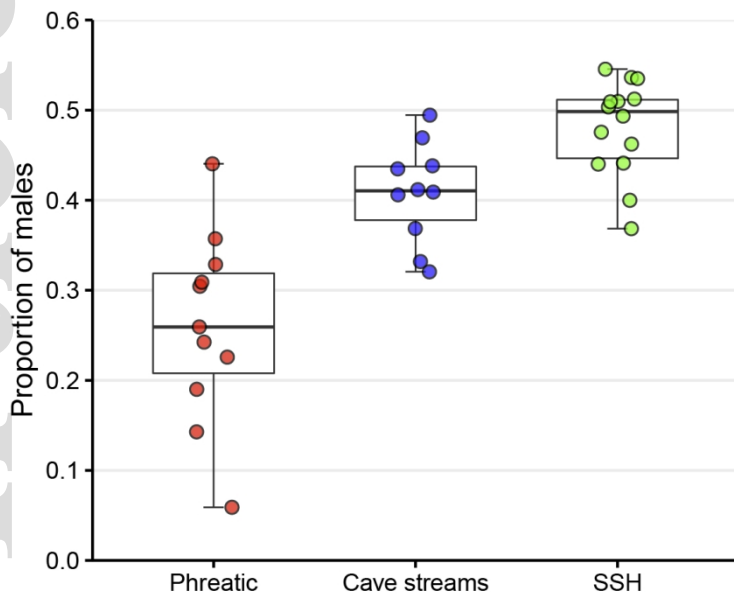


Fig. 1. Distribution of the adult sex ratio (ASR: proportion of males) for 35 *Niphargus* species from three habitat types (phreatic, cave streams and shallow subterranean habitats (SSH)).

The phylogenetic distribution of the ASRs is not explained by neutral evolution (Fig. 2, Table 2). An Ornstein-Uhlenbeck model, simulating natural selection, parametrized with three evolutionary attractors for species from phreatic, cave streams and shallow subterranean habitats respectively explains variation in the ASRs across the phylogeny tips substantially better than either Brownian motion model, or than an Ornstein-Uhlenbeck model with a single evolutionary attractor (Fig. 2, Table 2). Thus, ASR does not evolve at random, but seems to be subject to habitat-specific selection. A SURFACE analysis also implies three evolutionary attractors, but their distribution is different. All species from cave streams and shallow subterranean habitats were assigned to a single evolutionary attractor. In contrast, the 11 phreatic species were assigned to three evolutionary attractors. *N. gammariformis*, a species which lives in a single sulfide-rich cave, shared the same attractor as species from cave streams and shallow subterranean habitats; while *N. orcinus* had its own unique attractor (Fig. 2). The other nine species had an evolutionary attractor that differed from that of cave streams and shallow subterranean habitat species or the other two phreatic species. It is worth noting that at both collection sites the ASR was heavily female-biased for *N. orcinus* (0.063 and 0.056).

Table 2. Results of evolutionary modelling of the evolution of the adult sex ratio (ASR) of 35 *Niphargus* species. We tested models of random evolution (Brownian motion) using either a single or multiple rates, and selection models (Ornstein-Uhlenbeck) with either a single evolutionary attractor or one for each of the three habitats.

Model sex ratio evolution	AIC	AICw
Ornstein-Uhlenbeck, three evolutionary attractors	-76.9	1
Brownian motion, multiple rates	-61.0	$3.46 * 10^{-4}$
Brownian motion, single rates	-54.7	$1.45 * 10^{-5}$
Ornstein-Uhlenbeck, single evolutionary attractor	-51.2	$2.63 * 10^{-6}$

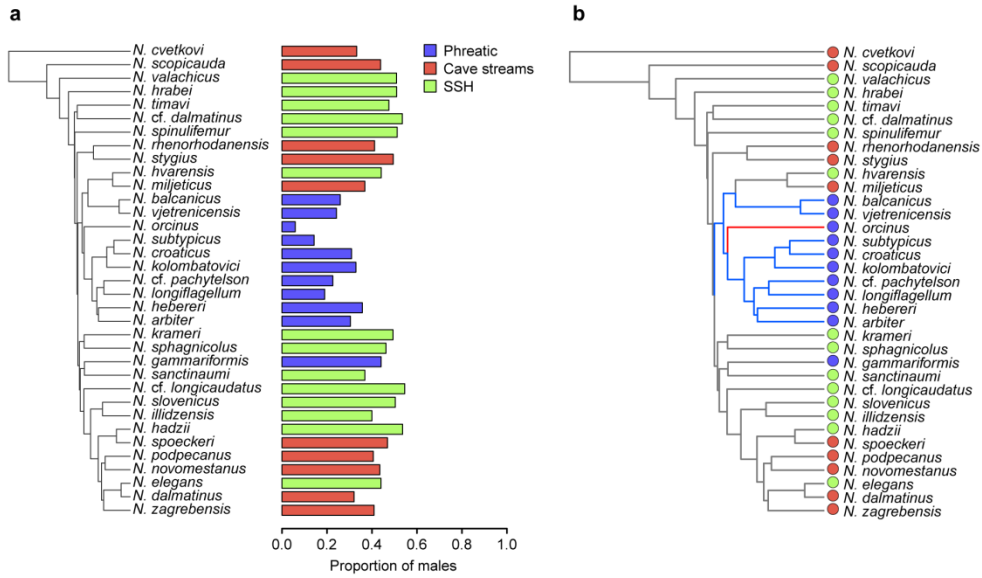


Fig. 2. Distribution of the adult sex ratio (ASR, proportion of males) of 35 *Niphargus* species on the phylogeny (left) and the results of modelling in SURFACE (right). The habitat of each species is colour coded. On the right, colours of the branches indicate distinct evolutionary attractors. Note that species from cave streams and shallow subterranean habitats share one evolutionary attractor (grey, proportion of males = 0.45), whereas species from phreatic habitats have three evolutionary attractors (grey (proportion of males = 0.45), red (proportion of males = 0.26) and blue (proportion of males = 0.057)).

Discussion

Accurate estimation of adult sex ratios (ASRs) is challenging, especially when taxa are difficult to access and sample (Ancona *et al.*, 2017). As far as we know, we have compiled the largest dataset of ASRs for subterranean species to date. Our results show two relatively robust patterns in the ASR along the ecological gradient from surface habitats to deep caves. First, females are generally the more numerous sex. None of the 35 species had a statistically significant male-biased ASR, while 12 had a significantly female-biased ASR (Table 1). Second, the female-bias increases with the distance from the surface, being lowest at the surface-subterranean boundary and strongest in phreatic lakes.

The bias towards adult females in *Niphargus* species from phreatic lakes is consistent with previous single-species studies on subterranean crustaceans that showed either a female bias or parthenogenesis (Culver & Holsinger, 1969; Vonk & Nijman, 2006; Borgonie *et al.*, 2011; Tilquin & Kokko, 2016; Carpenter, 2021). It also agrees with studies reporting a female-biased ASR in copepod crustaceans from the deep sea (Mednikov, 1961; Thistle & Eckman, 1990), which shares some key ecologically similarities with phreatic lakes (Martens & Danielopol, 1999). Notably, a female-biased ASR has also been reported for *Niphargus* species from interstitial habitats (deep layers of alluvial sediments), another subterranean habitat (Stock & Gledhill, 1977). In sum, it appears that a female-biased sex ratio is favoured in simple, isolated habitats.

The pattern that the proportion of adult males decreases along the ecological gradient from surface to deep caves is seen for individual species (Table 1), but also when correcting for phylogenetic relatedness (Fig. 1), and when modelling the evolution of sex ratios as a 'trait' (Fig. 2. Table 2). However, the latter analyses yielded a less clear result. The number of estimated evolutionary attractors ranged from two to four. In all cases, however, there was still a clear pattern for the evolution of a more strongly female-biased ASR in phreatic species than in species from cave streams or shallow subterranean habitats. The widest range in the ASR was 0.38 in species from deep phreatic lakes (0.06 -0.44) versus 0.17 in species from cave streams (0.32-0.49) and 0.18 in shallow subterranean species (0.37-0.55). This variation may be partly related to ecological differences among the caves sampled.

The observed female-biased ASR in cave *Niphargus* may be due to several possible mechanisms. Sex-specific mortality or parasite-induced feminization (e.g., Bulnheim, 1978) cannot be ruled out, but there is no a priori reason to link either of these mechanism to being deeper underground. Instead, we propose the following verbal model, to encourage further research to test its claims. We suggest that living in caves selects for a

female-biased offspring sex ratio via a mechanism that occurs in five stages. (1) Inbreeding arises when living in confined spaces at low densities. (2) Inbreeding exposes recessive deleterious alleles to selection in their homozygous form, leading to them being purged from the population. This lowers inbreeding depression. (3) As inbreeding depression weakens, kin selection favours mating with siblings (Parker, 1979; Puurtinen, 2011). (4) Once mating between siblings is common, there is selection for a female-biased offspring sex ratio due to local mate competition (Hamilton 1967) and kin-selection (for an explanation of how these are distinct yet complementary effects, see Frank 1990). (5) The environmental stability of subterranean habitats may eliminate potential long-term costs of habitual inbreeding (i.e., lower genetic variation reduces the ability to adapt to changing conditions). Consistent with our verbal model, reduced heterozygosity indicative of inbreeding occurs in many subterranean species (Sbordoni *et al.*, 2012; Konec *et al.*, 2015).

There are knowledge gaps about the basic biology of *Niphargus* species that challenge the scenario we have outlined. For example, it is not known how sex is determined and whether parents can directly control the offspring sex ratio. Sex determination in crustaceans is diverse and can depend on genetic and environmental factors (Charniaux-Cotton, 1960; Bulnheim, 1978; Watt & Adams, 1993; McCabe & Dunn, 1997; Rigaud *et al.*, 1997) or, as in tanaidaceans, even change over the life span (Holdich & Jones, 1983). Crucially, it is also unknown whether the sex ratio changes from birth to adulthood due to sex-specific mortality. Quantification of the birth sex ratios would directly test the proposed mechanism for the observed female-biased ASR.

Despite these limitations, our results reveal that *Niphargus* species are a promising model system to test sex allocation theory. Experimental manipulations could more precisely determine whether facultative responses to local ecological factors (environmental stability, food availability) drive sex ratio differences among habitats. Further, observational studies could test the assumptions of our verbal model, e.g., that populations with a more female-biased ASR have lower inbreeding depression and higher rates of sibling mating.

Furthermore, biased sex ratios are often linked with sex differences in other traits, notably male-male aggression, dispersal, body size and parental care (Trochet *et al.*, 2016; Jennions & Fromhage, 2017; Jennions *et al.*, 2017) and *Niphargus* species could provide an opportunity to test theoretical predictions using a comparative approach. Preliminary observations in *Niphargus* indicate that males are larger than females in species with an even ASR (Fišer C., pers. obs.). In phreatic *Niphargus* species with an excess of females, there is increased prevalence of brood care. These species have larger eggs, which likely take longer to develop in the maternal marsupium, and the newly hatched juveniles may then be larger and more independent than

juveniles of species from streams (Fišer *et al.*, 2013). A biased sex ratio may also have macroevolutionary consequences. *Niphargus* apparently evolved in several independent adaptive radiations (Borko *et al.*, 2021). Biased sex ratios lower the effective population sizes, and reduce genetic diversity, which potentially lowers rates of adaptation (Martin & Richards, 2019), which could increase the risk of extinction (but see Day *et al.*, 2016; Stern *et al.*, 2017).

In sum, using data from 35 species we found that the adult sex ratios in the subterranean amphipod genus *Niphargus* shows a trend to become increasingly female-biased across the ecological gradient from surface to deep phreatic locations. Further investigations of the underlying causes of this pattern could improve understanding of a range of evolutionary processes in subterranean environments, from the evolution of sex roles to macroevolutionary patterns of rates of speciation and extinction.

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