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MORPHOLOGICAL DIFFERENCES AMONG EYELESS AMPHIPODS IN THE GENUS *STYGOBROMUS* DWELLING IN DIFFERENT SUBTERRANEAN HABITATS

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ABSTRACT

The amphipod genus *Stygobromus* occurs in a variety of subterranean habitats in North America, including caves, phreatic (groundwater) lakes, and superficial subterranean habitats (seeps and epikarst). The habitats share the absence of light but differ in other features, such as pore size of the habitat, available food, and degree of seasonality. Measurements of body size, antennal size, and antennal segment number of type specimens were compared for 56 species occurring in the eastern United States. Except for differences in body size, differences among species in the four different habitats were not significant. Body size was related to relative pore size of the habitat, e.g., epikarst, with the smallest spaces, had the smallest species. However, in all habitats, there was one very large species (> 15mm); these enigmatic species apparently occupy a distinct ecological niche, perhaps being more predatory. Differences in relative antennal size showed no significant differences among habitats, and differences in number of antennal segments were marginally significant (*P* = 0.06) among habitat types and not in the predicted pattern. Differences among habitats in seasonality and available food seemed to be a minor part of the selective environment; absence of light seemed to be a major part of the selective environment.

KEY WORDS: Amphipoda, cave biotas, caves, evolution

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INTRODUCTION

Most of what is known about evolutionary ecology, e.g., life history and community dynamics, and adaptation of aquatic subterranean animals, e.g., the morphological changes accompanying isolation in subterranean habitats, comes from animals whose primary habitats are cave streams. One of the best studied groups of aquatic subterranean animals in this regard are the amblyopsid cave fish (Poulson, 1963; Poulson and White, 1969). For the most part cave amblyopsids live in cave streams, and Poulson convincingly demonstrated that as a group, the obligate subterranean species (stygobionts) in Amblyopsidae had elaborated extra-optic sensory structures, e.g., lateral line systems, no eyes and pigment, reduced metabolic rate, a small number of large eggs, and delayed reproduction. This, combined with the contemporaneous work of Christiansen (1961, 1965) on adaptation in Collembola and Barr (1967, 1968) on adaptation in carabid beetles living in caves, often the same ones that harbored cave amblyopsids, became the paradigm for adaptation to cave life, especially among North American biologists. The selective environment envisioned by Poulson, Barr and Christiansen was one of total darkness, more or less environmental constancy, and food scarcity. The suite of shared morphological, and by implication ecological and behavioral characteristics of cave organisms, the result of convergent and parallel evolution (Culver et al., 1995) is called troglomorphy (Christiansen, 1962). There are now countless papers that mention, with varying degrees of detail (Campanoro and Bruno, 2007; Souza et al., 2006),

that subterranean species share adaptive features originally described by Barr, Christiansen and Poulson.

While North American subterranean biologists focused almost exclusively on caves, European researchers, stimulated by the prescient essay by Racoviţă (1907, 2006), studied a variety of non-cave aquatic subterranean habitats. Most prominent of these were interstitial habitats such as the underflow of streams and rivers. Animals dwelling in these environments share many of the same characteristics of obligate cave dwellers, including eye and pigment loss and life history characteristics of K selected species in energy-poor environments. They differ from cave species in their morphological miniaturization (Coineau, 2000), the result of habitable spaces being very small in diameter, often less than 1 cm (Ward et al., 2000). These habitat differences led Botosaneanu (1986) to suggest a subdivision of aquatic subterranean habitats into "milieux permeable et grand" and "milieux permeable et petit." The commonly held view of the selective environment in both of these subterranean habitat types is nearly the same — no light, little food, and little temporal variation — except for the diameter of habitat spaces.

It is now well known that troglomorphic organisms occur in a wide variety of subterranean habitats (Culver and Pipan, 2009), ranging from deep ground water (phreatic) to very shallow seeps (hypotelminorheic), and the interstices in talus slopes (milieu souterrain superficiel). Some of these habitats, like deep ground water, are extreme environments like caves, with no light, little environmental variation, and little organic carbon. Superficial subterranean habitats (SSH), aphotic habitats less than 10 m from

the surface and with cavities considerably larger than their inhabitants, do not fit this paradigm (Culver and Pipan, 2008). A variety of habitats, including epikarst, talus slopes, regolith, superficial groundwater emerging in small seeps, and cracks and fissures (clinker) in lava, are aphotic, but with relatively high resources and temporal variability as a result of their close proximity to the surface. SSH's share two important characteristics with both large and small cavities — the absence of light and the presence of species both limited to (troglobionts for terrestrial species and stygobionts for aquatic species) and usually modified for subterranean life (troglomorphs). The two SSH's directly relevant to this study are epikarst and hypotelminorheic habitats.

Epikarst is a perched aquifer, and the major point of contact and transmission between surface and subterranean water, and it forms a more or less permanently saturated zone with a considerable volume of water close under the surface (Williams, 2008). Compared to streams that sink into caves from the surface, concentrations of dissolved organic matter are not as high in epikarst (1.1 mg/L compared to 7.6 mg/L for Organ Cave, West Virginia [Simon, Pipan, and Culver, 2007]), but they are higher than epikarst fed cave streams (0.7 mg/L for Organ Cave). Many caves and cave passages, if they have streams at all, have only epikarst streams. In Organ Cave, more than half of the more than 70 km of passage, is of this type. Seasonal variation in flow rates, measured as the drip rate of water from cave ceilings, is very high. Drip rates of water, however, were highly variable, ranging from < 1 ml min^{-1} to > 100 ml min^{-1} in Taborska jama, with the seasonal pattern dependent on rainfall (Kogovšek, 1990). Pore size of epikarst varies from perhaps as large as 1 m to 5 mm, the minimum size of a stalactite tube (Curl, 1972)

The hypotelminorheic is the most superficial of SSH's. Working in the Medvednica Mountains north of Zagreb, Croatia, Meštrov (1962) described a new subterranean habitat, which he termed the hypotelminorheic, with subterranean drainage basins of only a few thousand m^2 in area and depths of a meter or so that had several troglomorphic species. The habitat can occur in a wide variety of geologic settings anywhere outside of arid regions where there is a layer of impermeable sediment, typically clay, that "perches" a small aquifer (Culver et al., 2006). It is a very superficial habitat, usually less than 1 m in depth, and is underlain by clay which acts as an aquiclude. Most of the actual habitat for the animals is space between decomposing leaves and sediment, and they literally live in their food. Organic carbon has not been measured but is presumably high because of the concentration of decaying leaves. In Prince William Forest Park in northern Virginia, hypotelminorheic temperatures ranged from 1.8°C to 21.8°C, approximately 70 percent of the variation of surface waters in the same year of measurement (Culver and Pipan, 2009b). Pore size is the space between rotting leaves, in the range of 1 mm to 1 cm, although larger invertebrates such as amphipods can presumably force their way between leaves.

There are of course caves with large sinking streams and rivers that are highly variable and with high organic inputs, but this is not universal. All SSH's are variable with relatively high organic matter compared to deep, isolated caves; some caves are also variable with relatively high organic matter. Once again using Organ Cave, West Virginia, as an example, the stygobiotic fauna of cave streams is typically in the low resource epikarst-fed streams (Fong and Culver, 1994).

Given the different selective environment of SSH's, it is of considerable interest to determine the morphological differences among phylogenetically close species that occupy different kinds of subterranean habitats. If SSH's are a less extreme selective environment than caves, because SSH's have higher resource levels, then elaborated troglomorphic features, such as appendage lengthening, should be less accentuated than in caves. If, on the other hand, the selective environment of SSH's and caves are nearly identical because darkness is the primary selective factor, then elaborated troglomorphic features should be similar in these environments. If pore size is an important selective factor, body should reflect pore size (habitat size). It is these twin predictions — size determined by the selective environment of pore size and antennal shape (relative size) being determined either by either the selective environment of darkness or the selective environment of darkness and low resource levels — that is the focus of this paper.

The amphipod genus *Stygobromus* is an ideal taxon for this purpose. It is widespread in a variety of subterranean habitats in North America, with 132 described species, and 22 more in manuscript (Holsinger, 2009). All species are without eyes or visible pigment. It is especially amenable to morphometric analysis because Holsinger, who described or redescribed nearly all of the species, especially in the eastern United States (Holsinger, 1967, 1969, 1978, 2009) has provided equivalent morphological measurements for all the species.

Subterranean habitats of *Stygobromus* in the eastern United States fall into four categories: 1) phreatic pools and lakes, generally accessible via caves or wells in karst areas; 2) cave streams; 3) epikarst and associated drip pools; and 4) seeps. The first two are the lentic and lotic equivalents of subterranean habitats. The final two are SSH's, both quite distinct from streams and pools.

The habitat of *Stygobromus* in phreatic lakes and pools has the largest pore size of any of the four habitats; animals are benthic, living on the substrate. Except for rare chemoautotrophic caves, phreatic habitats are probably the lowest in organic carbon and other nutrients, because they are the most isolated from surface habitats. For the same reason, variability is likely the lowest of any of the four habitats.

Materials and Methods

As a standard for comparison, the body length, first antennal length, and number of primary flagellar segments of the first antennal segment of females in the type series of *Stygobromus* were used. Data were compiled from the original species descriptions and re-descriptions of Holsinger (1967, 1969, 1972, 1978, 2009). Body size, first antennal length, and number of flagellar segments have all been shown by Fong (1989) to be heritable and by Jones et al. (1992) to increase in the amphipod *Gammarus minus* Say 1818 during adaptation to life in cave streams and are assumed

to modify similarly for *Stygobromus*. Only females were analyzed because females typically outnumber males (Culver and Holsinger, 1969), for some species no males are in the type series, and there are often size differences between the sexes. For most species Holsinger gives the first antennal length as a ratio or range of ratios to body length. We used the mid-point of the range of ratios to convert the ratio to a length. We also used the mid-point of the range of number of flagellar segments. The number of females in the type series ranged from 1 to 100, but typically the number was less than 10. As is typical of K selected species, the majority of individuals are sexually mature, and at least the type specimen was always sexually mature. The differences in size should reflect differences in size at sexual maturity rather than differences in age or sexual maturity.

Stygobromus occurs throughout North America, mostly in the U.S., but only species east of the Mississippi River were analyzed because more information on habitat was generally available. No cladistic analysis of the genus using either morphology or DNA sequences has been done, but Holsinger (1967, 1978) has organized the species into species groups, which may represent monophyletic lineages. With few exceptions, species groups are based primarily on the morphological structure of gnathopod propodi, periopods 5, 6, and 7, pleonal plates, uropod 3, and telson. A total of 21 species groups and 7 unassigned species are described from eastern United States.

The 56 eastern U.S. species of *Stygobromus* were classified by habitat into five categories:

1) epikarst
2) hypotelminorheic and other superficial subterranean habitats not associated with caves and karst
3) cave streams
4) deep groundwater
5) generalist species not predominantly in any one of the above habitats.

Most species and all populations were found in a single habitat. Species for which the different populations that were found at least 80 percent of the time in one habitat, were classified as being in that habitat. Other species (3) were listed as generalists and were not further analyzed.

Body size among habitats was analyzed using ANOVA and pairwise comparisons among habitats using the Tukey-Kramer HSD test procedure. Because *Stygobromus*, like most crustaceans, shows indeterminate growth, antennal characters can best be compared by removing the effect of body size. To do this for the two antennal characters, Analysis of Covariance (ANCOVA) was done using natural log of body size as the covariate. For both ANOVA and ANCOVA, tests of residuals of untransformed data failed the Shapiro-Wilk test of normality, but residuals of natural log transformed data passed the Shapiro-Wilk test of normality, and so natural log transformed data was used throughout. Statistical analysis was done using JMP© 8.0 (SAS Institute Inc.). All tests used a type I error rate of $\alpha = 0.05$.

RESULTS

The basic data are shown in Table 1. A total of 40 (71 percent) of the 56 species of *Stygobromus* east of the Mississippi River are primarily associated with superficial subterranean habitats: 28 are found in epikarst, and another 12 are found in hypotelminorheic and related habitats. Only 9 of 56 were primarily associated with cave streams and four species were found primarily in phreatic habitats. An additional three can be classified as generalists. One of these, *S. alleghenensis*, is exceptionally general in its habitat, different populations being found in all four habitats, as well as occasionally in mines and surface streams. However, its occurrence in surface streams probably results from occasional washout through springs.

There was some evidence that some species groups recognized by Holsinger (1978, 2009) tended to be found in particular habitats. While the species groups are not the result of a cladistic analysis, they are the only available indication of likely phylogenetic relatedness and are assumed to be monophyletic. Of 13 species groups, three

had five or more species: the *emarginatus-*, *ephemerus-*, and *mackini-* species groups (Table 2). Two of the groups (*emarginatus* and *mackini*) had representatives in three or four of the habitats, but the *ephemerus* group was exclusively epikarstic. Each group is relatively wide ranging geographically, occurring in two to four states.

Body lengths differed among the four habitat groups (ANOVA, $F_{3,49} = 3.96$, $P = 0.013$), and habitat groups accounted for 19.5 percent of the total variance in body size. The Tukey-Kramer HSD test indicated two overlapping distinct groups: epikarst + hypotelminorheic + phreatic, and hypotelminorheic + phreatic + stream. Species from epikarst were the smallest, followed by hypotelminorheic, phreatic, and cave stream habitats (Fig. 1). The differences in body size were quite large; the mean for epikarst species was 5.3 mm and the mean for cave stream habitats was 9.0 mm. A striking and unexpected feature of all four habitat groups was the presence of a large species (> 15 mm): *S. stellmacki* (epikarst), *S. gracilipes* (stream), *S. pizzinii* (hypotelminorheic), and *S. grandis* (phreatic). These outliers are all the more striking because there were only two species between 11 and 15 mm in size. Only *S. baroodyi* and *S. emarginatus*, both cave stream dwellers, fall in this size range (Table 1). We compared the ANOVA results with and without the four species and found that the results are virtually identical – the largest difference being that R^2 increased to 20.4% from 19.5%.

The ANCOVA of antennal flagellar number assuming unequal slopes showed no significant differences among slopes ($F = 0.131$, $P = 0.94$) so that ANCOVA was done assuming equal slopes. The equal slopes ANCOVA gave some weak indication of differences among habitats (Table 3). Size had a highly significant effect on antennal flagellar number, and the effect of habitat, corrected for size, was marginally significant ($P = 0.056$ after the removal of the interaction term, and $P = 0.080$ with the interaction term included). Least squares means (adjusted to mean body size) were in the rank order of cave streams, hypotelminorheic, phreatic, and finally epikarst (Table 3B). Because the overall ANCOVA was only marginally significant; all pairwise comparisons were not significant.

Analysis of Covariance yielded non-significant results for the effect of habitat on antennal length (Table 4). As was the case for antennal flagellar number, an *F*-test indicated no differences among slopes ($P = 0.57$) so that ANCOVA was done assuming equal slopes. Size had a highly significant effect on antennal length, but the effect of habitat, corrected for size, was not significant either with the interaction term removed ($P = 0.43$) or with it retained ($P = 0.24$). Predicted means (adjusted to mean body size) were very similar to each other (Table 4B).

DISCUSSION

Overall, species of *Stygobromus* appear highly modified for subterranean life. Whatever their subterranean habitat, they lack eyes and pigment, and generally have a gracile appearance due in large part to their long appendages and antennae. For example, the length of the first antennae

Table 1. Body length, antenna I length, and antennal flagellar number for female *Stygobromus* found east of the Mississippi River. Habitats are described in the text.

Species	Taxonomic authority	Habitat	Female length	Antenna I	Mean flagellar number
abditus	Holsinger, 1978	Cave stream	6.0	3.0	18.0
ackerlyi	Holsinger, 1978	Cave stream	8.0	3.6	18.0
allegheniensis	(Holsinger, 1967)	Generalist	13.0	7.2	25.0
araeus	(Holsinger, 1969)	Hypotelminorheic	5.5	3.6	22.0
baroodyi	Holsinger, 1978	Cave stream	11.9	6.5	32.0
barryi	Holsinger, 1978	Cave stream	3.0	1.4	15.0
biggersi	Holsinger, 1978	Epikarst	7.0	3.9	15.0
borealis	Holsinger, 1978	Phreatic	4.0	2.2	10.5
carolinensis	Holsinger, 1978	Hypotelminorheic	4.5	1.9	12.0
conradi	(Holsinger, 1967)	Cave stream	8.2	4.1	17.0
cooperi	(Holsinger, 1967)	Epikarst	6.0	3.0	20.0
culveri	Holsinger, 1978	Epikarst	3.2	1.6	11.5
cumberlandus	Holsinger, 1978	Epikarst	4.5	2.0	11.0
dicksoni	Holsinger, 1978	Epikarst	6.7	2.7	14.5
emarginatus	(Hubricht, 1943)	Cave stream	14.0	7.0	23.0
ephemerus	(Holsinger, 1969)	Epikarst	5.0	1.8	9.0
estesi	Holsinger, 1978	Epikarst	5.8	2.9	17.5
exilis	Hubricht, 1943	Generalist	7.0	3.7	16.0
fecundus	Holsinger, 1978	Epikarst	4.0	1.8	12.0
fergusoni	Holsinger, 1978	Epikarst	7.0	3.7	19.5
finleyi	Holsinger, 1978	Epikarst	4.8	2.2	11.5
franzi	Holsinger, 1978	Hypotelminorheic	6.9	3.6	16.0
gracilipes	(Holsinger, 1967)	Cave stream	18.0	12.6	42.0
grandis	Holsinger, 1978	Phreatic	19.0	9.5	28.0
hayi	(Hubricht and Mackin, 1940)	Hypotelminorheic	10.0	5.0	23.5
hoffmani	Holsinger, 1978	Epikarst	5.5	2.3	12.5
hubbardi	Holsinger, 2009	Epikarst	2.3	1.2	10.5
indentatus	Holsinger, 1978	Hypotelminorheic	8.2	4.1	22.0
inexpectatus	Holsinger, 1978	Epikarst	7.7	3.3	15.0
interitus	Holsinger, 1978	Epikarst	4.2	1.7	10.0
kenki	Holsinger, 1978	Hypotelminorheic	5.5	2.5	15.0
leensis	Holsinger, 1978	Epikarst	3.7	1.7	11.0
mackini	Hubricht, 1943	Epikarst	10.0	5.2	18.5
mausi	Holsinger, 2009	Epikarst	2.5	1.1	8.0
minutus	Holsinger, 1978	Epikarst	2.3	0.8	8.0
morrisoni	(Holsinger, 1967)	Cave stream	8.0	4.2	17.5
mundus	(Holsinger, 1967)	Cave stream	8.2	3.3	18.0
nanus	Holsinger, 1978	Epikarst	2.2	1.0	8.0
nortoni	(Holsinger, 1969)	Epikarst	3.5	1.4	12.0
obrutus	Holsinger, 1978	Hypotelminorheic	3.6	1.5	11.0
parvus	(Holsinger, 1969)	Epikarst	3.0	1.7	10.0
phreaticus	Holsinger, 1978	Hypotelminorheic	7.0	3.3	17.0
pizzinii	(Shoemaker, 1938)	Hypotelminorheic	15.7		26.0
pollostus	Holsinger, 1978	Epikarst	2.5	1.1	8.0
pseudospinosus	Holsinger, 1978	Epikarst	7.0	3.9	15.0
putealis	(Holmes, 1909)	Phreatic	6.0	2.7	12.0
redactus	Holsinger, 1978	Epikarst	2.0	1.0	10.0
sextarius	Holsinger, 2009	Hypotelminorheic	3.3	1.6	12.5
smithi	Hubricht, 1943	Generalist	7.5	3.8	16.0
sparsus	Holsinger, 1978	Epikarst	4.7	2.6	18.5
spinatus	(Holsinger, 1967)	Epikarst	7.5	4.7	18.0
spinosus	(Hubricht and Mackin, 1940)	Hypotelminorheic	5.5	2.9	15.0
stegerorum	Holsinger, 1978	Phreatic	6.8	5.4	20.5
stellmacki	Holsinger, 1978	Epikarst	16.5	9.7	25.5
tenuis potomacus	(Holsinger, 1967)	Hypotelminorheic	9.9	5.0	19.5
vitreus	Cope, 1872	Epikarst	7.0	3.2	12.0

(Table 1) was typically 50 percent of body length, with a minimum of 35 percent and a maximum of 70 percent. These shared, troglomorphic features of all species of *Stygobromus* studied suggest that darkness is a major selective agent since darkness is the primary feature shared among habitats. Individual variation among species not accounted for by habitat of course may be the result of other factors, such as food availability, and phylogenetic inertia.

The most obvious difference among species of *Stygobromus* is size, and habitat type accounted for 20 percent of the variation in size. Size differences were in agreement with previous studies that have shown that crustaceans in interstitial habitats such as sand and gravel aquifers are smaller than phylogenetically related species from caves (Coineau, 2000). Culver and Ehlinger (1982) showed that, within cave streams, different sized isopods in the genus *Caecidotea* occupied the underside of different sized rocks,

Table 2.   Distribution of species, by habitat, for the three species groups of *Stygobromus* with five or more species, together with the ranges of the species groups.

Habitat	*emarginatus* group	*ephemerus* group	*mackini* group
Epikarst	4	8	5
Hypotelminorheic	2	0	1
Cave stream	3	0	1
Phreatic	0	0	1
Generalist	0	0	0
Range	Maryland, Virginia, West Virginia	Virginia, West Virginia	Alabama, Tennessee, Virginia, West Virginia

with larger isopods under larger rocks. The size of mature females was, in rank order, streams, phreatic, hypotelminorheic, and finally epikarst. Pore size of the habitats falls in more or less the same order except that streams and phreatic habitats are likely reversed (although both are relatively large). The overall correspondence between pore size and animal size suggests that pore size is an important selective factor, especially for epikarstic and cave stream species.

The occurrence of four large-sized species, one from each habitat as well as a habitat generalist, is striking and unexpected (Table 1, Fig. 1). They are from different species groups so it is unlikely to be a phylogenetic effect. One explanation is that these species occupy a distinct ecological niche that resulted from competition with other species. Alternatively, perhaps these large species of *Stygobromus* are predators of small *Stygobromus*.

If the selective environment for *Stygobromus* with respect to antennal size is the availability of organic matter and nutrients, then the order of relative antennal size should
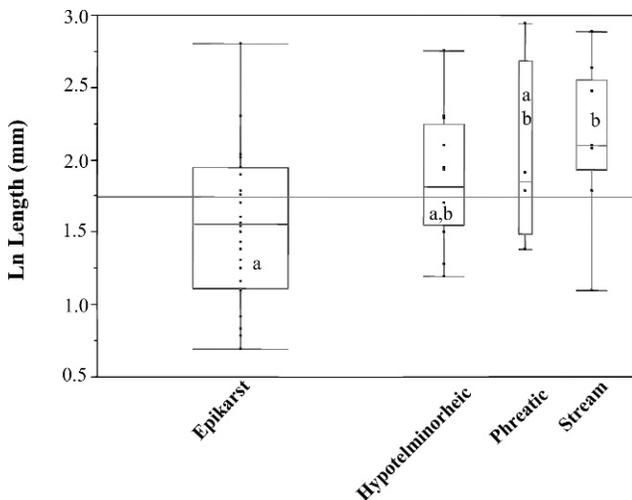

Fig. 1.   Box and whiskers plots of ln female body length for epikarst, hypotelminorheic, cave streams, and phreatic habitats. The line across the entire figure is the overall mean. The rectangles enclose the middle 50 percent of the data, the line across each rectangle is the group median, the "whiskers" are the minimum and maximum values. The width of the rectangles are proportional to sample size. Dots are individual data points. Plots with the same letter (a or b) do not differ according to the Tukey-Cramer HSD test.

be hypotelminorheic > streams ~ epikarst > phreatic. Simon et al. (2007) showed higher organic carbon levels in epikarst drips than in epikarst-fed streams. Streams are highly variable and can have either more, or less food and nutrient than epikarst (Simon and Benfield, 2001; Simon et al., 2007). Sinking streams have more organic carbon, but the biofilm utilizes epikarst organic carbon (Simon et al., 2003). In fact, there were no significant differences and even the non-significant order of size corrected antennal flagellar number: streams, hypotelminorheic, phreatic, and epikarst was not in agreement with this hypothesis of antennal size dependent on food availability. When the relative size of antennal lengths were analyzed, there were no significant differences at all, not even marginal ones (Table 4). Thus, there is no support for the hypothesis that differences in antennal elongation are the result of differences in food and nutrient supply in the different habitats.

If, on the other hand, the selective environment is the same in the four subterranean habitats, then differences in antennal length or flagellar number would be the result of different levels of adaptation, possibly the result of different lengths of time of isolation in caves and other subterranean habitats. This is the argument used by Poulson (1963) and many others since then to explain differences in morphology and physiology of fish in Amblyopsidae living in cave streams. It is possible that species of *Stygobromus* are all descended from a very small number of surface-dwelling ancestors and their shared troglomorphy is the result of a few cases of adaptation followed by dispersal.

Table 3.

A) Analysis of Covariance of ln of first antennal flagellar number, with ln of size as controlling variable.

Source	d.f.	SS	F	P
ln length	1	3.7248	85.83	<.001
habitat	3	0.2305	2.41	0.08
ln length * habitat	3	0.0125	0.13	0.94

B) Least squares means of ln first antennal flagellar number at the mean ln female length (1.75), and observed mean ln first antennal flagellar number for the four habitat types.

Habitat	Least squares mean	SE	Observed mean
Cave streams	2.835	0.061	3.046
Hypotelminorheic	2.769	0.050	2.833
Phreatic	2.657	0.088	2.797
Epikarst	2.652	0.034	2.537

Table 4.

A) Analysis of Covariance of ln of first antennal length, with ln size as controlling variable.

Source	d.f.	SS	F	P
ln length	1	8.9557	412.76	<.001
habitat	3	0.0948	0.39	0.240
ln length * habitat	3	0.0442	0.68	0.57

B) Least squares means of ln first antennal length at the mean ln female length (1.75), and observed mean ln first antennal length for the four habitat types.

Habitat	Least squares mean	SE	Observed mean
Cave streams	1.122	0.074	1.432
Hypotelminorheic	1.005	0.052	1.458
Phreatic	1.012	0.044	1.077
Epikarst	0.988	0.029	0.773

This is highly unlikely given the restricted ranges of nearly all subterranean species and reduced opportunities for dispersal (Culver et al., 2007; Trontelj et al., 2009). However, the role of time since isolation and phylogenetic inertia must await an analysis of phylogeny within *Stygobromus*. Given the partial association of habitat and species group (Table 2), the answer is likely to be both complicated and interesting.

We have not shown that there are no differences among habitats in antennal characteris correctors, only that we were unable to find significant differences. Tests of equivalence (Schuirmann, 1987) allow one to test directly the hypothesis that there are no significant differences between means, so that rejection of the null hypothesis indicates no differences between two groups. This test can be applied to our data but it requires that a biological statement of equivalent antennal lengths or segment numbers for pairs of habitats be specified. The actual test requires an estimate of reasonable bounds on the difference between two means and the confidence interval of that difference:

$$\bar{X}_1 - \bar{X}_2 \pm z_{1-\alpha/2} S.E.(\bar{X}_1 - \bar{X}_2)$$

where z is the 1-α/2 quantile for the standard normal distribution. The difficulty comes in estimating what the difference in means should be such that the difference is not biologically significant. This problem is worth further study and consideration.

With the exception of overall body size, differences in the morphology of *Stygobromus* in different subterranean habitats are small. Since the chief selective feature that superficial subterranean habitats share with caves is absence of light (Culver and Pipan, 2008), adaptation to subterranean life and the evolution of troglomorphy would seem to be the result of evolution in the dark. It is interesting to note in this context that strong evidence is accumulating that eye loss in cave animals involves direct selection, not neutral mutation (Jeffery, 2005).

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