


Body shape variation within the Southern Cavefish, *Typhlichthys subterraneus* (Percopsiformes: Amblyopsidae)

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Abstract The Southern Cavefish, *Typhlichthys subterraneus* Girard 1859, is one of the most fascinating stygobionts of the Amblyopsidae because of its undescribed diversity. Previous molecular analysis suggests the presence of at least ten distinct lineages in the Southeastern United States. Morphological variation for this group has not been quantified previous to this study. We quantified differences in body shape within the Southern Cavefish utilizing landmark-based geometric morphometrics. We found significant allometry of body shape (Relative Warps) across all putative lineages. We then performed an allometric correction to develop a size-independent morphospace. Principal components analysis indicated that the major axes of size-independent shape explained variation in relative head length to predorsal length, as well as head size and shape in both lateral and dorsal views. We examined if morphological variation corresponded to putative genetic lineages and three geographic variables (aquifer, HUC subregion, and ecoregion). We found shape differences among groups within some variables, but generally, body shape variation was not well explained by these variables. Instead, the dramatic body shape diversity

among individuals was explained by ontogeny. Poor agreement between morphology and lineages, as well as multiple geographic variables may be explained by convergent evolution of cave-adapted morphologies or cryptic morphology (i.e., no morphological characters to define diversity).

Keywords Allometry · Geometric morphometrics · Phenotypic diversity · Subterranean fish

Introduction

The amblyopsid fishes (Actinopterygii: Percopsiformes) are one of a few families of animals that include all three states associated with troglomorphy (i.e., morphological, physiological, and behavioral changes related to subterranean life). This family is restricted to North America and consists of species representing epigean (surface), stygophilic (facultative cave-dwelling), and stygobiotic (cave-obligate) forms (Woods and Inger 1957; Poulson 1963; Niemiller and Poulson 2010). Six genera comprise the family, four of which are stygobiotic. The described stygobionts include the Northern and Hoosier Cavefishes (*Amblyopsis spelaea* and *A. hoosieri*), the Ozark Cavefish (*Troglichthys rosae*), the Alabama Cavefish (*Speoplatyrhinus poulsoni*), the Southern Cavefish (*Typhlichthys subterraneus*), and *Typhlichthys eigenmanni* (no common name yet assigned). The Swampfish (*Chologaster cornuta*) and the Spring Cavefishes (*Forbesichthys* spp.) are epigean and stygophilic, respectively. Studies have detailed the higher phylogenetic relationships within the Percopsiformes as well as within the Amblyopsidae; the order and family have been recovered as monophyletic using both morphological and genetic analyses (Springer and Johnson

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2004; Dillman et al. 2011; Near et al. 2012; Niemiller et al. 2013; Betancur-R. et al. 2013; Borden et al. 2013; Grande et al. 2013; Armbruster et al. 2016).

The Southern Cavefish, *Typhlichthys subterraneus* Girard 1859, has an unusual geographic distribution throughout much of the southeastern United States, resulting in the broadest range known for a cavefish in North America; however, there is likely undescribed diversity contained within the species (Woods and Inger 1957; Poulson 1963; Swofford 1982; Niemiller and Poulson 2010; Niemiller et al. 2012). Uncertainty in the taxonomic status of *T. subterraneus* has led to three species being previously described (*T. osborni* Eigenmann 1905, *T. wyandotte* Eigenmann 1905, and *T. eigenmanni* Hubbs 1938) and subsequently synonymized (Woods and Inger 1957). However, there are numerous lines of evidence that indicate the existence of undescribed diversity, and *T. eigenmanni*, found west of the Mississippi, was recently recognized again as a distinct species (Niemiller et al. 2012; Chakrabarty et al. 2014), although the species has not been taxonomically redescribed with traditional characters (Charlton 1933, Parenti 2006).

Support for unrecognized lineages has developed from multiple lines of evidence, including the observable patterns of extreme endemism exhibited by North American cave-obligate taxa. Within the contiguous United States, 25% of the cave-obligate aquatic species reside in just six counties (<0.14% total land area). Sixty-one percent of stygobiotic and troglomorphic species and subspecies are found only in a single county (Culver et al. 2000). Low dispersal ability and physical isolation within hydrological units can lead to restricted ranges and high endemism in stygobionts (Trontelj et al. 2009). Prior to the recognition of *T. eigenmanni* as a separate species, the range of *T. subterraneus* included parts of three distinct karst regions: the Appalachians, the Interior Low Plateau, and the Ozarks (Boschung and Mayden 2004; Niemiller and Poulson 2010). Currently, the range of *T. eigenmanni* extends across the Ozarks in Missouri, Arkansas, and possibly into the northeastern tip of Oklahoma. *Typhlichthys subterraneus* is now restricted to east of the Mississippi River in Kentucky, Tennessee, Alabama, and extreme northwestern Georgia (Niemiller et al. 2016). Compared to the limited geographic distributions of other stygobionts, this vast range is extremely uncharacteristic for single stygobiotic species. Isolated hydrological units including aquifers and river basins act as physical confining units for many stygobionts (Gibert and Deharveng 2002; Trontelj et al. 2009), yet the distribution of *T. subterraneus* extends across many of the aforementioned hydrological boundaries.

In addition to an unlikely single species distribution, multiple divergent genetic lineages have been recovered

based on analyses of allozymes, as well as nuclear and mitochondrial genes (Swofford 1982; Niemiller and Fitzpatrick 2007; Niemiller et al. 2012). As many as 10 genetically distinct lineages may exist throughout the range of *T. subterraneus* (Niemiller et al. 2012); however, the number of delineated species differed with the alteration of three variables: (1) number of individuals, (2) number of populations, and (3) number of genes analyzed. Niemiller et al. (2012) focused on O'Meara's (2010) species delimitation technique, particularly the changes in lineage designation of a population when the aforementioned variables were altered. Though the lineage designation scheme provides a molecular hypothesis for the possible complex of species (Fig. 1), the number of evolutionary lineages remains undefined. Niemiller et al. (2012) found that genetic relationships were structured by surface hydrological basin and to a lesser extent surface ecoregion.

In addition to having an unusual geographic distribution and potentially distinct lineages, *T. subterraneus* also exhibits considerable body shape variation among similarly sized individuals (Fig. 2). Additionally, *T. subterraneus* exhibit body shape variation among dissimilarly sized specimens from the same locality such that there is a change in morphology and/or physiology of an organism through ontogenetic stages (Klingenberg and Zimmermann 1992; Klingenberg 1998). In small individuals of *T.*

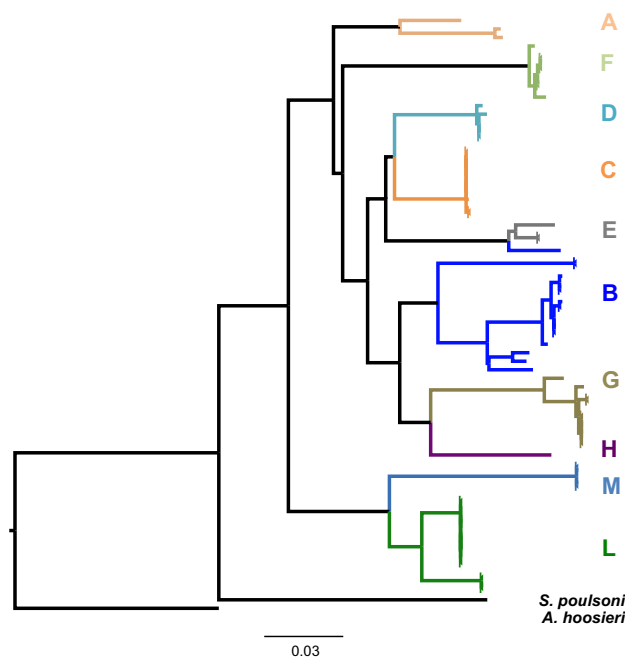
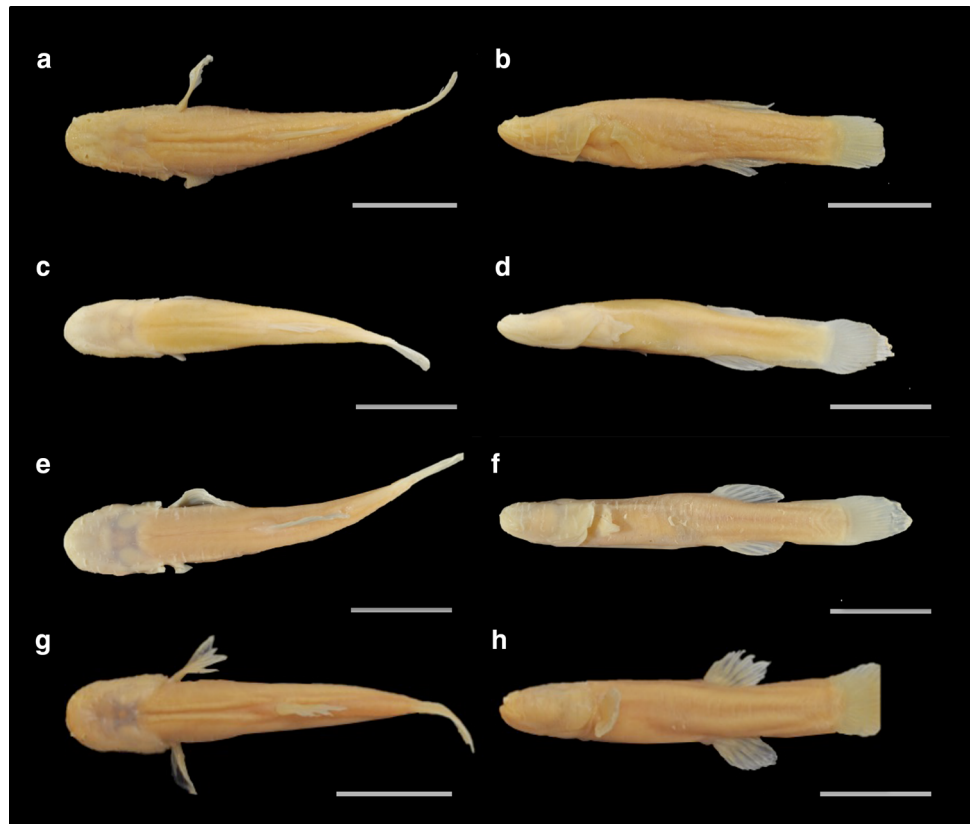


Fig. 1 Maximum likelihood, multilocus molecular phylogeny of the *Typhlichthys subterraneus* populations colored by genetic lineage (adapted from Niemiller et al. 2012). Outgroup taxa include *Speoplatyrhinus poulsoni* and *Amblyopsis hoosieri*. Scale bar unit expected substitutions per site

Fig. 2 Shape variation between adult *Typhlichthys subterraneus* specimens. Scale bars are 1 cm. Specimens are listed as follows: Cave locality, museum accession (standard length in mm): **a, b** Tally Ditch Cave, AUM 63190 [32.7], **c, d** Baugus Cave, AUM 57001 [32.3], **e, f** Camps Gulf Cave No. 2, AUM 56982 [34.1] and **g, h** L & N Railroad Cave, UF 35665 [39.1]



subterraneus, there is a considerable degree of rostral flaring and dorsoventral compression, creating the appearance of a shovel-shaped snout (Fig. 3). Retention of neotenic characters (such as rostral flaring and larger head to body ratios) is apparent between genera within the Amblyopsidae (see Niemiller and Poulson 2010; Armbruster et al. 2016); however, the shape changes between ontogenetic stages within the genera have not yet been quantified.



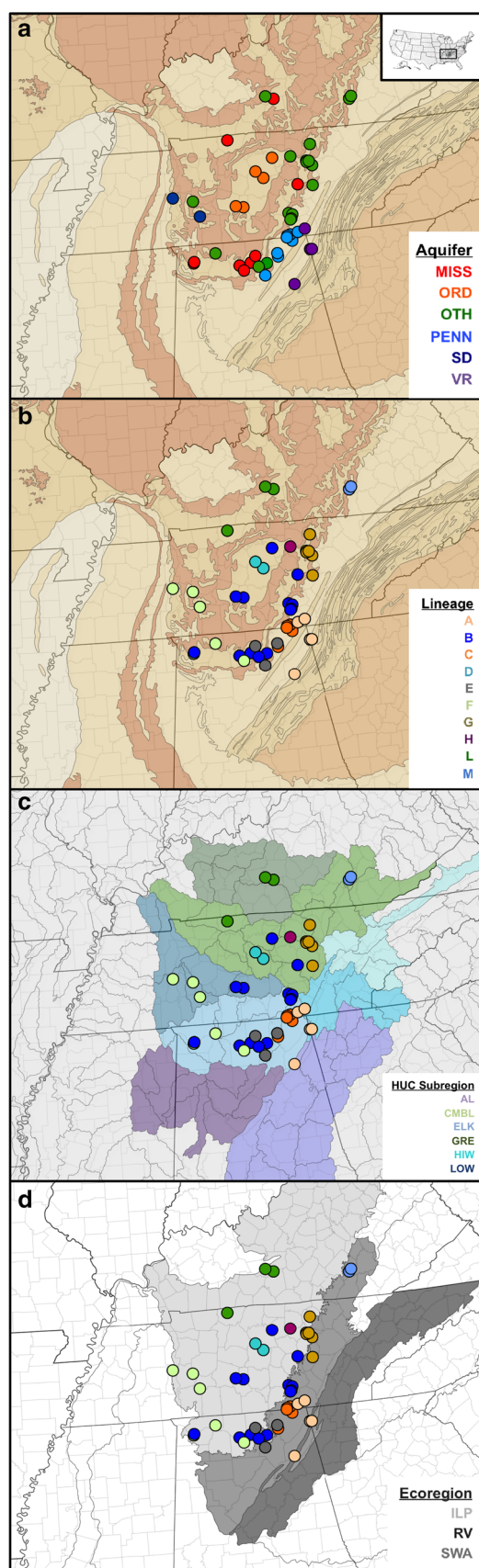
Fig. 3 An example of shape differences between juvenile and adult *Typhlichthys subterraneus*. Both specimens are from Hering Cave. **a** Juvenile with complete vent migration (20.1 mm standard length) and **b** adult (55.8 mm)

Our objective is to examine body shape variation within *T. subterraneus*. We evaluate size-independent body shape variation among genetic lineages and in relation to intuitive geographic variables that may influence cavefish distribution (i.e., aquifer, HUC subregion, and surface ecoregion). Additionally, we explore the effect of growth allometry by testing the ability of standard length to predict body shape.

Materials and methods

Specimen collection

Fieldwork occurred between fall 2012 and fall 2015. Fieldwork was concentrated in Kentucky, Alabama, Georgia, and Tennessee and across different geographic variables (Fig. 4a–d). Up to ten specimens were sacrificed at a single location by over-anesthetization using MS222. Fin clips were stored in RNA Later or 95% ethanol. Whole specimens were treated with 10% formalin solution and are stored in 70% ethanol in the Auburn University Museum of Natural History Fish Collection. We visited historical localities, as well as new caves with the hope of finding new populations. Three localities (Limrock Blowing Cave, Sells Cave, and Tally Ditch Cave) are newly sampled for



◀**Fig. 4** Distribution of *Typhlichthys subterraneus* populations utilized for this study colored by **a** aquifer association; aquifer rock types shown, **b** genetic lineage; aquifer rock types shown, **c** genetic lineage; HUC subregions shown and **d** by genetic lineage; ecoregions shown

this study with the rest (32 localities) genetically sampled in Niemiller et al. (2012; Table S1).

Shape investigation

We photographed museum specimens (Table S1) in dorsal and lateral orientation with a Nikon D90 digital SLR camera attached to a copy stand. We then utilized geometric morphometrics (GM) to describe biologically relevant body shape variation. GM landmark schemes for both dorsal and lateral views were adapted from Armbruster (2012). Body shape in dorsal view was quantified using 12 homologous, six sliding, five strictly Unbend landmarks (removed before analysis), and eight total landmarks used for the Unbend function ($N = 150$; Fig. 5a). Body shape in lateral view was quantified using 14 homologous, 12 sliding landmarks, two strictly Unbend landmarks (removed before analysis), and four total landmarks used for the unbend function ($N = 154$; Fig. 5b). We utilized sliding landmarks to quantify shape variation between homologous landmarks. These landmark schemes were digitized in tpsDig v. 2.16 (Rohlf 2010).

We used Generalized Procrustes Analysis to rotate, scale, and fit the specimen's shape onto a coordinate plane, which then provides coordinate points for each landmark. We then constructed covariance matrix and generated Relative Warps Analysis (RWA) using tpsRelw v. 1.53 (Rohlf 2013a). TpsRelw was utilized for its ability to incorporate sliding landmarks. RWA is a variable reduction technique that quantifies individual variation (Birch 1997), and it is identical to a Principal Component Analysis (PCA). The Unbend function was then utilized in tpsUtil v. 1.58 (Rohlf 2013b). This function fits user-specified points to a quadratic curve, effectively removing this type of specimen curvature, which may be associated with preservation effects (e.g., unnatural morphological variation).

Relative warps (RWs) were plotted against one another and then plotted against standard length (mm; Fig. 6a–f). We calculated correlation coefficients to determine the statistical degree to which standard length predicted axes of shape variation (i.e., RWs). We performed these comparisons to investigate whether aspects of shape variation corresponded to size and age of the fish. We used standard length to group the specimens in our study into three life history stages according to Poulson (1963): Juveniles with complete vent migration (10–20.49 mm standard length),

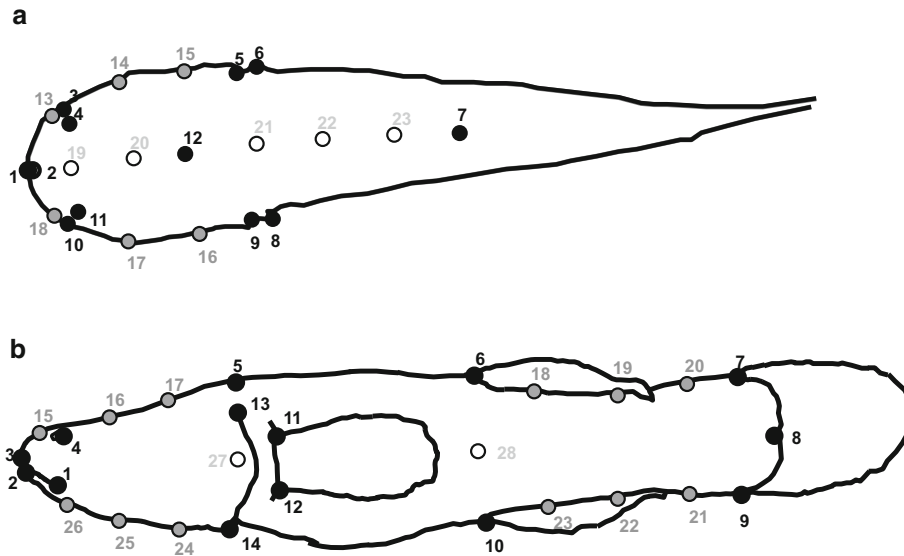


Fig. 5 Landmark schemes used for geometric morphometrics analysis. *Black circles* homologous landmarks, *gray circles* sliding landmarks, and *open circles* unbend landmarks strictly used for the unbend function and removed prior to analyses. **a** Dorsal view: 12 homologous landmarks (1–12), 6 sliding landmarks (13–18), 5

unbend landmarks (19–23), and 8 landmarks total used for the unbend function (2, 7, 12, 19–23), landmark 7 indicates the insertion of the dorsal fin, and **b** lateral view: 14 homologous landmarks (1–14), 12 sliding landmarks (15–26), 2 unbend landmarks (27, 28), and 4 landmarks total used for the unbend function (3, 8, 27, 28)

Juveniles in first annulus (20.5–30.49 mm), and adults (30.5–62 mm).

We concluded that standard length and thus age of the specimen has a profound effect on the shape of the specimen (see Results: [Pre-allometric correction](#)). To investigate relevant shape variation not associated with size, we performed a residuals analysis similar to that utilized in Sidlauskas et al. (2011). A standard linear regression using log centroid size on GM Procrustes Coordinates was performed in R v. 3.1.1. A Principal Components Analysis (PCA) was then implemented on the residuals of the regression. We then assessed the distribution of specimens in morphospace based on four variables: (1) aquifer association: Mississippian (MISS, $N = 59$), Ordovician (ORD, $N = 10$), Other Rocks (OTH, $N = 71$), Pennsylvanian (PENN, $N = 20$), Silurian Devonian (SD, $N = 4$), and Valley and Ridge (VR, $N = 10$) (US Geological Survey 2003; Fig. 4a), (2) the genetic lineage based on the adapted molecular phylogeny from Niemiller et al. (2012; (A) $N = 10$; (B) $N = 27$; (C) $N = 28$; (D) $N = 6$; (E) $N = 47$; (F) $N = 5$; (G) $N = 10$; (H) $N = 2$; (L) $N = 21$; (M) $N = 10$; Fig. 4b), (3) HUC subregion association: Alabama (AL, $N = 9$), Cumberland (CMBL, $N = 34$), Elk (ELK, $N = 98$), Green (GRE, $N = 21$), Hiwassee (HIW, $N = 1$), and Lower Tennessee (LOW, $N = 7$) (Seaber et al. 1987; Fig. 4c), (4) surface ecoregion association: Interior Low Plateau (ILP, $N = 101$), Ridge and Valley (RV, $N = 9$), and Southwestern Appalachians (SWA, $N = 61$) (Niemiller et al. 2012; Fig. 4d). For each variable, we performed an analysis of variance (ANOVA)

on the PCs to determine if significant differences ($p < 0.05$) existed among the groups within each variable. If significant differences were found, we used a Tukey's honest significant difference (HSD) post-hoc test to adjust for multiple comparisons and determine which groups were significantly different ($p < 0.05$).

Results

Pre-allometric correction

There was a significant relationship between SL and RW1 ($R^2 = 0.27$, $p < 0.001$) and RW2 ($R^2 = 0.03$, $p < 0.05$) in the dorsal view, which collectively explained 63.9% of shape variation (Fig. 6a–c). RW1 described variation in the head length and head width of the fish. The juveniles had more negative scores (narrow, elongate head) while the adults had more positive scores (wide, stout head). Thus, juveniles have longer, narrower heads than adults. Flaring of the rostral edges in juveniles can also be seen in the RW demonstration plots. RW2 had an inverse relationship with standard length; as standard length increases, head width increases but predorsal length shortens. Adults have wider heads with shorter predorsal lengths than juveniles.

In the lateral view, there was a significant relationship between SL and RW1 ($R^2 = 0.36$, $p < 0.001$). RW2 was attributable to preservation effects, thus we utilized RW3 for further analyses. RW1 and RW3 explained 52% of shape variation (Fig. 6d–f). RW1 described variation in the

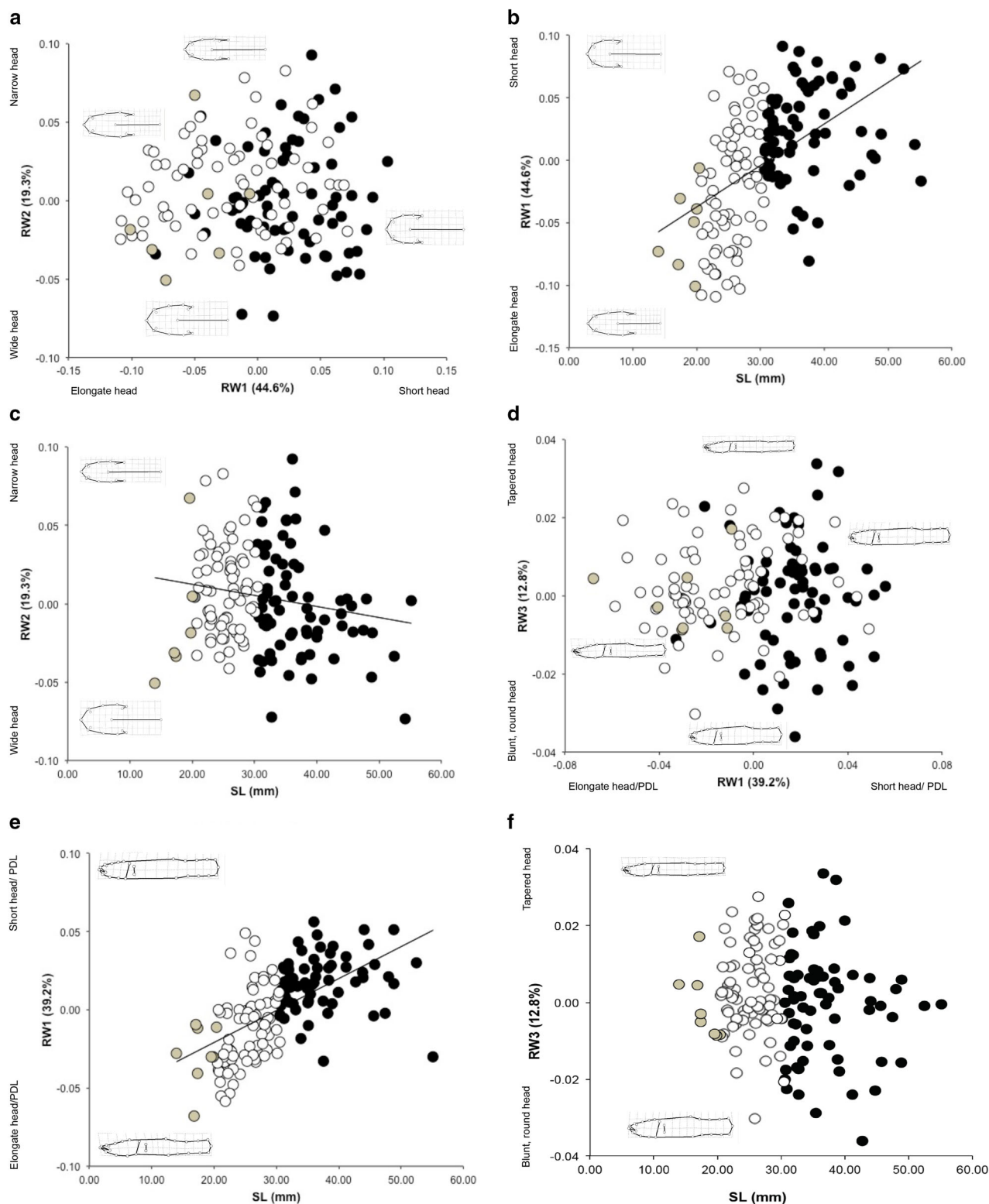


Fig. 6 Scatterplots of relative warps before allometric correction coded by life history stage. The *trendline* indicates the linear relationship between relative warp and standard length. **a–c** Dorsal

and **d–f** lateral views. *Gray circles* juvenile vent migration complete (10–20.49 mm), *open circles* juvenile first annulus (20.5–30.49 mm), *black circles* adults (30.5–62 mm)

head length to predorsal length ratio, as well as head depth. Juveniles have an elongate and dorsoventrally flattened head with a high head to predorsal length ratio. Adults have a blunt head with a small ratio of head to predorsal length. Though there was not a significant relationship, RW3 described variation in head length to predorsal length ratio ($R^2 = 0.01$, $p > 0.05$).

Allometric correction

Two biologically relevant PCs explained a total 62.5% of the variation among individuals in the dorsal orientation. PC1 explained 43.9% of the variation and described variation in relative predorsal length and head length. PC2 explained 18.6% of the variation and described variation in head width (Fig. 7). Additional PCs were excluded because they either explained minimal shape variation or explained preservation effects.

PC1 and PC3 in the lateral orientation explained a total of 47.6% variation. PC1, explaining a ratio between head length and predorsal length, described 38.1% variation (Fig. 7). PC3 explained 9.5% of the variation and described variation in head shape and depth. PC2 and the remaining PCs were excluded because they represented preservation effects.

Aquifer association plots

Results of ANOVA for the dorsal orientation indicated that there were significant differences in body shape among aquifers utilizing only PC1 ($F = 6.18$, $p < 0.001$). In contrast, body shape was not significantly different among aquifers based on the dorsal orientation PC2 ($F = 2.35$, $p > 0.05$). HSD test yielded significant results ($p < 0.05$) for three comparisons using PC1 (Table 1); however, no structuring among aquifers was visually distinguishable in the scatterplot of PC scores (Fig. 7a).

The ANOVA results for the lateral orientation indicated significant differences in body shape among aquifer associations only with PC1 (PC1: $F = 8.17$, $p < 0.001$; PC3: $F = 1.66$, $p > 0.05$). Five pairwise comparisons were significant based on PC1 (Table 2); however, none of the aquifers exhibited body shapes that were visually distinct (Fig. 7b).

Genetic lineages

ANOVA results indicated significant differences in dorsal body shape among genetic lineages for both PC1 ($F = 10.56$, $p < 0.001$) and PC2 ($F = 3.17$, $p < 0.01$). Eight comparisons from the Tukey's HSD post-hoc analysis yielded significant results (95% CI; $p < 0.05$) for PC1

in the dorsal orientation (Table 3). For PC2, two post-hoc comparisons were significantly different between genetic lineages. However, none of the genetic lineages were visually distinguishable from one another (Fig. 7c).

ANOVA results indicated that there were significant differences in lateral body shape among lineages using PC1 but not PC3 (PC1: $F = 13.4$, $p < 0.001$; PC3: $F = 0.89$, $p > 0.05$). In the lateral view, there were 11 significantly different comparisons utilizing PC1 (Table 4); however, none of the genetic lineages visually exhibited distinct body shapes (Fig. 7d).

HUC subregion

There were significant differences in both PC1 ($F = 9.53$, $p < 0.001$) and PC2 ($F = 2.54$, $p < 0.05$) among HUCs based on the ANOVA results in the dorsal orientation. Utilizing PC1, body shape was significantly different between five HUCs based on the Tukey's HSD post-hoc test (Table 5). Using PC2, there was one significant comparison (LOW and GRE), which was also significant using PC1. In lateral view, ANOVA results indicated significant differences in body shape among HUCs using PC1 ($F = 9.12$, $p < 0.001$) but not PC3 ($F = 1.7$, $p > 0.05$). Body shapes were significantly different in two comparisons utilizing PC1 (Table 6). Similar to results for aquifers and genetic lineages, body shapes were not distinguishable in scatterplots based on their HUC subregion (Fig. 7e, f).

Surface ecoregion

Significant shape variation was found among ecoregions using PC1 ($F = 28.53$, $p < 0.001$) but not PC2 ($F = 3.04$, $p > 0.05$) in the dorsal orientation. Differences in shape were significant between two comparisons utilizing the first PC (Table 7). In the lateral orientation, ANOVA results indicated that there was significant shape variation utilizing PC1 ($F = 24.7$, $p < 0.001$), but not utilizing PC3 ($F = 2.74$, $p > 0.05$). Results from Tukey's HSD post-hoc test indicated two comparisons with significant differences (Table 8). Visually, the ecoregions exhibited similar body shapes (Fig. 7g, h).

Discussion

Ontogenetic allometry

Growth allometry has not previously been quantified within the genus *Typhlichthys*. Our analyses revealed significant allometry in *T. subterraneus*. We examined confounding

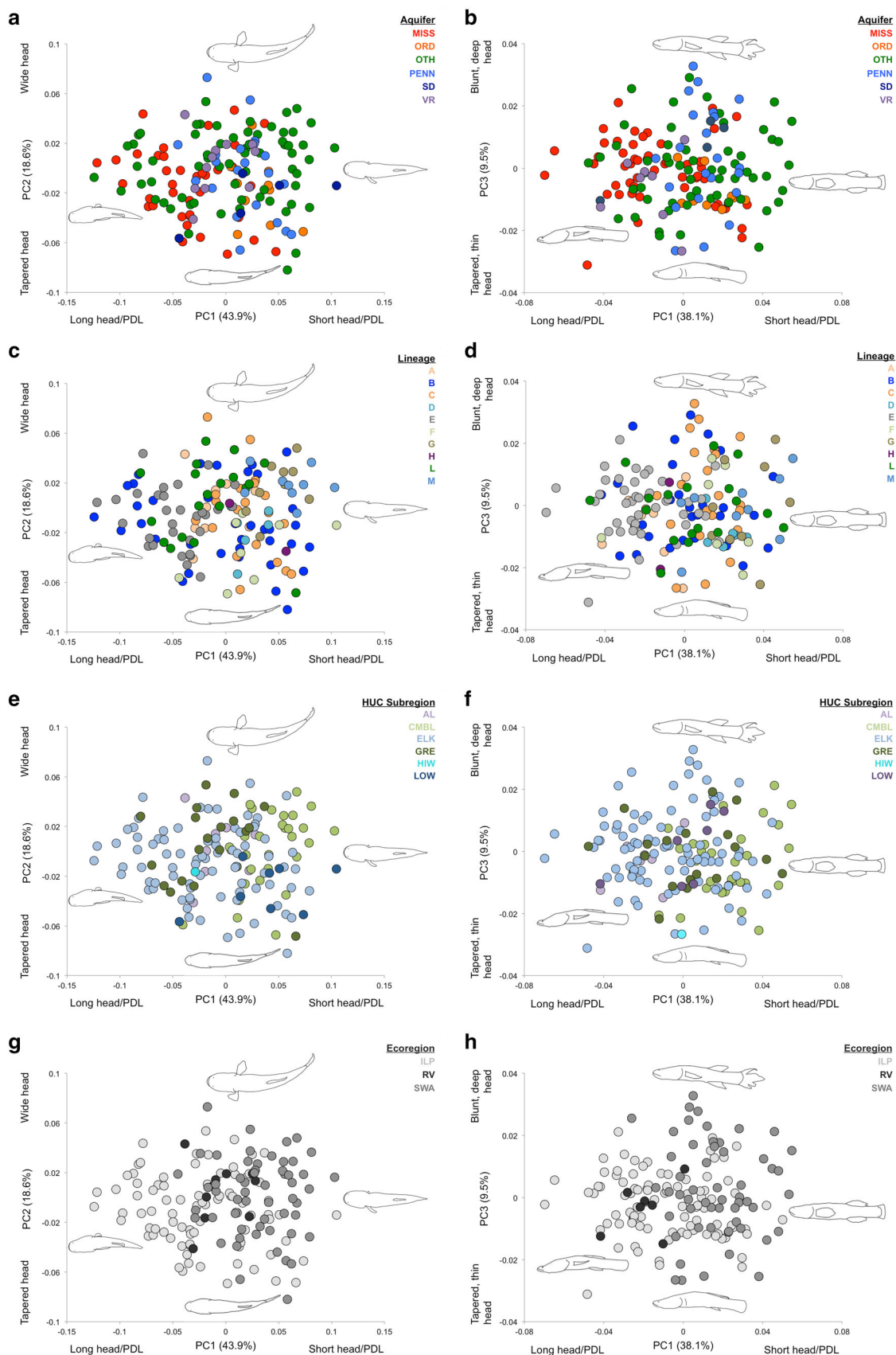


Fig. 7 Scatterplots of allometrically corrected PCA results. *Color coded by a, b* aquifer: Mississippian (MISS), Ordovician (ORD), Other Rocks (OTH), Pennsylvanian (PENN), Silurian Devonian (SD), Valley and Ridge (VR); *c, d* genetic lineage according to Niemiller et al. (2012, A–H, L, M); see Fig. 1 for phylogeny; *e, f* HUC subregion: Alabama (AL), Cumberland (CMBL), Elk (ELK), Green (GRE), Hiwassee (HIW), Lower Tennessee (LOW); and *g, h* ecoregion: Interior Low Plateau (ILP), Ridge and Valley (RV), South-western Appalachians (SWA)

effects growth allometry has on shape analyses. Standard length significantly predicted three of the four major axes of shape variation in the dorsal and the lateral orientations. Juveniles had elongate, dorsoventrally flattened heads, with higher ratios of head to predorsal length. Flaring of the rostral edges was also a noticeably juvenile trait. The shape of *T. subterraneus* juveniles is concordant with the shape of many troglomorphic vertebrate genera that retain neotenic

Table 1 Tukey's honest significant difference analysis *p* values for aquifer comparisons in the dorsal orientation

	Mississippian	Ordovician	Other rocks	Pennsylvanian	Silurian Devonian	Valley and Ridge
Mississippian		0.9945	0.1303	0.8890	0.9557	0.6242
Ordovician	0.0015		0.3104	0.8054	0.9987	0.5589
Other rocks	0.0008	0.5642		0.9538	0.3586	1.0000
Pennsylvanian	0.0011	0.9658	0.9164		0.7210	0.9869
Silurian Devonian	0.0551	1.0000	0.8841	0.9956		0.5021
Valley and Ridge	0.5674	0.3827	0.9518	0.7006	0.6849	

PC1 is below the diagonal; PC2 is above the diagonal. Significant values are bolded

Table 2 Tukey's honest significant difference analysis *p* values for aquifer comparisons in the lateral orientation

	Mississippian	Ordovician	Other rocks	Pennsylvanian	Silurian Devonian	Valley and Ridge
Mississippian		0.5932	1.0000	0.8014	0.8885	0.8375
Ordovician	0.0101		0.5436	0.2051	0.4049	0.9998
Other rocks	0.0000	0.9868		0.8306	0.9018	0.8038
Pennsylvanian	0.0005	0.9988	0.9996		0.9980	0.4144
Silurian Devonian	0.7746	0.9402	0.9893	0.9772		0.5606
Valley and Ridge	0.9997	0.0673	0.0410	0.0495	0.7864	

PC1 is below the diagonal; PC3 is above the diagonal. Significant values are bolded

Table 3 Tukey's honest significant difference analysis *p* values for genetic lineage comparisons in the dorsal orientation

	Lineage A	Lineage B	Lineage C	Lineage D	Lineage E	Lineage F	Lineage G	Lineage H	Lineage L
Lineage A		0.8423	0.9988	0.9161	0.9867	0.1739	0.9826	0.9976	1.0000
Lineage B	1.0000		0.9950	1.0000	0.9994	0.7196	0.1369	1.0000	0.2973
Lineage C	0.6891	0.4948		0.9953	1.0000	0.3783	0.6167	1.0000	0.9354
Lineage D	0.9126	0.9173	1.0000		0.9988	0.9917	0.3794	1.0000	0.6972
Lineage E	0.1119	0.0005	0.0000	0.0051		0.4389	0.3890	1.0000	0.7457
Lineage F	0.8193	0.7911	1.0000	1.0000	0.0006		0.0138	0.9988	0.0339
Lineage G	0.0437	0.0127	0.6663	0.9266	0.0000	0.9135		0.8999	0.9969
Lineage H	0.9608	0.9724	1.0000	1.0000	0.1716	1.0000	0.9998		0.9866
Lineage L	0.9999	0.9830	0.1286	0.5906	0.1554	0.3782	0.0023	0.8515	

PC1 is below the diagonal; PC2 is above the diagonal. Significant values are bolded

Table 4 Tukey's honest significant difference analysis *p* values for genetic lineage comparisons in the lateral orientation

	Lineage A	Lineage B	Lineage C	Lineage D	Lineage E	Lineage F	Lineage G	Lineage H	Lineage L
Lineage A		0.9666	0.5985	1.0000	0.9506	1.0000	0.9959	1.0000	0.9862
Lineage B	0.3484		0.9703	0.8805	1.0000	0.9999	1.0000	0.9995	1.0000
Lineage C	0.0243	0.7790		0.4524	0.9653	0.9446	0.9891	0.9776	0.9762
Lineage D	0.0173	0.4679	0.9880		0.8478	0.9985	0.9697	1.0000	0.9317
Lineage E	0.9998	0.0001	0.0000	0.0001		0.9998	1.0000	0.9993	1.0000
Lineage F	0.1284	0.9589	1.0000	0.9984	0.0018		1.0000	1.0000	1.0000
Lineage G	0.0000	0.0010	0.1287	0.9747	0.0000	0.5021		0.9999	1.0000
Lineage H	1.0000	0.9986	0.9112	0.6645	0.9979	0.9303	0.1482		0.9997
Lineage L	0.2561	1.0000	0.9830	0.7385	0.0003	0.9965	0.0116	0.9942	

PC1 is below the diagonal; PC3 is above the diagonal. Significant values are bolded

Table 5 Tukey's honest significant difference analysis *p* values for HUC subregion comparisons in the dorsal orientation

	Alabama	Cumberland	Elk	Green	Hiwassee	Low
Alabama		1.0000	0.7594	1.0000	0.9833	0.1345
Cumberland	0.0495		0.6114	0.9864	0.9914	0.0852
Elk	0.9713	0.0000		0.3144	0.9998	0.3659
Green	0.9895	0.0003	1.0000		0.9716	0.0393
Hiwassee	0.9953	0.6173	0.9997	0.9997		0.9980
Low	0.3015	1.0000	0.0122	0.0459	0.6979	

PC1 is below the diagonal; PC2 is above the diagonal. Significant values are bolded

Table 6 Tukey's honest significant difference analysis *p* values for HUC subregion comparisons in the lateral orientation

	Alabama	Cumberland	Elk	Green	Hiwassee	Low
Alabama		1.0000	0.9492	0.9961	0.4715	0.9873
Cumberland	0.0002		0.4528	0.9530	0.4356	0.9436
Elk	0.7441	0.0000		0.9966	0.2193	1.0000
Green	0.1920	0.0636	0.3932		0.3006	0.9998
Hiwassee	0.9718	0.9086	0.9997	1.0000		0.2835
Low	0.5509	0.1935	0.9435	0.9996	1.0000	

PC1 is below the diagonal; PC3 is above the diagonal. Significant values are bolded

Table 7 Tukey's honest significant difference analysis *p* values for ecoregion comparisons in the dorsal orientation

	Interior Low Plateau	Ridge and Valley	Southwestern Appalachians
Interior Low Plateau		0.2978	0.0694
Ridge and Valley	0.4309		0.9409
Southwestern Appalachians	0.0000	0.0289	

PC1 is below the diagonal; PC2 is above the diagonal. Significant values are bolded

Table 8 Tukey's honest significant difference analysis *p* values for ecoregion comparisons in the lateral orientation

	Interior Low Plateau	Ridge and Valley	Southwestern Appalachians
Interior Low Plateau		0.9698	0.0651
Ridge and Valley	0.4395		0.4602
Southwestern Appalachians	0.0000	0.0003	

PC1 is below the diagonal; PC3 is above the diagonal. Significant values are bolded

characters (Niemiller and Poulson 2010; Christiansen 2012; Fenolio et al. 2013); the adaptive value for most of these neotenic characters is unknown and warrants exploration. In contrast, blunt, wide heads with deep bodies characterized adult *T. subterraneus* specimens.

Shape variation independent of size

We discovered that the major axes of size-independent shape variation were head length to predorsal length ratio, head width, and head depth. Some aspects of shape variation could distinguish some aquifers, HUC subregions, and surface ecoregions; however, these differences were often based on a single axis of shape variation (i.e., PC1), rather than multiple axes of shape variation. There were large discrepancies in shape diversity exhibited by some groups within aquifers, HUC subregion, and surface ecoregions. For example, some HUC subregions (e.g., the Elk and Cumberland) and surface ecoregions (e.g., the Interior Low Plateau) are geographically expansive and encompass numerous caves, and therefore, may simply exhibit more body shape diversity due to larger sample size. However, none of these geographic variables sufficiently explained the body shape diversity within *T. subterraneus* due to large degrees of overlap in body shape among most groups.

Geographic unit distributions in morphospace

The Mississippian aquifer and other rocks aquifer have wider distributions across morphospace within scatterplots than the remaining four aquifers in both the dorsal and the lateral views (Fig. 7a, b). The comparably larger range across morphospace for these two aquifers is most likely due to the geographic distribution of the caves in the Southeastern United States; there are more caves associated with the Mississippian and other rocks aquifers than the remaining aquifers. When comparing aquifers (such as the Mississippian aquifer to the Silurian–Devonian aquifer), the aquifer with the greater geographic range will be associated with a greater range of caves (Fig. 4a). Since there are more caves associated with the Mississippian aquifer than the Silurian–Devonian aquifer, there are a greater number of samples from the Mississippian aquifer. Subsequently, a larger sample of specimens may possess more shape variation. Thus, the Mississippian aquifer has a wider distribution in morphospace when compared to another aquifer with fewer associated caves.

Similar to aquifer distributions in morphospace, two HUC subregions (the Elk and the Cumberland subregions) have wider distributions in morphospace when compared to the other four subregions (Fig. 7e, f). Caves are not evenly distributed among HUC subregions, thus sampling is

denser in some HUC subregions (Fig. 4c). Subsequently, some of the discrepancies in body shape diversity among HUC subregions are likely attributable to variation in sampling. For example, large diversity of body shape is apparent for both the Elk and Cumberland HUC subregions when compared to the remaining subregions.

Analogous to the aquifers and HUC subregions, the distribution of a surface ecoregion across morphospace is dependent on the geographic size of the ecoregion itself (Fig. 4d). For example, the Interior Low Plateau spans the largest geographic area and consequently encompasses the most caves, and therefore the largest morphological variation occurs within this ecoregion (Fig. 7g, h).

Possible explanations for morphological variation

Convergent evolution, sexual dimorphism, conditional variation, and phenotypic plasticity are several potential explanations for why the variation within *T. subterraneus* is dramatic, but does not correspond to their genetic lineage or the geographic variables that they inhabit. First, convergent evolution is prevalent in cave environments. Similarly harsh selective pressures (e.g., complete darkness and scarcity of resources) can cause morphological convergence on a phenotype that is advantageous such as being energy inexpensive. The phenotype of many cave-obligate vertebrate taxa converges on an eyeless, pigmentless form with neotenic characters (Niemiller and Poulson 2010; Christiansen 2012; Fenolio et al. 2013). Caves likely impose similar selection pressures on populations of *T. subterraneus* regardless of the aquifer, HUC subregion, or surface ecoregion in which the cave resides. Thus, both parallel and convergent evolution may result in similarly shaped *T. subterraneus* across their expansive range.

Furthermore, there may be functional sexual dimorphism within the Amblyopsidae. Eggs and yolk-sac fry have been observed in the buccal cavity of the Northern Cavefish, *A. spelaea*, and the Hoosier Cavefish, *A. hoosieri* (Eigenmann 1909; Niemiller and Poulson 2010; Chakrabarty et al. 2014). It is also hypothesized that the jugular positioning of the anus in the Amblyopsidae is for buccal brooding (Poulson 1963). If buccal brooding is family-wide, the head shape of the gender that broods the young may be different from that of the non-brooding parent due to physical and physiological demands from brooding the young. For example, the brooding parent might have a larger buccal cavity than the non-brooding parent, altering the head shape of the fish based on their sex; however, no *Typhlichthys* has ever been found with young, and it is unlikely that there is enough space in the buccal region to hold a clutch of eggs (Niemiller and Poulson 2010; Armbruster et al. 2016). Future directions should include

examination of reproductive organs to determine if there is sexual dimorphism.

Additionally, many caves are energy-poor environments such that they cannot support photosynthesizing primary producers (Poulson and Lavoie 2000). Some caves support invertebrate and vertebrate communities with autochthonous production via hydrogen sulfide associated bacteria (Roach et al. 2001), but most cave communities rely upon subsidies from the terrestrial environment such as leaf litter, organic waste, and carrion. Due to the nutrient-poor nature of caves, as well as the unpredictable nature of inputs from the terrestrial environment, some of the variation we observed in *T. subterraneus* may be due to variation in condition. Lastly, some of the variation in body shape could be phenotypic plasticity. For example, the cave systems included in this study vary greatly in their size and complexity, which can affect food chain length and food web composition (Post 2002; Post and Takimoto 2007). In the case of *T. subterraneus*, which feed primarily upon small crustaceans, head and jaw shapes may exhibit plasticity in response to prey exploitation if prey availability is heterogeneous among caves. Potential contributions of phenotypic convergence, sex-specific morphology, variable condition, and plasticity may partly explain our inability to sufficiently distinguish groups among genetic lineages, aquifers, HUC subregions, and surface ecoregions based on body shape.

Future taxonomic investigations

Our analysis of body shape variation within *T. subterraneus* may partly explain the history of taxonomic uncertainty with this taxon. Despite strong support that there are distinct lineages (Niemiller et al. 2012), we found that the dramatic body shape variation within the species did not correspond well with these lineages or intuitive geographic units that may constrain dispersal and thereby influence gene flow. We did identify several traits that distinguish individuals from some of these groups such as the head length to predorsal length ratio. Future taxonomic work should utilize multiple types of data to further investigate the unrecognized diversity within *T. subterraneus*, including additional molecular sequences, morphological characters, and their geographic distribution. For example, lineage A is the sole genetic lineage found in the Valley and Ridge aquifer; and although they overlap with other lineages in several axes of body shape variation, lineage A does have a limited distribution in morphospace associated with intermediate head length to predorsal length ratios (i.e., PC1). Lastly, our results emphasize the significant influence of allometry when comparing populations, and therefore, will need to be a central consideration in future comparative analysis.

Conclusions

Although shape alone cannot distinguish the intuitive geographic boundaries used in this study (e.g., aquifer, HUC subregion, and surface ecoregion), our results will assist taxonomic investigations by revealing discrete morphological characters that may be useful in discriminating some of the genetic lineages. Among numerous genetic lineages of *T. subterraneus* that span six aquifers, six HUC subregions and three surface ecoregions, the main determinant of body shape was body size. Our study provides further insight into the driving factors behind physical changes of troglomorphic fishes and specifically implicates size-structured processes or constraints that transcend lineages and numerous environmental factors.

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Compliance with ethical standards

Ethical statement This research is not in consideration or published elsewhere. Accession numbers of all specimens used in the analyses are provided in the supplementary material. All co-authors approve the submission of this manuscript. The authors declare that they have no conflict of interest. Specimens were collected in accordance with the Alabama Department of Conservation and Natural Resources Alabama conservation license (2016087218468680-8823) and the Georgia Department of Conservation of Natural Resources scientific collecting permit (29-WJH-14-120), and Auburn University IACUC PRN 2014-2451.

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