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Evolutionary transition from surface to subterranean living in Australian water beetles (Coleoptera: Dytiscidae) through adaptive and relaxed selection

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ABSTRACT

Over the last 5 million years, numerous species of Australian stygobiotic (subterranean and aquatic) beetles have evolved underground following independent colonization of aquifers by surface ancestors, providing a set of repeated evolutionary transitions from surface to subterranean life. We used this system as an 'evolutionary experiment' to investigate whether relaxed selection has provided a source of variability for adaptive radiations into ecosystems containing open niches and whether this variability underpins phenotypic evolution in cave animals. Linear and landmark-based measurements were used to quantify the morphology of subterranean species from different aquifers, compared to interstitial and closely related aquatic surface species. Subterranean dytiscids were observed to be morphologically distinct, suggesting they have a different lifestyle compared to their surface relatives. However, variation in the measured traits was much greater in the subterranean species, and unstructured, showing no evidence of clustering that would indicate adaptation to specific niches. Furthermore, a previously identified pattern of repeated nonoverlapping size variation in beetles across aquifers was not correlated with repeated body shape evolution. The observed variability across body shape and limb traits provides support for the hypothesis that relaxed selection and neutral evolution underlie the phenotypic evolution in these species.

Keywords: Dytiscidae; epigean; evolution; morphology; stygobiotic; subterranean

INTRODUCTION

Phenotypic evolution is generally assumed to result from natural selection, and even for traits that regress or are lost in evolution (e.g. eyes and pigment in cave animals), selectionist theories dominate the literature (Plate 1910, Huppop 2000, Borowsky and Wilkens 2002, Niven 2007, 2008, Protas *et al.* 2007, 2008, Borowsky 2008, Niven and Laughlin 2008, Stearns 2010, Moran *et al.* 2023; but see Wilkens 2020). It was recently proposed, however, that relaxed selection provides a source of variability for adaptive radiations into ecosystems containing open niches and that this variability, found in traits such as the viscerocranium in fishes, parallels the variability initially found during the early evolution of eye size and pigment traits in cave animals prior to their complete regression (Wilkens 2021). Wilkens (2020, 2021) has argued that the latter variability results from relaxation of purifying selection on genes that are no longer required in the absence of light. Evidence for neutral evolution and loss of function of genes associated with regressed traits (e.g. opsin genes) is strong (e.g. Springer *et al.* 1997, Niemiller *et al.* 2013, Emerling and Springer 2014, Tierney *et al.* 2015, Langille *et al.* 2022), but, despite this, the influence of relaxed selection on phenotypic evolution has not been widely accepted (reviewed by Culver *et al.* 2023). Ultimately, verification of a possible role for relaxed selection in phenotypic evolution requires systems

where the same evolutionary experiment is repeated under similar ecological and environmental conditions, and it is possible to contrast the variability in traits that are constructively evolving with those that are potentially regressing.

One such system has been identified in calcrete (carbonate) aquifers of central Western Australia and the Northern Territory, which contain a highly diverse subterranean diving beetle (Coleoptera: Dytiscidae) fauna (Watts and Humphreys 2009 and references therein; Austin *et al.* 2023), in addition to a variety of other subterranean aquatic organisms, referred to as stygofauna (Cooper *et al.* 2007, 2008, Guzik *et al.* 2008, Bradford *et al.* 2010, Karanovic and Cooper 2011, Abrams *et al.* 2012). Around 90 beetle species are known from 50 aquifers, with between one and three species per aquifer, and individual species are usually endemic to a single calcrete body (Cooper *et al.* 2002, Leys *et al.* 2003, Watts and Humphreys 2006, 2009, and references therein). The majority of species (75%) are likely to have evolved independently from widespread surface ancestral species (Leijs *et al.* 2012), providing an opportunity to study regressive and adaptive evolutionary processes in parallel at an 'unheralded comparative scale' (Tierney *et al.* 2018). There is also strong evidence for speciation underground from subterranean ancestors (25% of species), providing a unique comparison in trait evolution (Leys *et al.* 2003, Leijs *et al.* 2012, Langille *et al.* 2021, Cooper *et al.* 2023).

Within calcrete aquifers, beetle species show a repeated pattern of nonoverlapping size variation, but absolute sizes vary from aquifer to aquifer, consistent with their self-organization under a limiting similarity model (Vergnon *et al.* 2013). Under this model, two species must be sufficiently different to coexist (MacArthur and Levins 1967), in comparison to a model where pre-existing environmental niches drive the size differences. Individual species also appear to exhibit extensive body shape variation (e.g. Watts and Humphreys 2003, 2004, 2006, 2009). However, the latter has never previously been formally quantified in subterranean dytiscid species or compared with the shape of surface species. Based on behavioural observations, subterranean dytiscids crawl around in their aquifer habitat and are not active swimmers, unlike surface species, which generally have a streamlined body shape to improve their speed and ability to catch prey under water (Yee 2014, Miller and Bergsten 2016). For subterranean species, which are physiologically adapted to stygobiotic life and live permanently under water (Jones *et al.* 2019), we therefore predict that they may no longer need to maintain a streamlined body shape. However, whether natural selection has driven the evolution of alternative body shapes to enable their adaptation to their aquifer habitat is unclear. Additional traits which are likely to be adaptive for the subterranean beetles, based on studies of other cave animals, include elongated antennae and limbs (Poulson 1963, Juberthie and Massoud 1977, Jones *et al.* 1992, Culver *et al.* 1994, Turk *et al.* 1996, Pérez-González and Zaballos 2013, Liu *et al.* 2017, Balart-García *et al.* 2021).

Here we use a dataset of more than 100 Australian dytiscid beetles to test whether the transition from surface to subterranean life has resulted in convergent morphological changes, indicative of adaptive evolution, or resulted in variable, unstructured divergent morphology, potentially indicative of relaxed selection. We examine three specific aims and hypotheses:

(1) To investigate the differences in body shape and amount of morphological disparity among surface and subterranean species. We hypothesize that a strong adaptive selection pressure is exerted on the body shape of surface species to enable them to actively swim and catch their aquatic prey, evidenced by a narrow range of streamlined body shapes in this group. A release of this selection pressure in subterranean dytiscids would lead to a greater diversity of body shapes occupying the morphospace. Alternatively, similarly narrow ranges of body shapes in distinct regions of morphospace from the surface species would indicate adaptive evolution for novel niches.

(2) To investigate differences in sensory (antennae) and locomotory (limbs) appendages among surface and subterranean species. We hypothesize that subterranean species will show adaptive evolution of their sensory and locomotory appendages, compared to surface dytiscids, to enable them to find prey and mates in the dark. Given the findings in previous studies of subterranean animals, we predict that antennae will be longer, and limbs would be enlarged/elongated.

(3) To examine whether the repeated pattern of body size evolution (i.e. large, medium or small relative sizes) within aquifers corresponds to repeated body shape evolution. If body size relates to distinct niche partitioning, we might expect there to be distinct body shapes for each size class. Alternatively, if body shape is not adaptive, there will be no pattern to body shape variation across size classes.

MATERIALS AND METHODS

Overview of samples and the ecosystem

A total of 362 specimens were measured from the South Australian Museum (SAMA) Entomology collection, plus 13 published drawings (Watts and Humphreys 2003, 2004, 2006, 2009). This comprised 121 species (108 from SAMA and 13 from camera-lucida drawings) in Dytiscidae (Coleoptera), including 31 'surface' aquatic diving species, 88 'subterranean' aquifer-inhabiting species, and four 'interstitial' species (Fig. 1).

These classifications refer to their ecology: surface refers to diving dytiscid species that inhabit above-ground stream- or pond-related habitats; subterranean refers to species that live in underground aquifers (calcrete/fractured rock); and interstitial species reside in ephemeral water bodies such as seasonal drying streams. The subterranean calcrete fissure ecosystem exhibits a complex abiotic physicochemical environment, albeit with a dampened dynamic and temporal hydrology (Michael *et al.* 2005, Saccò *et al.* 2020b). Nevertheless, the hydrology of Australian subterranean ecosystems can vary significantly in terms of pH, salinity, and temperature due to slow-moving groundwater, episodic rainfall, and water body stratification (reviewed by Humphreys *et al.* 2009). These subterranean fissure ecosystems host diverse biological communities, including micro-organisms and invertebrates such as microbial and protozoan communities (discussed in Humphreys 2000, 2008), crustaceans such as Syncarida (e.g. Leijs *et al.* 2015) and amphipods (e.g. Cooper *et al.* 2007), and insects such as troglobiotic beetles (Carabidae) (e.g. Baehr and Main 2016) and stygobiotic beetles

Figure 1. Examples of the highly diverse subterranean diving beetle (Coleoptera: Dytiscidae) fauna: a surface diving dytiscid—*Paroster pallescens* (left), two subterranean calcrete dytiscids—*Paroster megamacrocephalus* and *Paroster arachnoides* (right), and an interstitial dytiscid—*Limbodessus occidentalis* (middle left).

(Dytiscidae) (e.g. Watts and Humphreys 2003, 2004, 2006, 2009).

A species list, including authorities for each species, is included in Supporting Information File S1. The selected dytiscid species are distributed across the genera *Limbodessus* Guignot, 1939, *Paroster* Sharp, 1882, *Allodessus* Guignot, 1953, *Gibbidessus* Watts 1978, *Neobidessodes* Hendrich & Balke, 2009, and *Uvarus* Guignot, 1939, in Dytiscidae, with over 90% of species distributed in the genera *Limbodessus* and *Paroster*. Among the 13 species sampled from drawings, five are in *Limbodessus*, five in *Paroster*, two in *Neobidessodes*, and one in *Exocelina* Broun, 1886. Species sampled from drawings were used for the aquifer triplet analysis only, as detailed below.

Digital imaging

Photographs of the dorsal view of the whole animal and close-up images of the antennae and the legs were taken with one Auto Montage system, which used algorithms to merge multiple photos in different focuses into one high-quality image. The imaging system was a Leica M205C microscope on a vertical track operated by Leica Application Suite v.3.8.0 attached to a Leica DFC500 camera. From the series of images with incremental focus, a stacked montage image was automatically produced in the Leica Application Suite v.3.8.0 (Fig. 2).

To maintain consistency and reduce the impact of specimen presentation orientations, all specimens were imaged with the scutellum or the anterior end of the middle suture of the elytra being the uppermost point. No colour calibration was used. No optical control was used except an automatic exposure time regulator at its default value. The specimens were presented as flat to the plane of the camera as possible to avoid horizontal tilts.

To capture as many details of the specimen as possible, we used optimizing steps for the multifocus within Leica Application Suite v.3.8.0. A step was defined as each focus plane. Each step varied between 0.01 and 0.05 mm. The outcome was high-resolution (4080 × 3072 pixels) TIFF image files with scale bars.
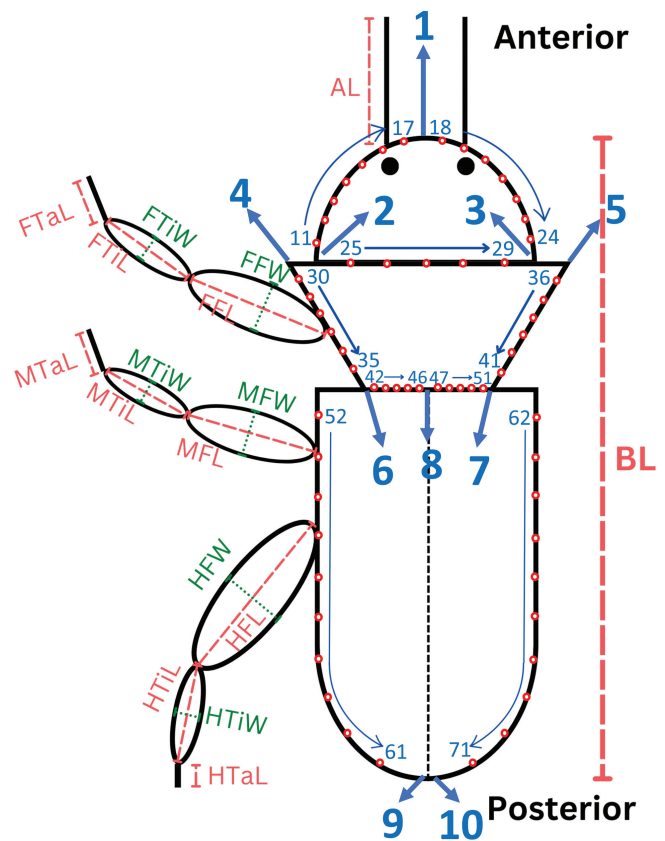
Figure 2. Illustration of the two morphometric methods used to capture morphological variation on a schematic dytiscid beetle. Body shape is defined by fixed landmarks (1–10) and semilandmarks (11–71) shown in blue. Body length, limbs, and antennae dimensions are captured with 17 linear measurements. Fixed landmarks are homologous and identifiable on all specimens. Semilandmarks describe a homologous curve. The definition of each landmark is given in Table 1. Length measurements are shown in red and width measurements in green, with acronyms explained in Table 2.

Table 1. Definitions of the fixed landmarks and semilandmarks to characterize body shape; illustrations are shown in Figure 2.

	Fixed landmarks	Semi-landmarks
Head	Three fixed landmarks including one landmark at the anterior end of the head [1], two landmarks at the posterior edge of the head [2] & [3]	Fourteen semilandmarks ([11] to [24]) to draw the anterior edge of the head, five semilandmarks ([25] to [29]) to draw the posterior edge of the head
Pronotum	Four fixed landmarks including two landmarks at the anterior end of the pronotum [4] & [5], two landmarks at the posterior end of the pronotum [6] & [7]	Twelve semilandmarks ([30] to [41]) to draw the shape of the left & right edge of the pronotum, ten semilandmarks to draw the posterior edge of the pronotum ([42] to [51])
Elytra	Three fixed landmarks including one landmark at the anterior end of the middle suture of the elytra [8], two landmarks at the posterior end of the left and right elytra [9] & [10]	Twenty semilandmarks ([52] to [71]) to draw the shape of the left and right elytra
Total	10 fixed landmarks	61 semilandmarks

Morphometrics

Two morphometric methods were used to capture the morphological variation among specimens. We quantified the morphology in the body outline of the specimens by using geometric morphometrics (landmark-based measurements). As the landmarking required a dorsal view of the beetles, we used 255 specimens of 99 species that were dorsally orientated and presented. The morphology of the three pairs of limbs and antennae were quantified using linear measurements for 107 species, each represented by one specimen.

The body shape dataset used both fixed homologous landmarks identifiable on all specimens and semilandmarks that constitute a homologous curve (Zelditch *et al.* 2012). Where possible, only male specimens were used to avoid any variation due to sexual dimorphism. Multiple specimens were selected to represent their species. However, some species were represented by a single specimen. For example, *Paroster extraordinarius*, the first and only known stygobiotic dytiscid species found in South Australia (Leys *et al.* 2010), was represented by one holotype specimen. In summary, a set of 71 landmarks were chosen to capture the outline shape of the dytiscids, illustrated in Figure 2 with definition described in Table 1. We used the software tpsDIG2 v.2.32 (Rohlf 2021) to manually place the fixed landmarks and semilandmarks on the photographs (Fig. 2). The landmarks, represented by two-dimensional coordinates, were exported in .TPS file format.

Body length, and dimensions of the limbs and antennae comprised a total of 17 measurements (in millimetres) recorded from the photographs with scale bars (Fig. 2; Table 2). When photographing, the length of the body, antennae and tarsi were recorded on-site using the default measuring tool implemented within Leica Application Suite v.3.8.0. The length and width of legs were later measured using the measuring tool in the GIMP v.2.10.32 software (The GIMP Development Team 2022). Both dorsally and ventrally presented specimens were used to capture the size of the features. As the antennae were usually curled out of the plane of the camera, up to three images were taken at different angles. The length of the antennae was recorded by the sum of the length of the 11 segments—the scape, the pedicel, and nine flagellomeres.

As the coxae of dytiscids are fused medially to the sternum and the trochanters are not always presented in the same orientation with the rest of the legs, neither coxae nor trochanters were recorded, leaving only femur, tibia, and tarsus (Fig. 2; Table 2).

Table 2. Description of the 17 linear measurements of the body length, antennae, and legs; acronyms refer to those given in Figure 2.

Linear measurements	Acronym
Body length	BL
Antennae length	AL
Forelimb femur length	FFL
Forelimb femur width	FFW
Forelimb tibia length	FTiL
Forelimb tibia width	FTiW
Forelimb tarsus length	FTaL
Midlimb femur length	MFL
Midlimb femur width	MFW
Midlimb tibia length	MTiL
Midlimb tibia width	MTiW
Midlimb tarsus length	MTaL
Hindlimb femur length	HFL
Hindlimb femur width	HFW
Hindlimb tibia length	HTiL
Hindlimb tibia width	HTiW
Hindlimb tarsus length	HTaL

Statistical analyses

All statistical analyses and visualizations were performed in the R Statistical Environment v.4.0.5 (R Core Team 2021) (hereinafter referred to as 'R'). The following R packages were used: *ape* v.5.5 (Paradis and Schliep 2019), *geomorph* v.4.0.0 (Adams *et al.* 2021, Baken *et al.* 2021), *stats* v.4.0.5 (R Core Team 2021), *ggplot2* v.3.3.6 (Wickham 2016), and *ggstatsplot* v.0.9.4 (Patil 2021). The R scripts arre included in the Supporting Information. All statistical tests were evaluated at a 5% cut-off.

Body size was measured as body length (Fig. 2). We used a Welch's unequal variances t-test to determine whether the body size of subterranean and surface species was different, implemented with the 'ggbetweenstats' function in the *ggstatsplot* R package (Patil 2021). Welch's t-test is a more reliable alternative to Student's t-test when there are differences in sample sizes and variances of two samples (Welch 1947). Since the subterranean group has a sample size approximately three times greater than that of the surface group, and the sample variances are likely to differ, a Welch's t-test was deemed more appropriate.

Landmark data: To remove the variation due to partially open elytra, we used a modified 'fixed.angle' R function in *geomorph* that rotates a set of landmarks relative to another set defined by an angle given by the user (Adams 1999). This method was modified to allow two sets of landmarks to rotate relative to a third fixed set (code in Supporting Information), and digitally close the elytra. The landmark coordinates were transformed into shape variables using the 'gpagen' function in the *geomorph* R package (Adams *et al.* 2021). This performed a generalized Procrustes superimposition, which removed scale, translation, and rotation information from the coordinates, leaving only shape variation (Zelditch *et al.* 2012). Sliding semilandmarks were permitted to slide along their tangent direction (i.e. along the curve) during superimposition using 'gpagen' in *geomorph* (Adams *et al.* 2021). To obtain the average body shape by species, we used 'aggregate', implemented in the R package *stats*.

To visualize shape variation among species, we used principal component analysis (PCA) using 'gm.prcomp' in *geomorph* (Adams *et al.* 2021). Plotting the PC axes visualized the morphospace of beetle body shape, and shape variation along each axis was plotted as a shape change from the mean body shape to the minimum and maximum of each axis, represented as a thin-plate spline (TPS) graph, using the 'plotRefToTarget' function in *geomorph*.

To determine whether there was a shape difference among the surface, subterranean, and interstitial habitat groups, an analysis of variance (ANOVA) using 'procD.lm' in *geomorph* was used, with significance evaluated through 1000 permutations. This ANOVA, sometimes called a Procrustes ANOVA when applied to landmark data, is a multivariate test designed to examine all variables simultaneously (Adams *et al.* 2021) and is equivalent to the nonparametric multivariate ANOVA (Anderson 2001). To examine whether the three habitat groups differed in terms of morphological disparity we applied a permutation procedure to compare the studied taxa, using 'morphol.disparity' in *geomorph* (Adams *et al.* 2021) with significance evaluated through 1000 permutations. Disparity was measured as Procrustes variance, which was the sum of the diagonal elements of the group covariance matrix divided by the number of observations in the group (Zelditch *et al.* 2012). This approach takes into account the number of observations in each group and scales the variance accordingly, such that uneven sampling does not affect the results.

To investigate whether the body shapes of the 15 sets of triplets were correlated with their size classes (i.e. small, medium, large), we created a subset of 44 subterranean species. This subset included 31 species that were pictured and measured in this study and 13 sampled species from published drawings (Watts and Humphreys 2003, 2004, 2006, 2009). The 44 species from 15 calcrete aquifers were chosen based upon the observed repeated pattern of nonoverlapping size variation (Watts and Humphreys 2009) that has previously been examined in the context of size-related niche evolution (Vergnon *et al.* 2013). One species (*Limbodessus insolitus*) is found in two adjacent aquifers. While absolute sizes vary from aquifer to aquifer, they often present as triplets of small, medium, and large species. We used a PCA to ordinate the body shape data for these species to visualize (i) shape variation between species within aquifers, and (ii) examine any patterns of replicated body shape patterns among all aquifers.

Linear measurements: To standardize the linear measurements for body size differences, making the data equivalent to the landmark shape data, we divided all linear variables by body length, except the widths of limbs. To highlight the robustness of the limb, by using the length–width ratio, we divided the widths of limbs by the relative length of that limb. As with the landmark data, 'aggregate', implemented in the R package *stats*, was used to obtain species means for all variables, and a PCA was used to visualize the variation among species for all variables.

To avoid biases in antenna and limb morphology across multiple genera, a subset of the genera *Limbodessus* and *Paroster* was analysed, as these two genera contain more than 90% of the studied species. To determine whether the two habitat groups (surface and subterranean) had different-sized appendages (relative to body size), Student's t-tests were conducted, with null hypotheses assuming that there was no difference between the means of the groups. Here, a t-test was performed to examine whether the surface and subterranean groups differed for each variable. Statistical results and visualization were simultaneously produced using the *ggstatsplot* package in the form of violin boxplots with highlighted outliers. A violin plot is a combination of both a box plot and a kernel density plot, which displays the full distribution of the data.

To determine whether there were differences in average dimensions and disparity in all three pairs of limbs among the surface, subterranean, and interstitial habitat groups, we used the same ANOVA and disparity approaches described above for the body shape data.
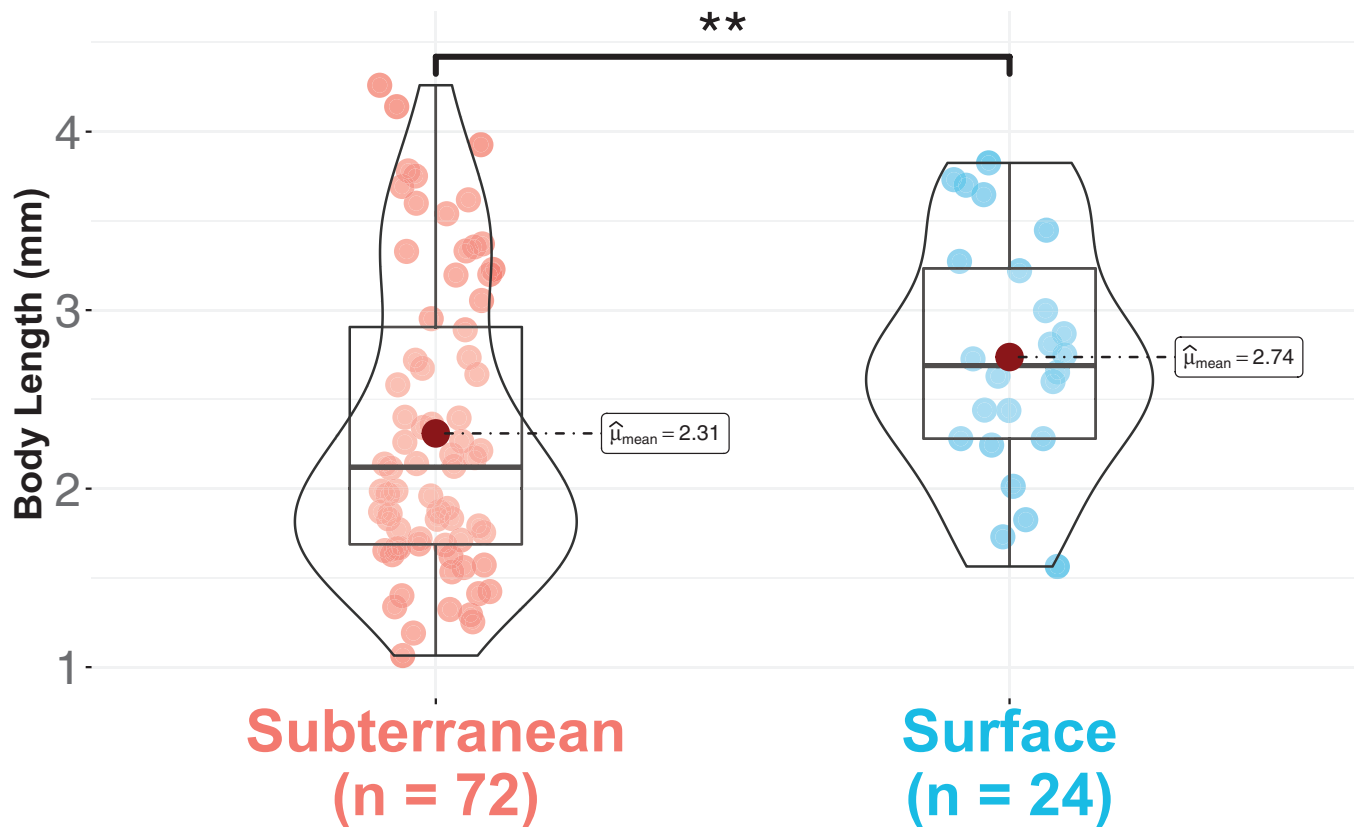
RESULTS

Body size

Body size was significantly different between the surface and subterranean species, as shown by data from 96 species $[t_{welch}(49.19) = -2.62, P = .01, \hat{g}_{Hedges} = -0.57;$ Fig. 3]. The surface group was on average larger (μ_{mean} = 2.74 mm, N = 24), and subterranean group smaller in size (μ_{mean} = 2.31 mm, N = 72). The four interstitial species had an average body size of 2.08 mm (σ = 0.178).

Body shape

There was a significant difference in body shape among the habitat groups as inferred from data of 98 species ($F_{2,96}$ = 37.1, P = .001) (Fig. 4). The morphospace of dytiscid species is visualized by the first three PC axes (Fig. 4). The first axis (PC1, 64.3%) describes the continuity of the body shape outline, ranging from a rounded and streamlined body outline at the minima of PC1, to an hourglass-shaped body outline with the narrowest point being the posterior edge of the pronotum at the maxima of PC1. The second axis (PC2, 20.4%) describes proportional differences between the head, pronotum, and elytra; dytiscids with a larger head, larger pronotum, and smaller elytra at the maxima of PC2 are contrasted with a more typical proportioned body (minima). Variation at the maxima is driven by an outlier from the subterranean group (*Paroster macrocephalus*). The third axis (PC3, 5.2%) demonstrates similar shape variation to PC2, and is also driven by an outlier (*Paroster arachnoides*; Fig. 1).

The PCA morphospace shows that subterranean dytiscids occupy a larger region than surface dytiscids and are mostly distinct

Figure 3. Violin plot of the pairwise Welch's t-test results of body length between subterranean and surface habitat groups $[t_{\text{Welch}}(49.19) = -2.62, P = .01]$.

in shape; the body shape outlines of interstitial species occupy the intermediate position in the morphospace between surface and subterranean dytiscids (Fig. 4; Supporting Information Figs S1, S2). The test for morphological disparity supports these observations (Fig. 4C). A significant difference was observed between the Procrustes variances (PVs) of surface and subterranean groups ($P = .012$), where the subterranean group exhibits a higher diversity of body shapes than the surface group ($PV_{\text{sub}} = 3.516 \times 10^{-3}$; $PV_{\text{sur}} = 1.271 \times 10^{-3}$). Interstitial species occupy a smaller region of morphospace ($PV_{\text{int}} = 1.089 \times 10^{-3}$), but this group has only four species sampled.

Antennae and limbs

The t-test shows there are significant differences in antennae length between subterranean and surface species as shown by data from 96 species $[t_{\text{Welch}}(39.76) = 3.93, P = 3.33 \times 10^{-4}, N = 96, \hat{g}_{\text{Hedges}} = 0.91;$ Fig. 5]. The subterranean group exhibits longer antennae relative to body size ($\mu_{\text{mean}} = 0.35, N = 72$), and the surface group exhibits shorter antennae relative to body size ($\mu_{\text{mean}} = 0.32, N = 24$). The violin plot (Fig. 5) also highlights one subterranean species (*Paroster microsturtensis*) as an outlier displaying the longest antennae length relative to body size; and the distribution range of the subterranean group is slightly broader than that of the surface group.

The t-tests also showed a significant reduction in the width of all three pairs of limbs in the subterranean group compared to the surface species (Fig. 6). The subterranean group exhibits a shorter forefemur and foretibia in length (Supporting

Information Fig. S3A, C); there was no significant difference in the relative total length of three pairs of limbs (Figs S3E, S4E, S5E); more outliers are present in the subterranean group than in the surface group (Figs S3A–E, S4A–E, S5A–E).

The PCA morphospace of limb length as shown by data from 60 species (Fig. 7A) reveals a similar pattern to that of body shape (Fig. 4A). For PC1 (50.6%), forelimb femur length (FFL) loads strongly in the positive direction and forelimb tibia width (FTiW) in the negative direction. For PC2 (12.0%), the hindlimb tibia length (HTiL) and midlimb femur width (MFW) are the strongest loadings with opposite effects.

MANOVA of all limb variables found that there was a significant difference among the three habitat groups (surface, subterranean, and interstitial) ($F_{2,57} = 7.20, P = .001$). Morphological disparity in legs among the three habitat groups was different (Fig. 7C). Specifically, a significant difference was observed between the surface and subterranean groups ($P = .022$), where the subterranean group exhibits a higher diversity of leg size variation than the surface group ($PV_{\text{sub}} = 21.40 \times 10^{-2}$; PV_{sur} 8.55×10^{-2}). Interstitial species occupy a smaller region of morphospace ($PV_{\text{int}} = 1.86 \times 10^{-2}$), but this group has only three species sampled. Pairwise disparity analysis indicates that the diversity of leg parameters is greater in subterranean dytiscids than in interstitial dytiscids and surface dytiscids.

### Body-size triplets in calcrete aquifers

Morphospace for 15 sets of subterranean dytiscid species that are known to exhibit body size variation within the same calcrete
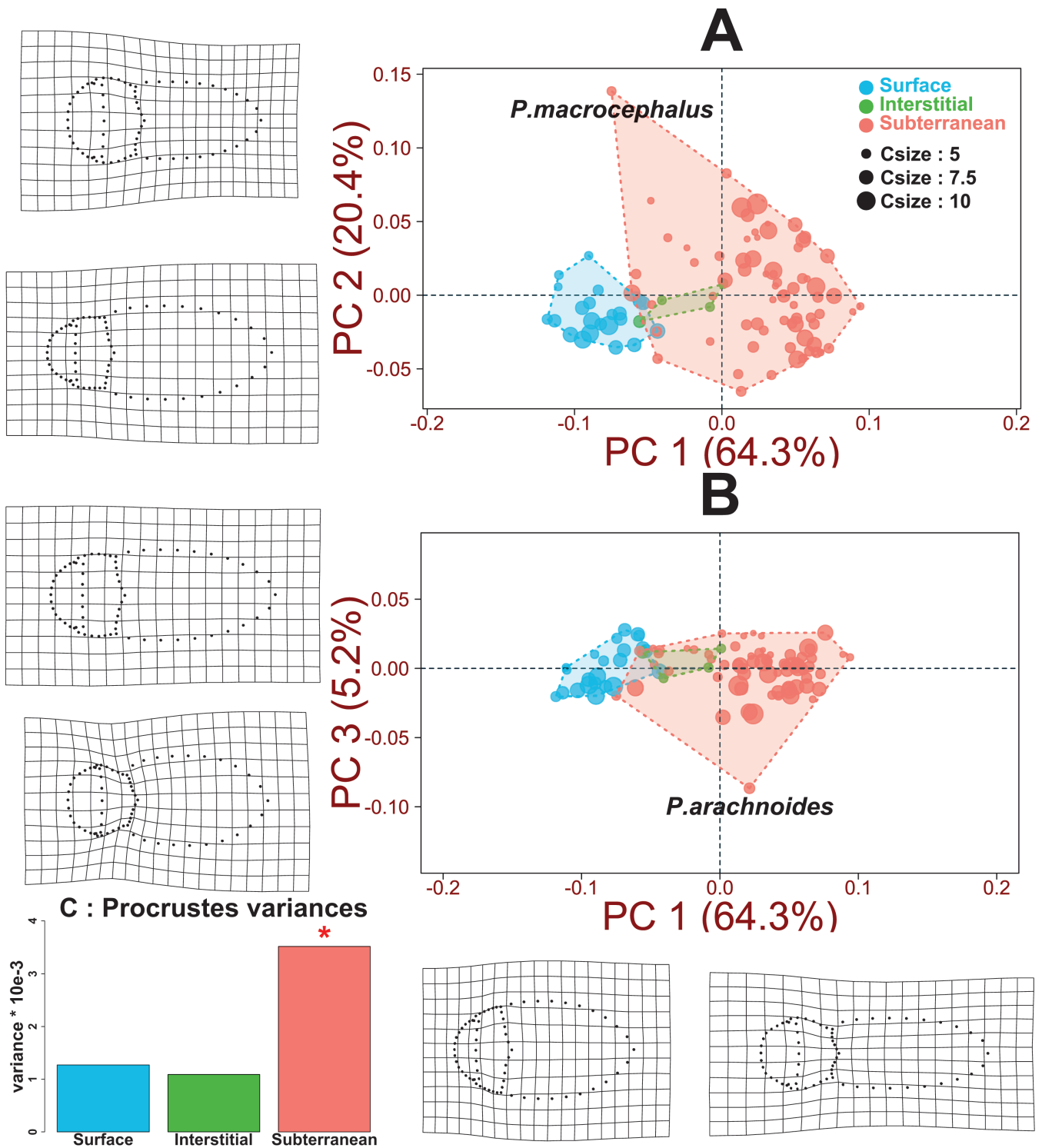
Figure 4. Morphospace of the body shape outline of surface, interstitial, and subterranean dytiscids, defined by the first three principal component axes (totalling 89.9% of the variance): PC1 (64.3% of the total variance), PC2 (20.4%), PC3 (5.2%). Each point represents one species. Point size represents the centroid size of each species. Thin-plate spline grids illustrating the shape change from the mean shape to the extreme ends of each axis are given besides the axis. A, morphospace constituted by PC1 and PC2. B, morphospace constituted by PC1 and PC3. The shape of the centre (0,0) of the PCA is represented by a thin-plate grid diagram with equal-sized grid squares, whereas the shapes of the extreme points are represented by distorted thin-plate grid diagrams with different-sized grid squares. C, bar chart demonstrating the Procrustes variance of each habitat group. The subterranean group (red) has the greatest Procrustes variance, which is significantly different from the other two groups.

aquifer is shown in Figure 8. Each triplet of species is coloured to represent individual aquifers (see Watts and Humphreys 2009) and symbol shape refers to whether it is identified as a small, medium, or large species within the aquifer. Visual inspection of the distribution of species reveals no orderly pattern of shape variation relative to these size triplets; large species do not
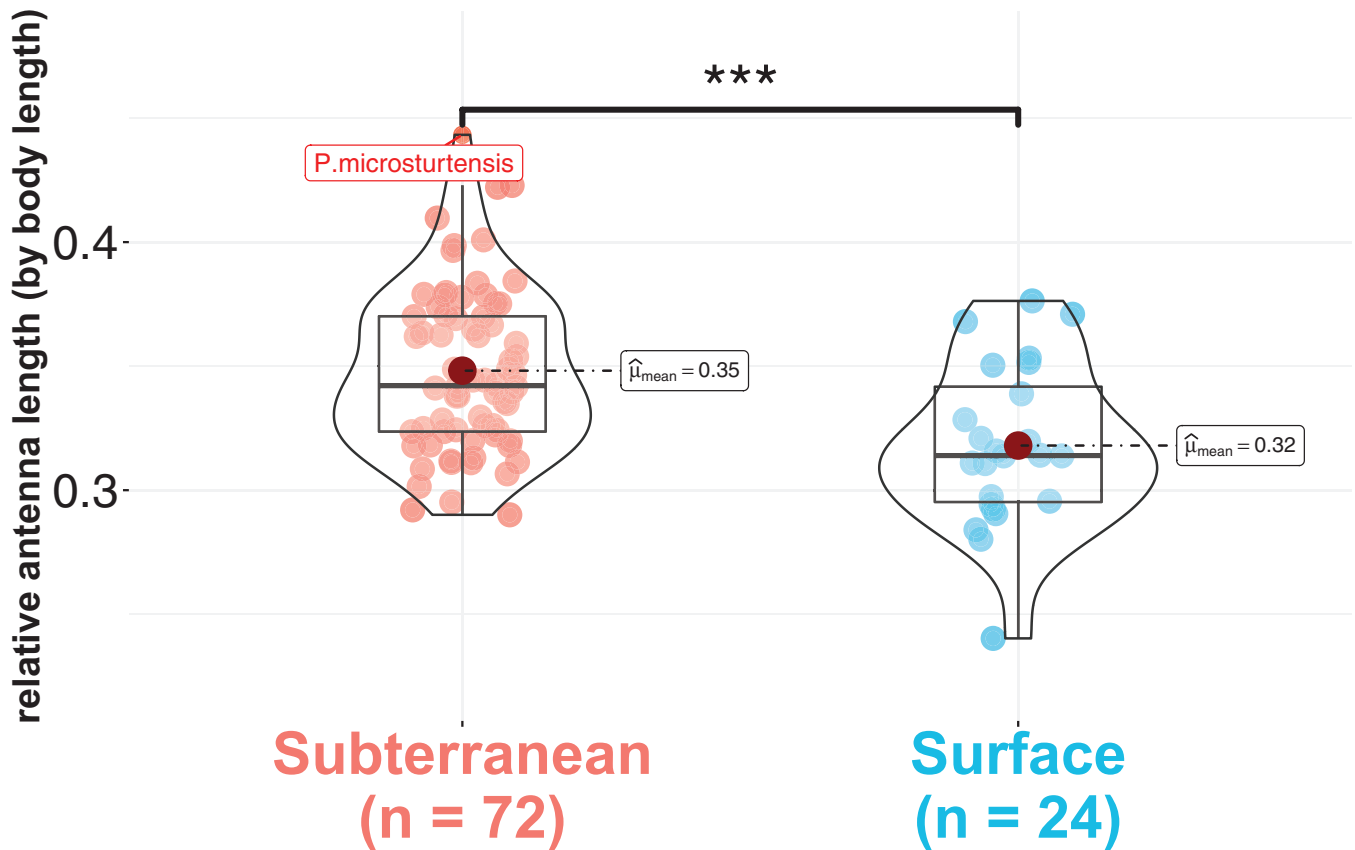
Figure 5. Violin plot of the pairwise Welch's t-test results of antennae length between subterranean and surface habitat groups $[t_{Welch}(39.76) = 3.93, P = 3.33 \times 10^{-4}, N = 96]$. One outlier is present in the subterranean group (*Paroster microsturtensis*). Antennae lengths are scaled by the body length of each individual specimen.

resemble each other, although they occupy a narrow region of the morphospace. Similarly, medium species are very varied in body shape, as are the small species. For each aquifer, the difference in shape between species is also variable and appears to have no pattern in morphospace.

DISCUSSION

Through a quantitative analysis of body shape and appendage size across more than 100 Australian dytiscid beetles, we inferred how these traits change with the transition from surface to subterranean life. Our results for body shape and limb morphology generally reveal patterns of variable, unstructured divergent morphology, and much greater disparity among the subterranean species compared to the surface species. These observations, coupled with the lack of distinct groups of species with similar body shapes or body sizes, is consistent with an hypothesis of relaxed selection. In contrast, the divergent antennae morphology suggests this trait is the result of selection towards longer antennae in the subterranean species. We discuss our three specific aims and hypotheses below.

Body shape disparity

To overcome the hydrodynamic resistance force (water drag force), most fully aquatic animals, from vertebrates such as fish and aquatic mammals, to invertebrates such as giant water bugs

(Hemiptera; Belostomatidae) and aquatic beetles, have evolved a rounded, streamlined body shape, and other adaptations such as fish slime and the microsculptures on the elytra of aquatic beetles (Rosen 1970, 1971, Wolfe and Zimmermann 1984, Lauder *et al.* 2007, Azuma, 2012, Sun *et al.* 2012, Xu *et al.* 2012). Wind-tunnel tests of aquatic beetles were conducted by Nachtigall (1980), and showed that a streamlined body shape was optimal for reduced drag force at a high swimming speed (~0.3 m/s) for beetles. Our results support this biomechanical expectation on their body shape, since we observed that the surface beetles have a very narrow range of morphologies, all strongly streamlined in shape.

Conversely, our results on body shape of the subterranean species showed they present much greater diversity in shape morphology, more than double that observed in surface species. The variation was widespread in morphospace with no apparent structure; that is, no visible clustering of species with similar body shapes that would suggest repeated parallel adaptations for specific different niches in the subterranean environment. Specifically, the lack of any structure of species in morphospace, particularly with respect to size (see discussion on body size triplets below), suggests that niche partitioning and selection are unlikely to explain the shape patterns we observed. Instead, the morphospace pattern we observed is similar to that produced under a Brownian motion model in theoretical morphospace (Stayton 2020). It is also concordant with the
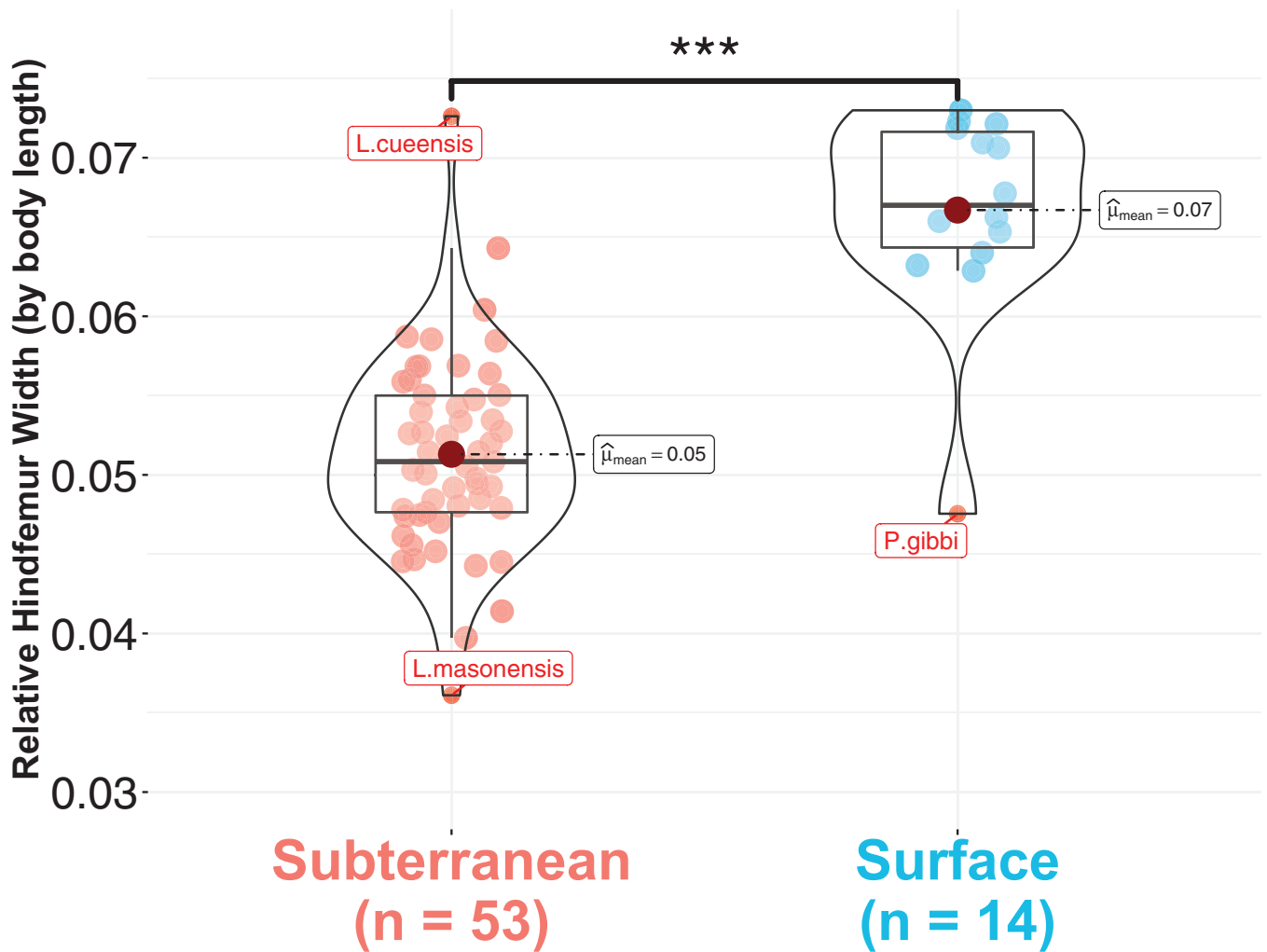
Figure 6. Violin plot of the pairwise Welch's t-test results of relative hindlimb femur width between subterranean and surface habitat groups $[t_{\text{Welch}}(19.43) = -7.88, P = 1.79 \times 10^{-7}, N = 67]$. Subterranean dytiscids exhibit thinner femurs and tibiae across all three pairs of limbs.

extensive variation found in traits such as the viscerocranium of fishes that have radiated into ecosystems containing open niches (Wilkens 2021). We propose that the greater diversity of body shapes in subterranean dytiscids arises because the requirement for streamlined swimming is absent in the aquifer environment, and body shape diversity has occurred through genetic drift and neutral evolution (Kimura 1983). The role of neutral processes during the evolution of the stygobiotic dytiscid beetles has also recently been supported by the study of genes specifically involved in phototransduction, which show parallel decay (pseudogenization) across stygobiotic species compared to surface species (Tierney *et al.* 2015, Langille *et al.* 2021, 2022).

Subterranean dytiscids also occupied a predominantly novel area of morphospace that pertains to an increasingly flattened body plan with the more disparate species having an hour-glass shape, with a disjunct waist between mesothorax and hindbody (Fig. 4; Supporting Information Figs S1, S2). This shape includes tightly fitted, locked, or fused elytra, which are observed in nearly all carabids and many other terrestrial beetles (Forsythe 1987, Evans 1994). In Scaritini Carabidae, a disjunction between the pronotum and hindbody appears to provide greater flexibility and enables large angular, unrestricted locomotion in confined

environments, for example during burrowing (Forsythe 1987 and references therein). Having this hour-glass shape, subterranean dytiscids could be hypothesized to live a benthic and crawling lifestyle (as documented by Jones *et al.* 2019), rather than an obligatory nektonic and swimming lifestyle. Whether this body shape is thus an adaptation to terrestrial crawling locomotion, or represents the standard, pleisomorphic body shape of terrestrial beetles, remains to be studied. However, this form–function relationship does not explain the great diversity in body shapes we observed among subterranean species, ranging from the streamlined shape similar to surface species through to a strongly hour-glass form.

Within-species variation was not considered in our study, due to the focus on among-species diversity and sampling from many unique aquifers. Furthermore, the role of phenotypic plasticity is probably underplayed in these systems (Culver *et al.* 2023) and has been shown to be very important in the colonization of cave systems (Romero 2009, Gore *et al.* 2018, Bilandžija *et al.* 2020). In our study system, we would expect that under neutral evolution, there should be greater within-species variation in the subterranean species compared to surface species. However, few species are adequately sampled at the intraspecific level to
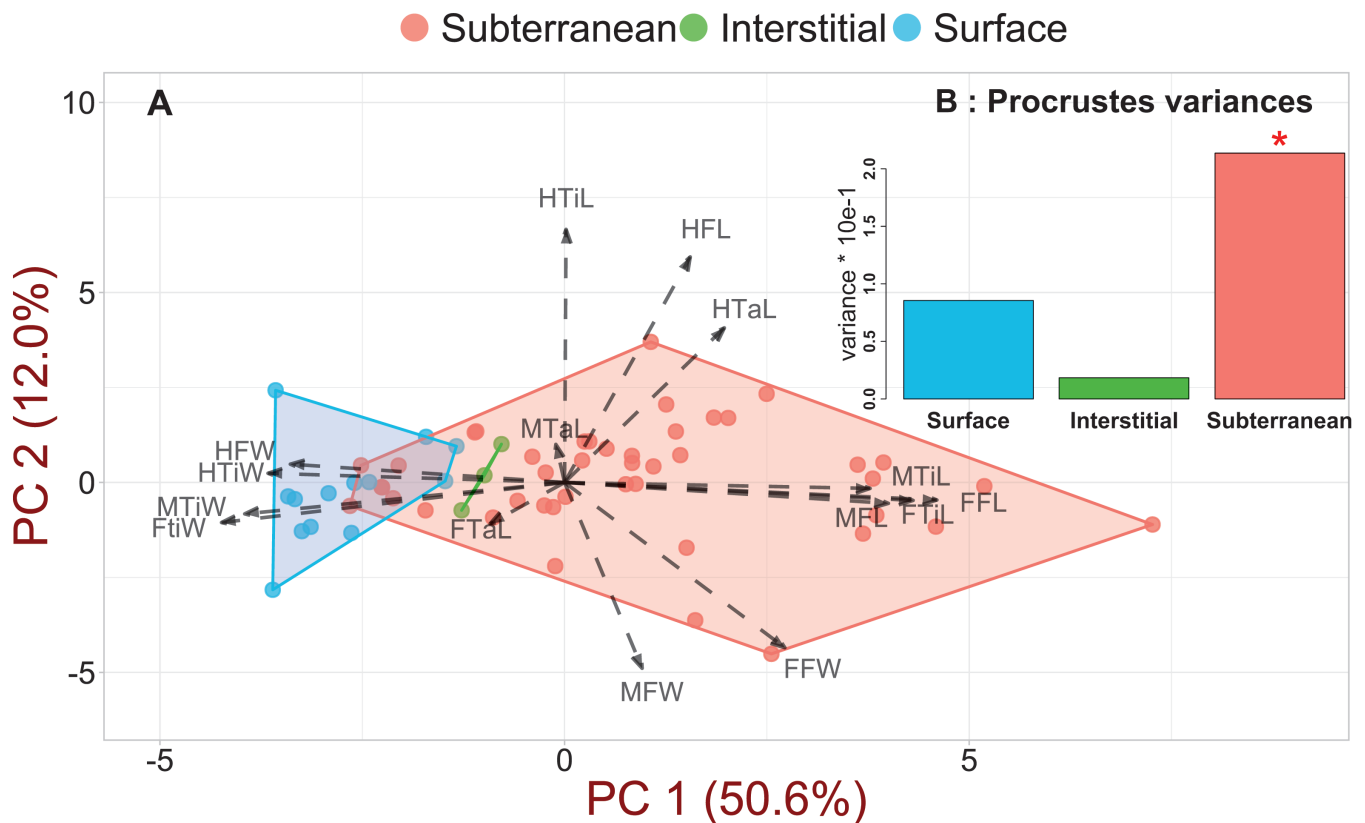
Figure 7. A, PCA of the legs (lengths and widths) of the surface, interstitial, and subterranean dytiscids, defined by the first two principal component axes (totalling 62.7% of the variance): PC1 (50.6% of the total variance), PC2 (12.0%). Each point represents one species. Loadings of each variable on the PCs are shown by the overlaid arrows. B, morphological disparity shown in a barplot.

currently enable such a comparative study, though it would be of considerable interest to further verify the role of neutral processes during phenotypic evolution.

Sensory and locomotory appendages

Our results showed that subterranean species have substantially increased the size of their antennae, but we also observed greater disparity in antennae length in the subterranean species compared to the surface species. Antennae elongation has been observed in stygobiotic crustaceans (Jones *et al.* 1992, Culver *et al.* 1994, Turk *et al.* 1996), troglobiotic millipedes (Liu *et al.* 2017), and beetles [*Speonomus longicornis* (Leiodidae)] (Balart-García *et al.* 2021). Long antennae can be potentially beneficial by having more surface area for sensilla, a wider sensory detection range, and tactoreceptors (touch receptors) (Kaneko 1994, Balke *et al.* 1997, Baker 2001, Miller and Bergsten 2016, Shaverdo *et al.* 2020). Further research at the microstructure level is needed to investigate whether longer antennae mean more sensilla and whether sensilla density is increased irrespective of antennae length. This research would provide more evidence to suggest that the longer antennae in subterranean dytiscids is an adaptive trait.

In other subterranean systems, there are documented adaptations of cave species to enhance their ability to find prey and mates in the dark. These include the vibration-detecting structures in the lateral line system and increases in taste buds of stygobiotic fish (Amblyopsidae and Characidae) (Poulson 1963,

Jeffery 2001), an increase in the number of chemoreceptors and the length of sensory pegs of troglobiotic cave carabid beetles (Carabidae) (Juberthie and Massoud 1977), and modifications of the antennomeres in endogean carabid beetles (Carabidae) (Pérez-González and Zaballos 2013). Using phylogenomic studies, Balart-García *et al.* (2021, 2022) have also shown considerable evolutionary modifications to chemosensory genes in subterranean beetle lineages, including dytiscids, associated with their adaptation to life underground. However, the sensory evolution of subterranean dytiscids is an area of research that requires further attention at both the phenotypic and the molecular level.

Limbs of subterranean species were similar in relative length to surface species, but generally much narrower. Similar to the body shape results, the diversity in limb morphology was much greater in the subterranean species. Since the limbs of the aquatic surface beetles are involved in different functions, namely swimming (hindlimbs) and prey-catching (forelimbs), we discuss these functions separately below.

To provide thrust, the hindlegs of aquatic diving beetles, especially in surface dytiscids, are not only broadly flattened, but also have many rowing appendages and hairs (setae) for effective water paddling (Nachtigall 1974, Azuma 2012). Water paddling is a reciprocal motion that consists of both a power strike and a recovery stroke. During the power stroke, hindlegs and appendages of diving beetles are fully stretched, while in the recovery stroke, the broad sides are turned up 90° and tarsal sections are folded
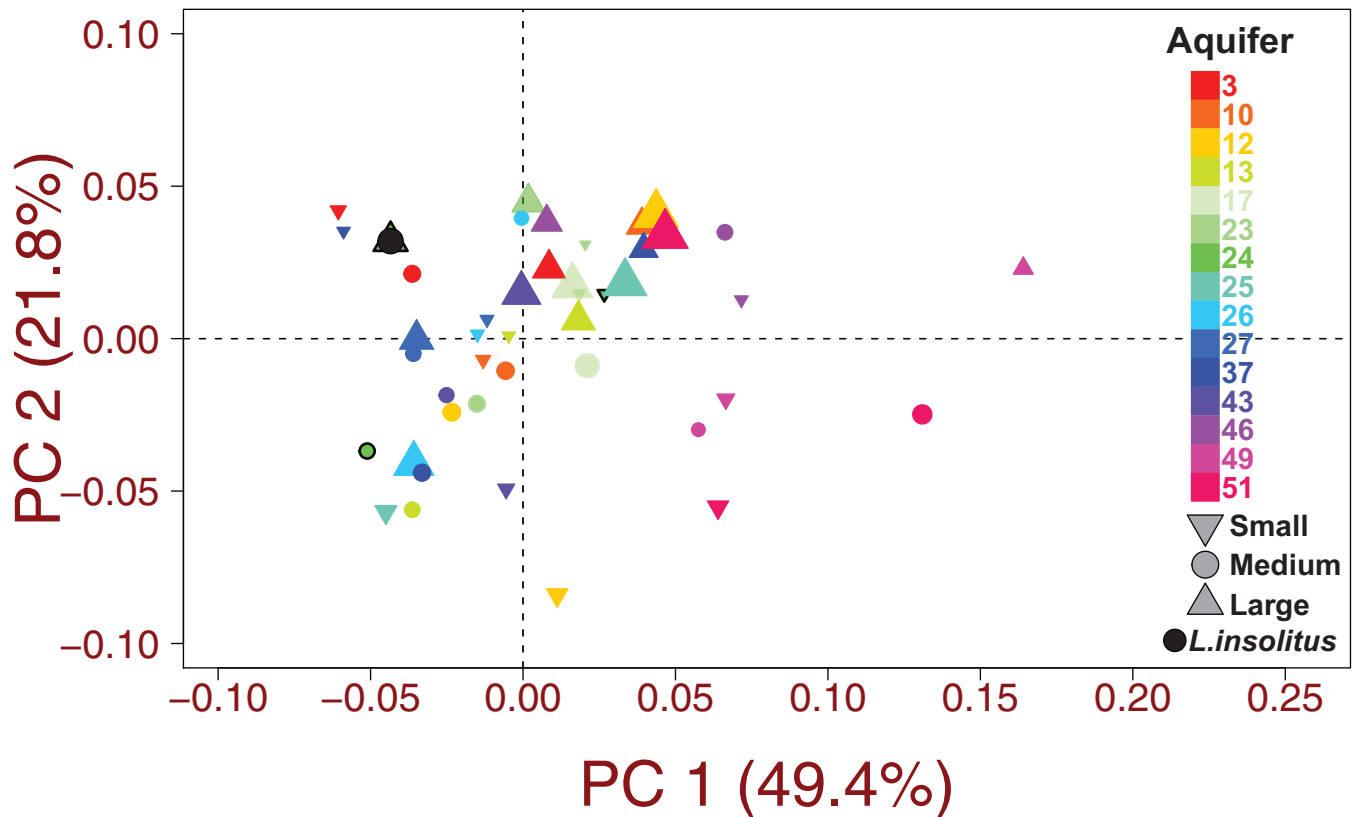
Figure 8. PCA of body shape for triplet analysis of 15 sets of subterranean dytiscids, defined by the first two principal component axes (totalling 71.2% of the variance): PC1 (49.4% of the total variance), PC2 (21.8%). Each point represents one species. Point size represents the centroid size of each species. Colours represent individual aquifers (see Watts and Humphreys 2009). Down-pointing triangles represent the smallest species, up-pointing triangles represent the largest, and circles represent medium-sized species in the calcrete aquifer.

to reduce unfavourable drag (Azuma 2012). Water paddling is evident here by the observation that surface species have robust hindlimbs, and is consistent with existing literature (Miller and Bergsten 2016). Interestingly, our analyses also showed that subterranean dytiscids exhibited slender hindlegs, congruent with the hypothesis that they no longer require efficient swimming abilities. Slender hindlimbs further imply that these subterranean species are likely to be walking (crawling) in the calcrete aquifers. Unlike the modified paddle-shaped hindlegs for swimming of surface aquatic species (Nachtigall 1974, Azuma 2012), thin legs have been frequently observed in terrestrial and some aquatic insects and are likely to be the most common and primitive leg morphology in insects that walk (crawl) or run on various surfaces (Chapman 1998, Snodgrass 2018). Thin legs are exhibited in crawling terrestrial insects including most cockroaches (Blattodea), most earwigs (Dermaptera), terrestrial true bugs (Hemiptera), and beetles (Coleoptera), such as ground beetles (Carabidae) and wrinkled bark beetles (Rhysididae) (Holmes *et al.* 2006, Krell 2008). Evidence of crawling water beetles with slender legs has also been observed in Haliplidae, a closely related family to Dytiscidae (Roughley 2000). Therefore, the observed difference in leg width among subterranean dytiscids could be related to a terrestrial or benthic crawling and scavenging lifestyle.

In contrast to hindlimbs, forelimbs are also known to be used to catch prey (Miller and Bergsten 2016). Our analyses showed that both forelimbs and midlimbs of subterranean dytiscids are

thinner than those of surface species. Additionally, there is reduction in the pronotum (Fig. 4), which is where muscles of the forefemur are attached (Larsén, 1966, Balfour-Browne 1967). Improved predation and grip in insects have shown extreme adaptations, for example the elongation of coxae and spines in Mantodea (mantises) to narrow the gap through which the prey might escape and therefore increase the chance of catching prey (Loxton and Nicholls 1979). Modifications of forelimbs are also present in surface dytiscids, for example the spines along the femur and tibiae in some species (e.g. *Allodessus bistrigatus*), and the tarsal sucking discs, which are considered beneficial for both grasping prey and grasping females when mating (Miller and Bergsten 2016). Under such circumstances, a wide and robust forelimb indicates a stronger grasping force and would suit a predative lifestyle. Trophic dynamics studies by Bradford *et al.* (2013) and Saccò *et al.* (2020a) suggested that subterranean dytiscids are likely to feed on small amphipods, suggesting they are potentially at the top of the trophic level in these calcrete aquifers. Therefore, these observations suggest that the subterranean species have a lower prey holding strength, and thus could be scavengers.

Repeated evolution of body size morphs within aquifers

Our final aim related to the previously reported repeated body size evolution in calcrete aquifers (Vergnon *et al.* 2013). Within aquifers, species of subterranean dytiscids show a repeated pattern of nonoverlapping size variation, presenting often as

sets of size 'triplets' (small, medium, and large), although absolute sizes vary from aquifer to aquifer (see fig. 9.2 in Austin *et al.* 2023). This pattern is consistent with the theory of self-organization under a limiting similarity model, where two species must be sufficiently different to coexist (MacArthur and Levins 1967, Vergnon *et al.* 2013, Scheffer *et al.* 2015), rather than a model where pre-existing environmental niches drive the size differences. We hypothesized that if body size relates to distinct niche partitioning, we might expect there to be distinct body shapes for each size class. We found that this was indeed the case within aquifers, where species of different sizes are also different in body shape. However, we found that there was no similarity of body shape related to body size between aquifers (Fig. 8). It is evident that within aquifers for these different sized triplets, the shapes of the small, medium, and large species were seemingly randomly distributed in morphospace, and between calcretes we observed no similar patterning. Therefore, while individual aquifers present a pattern of size partitioning, body shape does not evolve in a predictable manner, as would be assumed under an adaptive model. This result lends further support to the conclusions regarding body shape diversity discussed above, which appears to have diversified under relaxed evolution.

Finally, it is evident that the diversity of body shapes among surface or subterranean dytiscids is not a result of evolutionary allometry, and therefore body size and shape are not coupled in the evolutionary history of this group. Evolutionary allometry is well studied with respect to vertebrate lineages while static (within species) and ontogenetic (during development) allometry is more often examined in insects (e.g. Sherratt *et al.* 2022). Unlike vertebrates, it appears that evolutionary allometry plays a smaller role in insect evolution (e.g. Klingenberg and Zimmermann 1992, Harvey and Sherratt 2023), where size and shape are less strongly coupled during speciation, and this is likely to be due to different mechanisms that underpin insect growth and the determinants of final body size (Stern and Emlen 1999). Further research into the role of evolutionary allometry in insect diversity is encouraged.

CONCLUSION

Overall, our results suggest that the modifications to limb and antennae morphology in subterranean dytiscids reflect a niche shift during colonization of the aquifers by surface species, and probably represent adaptations to their calcrete/fractured rock habitat (enhanced crawling ability) and life in complete darkness (improved sensory systems). However, the distinct hourglass body shape of many subterranean species, in contrast to the streamlined shape of surface species, and considerable variation in body shape and limb proportions among species, despite their similar ecological and environmental conditions, suggests an important role for relaxed selection during the phenotypic evolution of this group.

SUPPLEMENTARY DATA

Supplementary data are available at *Biological Journal of the Linnean Society* Journal online.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

DATA AVAILABILITY

Morphometric data associated with this study are available on Figshare (10.25909/23118254). Private link for review: https://figshare.com/s/4c5275a1f4b22bbe46cb.

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