



New insights into the evolution of the surface antennal sensory equipment in free-living and cave-dwelling beetles (Leiodidae: Leptodirini)

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† The present work is dedicated to the late Ignacio Ribera.

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Abstract

The stable environment of subterranean realms is characterized by constant darkness, temperature and humidity, and scarcity of resources. This led to similar adaptations in different lineages of animals, such as the reduction of eyes and pigmentation. It is common textbook knowledge that blindness in cave insects is compensated for by transformations of other sensorial structures, especially the antennae with their rich array of sensilla. We tested this hypothesis with 33 species of Leiodidae of the tribe Leptodirini (Coleoptera) with and without eyes and from hypogean and epigeal environments. We documented and compared the number, types, arrangement and density of smooth and furrowed antennal sensilla on certain flagellomeres. Our statistical analysis that took effects of body size and phylogeny into consideration showed that (1) the number of these sensilla does not differ between hypogean or epigeal beetles; (2) the same applies to length and diameter of the antennal sensilla; (3) there is a difference in density, but unexpectedly it is lower in hypogean species. Our finding thus contrasts with widely accepted earlier interpretations for those external antennal sensilla in the studied Leptodirini, showing that sensillar patterns are scarcely affected in these subterranean beetles if at all, and even less dense in blind and cave-living species. Our results thus add a new facet to the evolution of cave animals.

Keywords

Beetles, Caves, Speleology, Subterranean

1. Introduction

Dark and humid subterranean systems are highly specialized habitats for various forms of life (Racovitza 1907; Romero 2009; Gross 2012). The reduced food resources, limited space and overall stability of the cave environment makes the inhabitants of these ecosystems ideal model organisms for various fields of research, including ecological adaptations, adaptive processes, changes in the circadian rhythm, or phenomena occurring in humans such as albinism or autism (Protas et al. 2006; Gross 2012; Beale et al. 2016; Yoshizawa et al. 2018; Mammola 2019). Another major reason for the attractiveness of hypogean animals or troglobionts for solving basic evolutionary and ecological questions are their rather uniform adaptations towards the dark and confined environment. This comprises reduced or completely absent eyes and the loss of pigmentation (e.g. Luo et al. 2018). Cave adaptations that are summarized under the term troglomorphy evolved several times independently in various groups within cavernicolous species (Christiansen 1962; Moldovan 2004; Howarth and Moldovan 2018; Fišer 2019). The legs and sensory appendages are elongated in troglotic insects in order to compensate for the loss of visual information. This can potentially increase the number of tactile and olfactory sensors that help in orientation and in finding food or potential mating partners. This concept of sensory compensation in blind animals dates back to Charles Darwin, who stated in his “On the origin of species” that “natural selection will often have affected other changes, such as increase in the length of the antennae or palpi, as a compensation for blindness” (Darwin 1859). This can be considered as generally accepted textbook knowledge today (Jeannel 1911; Crowson 1981; Gunn 2004; Moldovan 2004, 2012, 2018; Howarth and Moldovan 2018; Fišer 2019).

The most successful group of organisms in terms of total species number but also troglotic specialists are insects (Gunn 2004; Stork 2015; Culver and Pipan 2019). It has been suggested that the reduction of the compound eyes in this group is compensated for by the elongation of the antennae and an increased density and elongation of its sensilla (Gunn 2004; Romero 2009; Moldovan 2012; Howarth and Moldovan 2018; Fišer 2019). The antennae of all ectognathous insects are composed of the basal scapus and pedicellus, and the flexible flagellum, which is almost always by far the longest antennal element (Beutel et al. 2014). The scapus is the only segment with intrinsic muscles and the pedicellus contains a chordotonal organ (Johnston’s organ). The flagellum is always composed of several or many segments in adults, and usually bears most of the sensorial structures (Fig. 1). The antennal vestiture is mostly formed of hair-like structures, articulated setae, but can also include variously shaped specialized sensilla that together fulfill an entire spectrum of functions, including tactile, olfactory, humidity-sensitive or chemoreceptive ones (Lucarelli and Sbordoni 1978; Zacharuk 1985; Hansson 1999). It is widely assumed that subterranean or hypogean insects have more and lon-

ger sensilla on their antennae (e.g. Gunn 2004; Moldovan 2004; Romero 2009; Howarth and Moldovan 2018; Culver and Pipan 2019; Fišer 2019), even though only few original studies have investigated this phenomenon in a quantitative way (e.g. Juberthie and Massoud 1977; Peck 1977; Buzilă and Moldovan 2000). These studies used a limited number of species and corrected neither the retrieved data for body size nor for phylogenetic constraints.

With more than 900 described subterranean species, Leptodirini of the polyphagan family Leiodidae is the second largest radiation of subterranean insects (Jeannel 1926; Perreau 2000, Gunn 2004; Deharveng and Bedos 2018; Faille 2019) after a specialized tribe of the adephagan ground beetles (Carabidae), the Trechini. The highly diverse tribe Leptodirini is mainly restricted to the western Palaearctic and its species inhabit leaf litter, deep soil, caves and other environments (Fresneda et al. 2011). Leptodirini have been used in several studies on morphological, physiological and ecological cave adaptations (e.g. Friedrich et al. 2011; Cieslak et al. 2014; Balart-García et al. 2021). Remarkably, the tribe differs from other lineages with hypogean representatives (e.g. Ptomaphagini, Leiodidae) by the occurrence of complete eye reduction even in most surface-living species (Faille 2019). This allows a comparison between blind epigeal and blind hypogean beetles.

We studied the relatedness between the length of antennal segments and their smooth and furrowed sensilla in 33 epigeal and hypogean species of Leptodirini, and evaluated the retrieved data in a statistical context. In contrast to earlier studies, we also took body size and phylogenetic constraints into account in our analyses. The latter correction addresses the non-independence of sampled specimens due to various degrees of phylogenetic relatedness. As a result, any similarity based on close phylogenetic relationships between the included species will not affect the correlation between the studied parameters and ecological traits.

Based on the previous knowledge and studies, we thereby tested the following hypotheses: 1) the number of smooth and furrowed sensilla on the antennae of species of Leptodirini is comparatively higher in troglotic species. 2) the same applies to the density, diameter and length of the individual sensilla. 3) the antennae of epigeal species are shorter and have a smaller surface.

As an additional and independent step, we also mapped the studied traits on a molecular tree in order to illustrate phylogenetic and evolutionary aspects of the morphological modifications.

2. Materials and Methods

Species examined: The present study is based on 33 species of Leptodirini. As we relied on rare museum materials and the performed experiments are destructive and alter the sample (critical point drying, sputter coating),

we used only a single specimen per species. Table S1 provides a detailed list including the source and collection accession numbers. The body length of the species is provided in Table S2.

Antennal segments and sensilla: Leiodids generally have nine flagellomeres (Fig. 1A), a ground plan feature of Coleoptera (e.g. Beutel et al. 2014). The present study compares the sensilla on flagellomeres V–VIII (Fig. 1A). These were chosen as the number of sensilla increases on the distal segments (Staudacher et al. 2005; Fig. 1A). We thus expected them to be more informative than the basal ones. The apical flagellomere (IX on Fig. 1A) was excluded as it is so densely covered that individual sensilla cannot be properly separated (see Fig. S1 for detailed information and documentation). We distinguished between furrowed and smooth sensilla. Furrowed or fluted hair-like sensilla (blue in Fig. 1) and bear longitudinal grooves and were classified as “sensilla chaetica” and “sensilla trichodea” by (Buzilá & Modovan 2000; Schneider 1964). These sensilla act as mechanical and chemical receptors. The second type are sensilla with a smooth surface (red in Fig. 1) that resemble typical sensilla *basiconica sensu Schneider (1964)* (same term also used in Buzilá and Modovan 2000) and act as chemoreceptors, hygro-sensitive or thermal sensors. The sensilla were identified and counted on flagellomeres V–VIII of all species in the list (all raw data are presented in Table S3).

Ecological traits: The studied species were categorized into different groups based on (1) the absence or presence of eyes (eyes developed vs eyeless/blind); the epigeal group contains edaphic (i.e. soil-dwelling) species: *Adelopsella bosnica*, *Bathysciola pusilla*, *Besuchetiola priapus*, *Karadeniziella omodeoi*, *Notidocharis calabrezi*, *Platycholeus hamatus*. (2) hypogean (living underground) or living in epigeal habitats (on the surface). The “hypogean” group only includes species inhabiting underground (caves and/or Milieu Souterrain Superficiel [MSS]), whereas the “epigeal” group includes species dwelling in non-cave environments. The term “subterranean” used in the text consists of a broad range of environments that range from caves to deep soil and MSS. The epigeal species occur in organic matter of living vegetation, loose plant material and wood debris. Table S1 provides the coding of these characters for all studied species.

Scanning electron microscopy (SEM): We modified the protocol recommended by Schneeberg et al. (2017) for cleaning surfaces of our beetles: specimens were transferred from FAE into 70% ethanol, followed by 0.5% Triton X100 (14 h), 5% KOH (14 h), glacial acetic acid (3 × 15 min), distilled water (multiple times until the specimens appeared clean), and finally 70% ethanol. Subsequently, they were dehydrated and dried in an Emitech K850 at the critical point. Samples were attached to a rotatable specimen holder (Pohl 2010) or stubs, then sputter-coated with gold (Emitech K500; Quorum Technologies Ltd., Ashford, UK). SEM observation and im-

aging was performed with an FEI (Philips) XL 30 ESEM at 10 kV.

Phylogenetic analyses: The taxon sampling for the analyses of molecular data comprises 24 species of Leiodirini. Additionally, we included six outgroup terminals. The tree was rooted using *Catops picipes* (Fabricius, 1787), a representative of the Cholevini, another tribe of Leiodidae (Fresneda et al. 2011). DNA sequences of specimens used in this study were compiled from previous publications (Ribera et al. 2010; Fresneda 2011; Cieslak et al. 2014; Faille et al. 2016). In cases where we did not have molecular data for the same species, we selected another one from the same genus (marked with sp. in Fig. 2). We compared 5 fragments of seven genes, four mitochondrial and two nuclear ones: (1) 3' end of cytochrome c oxidase subunit 1 (*cox1*); (2) 5' end of the large ribosomal unit plus the Leucine transfer plus the 3' end of NADH dehydrogenase subunit 1 (*rrnL+trnL+nad1*); (3) 5' end of the small ribosomal unit; (4) 18S rRNA (*SSU*); 5) an internal fragment of the large ribosomal unit, 28S rRNA (*LSU*). The sequences were aligned using MAFFT online v.7 with the Q-INS-i algorithm (Katoh and Standley 2013). Maximum likelihood analyses were performed with a data matrix combined with RAxML GUI (Silvestro and Michalak 2012; Stamatakis 2014), with four partitions corresponding to the fragments *cox1*, *rrnL + trnL + nad1*, *SSU* and *LSU*, with the evolution model GTR + I + G and the default values for the other parameters (Stamatakis et al. 2008).

Morphometrics: In order to be able to calculate the surface of an antennomere (which is a complex 3D structure) from 2D images, the studied flagellomeres were considered as cylindrical. Their lengths and diameters were measured with Adobe Illustrator CS6 (Adobe Inc., California, USA) with the “pencil” function on the SEM images in Adobe Illustrator and then calculated with the respective scale bar to 2 decimal places. The lengths were measured between upper and lower mid points of the flagellomeres, the diameters based on the width of the middle part of the segments (all raw data presented in Table S3 and Table S4). The surface area was calculated with the following formula: surface area = $2\pi \times 0.5 \text{ width} \times \text{heights}$.

For the assessments of average lengths and basal diameters of the furrowed sensilla, three of them from flagellomeres VIII of each species were chosen and measured in the same way as the length of the antennomere. The density of sensilla was calculated by dividing the number of sensilla by the surface area, and it has the unit of “Number per 500 μm^2 ”.

Statistics: We ran two sets of tests for epigeal/hypogean taxa (11 epigeal and 22 hypogean) and blind epigeal/blind hypogean (5 epigeal and 22 hypogean) taxa, in order to make sure that the observed differences were not affected by differences in species with or without eyes. As a first step, the measurements were checked for parametric test assumptions (normality of residuals, equality

of variances and absence of outliers) for both levels of groupings, and parametric one-way Anova, and non-parametric Kruskal-Wallis tests were carried out accordingly for each measurement. Where possible, data were log- or sqrt- transformed to meet parametric test assumptions. We estimated the significance of the divergence of each measurement between epigeal and hypogeal, and blind epigeal and blind hypogeal groups.

In order to account for the size of the animals, we ran Ancovas as a second step for each measurement separately, where the body length was included as covariate for all 33 species. When necessary, the measurements were log- or sqrt- transformed to meet the parametric test assumptions (e.g. linearity, homogeneity of regression slopes, normality and homogeneity of variances of residuals, as well as absence of outliers). Anova/Ancova and Kruskal-Wallis tests were performed using the package “rstatix” in R.

As a next step, we repeated the Ancova tests in a phylogenetic framework to account for the non-independence of the data due to phylogenetic relatedness (Adams & Collyer 2018a, b). This was achieved by using the “Im.rpp” function in package “RRPP” by supplying the phylogenetic covariance matrix, thus computing the linear model by using a randomized residual permutation process. This method is known to be unsusceptible to type I error rates (Adams and Collyer 2018a, b). The number of permutations was 10000. For this analysis, we used a reduced dataset (24 species) as molecular data were not available for all species in the sample. We did not reduce the number of species for the first two types of data (uncorrected, corrected for body size) to those with molecular data available, to increase statistical power in these approaches. The species lacking molecular data that we excluded from the phylogeny + body size correction are: *Adelopsella bosnica*, *Bathysciola pusilla*, *Besuchetiola priapus*, *Karadeniziella omodeoi* (sighted epigeal) and *Bathysciotes khwenhuelleri khwenhuelleri*, *Halbherria zorzii*, *Neobathyscia mancinii*, *Patriziella sardoa*, *Tismanella chappuisi chappuisi* (blind hypogeal). The raw data of all statistical results are provided in Table S5.

Evolutionary mapping: As an additional step that is completely independent from the analyses described above, we checked whether the measured traits were conserved phylogenetically by measuring the Pagel’s lambda phylogenetic signal (Pagel 1999), which varies from 0 to 1, with 0 indicating phylogenetic independence, and 1 phylogenetic conservatism. For that we used function “phylosig” in package “phytools”. The traits were size-corrected by regressing them on body size and extracting the residuals. Residuals were afterwards treated as size-corrected measurements and phylogenetic signal was computed for each of them separately. We also mapped size-corrected residuals onto phylogenies using the “plotBranchbyTrait” function in “phytools”. For traits which did not correlate with body size (e.g. total number of grooved sensilla and total number of smooth sensilla), we used the raw measurements to map them onto the phylogeny.

3. Results

Among all studied 33 species, the antennae were 11-segmented (Fig. 1A). Compound eyes were present in 6, whereas 27 were blind, and 11 were epigeal and 22 hypogeal. The **body length** of hypogeal species was on average 3241.93 (± 1023.62), and in the epigeal ones 1832.94 (± 747.74) (Fig. 3B). The blind epigeal beetles were on average 1563.31 (± 841.74) μm long (Fig. 3B; detailed values for every species in Table S2).

The result of the **phylogenetic analyses** is provided in Fig. 2. Leptodirini were recovered monophyletic, with *Platycholeus* sp. as sister to the rest of the tribe, as already suggested earlier (Fresneda et al. 2011). The overall topology and the supported nodes are in agreement with previous works (e.g. Ribera et al. 2010; Cieslak et al. 2014; Faille et al. 2016). The taxon sampling for the uncorrected and body-size corrected analyses comprises 33 species while the one for the phylogeny and body size corrected contains only the 24 species for which we had molecular data.

The **combined area** of the flagellomeres V–VIII varied between 13232.37 μm^2 and 520174.73 μm^2 in the studied beetles (detailed values for every species in Table S3). It was on average 137861.45 (± 118357.69) μm^2 in the hypogeal ones, 32582.76 (± 16878.32) μm^2 in the epigeal ones, and 30054.08 (± 9420.31) μm^2 in blind epigeal ones. Details on the individual segments can be found in Table S3.

We found significant differences in the area between the epigeal and hypogeal ($p = 0.00002$; Fig. 3A) and the blind hypogeal and blind epigeal species, respectively ($p = 0.0006$; Fig. 3A). After size correction, the significant differences remained stable in both cases (epigeal/hypogeal $p = 0.001$; Fig. 3A, blind epigeal/blind hypogeal $p = 0.02$; Fig. 3A) (Table S5). After size and phylogenetic corrections with a reduced data set (only species with molecular data; see M&M), epigeal/hypogeal groups remained significantly different ($p = 0.01$; Fig. 3A) while no differences were found between the blind hypogeal and blind epigeal individuals.

The **combined length of flagellomeres V–VIII** varied between 118.03 μm and 2215.73 μm (detailed values for every species in Table S3). In the hypogeal species it was on average 693.80 (± 476.00) μm , in the epigeal ones 204.31 (± 73.09) μm , and in the blind epigeal ones 187.61 (± 42.89) μm . Details about the individual segments can be found in Table S3. We found significant differences in both groupings (Fig. 3A; Table S5). After size correction, there were significant differences between the hypogeal and epigeal ($p = 0.002$; Fig. 3A) species, and blind hypogeal and blind epigeal ones ($p = 0.04$; Fig. 3A). After size and phylogenetic corrections, there were only significant differences between the hypogeal and epigeal groups ($p = 0.02$; Fig. 3A). Values and significances for the individual flagellomeres are found in Table S5.

The **total number of sensilla** on all studied flagellomeres varied between 73 and 391 in the studied beetles (detailed values for each species in Table S3). All studied species had only furrowed (*sensilla chaetica* and *sensilla trichodea*) and smooth sensilla (*sensilla basiconica*) [Buzilă and Modovan 2000; Schneider 1964]. While furrowed sensilla were observed on all studied flagellomeres, the smooth type is only present on the distal ones. In hypogean species the total number of sensilla was on average 177.23 (± 64.09), in the epigeal ones 127.27 (± 43.37), and in the blind epigeal ones 118.80 (± 16.08) (Fig. 3C). Our analyses only revealed significant differences be-

tween both groups in the analyses without taking size or phylogeny into account (Fig. 3A). The same is true for individual flagellomeres, except VIII, where the total number was not significantly different between the blind epigeal and hypogean groups (Table S5). The results with furrowed or smooth sensilla treated separately can be found in Table S5.

The **density of all sensilla** on flagellomeres V–VIII varied between 0.47 / 500 μm^2 and 5.82 / 500 μm^2 (detailed values for every species in Table S3). In hypogean species it was on average 1.73 (± 0.76) / 500 μm^2 , in the

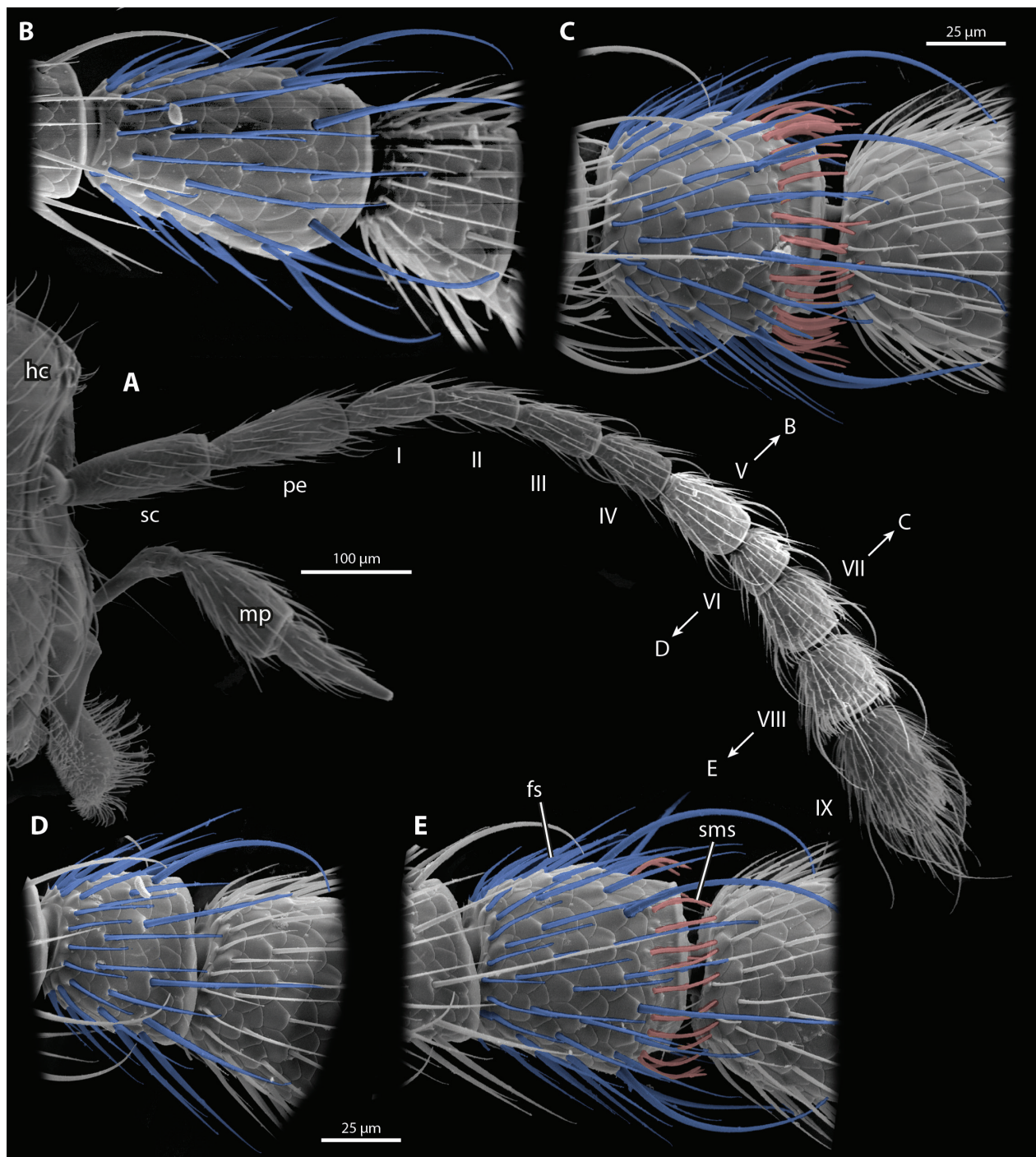


Figure 1. Scanning electron micrographs of the antenna and the studied flagellomeres of *Adelopsella bosnica* (Reitter, 1884). Furrowed sensilla (fs) in blue, smooth ones (sms) in red.

epigean ones $4.30 (\pm 0.98) / 500 \mu\text{m}^2$ (Fig. 3D), and in the blind epigean ones $4.17 (\pm 0.93) / 500 \mu\text{m}^2$ (Fig. 3D). In the uncorrected raw data, we found significant differences between the epigean and hypogean species ($p = 0.00000002$; Fig. 3A), and the blind epigean/ blind hypogean ($p = 0.00002$; Fig. 3A). After size correction, significant differences between both groupings were still obtained ($p = 0.000004$ and $p = 0.002$), and they remained also stable after taking size and phylogenetic constraints

into account ($p = 0.0004$; 0.009 ; Fig. 3A). The results for the density of all sensilla on the individual flagellomeres and for furrowed sensilla alone are presented in Table S3 and Table S5.

The **length of the furrowed** sensilla on flagellomere VIII varied between $29.94 \mu\text{m}$ and $203.87 \mu\text{m}$ (detailed values for every species in Table S3). In the hypogean species, it was on average $116.20 (\pm 44.60) \mu\text{m}$, in the

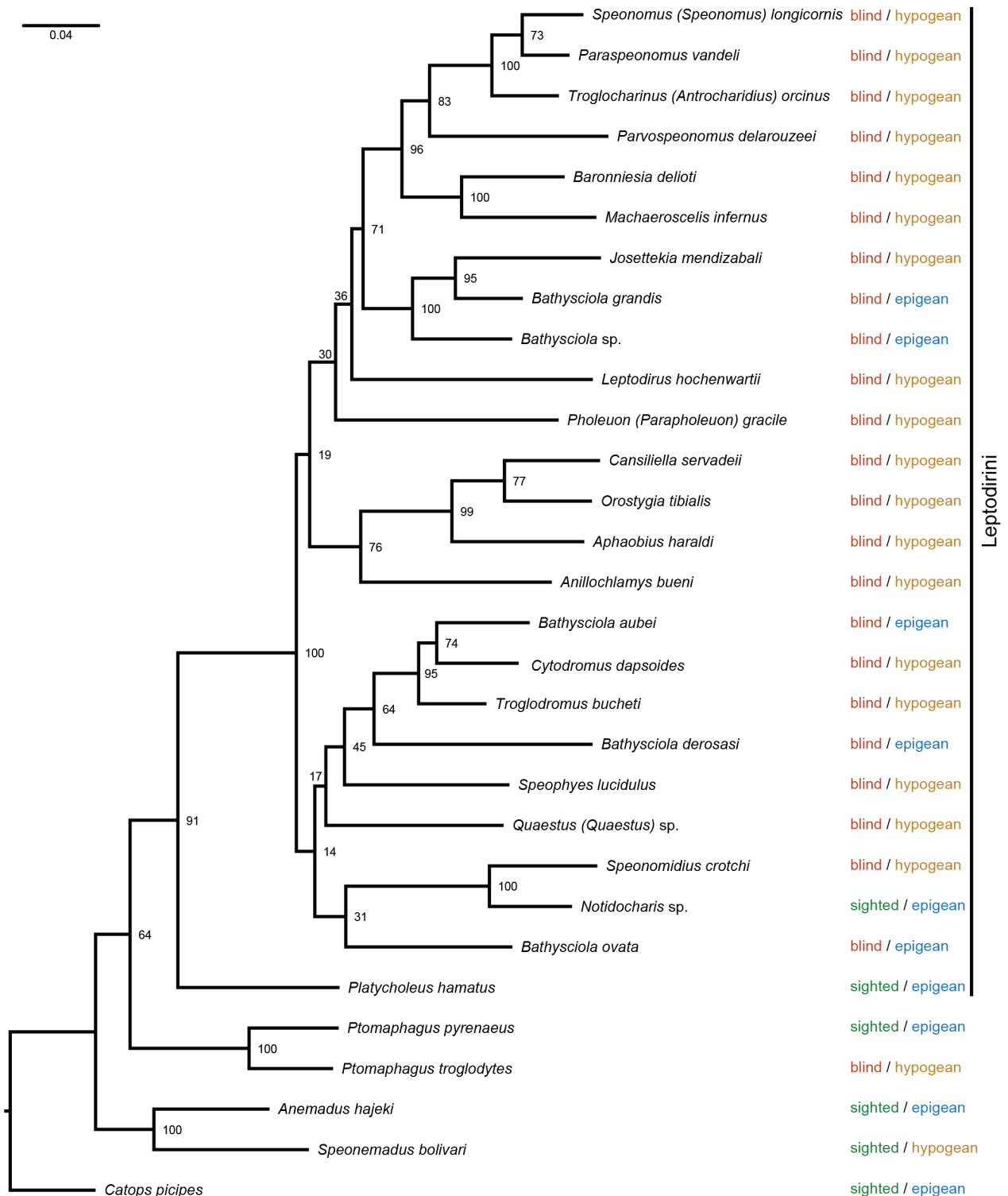


Figure 2. Phylogram obtained with RAxML and the combined mitochondrial and nuclear data. Above nodes, bootstrap support values.

epigean ones 52.81 (± 19.51) μm , and in the blind epigean ones 49.79 (± 17.90) μm . In the uncorrected raw data, we found significant differences between the epigean and hypogean species ($p = 0.00004$), and also between the blind

epigean and blind hypogean beetles ($p = 0.001$) (Fig. 3A; Table S5). After taking size and phylogeny into account, no significant differences between any of the groups were revealed.

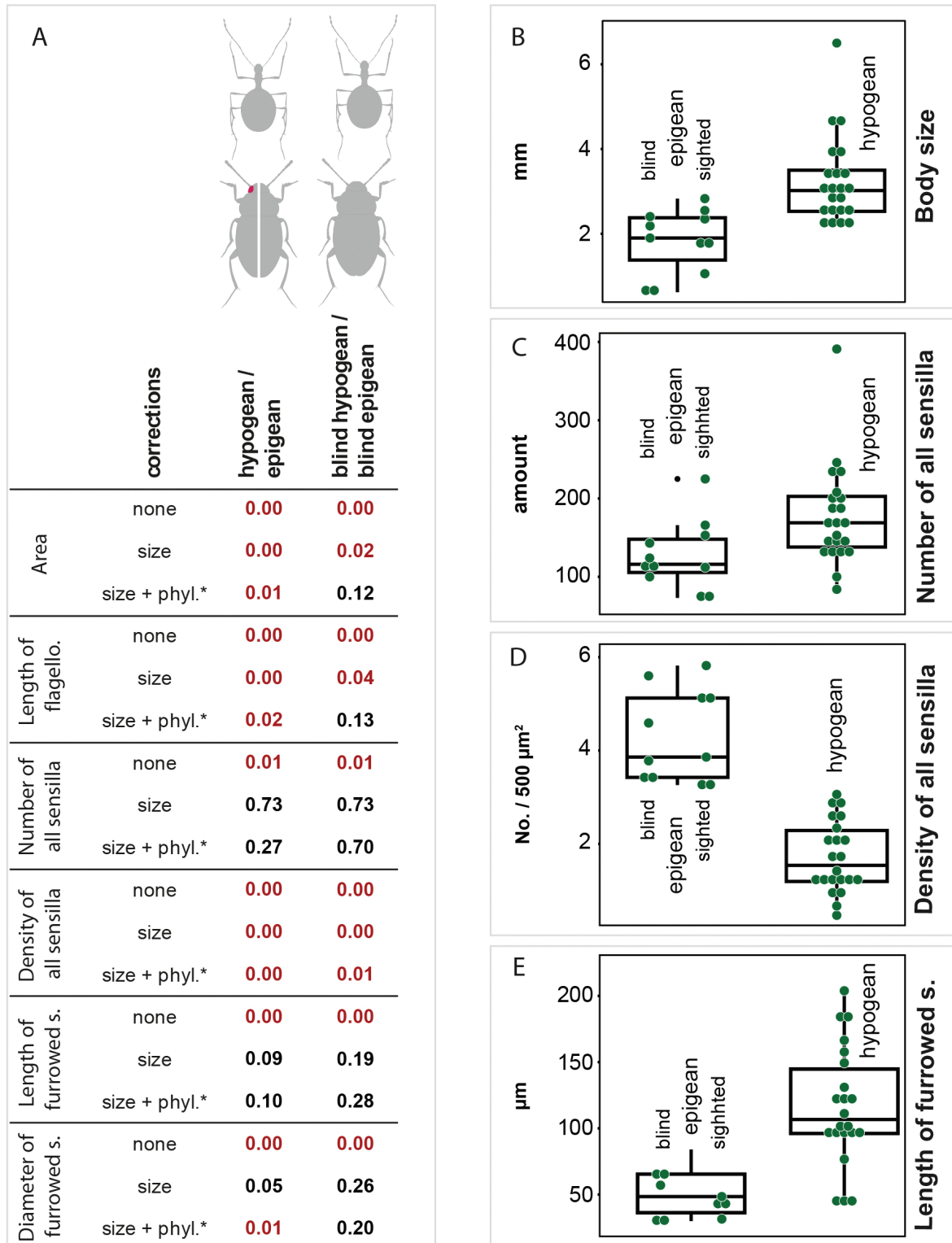


Figure 3. Results of the statistical analyses for selected traits (raw data provided in Table S5). **A** p -values for different traits with no correction (none), size correction (size) and size + phylogenetic correction (size + phyl.); significant correlations in red and bold. **B** median body size between the studied hypogean and epigean species. **C** median number of sensilla between the studied hypogean and epigean species. **D** median density of sensilla for the studied hypogean and epigean species. **E** median length of the furrowed sensilla for the studied epigean and hypogean species. The outer horizontal lines of the box represent 25–75 percent quartiles, the vertical lines drawn from the box represent standard deviations.

The **diameter of the furrowed** sensilla ranged between 1.63 and 5.91 μm (detailed values for every species in Table S3). In the hypogean species it was on average 3.38 (± 1.04) μm , in the epigean ones 2.15 (± 0.39) μm , and in the blind epigean ones 2.09 (± 0.22) μm . The significant differences we found between the studied groups was between the hypogean / epigean ($p = 0.00004$; Fig. 3A) and the blind hypogean / blind epigean taxa ($p = 0.001$;

Fig. 3A). After phylogenetic correction the difference between epigean/hypogean species was still significant $p = 0.01$; Fig. 3A), but not any more after removing species with eyes.

In an additional step, we mapped the studied traits on a molecular tree in order to assess their **evolutionary and phylogenetic relevance**. Fig. 4 shows the mapping on the

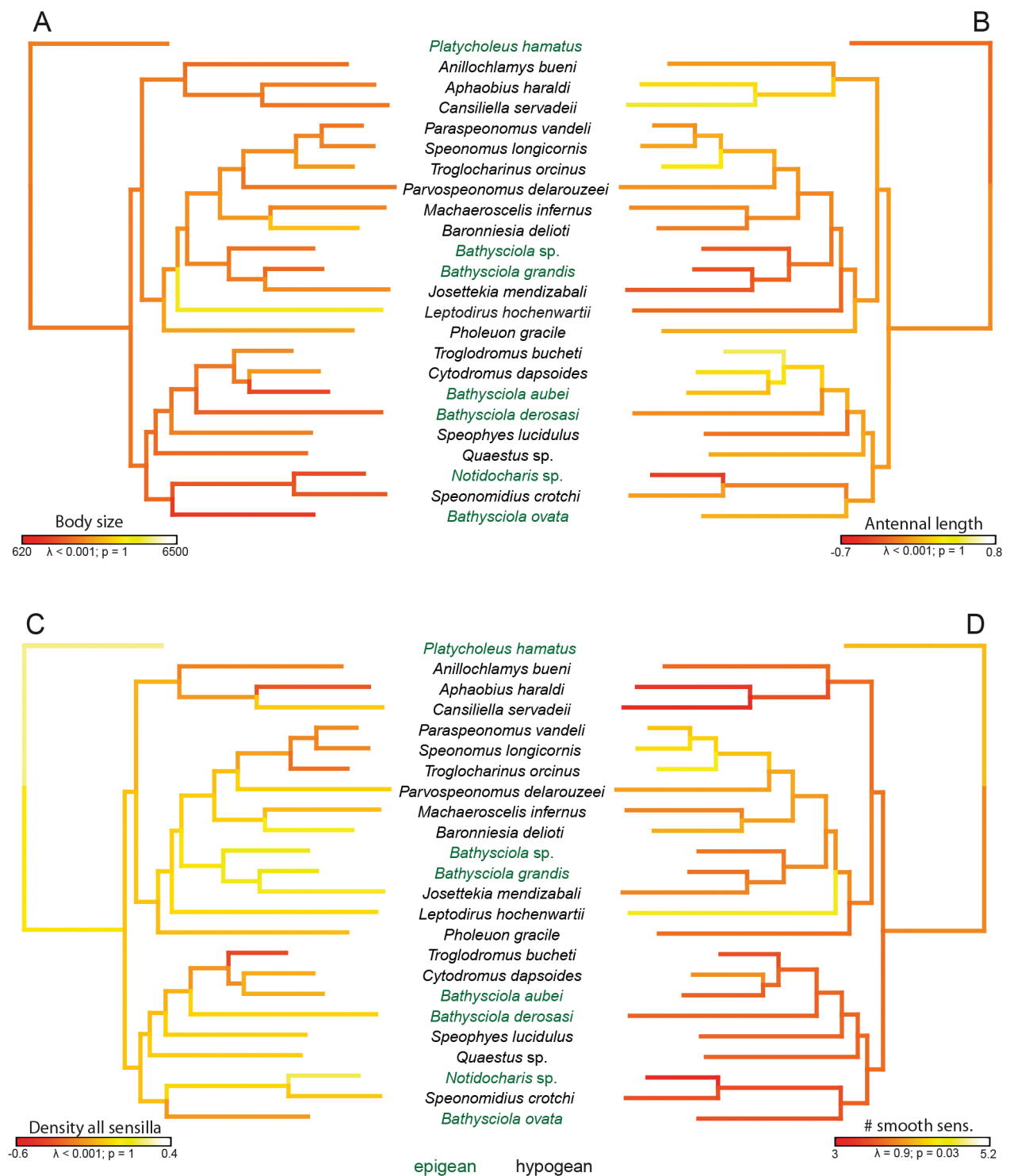


Figure 4. Morphological trait distribution on the tree. Traits were mapped onto phylogenies using the “plotBranchbyTrait” function in the phytools package in R. Colors correspond to smaller trait values (dark red) to larger trait values (light yellow). **A** body size; **B** size-corrected residuals of antennal length; **C** size-corrected residuals of density of all sensilla; **D** number of smooth sensilla (log-transformed).

tree for the body size ($\lambda < 0.001$; $p = 1$), antennal length (size-corrected measurements, $\lambda < 0.001$; $p = 1$), density of all sensilla (size-corrected measurements, $\lambda < 0.001$; $p = 1$), and number of smooth sensilla (raw log-transformed measurements, $\lambda = 0.9$; $p = 0.03$). With the exception of the number of smooth sensilla, all these studied traits were not significantly correlated with phylogeny according to the Pagel's Lambda test (λ), i.e. we could not identify a phylogenetic signal. The number of smooth sensilla decreases twice independently in the clades (*Notidocharis* sp. + *Speonomidius crotchi*) + *Bathysciola ovata* and *Aphaobius haraldi* + *Cansiliella servadeii*. An increase occurs in the clade *Troglocharinus orcinus* + (*Speonomus longicornis* + *Paraspeonomus vandeli*).

4. Discussion

The Leptodirini are particularly well suited for the present study as the large majority of the species – including those that live above ground – are eyeless (Fresneda et al. 2011). It thus allows a comparison between eyeless hypogean and eyeless epigeal species. However, the general scarcity of sighted species impedes a comparison between species with and without eyes. Our taxon sampling only contains six species with eyes, which is not sufficient for a sound statistical analysis. Therefore, in the following discussion, we pay more attention to the comparison between blind epigeic and blind hypogeic species.

Our results on smooth and furrowed sensilla patterns are in contrast to current hypotheses about the sensorial equipment in cave insects. For example, it is widely accepted in textbooks (e.g. Gunn 2004; Romero 2009; Moldovan 2012, 2018; Protas and Jeffery 2012; Chapman 2013; Culver and Pipan 2019; Howarth and Moldovan 2018; Fišer 2019, and original studies (e.g. Peck 1977; Juberthie and Massoud 1980) that cave-dwelling insects have a higher number of antennal sensilla than epigeal ones. Our uncorrected inter-specific comparisons show that there is indeed a difference in the number of sensilla (Fig. 3A). However, this effect dissolves after corrections for body size only, and also for body size and phylogeny combined. We included these corrections in order to assure that our results are not affected by body size and potential relationships, which might have obscured the correlation with the ecological traits. Our results thus imply that the higher number of sensilla is indeed an allometric effect. We found the same pattern in the comparison of blind epigeal / blind hypogeal species and it also applies to both furrowed and smooth sensilla.

In addition to external sensilla, leiodid beetles are also equipped with partially internal sensory Hamann's organs (Accordi and Sbordoni 1978), hygroreceptors according to Lucarelli and Sbordoni (1978). These are located on the flagellomeres V, VII and VIII. They comprise external rings on the distal surface that connect to internal tunnels that contain the internal parts of the Hamann's organ and the associated sensilla (Peck 1977; Accordi and Sbordoni

1978). As only single specimens from museum material of most species were available to us, and previous studies required complex preparation and fixation to study Hamann's organ (Peck 1977), we did not include them in our study. We therefore cannot exclude that there are differences between hypogean and epigeal species in the internal sensory system as suggested by Peck (1977).

The density of antennal sensilla turned out as the only trait with significant differences between both studied categories after accounting for size and phylogenetic position (Fig. 3A). Intuitively, current interpretations and the available literature suggest that blind hypogean species have a denser arrangement of tactile or olfactory sensilla to compensate for the loss of vision (e.g. [Wilkens et al. 2000; Howarth and Moldovan 2018]). However, remarkably, we found the exact opposite, i.e. a lower density in hypogean species than in epigeal ones (Fig. 3D). To our knowledge, the only study specifically addressing the density of antennal sensilla in blind or cave insects is the one of Juberthie and Massoud (1980), who stated that cave species have a denser vestiture of sensilla than epigeal ones. A similar result was found in the basal hexapod order Collembola (springtails) (Deharveng 1988; Jantarit et al. 2019). An explanation for this observation might be differences in body size (with hypogean species on average larger than epigeal ones) and the significantly longer flagellomeres with a larger surface area in the hypogean species (Fig. 3A). In the comparison between blind epigeal and blind hypogeal species, both areas and length of flagellomeres were not significantly different in our sample after size and phylogenetic correction. Our results thus imply that in the studied hypogean species a similar number of sensilla is distributed over a larger area, which results in a lower density. An opposite effect was observed in bumblebees, where larger individuals also have denser arrangements of sensilla (Spaethe et al. 2007). In addition to these allometric factors, it was shown in several studies that sociality, diet, sex, and sensitivity to odors can also affect the density of antennal sensilla (Gill et al. 2013; Fialho et al. 2014; Polidori et al. 2020).

Another widespread assumption is that antennal sensilla are elongated and widened in troglobitic species, compared to those of epigeal relatives (Juberthie and Massoud 1980; Moldovan 2004, 2012; Howarth and Moldovan 2018; Fišer 2019). Our initial and uncorrected results suggested significant differences of the length of the furrowed sensilla in hypogean / epigeal or blind epigeal / blind hypogeal species. However, after accounting for body size and the phylogenetic placement, the only trait that remained significantly different is the diameter of the sensilla in the comparison between epigeal / hypogeal species. In this context, we would like to point out that the phylogenetic + body size correction was done with a reduced data set, as we did not have molecular data for all species. We only found a significant difference between epigeal and hypogeal but not between blind epigeal and blind hypogeal. This might imply that the observed difference in the diameter might be rather linked to the presence or absence of eyes than to an epigeal or hypogeal

lifestyle. Unfortunately, our data set does not contain a sufficient number of sighted species for a valid statistical evaluation. Apparently, Leptodirini alone with its limited number of species with well-developed eyes is not the best group to address this question.

It is also commonly stated in textbooks that blind or cave species have longer antennae with an increased surface area compared to sighted and epigean relatives (e.g. Crowson 1981; Gunn 2004; Moldovan 2004, 2012, 2018; Chapman 2013; Howarth and Moldovan 2018; Fišer 2019). This hypothesis is confirmed by our analysis. We found significant differences in the flagellomere length and surface area between hypogean and epigean species ($p = 0.02$; Fig. 3A) ($p = 0.01$; Fig. 3A) after correction for size and phylogeny. Interestingly, this was not observed after the same corrections in the comparison between blind representatives of both groups. Whether this is also rather correlated with the absence of eyes than with a hypogean life style has to be clarified by future research. The studied hypogean species were significantly larger than the epigean ones (Fig. 3B), which is in agreement with the general observation that troglomorphic animals often have an increased body size compared to epigean relatives (e.g. Howarth and Moldovan 2018). In the studied beetles, the observation of longer antennae with larger surface area in hypogean species is thus also affected by allometric patterns. Similar results were also found by Faille (2006) in the carabid genus *Aphaenops* and in the leiodid species *Speonomus hydrophilus*, where the length of the antennae varies independently from the rest of the body (Juberthie et al. 1980). Faille (2006) could also show that there is a strong evolutionary pressure on the length of individual flagellomeres. As we only measured selected flagellomeres (V–VIII), we cannot fully exclude that the other may be elongated.

In summary our data show that widely accepted hypotheses concerning the antennal sensory equipment of cave beetles (e.g. increased number, density or length of sensilla) do not apply to the external antennal sensilla of the studied Leptodirini when the data are corrected for body size and/ or body size and phylogeny. Our study underlines the importance of including allometric and phylogenetic corrections. We also found indications that many of the observed correlations such as the increased area and length of the studied flagellomeres or the larger diameter of the sensilla might rather be correlated to the reduction of eyes than the hypogean lifestyle. However, we have to concede that our taxon sampling was too limited for a sound statistical interpretation concerning species with functional compound eyes. Another problem is the formulation of categories such as “sighted” as this would comprise species with large compound eyes but also those with only a few ommatidia. The studied sighted Leptodirini have relatively small compound eyes compared to the epigean *Catops picipes* of Cholevini. The inclusion of an additional category such as microphthalmia (strongly reduced eyes; Růžička and Perreau 2017) will also not solve the issue as the boundaries between microphthalmia and fully developed eyes would still be subjective and artificial as different degrees occur in

the reduction of eyes. We therefore recommend for future analyses to use the eye diameter or surface area as a discrete or continuous covariate in any statistical analyses. This would solve the problem of artificial and subjective categories and would account for the full dynamic and continuum of the presence and partial reduction of insect compound eyes.

In addition to assessing correlations between sensilla patterns and ecological preferences, we also mapped the studied traits on a phylogenetic tree in order to track possible evolutionary transformations and to test for potential phylogenetic signal. Our results show that no studied trait with the exception of the number of smooth sensilla is phylogenetically informative in our analysis. These sensilla function as chemoreceptors, or as hygrosensitive or thermal sensors (Schneider 1964). Our analyses suggest that this character complex might not be correlated with the habitat, but it is the only trait that shows a significant phylogenetic correlation ($\lambda = 0.9$, $p = 0.03$).

We can show that there are some trends in the studied species, but the taxon sampling is too restricted to identify any concrete apomorphies for subclades in the Leptodirini. It is noteworthy that some hypogean beetles make use of ultraspecialized habitats, such as for instance semi-aquatic (“cave hygropetric”) species of *Cansiliella* included in our study (Delić et al. 2023; Dorigo et al. 2007; Sket 2004). These beetles feed on fine substrates in water films on the wall, and their modified mouthparts are assumed to be adapted to the specific feeding habits (e.g. Moldovan 2004). Due to their highly unusual lifestyle, thorough examination of their antennal sensory system in comparison with other hypogean beetles would be of peculiar interest. Moreover, highly troglomorphic genera such as *Anthroherpon* with distinctly modified morphological characters are likely to provide additional information of the evolution of sensory systems among subterranean beetles. Our results thus show that the study of antennal sensory equipment could provide interesting insights of morphological traits in relation to the habitats. However, a finer sampling would be required to specifically address the impact of convergence on the morphological evolution of the group.

5. Conclusion

Our study with a sample of Leptodirini demonstrates that blind and cave-dwelling species of this tribe do not have more or longer external antennal sensilla, but that they are rather less dense than in their sighted and epigean relatives. As it was shown that the sensitivity increases with the number of olfactory (Ochieng and Hansson 1999; Spaethe et al. 2007) or tactile sensors (Staudacher et al. 2005), we assume that the sensorial perception via antennal sensilla is not improved in these categories in blind or cave-dwelling species of Leptodirini. As we did not study multiple individuals from different populations of the same species, we cannot account for intraspecific

variations. However, our study challenges the widely accepted hypothesis that all cave-dwelling species compensate for the loss of visual input with an increased sensory capacity of the antennae.

6. Declarations

Competing interests: The authors declare that they have no competing interests.

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Contributions: XL, IR and BW designed the work; XL, MG, AF, IR, and BW performed research; XL, MG, AF, IR and BW performed the analyses; XL, AF, RB, and BW contributed to the discussion and helped to structure the manuscript; XL and BW wrote the original draft of the manuscript; XL, RB and BW managed the collaborative work and critically revised the manuscript. All authors read and approved the final manuscript.

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Supplementary Material 1

Figure S1

Authors: Luo XZ, Gabelaia M, Faille A, Beutel R, Ribera I, Wipfler B (2023)

Data type: .pdf

Explanation notes: SEM images of the 9th flagellomeres of *Baronniesia deliotti* (A), *Bathysciola ovata* (B), *Ptomaphagus pyrenaicus* (C).

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Link: <https://doi.org/asp.81.e98166.suppl1>

Supplementary Material 2

Tables S1–S5

Authors: Luo XZ, Gabelaia M, Faille A, Beutel R, Ribera I, Wipfler B (2023)

Data type: .zip

Explanation notes: **Table S1.** Sampling information of the studied specimens. — **Table S2.** Body lengths of the studied specimens. — **Table S3.** Original data for statistical analyses. — **Table S4.** Lengths of the studied antennomeres. — **Table S5.** Results of statistical analyses. Effects in italic font represent χ^2 values.

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