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6 **Fundamental research questions in subterranean biology**

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74

75 **Running title:** Scanning the horizon of subterranean biology

76

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82

83 **ABSTRACT**

84 Five decades ago, a landmark paper in *Science* titled *The Cave Environment* heralded caves as
85 ideal natural experimental laboratories in which to develop and address general questions in
86 geology, ecology, biogeography, and evolutionary biology. Although the ‘caves as laboratory’
87 paradigm has since been advocated by subterranean biologists, there are few examples of studies
88 that successfully translated their results into general principles. The contemporary era of big
89 data, modelling tools, and revolutionary advances in genetics and (meta)genomics provides an
90 opportunity to revisit unresolved questions and challenges, as well as examine promising new

91 avenues of research in subterranean biology. Accordingly, we have developed a roadmap to
92 guide future research endeavours in subterranean biology by adapting a well-established
93 methodology of ‘horizon scanning’ to identify the highest priority research questions across six
94 subject areas. Based on the expert opinion of 30 scientists from around the globe with
95 complementary expertise and of different academic ages, we assembled an initial list of 258
96 fundamental questions concentrating on macroecology and microbial ecology, adaptation,
97 evolution, and conservation. Subsequently, through online surveys, 130 subterranean biologists
98 with various backgrounds assisted us in reducing our list to 50 top-priority questions. These
99 research questions are broad in scope and ready to be addressed in the next decade. We believe
100 this exercise will stimulate research towards a deeper understanding of subterranean biology and
101 foster hypothesis-driven studies likely to resonate broadly from the traditional boundaries of this
102 field.

103

104 *Key words:* biospeleology, cave biology, expert opinion, groundwater, horizon scanning,
105 research questions, stygofauna, troglobionts.

106

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123 **I. INTRODUCTION**

124 In the era of the Internet, social media, and open-access mega-journals, the amount of accessible
125 scientific information is overwhelming (Landhuis, 2016; Wakeling *et al.*, 2016; Fire & Guestrin,
126 2019; Jarić *et al.*, 2020). It is estimated that more than 50 million peer-reviewed scientific papers
127 exist (Jinha, 2010) and about 1.5 million new articles are published every year (Laurance *et al.*,
128 2013). To capitalize on the volume of this information and make the most of it (e.g. Ioannidis,
129 2005; Jeschke *et al.*, 2019), it is becoming increasingly important for scientists to explore
130 effective ways to capture the latest advances in their field or related fields of research. Horizon
131 scanning – i.e. the systematic examination of information to identify emerging issues and
132 opportunities in a given research area – has become a useful tool to summarize and determine
133 research priorities and agendas (Sutherland *et al.*, 2011). The most important questions in
134 ecology (Sutherland *et al.*, 2013; McGill *et al.*, 2019), island biogeography (Patiño *et al.*, 2017),

135 and microbiology (Antwis *et al.*, 2017), the annual identification of emerging issues in global
136 conservation (Sutherland *et al.*, 2020), as well as the 100 articles that every ecologist should read
137 (Courchamp & Bradshaw, 2018), are all instructive examples where horizon scanning has
138 successfully synthesized trends or highlighted the most promising future research avenues.

139 Fifty years ago, in a landmark *Science* paper titled *The Cave Environment*, Poulson &
140 White (1969) heralded caves as ‘natural laboratories’, i.e. simplified settings that can be used to
141 understand the principles governing the dynamics of more complex environments. Characterized
142 by stringent environmental constraints and simple communities, subterranean habitats have been
143 regarded as ideal systems for investigating many of the unresolved questions in ecology,
144 biogeography, and evolutionary biology (Juan *et al.*, 2010; Sánchez-Fernández *et al.*, 2018;
145 Mammola, 2019). Scientists have also studied subterranean organisms to understand human
146 diseases such as autism (Yoshizawa *et al.*, 2018), diabetes (Riddle *et al.*, 2018), and cancer
147 (Gatenby, Gillies & Brown, 2011), to investigate the engineering potential of biologically
148 inspired materials (Lepore *et al.*, 2012), and to discover new drugs and pharmaceutical products
149 (Cheeptham *et al.*, 2013). Others have even looked at caves through the lens of astrobiology,
150 showing that the subterranean microbiome might hold clues to life beyond Earth (Northup *et al.*,
151 2011; Popa *et al.*, 2011).

152 Although the ‘caves as laboratory’ paradigm is often advocated by subterranean
153 biologists, examples of studies that have successfully translated their results into general
154 principles remain few in number. Five decades after Poulson & White (1969), subterranean
155 biology is entering a new research era dominated by big data (Zagmajster *et al.*, 2019),
156 modelling tools (Flôres *et al.*, 2013; Mammola & Leroy, 2018), and increasingly cheaper
157 molecular approaches (Pérez-Moreno, Iliffe & Bracken-Grissom, 2016; Lefébure *et al.*, 2017).

158 Concomitantly, we are facing a global crisis that is negatively impacting subterranean
159 biodiversity (Mammola *et al.*, 2019b; Boulton, 2020). Therefore, the time is ripe to review the
160 outstanding challenges faced by this broad-in-scope discipline, as well as promising new
161 research avenues where subterranean-based studies may be helpful in answering general and
162 broadly scoped questions. Because gathering multiple views on such an extensive subject is
163 difficult, we relied on the well-established methodology of horizon scanning to identify 50
164 fundamental, but unresolved questions in subterranean biology. With this intellectual exercise,
165 we aimed to develop a roadmap that will guide future research endeavours and stimulate
166 hypothesis-driven studies likely to resonate beyond the boundaries of this discipline.

167

168 **II. HORIZON SCANNING PROTOCOL**

169 **(1) Initial list assembly**

170 We used horizon scanning methodology (Sutherland *et al.*, 2011) and adapted the approach
171 developed by Patiño *et al.* (2017) to identify priority research questions in subterranean biology.
172 Survey coordinators (S.M. and P.C.) identified seven subject areas within the subterranean
173 biology discipline (Table 1), namely: (1) Adaptation, (2) Origin and evolution, (3) Community
174 ecology, (4) Macroecology and biogeography, (5) Conservation biology, (6) Microbiology and
175 applied topics, and (7) Other topics. We included the latter subject area to cover additional topics
176 or ideas that departed from the six core subject areas and may have been overlooked. For each
177 subject area, survey coordinators invited a senior researcher (highlighted with asterisks in Table
178 1) to act as panel coordinator, with the task of establishing an international panel of experts to
179 identify and formulate a set of fundamental questions. Each panel coordinator selected and
180 invited three or four members to join their panel, which included at least one early-career

181 scientist (i.e. a postdoc or researcher with less than 10 years of experience) to obtain a multi-
182 generational perspective on the different topics. Survey coordinators encouraged panel members
183 to consult broadly with colleagues and select additional researchers to join their panels if deemed
184 important in providing complementary expertise. In assembling the panels, our goal was to
185 maximize multidisciplinary, while ensuring that research interests within the seven panels
186 covered a broad array of geographic areas, model organisms, and networks of international
187 collaborators. Members of each panel identified at least 20 questions that they viewed as
188 fundamental within their subject area and thus likely to advance the field significantly.

189 In total, we assembled 258 questions, which were screened for duplication or ambiguity
190 by the survey coordinators. In this phase, survey coordinators purged most subterranean-specific
191 jargon from questions and homogenized wording to ensure that all questions were presented in a
192 clear and straightforward manner. Therefore, throughout the survey we operated under the
193 assumption that all questions were characterized by a similar degree of readability (Plavén-
194 Sigray *et al.*, 2017). After the cleaning procedure and removal of duplicate questions, we
195 assembled a final list of 211 survey questions (hereafter ‘List #1’). In assembling List #1, we
196 subsumed questions identified by the panel focusing on ‘Other topics’ into the six main subject
197 areas.

198

199 **(2) Voting procedure and selection of 50 top-priority questions**

200 We subjected List #1 to an initial round of online voting by all panel members (Survey #1) to
201 select the most voted 20 questions for each of the six subject areas (Fig. 1). Voting was a binary
202 choice, whereby participants scored each question as either of ‘major’ or ‘minor’ importance.
203 We randomized question order for each participant. We repeated this voting protocol in all

204 subsequent online surveys. Each panel member voted on all questions irrespective of subject
205 area, although votes by panelists on their subject area were disregarded in the final ranking of
206 Survey #1. As a result, survey coordinators culled List #1 to the 120 most-voted questions (20
207 questions from each of six subject areas), referred to as List #2, thus balancing the number of
208 questions in subsequent voting rounds.

209 We then subjected List #2 to online voting (Survey #2) by inviting a broad community of
210 subterranean biologists including *ca.* 170 members of the International Society on Subterranean
211 Biology (ISSB), *ca.* 50 members of the European Cave Organism Network, *ca.* 100 members of
212 the Anchialine mailing list, as well as other working groups and email listservs related to
213 subterranean biology that we could identify (e.g. national biospeleological groups). Note that
214 members of these different groups often overlapped and some of the emails were no longer
215 active. We estimated that Survey #2 reached an upper boundary of between 200 and 250 unique
216 recipients. Of these, 133 recipients completed the online survey.

217 At the end of Survey #2, we gave participants the opportunity to submit one additional
218 question if they felt this question was missing from List #2. Thus, 25 additional questions were
219 added to the third list of questions (List #3). Questions in List #3 were voted on by all panel
220 members (Survey #3), and then ranked (by percentage of ‘major importance’ votes per question)
221 together with the 120 questions from List #2. Finally, we selected the highest ranking questions
222 to assemble a list of 50 top-priority questions.

223

224 **(3) Caveats on interpretation**

225 Some general caveats should be recognized when interpreting the results of any horizon scanning
226 survey (e.g. Sutherland *et al.*, 2011, 2013; Seddon *et al.*, 2014; Patiño *et al.*, 2017). First, the

227 background knowledge and intellectual passions of the experts involved may introduce
228 subjectivity in the formulation of the initial list of topics and questions. Second, subjectivity
229 likely plays a role throughout the voting process, as any voting outcome may be affected by the
230 interests of a particular group of participants. In our case, potential biases in the composition of
231 subterranean biologists sampled may have influenced the final selection of the top-priority
232 questions to an extent difficult to quantify precisely. For example, questions related to
233 microbiology received the lowest share of ‘major importance’ votes (mean \pm SD: 0.69 ± 0.01). It
234 is understood that microbiology topics are not less important or timely, it is simply that
235 microbiologists are probably underrepresented in the subterranean biology community. Also, an
236 imbalance in the expertise of participants may explain the substantial difference in how the
237 highest priority questions were parsed across the six subject areas – from four in ‘Community
238 ecology’ to 12 in ‘Conservation biology’.

239 To address these potential shortcomings, we adopted four countermeasures. First, we
240 increased the survey audience, by addressing the questionnaire to different groups and
241 associations of subterranean biologists. Second, we diversified the expertise of panel members
242 by including early-stage to mid- and late-career researchers from different disciplines, research
243 laboratories, and geographic areas. Third, we included a seventh panel (‘Other topics’)
244 specifically to fill the gaps in the initial composition of proposed questions. Indeed, it has been
245 argued that in horizon scanning, the initial division into subject areas may limit lateral thinking
246 (Sutherland *et al.*, 2013). Finally, by allowing voters to suggest additional questions when voting
247 in the survey, we were able to capture the range of priority topics better.

248 We are confident these practices minimized some of the biases inherent to this study.
249 Importantly, we believe this 50 top-priority survey served to highlight some of the most timely

250 and challenging areas of interest in current and future research, rather than providing a
251 comprehensive synthesis of research needs in modern subterranean biology.

252

253 **III. SUMMARY OF THE HORIZON SCAN**

254 In Survey #1, the percentage of ‘major importance’ votes ranged between 89% (top-voted
255 question) and 4% (least-voted question). In the extended online voting (Survey #2), 133 voters
256 participated, of which 71% identified ‘subterranean biology’ as their primary field of research.
257 Although voters’ gender was slightly skewed toward males (76 men *versus* 57 women),
258 deviation from the 1:1 male:female ratio was not significant ($\chi^2 = 2.71$; d.f. = 1; $P = 0.10$),
259 indicating that our sample was not gender-biased. 45% of survey voters were experienced
260 researchers, with an academic age of more than 10 years since they earned their PhD, while 29%
261 were researchers within 10 years from their PhD. PhD and undergraduate students accounted for
262 16% of voters. The remaining 10% of participants were other professionals, such as research and
263 field technicians or recreational cavers.

264 During Survey #2, participants suggested 28 additional questions; three questions were
265 duplicates and were thus excluded. The remaining 25 questions were evaluated during Survey
266 #3, and three made it to the 50 top-priority list. The lower threshold for questions was 67% of
267 ‘major importance’ votes, whereas the top-voted question garnered 91% votes (Fig. 1).

268 In the following, we present the 50 top-priority questions in subterranean biology
269 according to the results of Surveys #2 and #3 (the full list of questions is provided as online
270 supporting information in Appendix S1). For clarity, questions were compiled into our six
271 subject areas. We provide information about each question’s final rank (#) and percentage of
272 ‘major importance’ votes received (%), and highlight the three questions suggested by the

273 Survey #2 participants with an asterisk (*). A glossary of terms is available in Table 2.

274

275 **IV. ADAPTATION**

276 Q1 – What are the drivers of adaptive evolution in caves? [#1; 91%]

277 Q2 – What are the main constraints to subterranean adaptation? [#4; 83%]

278 Q3 – What are the degrees of adaptive plasticity of organisms across different subterranean
279 environments? [#9; 78%]

280 Q4 – Which traits of subterranean organisms should be considered as ‘adaptive’? [#11; 78%]

281 Q5 – How have morphological and behavioural traits co-evolved in subterranean organisms?
282 [#14; 76%]

283 Q6 – What is the level and nature of reproductive isolation between cave and surface populations
284 and what reproductive barriers are typically involved? [#19; 75%]

285 Q7 – Do similar traits evolve repeatedly in subterranean organisms due to changes in the same
286 genes, genetic pathways, and/or developmental processes? [#23; 73%]

287 Q8 – Have subterranean species evolved a distinct set of convergent behaviours? [#26; 72%]

288 Q9 – Are there common developmental pathways that promote or constrain subterranean
289 adaptation? [#29; 72%]

290 Q10 – Do traits that constitute reproductive isolation evolve in the same way across independent
291 closely related subterranean populations or species? [#42; 70%]

292

293 The morphology of subterranean organisms, which show bizarre convergent adaptations even
294 across different animal phyla, has historically attracted the attention of generations of scientists
295 (Juan *et al.*, 2010) including Charles Darwin (1859). Therefore, it is no surprise that subterranean

296 biologists participating in this survey greatly valued the role of subterranean habitats as natural
297 laboratories for the study of adaptive evolution. Ten questions focusing on adaptation were
298 included in our top-50 list (Fig. 1).

299 Colonization of suitable habitat is the initial event leading to subterranean adaptation
300 (details in Section V). Whatever the mode or pathway, colonizers often experience a significant
301 change upon entering the subterranean environment (i.e. complete darkness), which results in
302 visual sensory deprivation, challenges in locating mates and food, limited or modified food
303 resources, and physical barriers to dispersal. Adaptive responses to these factors may involve the
304 action of selection on plastic traits already existing in the colonizers (i.e. phenotypic plasticity;
305 Bilandžija *et al.*, 2020), standing genetic variation, or new beneficial mutations. Understanding
306 which of these environmental factors and adaptive responses play a primary role in subterranean
307 adaptation, either acting alone or in various combinations, was the most important question (Q1)
308 in our survey, selected by 91% of participants. Yet, given that some higher taxa are missing or
309 understudied in caves (Culver & Pipan, 2019), it remains unclear what are the main constraints
310 to subterranean adaptation (Q2) and whether specific exaptations facilitate successful
311 colonization events (see also Q11 in Section V). Resolving how many phenotypes of
312 subterranean dwellers depend on genetic and developmental constraints (Q9), or reflect
313 entrapment at local peaks in adaptive landscapes or recent invasions with insufficient time for
314 selection to alter traits, is one of the future challenges for evolutionary biologists.

315 Additional high-priority questions were focused on subsequent refinements of the initial
316 adaptive responses, such as the repertoire of adaptive plasticity (Q3), the degree to which pre-
317 existing genetic variation contributes to subterranean phenotypes, and which traits of
318 subterranean organisms can be considered as adaptive (Q4). Historically, reduction or loss of

319 traits such as eyes and pigmentation was thought to be driven by random mutations and genetic
320 drift or by natural selection, either directly or indirectly. This controversy has continued to the
321 present, with strong adaptationist (Carlini & Fong, 2017) and non-adaptationist (Wilkens &
322 Strecker, 2017) viewpoints. Depending on the species or ecological context, it is possible that all
323 of these mechanisms have roles in subterranean adaptation. Resolving this debate will require
324 explanations at the molecular, cellular, and developmental levels in multiple lineages (Jeffery,
325 2005), and the integration of this information to infer whether convergent traits evolve repeatedly
326 in subterranean animals due to changes in the same or different genes, genetic pathways, and
327 developmental processes (Q7). Answers to all these questions will contribute to our
328 understanding concerning why some species adapt rapidly and evolve when facing new
329 environmental conditions, inside or outside caves, which is a critical question given global
330 climate change (Walther *et al.*, 2002). In turn, this could provide insights about adaptive
331 processes occurring in other ecological settings with a similar set of environmental conditions
332 (e.g. permanent darkness, constancy in climatic conditions, food scarcity), such as deep-sea
333 habitats (Trontelj, Borko & Delić, 2019; Mammola, 2020).

334 Once survival in a subterranean habitat is ensured, the successful colonizers are subject to
335 adaptive morphological and behavioural (co-)evolution (Q5). Many behavioural changes are
336 probably influenced by the essential requirements of finding food and mates in darkness, and
337 may be convergent across different subterranean lineages (Q8). Also, some subterranean animals
338 suddenly attain a new status at the top trophic level and predator release occurs. For example, in
339 the Mexican tetra, *Astyanax mexicanus* (De Filippi) (Actinopterygii: Characidae), the workhorse
340 of adaptive evolution studies in caves (Jeffery, 2009; Wilkens & Strecker, 2017; Torres-Paz *et*
341 *al.*, 2018), this new ecological status of an apex predator facilitated the evolution of a range of

342 behaviours that may not be sustainable in a predator-limited surface environment (Yoshizawa *et*
343 *al.*, 2010; Hyacinthe, Attia & Rétaux, 2019).

344 Most subterranean organisms may also face subsequent invasions of their habitats by new
345 colonizers, of both former surface-dwelling conspecifics (if they are still extant) and other
346 competing species (e.g. Howarth *et al.*, 2007; Wynne *et al.*, 2014). Therefore, to understand
347 subterranean adaptations fully, it is crucial to explore the degree and nature of reproductive
348 isolation between the subterranean-adapted lineages and invading surface conspecifics (Q6). The
349 majority of subterranean animals probably arose through the process of ecological speciation in
350 which reproductive isolation evolved as a response to divergent selection between environments
351 (Niemiller, Fitzpatrick & Miller, 2008; Mammola *et al.*, 2018). Thus, many subterranean
352 adaptations should at least indirectly favour non-random mating between individuals of the
353 derived subterranean and ancestral surface populations. Understanding this will help to address
354 whether traits that constitute reproductive isolation evolve in the same way in independent
355 closely related subterranean populations or species (Q10), and therefore whether and how often
356 parallel speciation occurs in the subterranean realm. Ultimately, this would shed new light
357 concerning the intriguing hypothesis on the predictability of evolution (Blount, Lenski & Losos,
358 2018).

359

360 **V. ORIGIN AND EVOLUTION**

361 Q11 – Which traits present in surface species (exaptations) facilitate successful subterranean
362 colonization and adaptation? [#12; 77%]

363 Q12 – How do, and which, patterns of subterranean species diversification vary across taxa and
364 habitats? [#13; 77%]

365 Q13 – What evolutionary processes most commonly triggered radiations of subterranean
366 organisms? [#15; 76%]

367 Q14 – Do subterranean organisms lack genetic variation and thus the ability to adapt to a
368 changing environment? [#16; 75%]

369 Q15 – Does the timeline of subterranean evolution differ among taxa, types of subterranean
370 habitats, different biogeographic areas, and different ecological settings? [#22; 74%]

371 Q16 – What are the impact(s) of biotic and abiotic factors on speciation? [#28; 72%]

372 Q17 – Why are some lineages successful at colonizing subterranean habitats while others are
373 not? [#35; 71%]

374 Q18 – How old are subterranean species? [#36; 71%]

375 Q19 – The role of evolutionary processes (convergence/divergence/evolutionary
376 stasis/parallelisms) in subterranean organisms: what are the most common evolutionary
377 processes? [#40; 70%]

378 Q20 – Are shallow subterranean habitats a gateway to colonize deep zones and is the evolution
379 of deep subterranean species conditioned with a colonization of shallow and later deeper zones?
380 [#41; 70%]

381 Q21 – What is the rate of evolution of different subterranean traits and does the degree of
382 subterranean adaptation correlate with duration of subterranean inhabitation? [#44; 69%]
383

384 Subterranean animals have long interested biologists as evolutionary models. Studies of these
385 species have endeavoured to improve our understanding of evolution, its repeatability at the
386 phenotypic (Friedrich, 2013; Porter & Sumner-Rooney, 2018), physiological (Jones, Cooper &
387 Seymour, 2019), and molecular level (Leys *et al.*, 2005; Bilandžija, Četković & Jeffery, 2012;

388 Niemiller *et al.*, 2013), its reversibility (Copilaş-Ciocianu *et al.*, 2018), and the role of drift in
389 morphological changes (Martínez *et al.*, 2017; Wilkens, 2020). The eleven questions identified
390 highlight how, despite advances in the application of genetic tools and techniques in the last 50
391 years, fundamental questions regarding the origin and evolution of subterranean animals remain
392 unanswered.

393 Two high-ranked questions (Q11 and Q17) focused on the traits that enable species to
394 successfully colonize and adapt to subterranean habitats. Additional questions focused on the
395 most common evolutionary processes (Q19), and the influence of biotic and abiotic factors (Q16)
396 that lead to different patterns of diversification across subterranean lineages (Q12). Important
397 subterranean radiations are known in all major taxonomic groups (Deharveng & Bedos, 2019),
398 but only a few of them have been well documented. These include Amphipoda (Zakšek *et al.*,
399 2019), Collembola (Lukić *et al.*, 2019), and Coleoptera (Leys *et al.*, 2003; Faille *et al.*, 2010;
400 Njunjić *et al.*, 2018). Which evolutionary processes best explain these radiations remains highly
401 debated (Q13) and it would be particularly interesting to compare and contrast radiations of
402 surface-dwelling plants and animals (Gillespie *et al.*, 2020) with subterranean-adapted species to
403 determine if any universal patterns exist. For many animal groups, subterranean species are
404 commonly assumed to have evolved from surface species (Barr & Holsinger, 1985; Peck &
405 Finston, 1993), but recent phylogenetic studies suggest that this assumption may not always
406 apply (Faille *et al.*, 2010; Juan *et al.*, 2010; Leijs *et al.*, 2012). Speciation and diversification may
407 also occur within the confines of a subterranean habitat, a process referred to as ‘endogenous
408 diversification’ (Trontelj, 2019). Moreover, some phylogenetic studies suggested that
409 subterranean colonization is not an evolutionary dead end and surface species may actually arise
410 from subterranean ancestors (Prendini, Francke & Vignoli, 2010; Niemiller *et al.*, 2013; Copilaş-

411 Ciocianu *et al.*, 2018). However, cases of endogenous speciation and ‘subterranean to surface’
412 reversals are potentially confounded by extinction of surface lineages (Juan *et al.*, 2010).
413 Therefore, new approaches are needed that avoid reliance on phylogenetic methods alone to
414 improve our understanding of these patterns.

415 Genetic variation enhances the ability of species to adapt and diversify. Additionally, it
416 has been shown that some subterranean species may contain high levels of neutral genetic
417 variation (Buhay & Crandall, 2005; Guzik *et al.*, 2009), but it is still unclear whether neutral
418 mutations equates to high levels of adaptive genetic variation. This underpins the question
419 whether subterranean species lack the ability to adapt to changing environments (Q14), including
420 increasing temperatures and the introduction of new pathogens (Mammola *et al.*, 2019c). Such
421 hypotheses are obviously not exclusive to the subterranean environment. However, this
422 ecosystem does provide numerous examples of how low genetic variation was hypothesized to
423 be related to low adaptive capacity, a phenomenon more common underground than at the
424 surface (Konec *et al.*, 2015; Lefébure *et al.*, 2017; Fumey *et al.*, 2018).

425 Understanding the timeline and direction of subterranean evolution, as well as the age of
426 subterranean species, featured prominently in several questions (Q15, Q18, Q20, Q21).
427 Advances in molecular clock calibration (Drummond *et al.*, 2006) and genomic analyses (Pérez-
428 Moreno *et al.*, 2016) are considerably promising and permit the development of robust time trees
429 (Pons *et al.*, 2019). However, these analyses are limited by the availability of extant and fossil
430 taxa and the extinction of surface relatives; the latter makes it difficult to pinpoint the initial
431 colonization time of a subterranean habitat by a given species. This is particularly important for
432 ancient lineages of specialized subterranean organisms with marine origin, which often lack
433 surface-dwelling relatives and/or show low levels of fossilization (Pérez-Moreno *et al.*, 2016).

434 This is unfortunate because many of these basally branching lineages are required to reconstruct
435 trait evolution of major animal lineages (e.g. Johnson *et al.*, 2012; Khodami *et al.*, 2017; Lozano-
436 Fernandez *et al.*, 2019).

437 The genetic basis underlying evolution of subterranean traits, and how they are shaped by
438 natural selection and/or neutral processes, are key factors in determining rates of subterranean
439 evolution (Q21). Considerable advances have been made through the study of model
440 subterranean species, especially *Astyanax mexicanus* and the freshwater isopod *Asellus aquaticus*
441 (L.) (Protas & Jeffery, 2012). These species have several independent and recently evolved
442 subterranean populations, as well as extant surface populations, which can be hybridized in the
443 laboratory. Their features allow for the dissection of genes and mutations responsible for traits
444 related to subterranean life and provide information on the processes (e.g. selection or neutral
445 evolution) that shape their evolution. The role of neutral processes in the evolution of
446 subterranean animals has also been explored using alternative model systems (e.g. dytiscid
447 beetles and amblyopsid cavefishes). In both cases, species have been evolving underground for
448 millions of years, which is sufficient to enable the fixation of deleterious mutations in genes
449 under relaxed selection (Niemiller *et al.*, 2013; Tierney *et al.*, 2018). These model organisms
450 offer great potential to investigate major questions on the origin and evolution of subterranean
451 animals using comparative genomics, and thus may provide insights for similar processes in
452 other, non-subterranean, settings.

453 **VI. COMMUNITY ECOLOGY**

454 Q22 – What are the main ecological and ecosystem services provided by subterranean
455 populations and communities? [#20; 75%]

456 Q23 – What are the key food-web processes influencing subterranean community dynamics?
457 [#24; 73%]

458 Q24 – How do stochastic events interact with long-term trends in subterranean ecosystems?
459 [#30; 72%]

460 Q25 – How do basic life-history characteristics differ among subterranean communities and
461 between subterranean and surface communities? [#33; 71%]

462

463 Subterranean habitats are well-suited systems to address general problems in community ecology
464 (Mammola, 2019). Foremost, caves are often semi-closed environments extensively replicated
465 across the Earth (Culver, 1970; Culver & Pipan, 2019; Itescu, 2019; Mammola, 2019). Second,
466 subterranean communities generally exhibit lower diversity and abundance of organisms than
467 surface ones and are characterized by a bottom-truncated functional diversity (Gibert &
468 Deharveng, 2002), allowing us to disentangle the effect of abiotic conditions and biotic
469 interactions in filtering species possessing specific traits within the community (Cardoso, 2012).
470 Third, caves have some conspicuous environmental gradients from the surface towards the
471 subsurface (Howarth, 1982; Tobin, Hutchins & Schwartz, 2013; Mammola *et al.*, 2019d),
472 offering a mosaic structure of subterranean microhabitats defined by distinct habitat-filtering
473 properties (Trontelj, Blejec & Fišer, 2012; Mammola *et al.*, 2020).

474 Four questions in community ecology made it to the top-50 list. This result reflects a
475 general trend in subterranean biology, where researchers have primarily focused on caves as

476 model systems for evolutionary studies (Juan *et al.*, 2010), and secondarily used caves as
477 convenient settings to address fundamental ecological questions (Mammola, 2019). Yet, these
478 four questions fell within general and timely areas of current ecological research (see Sutherland
479 *et al.*, 2013).

480 The top-ranked question underscored the importance of services provided to humans by
481 subterranean species and ecosystems (Q22), rather than on theoretical aspects of community
482 ecology. Examples of ecosystem services provided by subterranean ecosystems include
483 pollination, seed dispersal, and agricultural pest control by bats (Kunz *et al.*, 2011; Medellin,
484 Wiederholt & Lopez-Hoffman, 2017), provision of clean water (Griebler & Avramov, 2015),
485 serving as a source for new pharmaceutical products (Cheeptham *et al.*, 2013), and even cheese
486 production (Ozturkoglu-Budak *et al.*, 2016). While services with direct benefit to humans have
487 received some attention, values provided by subterranean ecosystems extend far beyond direct
488 human needs. In light of emerging conservation issues associated with subterranean ecosystems
489 (Mammola *et al.*, 2019b), investigating ecological services and links between above- and below-
490 ground diversity in ecosystem functioning is crucial.

491 Two questions called for more research into life-history characteristics (e.g. growth rates,
492 age and size at sexual maturity, longevity, and survival rates; Q25) and food-web specificities of
493 subterranean communities (Q23). Interactions among life-history traits determine the fitness of
494 each population, while interactions between populations and the environment dictate the
495 distribution of species (Stearns, 1992). Only a few studies have described life histories of
496 subterranean species, and this is partially explained by the challenges of captive breeding and the
497 technical problems and effort necessary to conduct *in situ* comprehensive studies (Vonk &
498 Nijman, 2006; Voituron *et al.*, 2011; Venarsky, Huryn & Benstead, 2012; Riesch *et al.*, 2016;

499 Simon *et al.*, 2017). Consequently, the lack of knowledge on cave species traits limits our
500 understanding of evolutionary and ecological processes occurring in subterranean ecosystems.

501 Energy limitation is considered a primary mechanism influencing both evolutionary and
502 ecological processes in subterranean environments (Venarsky & Huntsman, 2018). However, a
503 more nuanced understanding of subterranean food-web dynamics (Q23) will require other
504 research actions, including to (i) understand the spatial and temporal dynamics of energy
505 resources; (ii) compare resource quality with consumers' physiological requirements; and (iii)
506 compare consumption rates with resource availability in subterranean habitats with different
507 environmental conditions (e.g. terrestrial *versus* aquatic, fresh *versus* salt water, and detrital
508 *versus* chemolithoautotrophic food webs).

509 Finally, understanding the role of stochastic events in caves was highlighted as a deficient
510 area in community ecology (Q24). Given that these events are increasing in frequency amid the
511 environmental crisis of the new millennium (Rahmstorf & Coumou, 2011), the study of
512 stochastic phenomena has emerged as a central topic in ecology (Scheffer *et al.*, 2001). Recent
513 papers used groundwater crustaceans to elucidate some of the mechanisms by which earthquakes
514 affect the composition and structure of biological communities (Galassi *et al.*, 2014; Fattorini *et*
515 *al.*, 2017; Fattorini, Di Lorenzo & Galassi, 2018; Morimura *et al.*, 2020). Additional studies have
516 focused on the effect of other events, such as heavy precipitation (Calderón-Gutiérrez, Sánchez-
517 Ortiz & Huato-Soberanis, 2018) and flooding (Pacioglu *et al.*, 2019). Although it may seem
518 counterintuitive to study stochastic environmental shifts in caves, as they have been traditionally
519 perceived as stable ecosystems, these examples show how caves may represent promising model
520 systems for quantifying the impacts of abrupt environmental shifts driving ecosystem evolution
521 (Mammola, 2019).

522 **VII. MACROECOLOGY AND BIOGEOGRAPHY**

523 Q26 – What drives subterranean patterns of phylogenetic and functional diversity? [#21; 75%]

524 Q27 – Would the use of novel molecular methods (e.g. metabarcoding, environmental DNA)
525 provide new insights on subterranean biodiversity patterns and affect known patterns? [#27;
526 72%]

527 Q28 – What is the species richness pattern of subterranean organisms globally? [#31; 72%]

528 Q29 – What factors drive the relative importance of speciation, extinction, and dispersal in
529 shaping subterranean diversity patterns across regions? [#34; 71%]

530 Q30 – Are current subterranean biodiversity patterns best explained by history of colonization of
531 surface ancestors or by *in situ* speciation and dispersal in subterranean habitats? [#39; 70%]

532 Q31 – How can sampling effort be standardized so that comparisons of species richness are
533 unbiased? [#43; 69%]

534

535 Over the last 20 years, research in subterranean ecology is shifting from local to landscape
536 studies aiming to document and understand biodiversity patterns at regional to global scales
537 (Zagmajster *et al.*, 2019). This transition is not without difficulties, as it requires linking
538 biodiversity patterns to eco-evolutionary processes with little to no possibility for manipulative
539 experiments. Six questions in ‘Macroecology and biogeography’ were identified in the top-50
540 list (Fig. 1). These questions mirror the main challenges faced when documenting and
541 understanding broad-scale biodiversity patterns at the surface. The first challenge is assembling
542 the data required to bring out the characteristic features of biodiversity patterns at such broad
543 scales, while ensuring these patterns are not biased by sampling effort (Q28, Q31). Secondly, to
544 combine multiple sampling techniques, species identification methods (e.g. morphological and

545 DNA-based identification), and biodiversity metrics (e.g. alpha, beta, and gamma diversity) in a
546 meaningful way to elucidate the many facets of biodiversity patterns (e.g. taxonomic,
547 phylogenetic, and/or functional diversity; Jarzyna & Jetz, 2016) (Q27, Q26). Lastly, the relative
548 contributions of different evolutionary processes (Q29) and diversification hypotheses (Q30) in
549 shaping biodiversity patterns should be fully examined.

550 The publication of global subterranean diversity maps and databases is a recent
551 phenomenon (Culver & Pipan, 2019; Zagramajster *et al.*, 2019). While diversity maps are
552 informative as they portray differences in species richness among regions or countries, we still
553 lack global maps showing species richness for spatial units of equal area [but see Zagramajster,
554 Culver & Sket (2008), Niemiller & Zigler (2013), and Eme *et al.* (2015) for examples of
555 regional- and continental-scale diversity maps]. Several approaches have been developed to
556 minimize differences in species richness due to sampling bias (Q31). This issue is particularly
557 germane to difficulties in sampling subterranean habitats. For example, sampling protocols were
558 typically standardized among sites and completeness of species inventories were assessed using
559 accumulation and rarefaction curves (Zagramajster *et al.*, 2008; Dole-Olivier *et al.*, 2009; Wynne
560 *et al.*, 2018). Also, observed species richness patterns were tested for robustness using species
561 richness estimators (Zagramajster *et al.*, 2014), or complemented with species richness predictions
562 modelled from environmental data (Mokany *et al.*, 2019).

563 Beyond accounting for sampling biases, molecular methods are increasingly useful in
564 understanding subterranean biodiversity patterns (Q27). For example, a recent study comparing
565 latitudinal patterns of crustacean species range size obtained from morphology- and DNA-based
566 species delimitation showed that the pattern of increasing median range size at higher latitudes
567 was more evident when delimiting species with DNA (Eme *et al.*, 2018) (Fig. 2). As sequencing

568 becomes increasingly applied to subterranean taxa, environmental DNA sampling and
569 monitoring may be also used to detect these species in areas difficult to access (Gorički *et al.*,
570 2017; Niemiller *et al.*, 2018), thus resulting in more accurate maps of their distributions. To our
571 knowledge, patterns of phylogenetic and functional diversity at continental to global scales have
572 not been documented for any subterranean taxon (Q26), despite the growing knowledge of
573 phylogenetic relationships and species traits (Morvan *et al.*, 2013; Fernandes, Batalha &
574 Bichuette, 2016; Fišer *et al.*, 2019; Mammola *et al.*, 2020). Documenting these patterns will
575 further underscore the relative importance of dispersal, extinction, and different speciation
576 modes in shaping geographic variation of species richness. Given the differences in global
577 diversity patterns between subterranean and surface habitats, comparing the two systems might
578 help further to elucidate the key drivers of diversity.

579 Recent macroecological studies have shown that historical climatic variability, spatial
580 heterogeneity, and energy contribute to species richness patterns of subterranean taxa in Europe.
581 However, the contributions of these factors vary regionally and across taxa (Eme *et al.*, 2015;
582 Bregović & Zagmajster, 2016; Bregović, Fišer & Zagmajster, 2019; Mammola *et al.*, 2019a). At
583 a landscape scale, linking environmental factors with speciation, extinction, and dispersal
584 dynamics (Q29), as well as diversification processes (Q30), remains challenging and requires the
585 use of phylogenetic methods and a large number of specimens for DNA analysis (Stern *et al.*,
586 2017). Yet phylogenetic methods encompass uncertainties that are highly sensitive to sampling
587 bias and the confounding effect of extinction, both obscuring the inference of transitions to
588 subterranean life. To ameliorate this, genes that lose their function soon after the transition
589 should be used (Lefébure *et al.*, 2017) (see also Section V).

590

591 **VIII. CONSERVATION**

592 Q32 – How does climate change affect subterranean-adapted organisms? [#2; 84%]

593 Q33 – What are the effects of pollution on subterranean-restricted microorganisms, arthropods,
594 and vertebrates? [#3; 84%]

595 Q34 – What is the impact of above-ground disturbance on subterranean environments and their
596 fauna? [#5; 82%]

597 Q35 – How can we evaluate the ecological status of subterranean ecosystems? [#6; 80%]

598 Q36 – How can we protect subterranean-adapted species from invasive species? [#7; 80%]

599 Q37 – How can we combine policy, education, research, and management to safeguard
600 subterranean biodiversity effectively? [#8; 80%]

601 Q38* – What factors determine the size and location of effective protected areas in subterranean
602 environments? [#10; 78%]

603 Q39* – How can we effectively involve governments and key stakeholders in the conservation of
604 caves and other subterranean systems? [#17; 75%]

605 Q40 – What would be the best monitoring protocols to quantify long-term changes in the
606 distribution and abundance of subterranean invertebrates? [#18; 75%]

607 Q41 – How do we address the lack of knowledge (biodiversity shortfalls) about the biology of
608 subterranean species to enhance proper conservation measures? [#25; 73%]

609 Q42 – Can subterranean-adapted organisms be used as bioindicators of the health of
610 subterranean ecosystems? [#45; 69%]

611 Q43 – How does the use of caves by humans (e.g. tourism, religious, therapeutic, and
612 recreational activities) affect subterranean ecosystems? [#48; 68%]

613

614 Ecosystems are experiencing biodiversity loss at an unprecedented rate worldwide (Barnosky *et*
615 *al.*, 2011; Dirzo *et al.*, 2014; IPBES, 2018; Cardoso *et al.*, 2020). Thus, conservation and
616 management of cave biological diversity is of the utmost concern among subterranean biologists
617 (Mammola *et al.*, 2019*b*). Conservation questions comprised most of the questions (24%) in our
618 top-50 list (Fig. 1). Of these, 10 questions were part of the initial List #1, while two additional
619 questions were suggested by survey participants. Three questions (Q32, Q33, and Q36)
620 highlighted three of the greatest threats to biodiversity worldwide – climate change (Ripple *et al.*,
621 2019), pollution (Ripple *et al.*, 2017), and invasive alien species (Pyšek *et al.*, 2020) – whose
622 effects are pervasive also underground (Mammola *et al.*, 2019*b*). Additional questions were
623 centred on the impacts of above-ground disturbance (Q34) and human activities (Q43) on
624 subterranean habitats. All these threats can be combined and described as ‘habitat loss and
625 degradation’, which is one of the most important drivers of biodiversity loss globally (IPBES,
626 2018). Subterranean habitat loss and degradation is primarily due to surface activities, such as
627 agricultural expansion and intensification, urbanization, and mining activities (Reboleira *et al.*,
628 2013; Mammola *et al.*, 2019*b*; Castaño-Sánchez, Hose & Reboleira, 2020). Human activities
629 inside caves may also constitute localized threats, with recreational use and tourism activities
630 being of particular concern (Fernandez-Cortes *et al.*, 2011; Faille, Bourdeau & Deharveng,
631 2015). In certain areas, people are even poaching rare invertebrate species for private collections
632 (Simičević, 2017), as in the discussed case of *Anophthalmus hitleri* Scheibel (Coleoptera:
633 Carabidae) (Berenbaum, 2010).

634 Evaluating, understanding, and mitigating these threats are primarily hampered by our
635 scarce knowledge of subterranean organisms’ biology (Q41), especially life-history traits (see
636 Q25 in Section VI). Understanding changes in species’ abundance and distribution will be

637 crucial to halting biodiversity loss in subterranean habitats. Studies aimed at identifying
638 bioindicator species (Q42) to help bolster long-term monitoring programs (Q40) are needed.
639 Additionally, improved sampling procedures and characterizing cave communities in previously
640 undocumented areas would both enhance our knowledge of subterranean biodiversity (Mammola
641 *et al.*, 2019b) and improve the effectiveness of conservation measures (Q41).

642 Furthermore, it is crucial to adopt innovative approaches to safeguard subterranean
643 biodiversity (Q37), as well as to determine the size and location of effective protected areas
644 (Q38). Standardized systematic sampling techniques have been applied to terrestrial (Wynne *et*
645 *al.*, 2018, 2019) and aquatic subterranean invertebrate species (Dole-Olivier *et al.*, 2009); to be
646 optimally beneficial to conservation and monitoring, these techniques will need to be further
647 scrutinized across a large breadth of taxa and systems. Recently, a cave vulnerability assessment
648 protocol has been developed for bat cave roosts (Tanalgo, Tabora & Hughes, 2018) and, if
649 refined, would hold promise for use with other subterranean animals.

650 Protected areas are the most crucial measure to safeguard specific subterranean habitats
651 and the sensitive animal populations they often support (Q38). Indices have been developed for
652 site selection and conservation prioritization (e.g. Borges *et al.*, 2012; Rabelo, Souza-Silva &
653 Ferreira, 2018; Strona *et al.*, 2019; Fattorini *et al.*, 2020) which are often based on
654 complementarity, flexibility, and irreplaceability principles (Michel *et al.*, 2009). Yet, rigorous
655 geospatial analysis is still rarely applied when the extents of protected areas are being
656 determined. Further considerations should include managing lands upslope from caves or entire
657 watersheds supporting sensitive subterranean habitats. If a species-level approach is taken for
658 establishing a protected area, it would be reasonable to protect the land at the hydrogeologic unit
659 (i.e. watershed or karst/volcanic unit) level – as animals are expected to use mesocaverns or

660 unconsolidated sediments for dispersal (Howarth, 1983; Malard *et al.*, 2017; Trontelj, 2019).
661 Importantly, such an approach should be based on the most accurate estimation of the relevant
662 animal's distributional range.

663 While effective legislation and/or management plans exist for some subterranean species
664 and some regions of the world, overall management policies for most regions of speleological
665 importance are lacking (Q39). Only a few countries have national cave protection laws. For
666 example, the United States Federal Cave Protection Act of 1988 has been used as a tool to
667 manage caves on federally owned lands, while Brazil requires geological and biological
668 assessments of caves and stipulates mitigation of any human activities that may negatively
669 impact cave natural resources. In any case, to be fully operational, such legislative and
670 management tools need to be based on the best available science including a comprehensive
671 knowledge of fauna distribution (Brooks, Da Fonseca & Rodrigues, 2004; Samways *et al.*, 2020)
672 and traits of the species of concern (Chichorro, Juslén & Cardoso, 2019; Fattorini *et al.*, 2020).
673 Importantly, management plans will require both financial, governmental, and local community
674 support for their implementation. Unfortunately, most countries lack the capacity or legislation to
675 protect and conserve sensitive subterranean resources.

676

677 **IX. MICROBIOLOGY AND APPLIED TOPICS**

678 Q44 – What is the role of Bacteria, Archaea, fungi, and viruses in nutrient cycling in
679 subterranean systems? [#32; 71%]

680 Q45 – How adaptable are cave microorganisms to changing environmental conditions (e.g.
681 climate change)? [#37; 70%]

682 Q46 – How do other organisms (humans and other animals), and their activities (e.g. visiting

683 humans and global climate change) influence cave microbiome diversity patterns? [#38; 70%]
684 Q47 – How does the range of energy sources and quantity influence the diversity of subterranean
685 microbiota? [#46; 68%]
686 Q48 – What are the limiting nutrients for subterranean microbiota and how do they affect overall
687 subterranean microbial diversity? [#47; 68%]
688 Q49 – How do subterranean microorganisms cycle key elements – nitrogen, iron, carbon, sulfur,
689 and phosphorus? [#49; 67%]
690 Q50* – What is the role of microorganisms in cave-formation processes (speleogenesis) in
691 subterranean environments? [#50; 67%]
692
693 Without a doubt, topics such as adaptation, origin and evolution, community dynamics, and
694 biogeographic distribution patterns are similarly important and actively targeted in microbial
695 ecology (Antwis *et al.*, 2017). However, research in macroecology and microbial ecology is
696 often conducted separately rather than hand-in-hand. For nearly 200 years, subterranean
697 ecosystems have been studied from a macroscopic perspective. Subterranean microbiological
698 research is a relatively new discipline with most research having been conducted since the
699 middle of the last century (Griebler & Lueders, 2009). A modern ecosystem approach to
700 subterranean biota requires consideration across all trophic levels and scales (Hershey & Barton,
701 2019), especially since the 1980s, when the first cave ecosystems fully sustained by *in situ*
702 chemosynthetic primary production were discovered (Sarbu, Kane & Kinkle, 1996; Kumaresan
703 *et al.*, 2014).

704 The seven questions on the top-50 list address general problems that have been frequently
705 examined for various subterranean ecosystems, such as alluvial aquifers, however, less

706 systematically for cave environments. Three questions focused on the active role of
707 microorganisms in nutrient cycling (Q44, Q49) and how nutrient limitations influence microbial
708 diversity (Q48). Although we know that microbes rule the subsurface in terms of element cycles
709 (Ortiz *et al.*, 2014; Kimble *et al.*, 2018) and constitute the basis of the food web, we still lack
710 detailed information on conversion rates and growth kinetics. In addition, subterranean
711 organisms often persist with limited energy resources. Thus, understanding their specific
712 adaptations would help advance our understanding of adaptive strategies for microorganisms in
713 other ecosystems (e.g. mountain-summit and deep-sea habitats). Additionally, the role of viruses,
714 which only recently has been recognized as ‘tremendous’ for groundwater ecosystems (Griebler,
715 Malard & Lefébure, 2014), has not been investigated for terrestrial subterranean systems (Q44).

716 Two questions further addressed the resistance and resilience of cave microbial
717 communities to disturbance from changes in environmental conditions (Q45) (Cavicchioli *et al.*,
718 2019), and the impacts of other organisms (in particular, humans; Moldovan *et al.*, 2020;
719 Martínez *et al.*, 2020) on microbial diversity (Q46). These questions also were related to
720 conservation issues from a microbiological perspective. The adverse impacts of the fungus
721 *Pseudogymnoascus destructans* that causes white-nose syndrome in North American bats is a
722 prominent example. To date, *P. destructans* occurs in 38 U.S. states and seven Canadian
723 provinces (see <http://www.whitenosesyndrome.org>), which raises serious concerns for the
724 conservation of hibernating bat species and the ecosystem services they provide (Kunz *et al.*,
725 2011; Boyles *et al.*, 2011; Medellín *et al.*, 2017; Mammola *et al.*, 2019b). The fungus is an
726 opportunistic environmental pathogen, which can remain in the subterranean environment and
727 contribute to the cave microbiome even in the absence of its host (Lorch *et al.*, 2013).

728 It has been hypothesized that microbial communities with high diversity and functional

729 redundancy do not select for ecosystems poor in energy and stable in environmental conditions
730 (Griebler & Lueders, 2009). Thus, the introduction of novel species may have a destabilizing
731 effect on a cave's biological equilibrium (Q46). The same is true for the introduction of
732 contaminants, such as organic compounds and nutrients that provide additional energy. We are
733 only beginning to understand whether and how energy–diversity relationships known from
734 macroecology apply to complex natural bacterial communities (Q47). In fact, there is a growing
735 body of evidence that diversity–productivity relationships also drive microbial communities
736 (Smith, 2007), but this question has not been examined systematically in subterranean
737 ecosystems yet.

738 Finally, Q50 points to the potential contribution of microorganisms in speleogenetic
739 processes, such as weathering and rock formation *via* inducing precipitation. Specifically, in
740 terms of (inorganic) carbon cycling in face of climate change, the role of microbes in the
741 formation of caves may be of great relevance, and has yet to be fully examined.

742

743 **X. CONCLUSIONS**

744 (1) The 50th anniversary of Poulson & White's (1969) article was the perfect time to reflect on
745 milestone scientific achievements obtained in the natural laboratories offered by caves, while
746 also delineating the most important research priorities for years to come. We have shown how
747 subterranean biology has contributed strongly to general scientific questions *via* the study of
748 evolutionary and ecological processes along the vertical dimension (i.e. the evolutionary
749 transition from the surface to the subsurface). These accomplishments resonate with the
750 sentiments of Poulson & White (1969) and we anticipate that biologists will continue to unravel
751 the mysteries of subterranean ecosystems and contribute to scientific knowledge more broadly,

752 insofar as revolutionary advances in approaches and technologies continue to foster and nurture
753 novel paradigms.

754 (2) There is a significant lack of knowledge concerning eco-evolutionary processes underlying
755 biodiversity patterns along the horizontal gradient (i.e. within subterranean habitats). This is
756 largely driven by a paucity of functional ecology studies, the weakness of trait-based approaches
757 (Cardoso, 2012; Fernandes *et al.*, 2016; Fišer *et al.*, 2019; Mammola *et al.*, 2020), and the lack of
758 robust systematic sampling techniques for most taxonomic groups (Wynne *et al.*, 2019).

759 Bridging these gaps will significantly influence how we address and prioritize future research on
760 the conservation and ecosystem services of subterranean habitats (e.g. Fattorini *et al.*, 2020), as
761 emphasized by the large number of unresolved questions in conservation biology (representing
762 nearly 25% of the top-50 list).

763 (3) We also invite scientists to redouble their efforts to understand the diversity of subterranean
764 life across all its components, with a special focus on linking macroscopic and microbial ecology
765 (Foulquier *et al.*, 2011; Mermillod-Blondin, 2011). This will enable us to achieve a mechanistic
766 understanding of subterranean eco-evolutionary processes and ecosystem function. This
767 information will be critical in guiding future policy decisions as human activities and global
768 environmental change increasingly impact and strain the subterranean realm.

769 (4) There is a concern that simple voting exercises such as this one may favour general over
770 specific questions. Perhaps as a result of this bias, some of the top-voted questions appear to be
771 broad in scope (e.g. Q1, Q2, and Q32). While these questions were able to capture important
772 general lines of inquiry, specific questions may be more useful for setting applied agendas.

773 Therefore, we invite interested readers to consult Appendix S1, which contains our complete list
774 of 120 questions.

775 (5) While the ‘caves as laboratory’ paradigm is an effective way to frame broadly scoped studies,
776 we recognize the top-50 list of questions primarily pertains to unresolved issues within the
777 borders of subterranean biology. Yet subterranean habitats offer much more. Deep subterranean
778 habitats are one of the few natural systems defined by highly stable and homogenous climatic
779 conditions tantamount to those maintained in a laboratory (Sánchez-Fernández *et al.*, 2018).
780 These systems have an island-like nature (Itescu, 2019), and often support communities
781 characterized by highly specialized organisms interacting in simplified ecological networks
782 (Mammola, 2019). By extension, a robust understanding of these rather simplified settings may
783 enable researchers to disentangle the complexities of more diverse systems (e.g. deep-sea
784 habitats).

785 (6) Ultimately, all these features point at subterranean ecosystems as ideal settings in which to
786 tackle general questions. We strived to provide examples of how some of our survey questions
787 may aid in addressing non-cave specific agendas. Our hope is that this horizon scan exercise both
788 underscores the importance of caves for addressing a range of eco-evolutionary questions, as
789 well as stimulates researchers to redouble their efforts to address some of these lingering
790 questions in subterranean biology.

791

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821

822 **XII. REFERENCES**

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1322 **XIII. SUPPORTING INFORMATION**

1323 Additional supporting information may be found online in the Supporting Information section at
1324 the end of the article.

1325 **Appendix S1.** Questions from List #2 (i.e. 120 questions selected from List #1 during Survey#1)
1326 and List #3 (i.e. 25 additional questions suggested by Survey #2 participants) ranked based on

1327 the percentage of 'major importance' votes.

1328

1329 Table 1. Subject areas, general topics addressed, panel member composition (*= panel
 1330 coordinator; °= postdoc or early career researcher), and number of questions included in the top-
 1331 50 list out of the total retained in List #1. Panel members are listed alphabetically by surname.
 1332

Subject area	General topics	Panel members	Number of questions
Adaptation	Morphological, physiological and behavioural adaptations to the subterranean environment	Žiga Fišer°, Daniel W. Fong, Tanja Pipan*, William R. Jeffery, Jure Jugovic	10 out of 43
Origin and evolution	Cave ontology and past climate change, migration–speciation–extinction dynamics, and speciation and diversification	Steven J.B. Cooper*, Matthew Niemiller, Alejandro Martínez°, Meredith Protas	11 out of 36
Community ecology	Population dynamics, community assembly, biotic interaction, trophic webs, and energy flows	Rodrigo L. Ferreira*, Cene Fišer, Thais G. Pellegrini°, Michael Venarsky°	4 out of 32
Macroecology and biogeography	Global diversity patterns (taxonomic, phylogenetic, functional), biogeography theory, and diversity drivers	Maria E. Bichuette, David Eme°, Florian Malard*, Maja Zgamažster°	6 out of 32
Conservation biology	Climate change, habitat loss, invasive species, conservation and management policies, and show-cave-related issues	Isabel R. Amorim°, Paulo A. V. Borges*, Louis Deharveng, J. Judson Wynne, Ana Sofia P. S. Reboleira	12 out of 37
Microbiology and applied topics	Microbial communities, industrial and pharmaceutical potential, epidemics, and exobiology	Naowarat Cheeptham, Thomas M. Lilley*, Melissa B. Meierhofer°, Diana E. Northup	7 out of 31
Other topics	Any topic falling outside the scope of the six core subject areas	David C. Culver*, Christian Griebler, Johanna Kowalko, Raoul Manenti°	n/a (merged within the other subject areas)

1333

1334 Table 2. Glossary of terms.

1335

Term	General definition
Cave	A human-accessible subterranean space, either a single chamber or series of chambers, formed within different substrata (Curl, 1964). Note that a cave is just one among the wide variety of subterranean habitats (see definition below).
Exaptation	A trait shaped by selection or neutral evolution co-opted for a new function (Gould & Vrba, 1982).
Speleogenetic process	The process of water dissolving surrounding rock, gradually forming passages that evolve into cave systems (Audra & Palmer, 2011).
Subterranean habitat(s) / ecosystem(s)	The breadth of underground voids of different sizes, either dry or filled with water, sharing two main ecological features: the absence of sunlight and buffered climatic conditions. Examples of subterranean habitats include caves, groundwater, anchialine systems, artificially excavated underground voids, shallow subterranean habitats, as well as deep maze of fissures and pore spaces with size prohibiting human entry (Culver & Pipan, 2019).

1336

1337 **FIGURE LEGENDS**

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1339 **Fig. 1.** Survey workflow, summary statistics of survey participants, and the breakdown by
1340 subject area of the 50 highest priority research questions.

1341

1342 **Fig. 2.** The relationship between median range size (maximum linear extent) per latitudinal band
1343 and latitude for 147 European groundwater species of Niphargidae (Amphipoda) and Aselloidea
1344 (Isopoda) delimited using morphology (A) and a molecular species delimitation method (B).
1345 Molecular delimitation was performed by a Bayesian implementation of the Poisson tree
1346 processes (Zhang *et al.*, 2013) approach based on molecular phylogenies inferred from 2883
1347 cytochrome *c* oxidase subunit I sequences. Black horizontal bars, dots, and boxes show the
1348 median, average, and interquartile range, respectively, for 0.9° latitudinal bands. The maximum
1349 length of each whisker is up to 1.5 times the interquartile range. Trend lines (with 95%
1350 confidence intervals) represent the fit of a gamma generalized linear model to the averages of
1351 latitudinal bands and its quadratic (A) and cubic (B) term. Data re-analysed from Eme *et al.*
1352 (2018).

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