Between darkness and light: spring habitats provide new perspectives for modern researchers on groundwater biology

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Abstract

21 Springs are interfaces between groundwater and surface habitats and may play an important role
22 in the study of subterranean animals. In this systematic evidence review, we explore whether
23 observations of stygobionts in springs are relevant and more common than observations of
24 epigean animals in groundwater.
25 We searched the Web of Science database for papers on groundwater fauna and spring fauna. For
26 each paper we found, we recorded whether the paper reported the occurrence of typical
27 stygobionts in springs, of surface animals in groundwater, or of the same taxa in both habitats. If
28 so, we recorded how many such species were reported. We also recorded the scientific discipline
29 of each study and the year of publication.
30 Our search yielded 342 papers. A considerable number of these papers reported stygobionts in
31 springs: 20% of papers dealing with groundwater fauna and 16% of papers dealing with spring
32 fauna reported the occurrence of stygobionts in spring habitats. Both the number of papers that
33 mentioned stygobionts in springs, and the number of stygobiont species that were documented in
34 springs, were higher than equivalent measures for the occurrence of surface fauna underground.
35 We also detected a positive relationship between year of publication and the number of reports of
36 stygofauna in springs.

37 To broaden the insights from biological research on underground environments, we suggest that
38 springs should be considered not only as simple sampling points of stygobionts but also as core
39 stygobiont habitats.
40
41
42 **Introduction**
43 The zoologist Lazăr Botoşăneanu (1998) defined springs as the "doors on River Styx," the river

44 of the Greek mythological underworld. Other biologists who study subterranean environments and

45 groundwaters similarly consider springs to be openings that allow them to see the inhabitants of

46 an otherwise inaccessible environment (Culver et al. 2012; Fiasca et al. 2014; Galassi et al. 2014;

47 Pipan & Culver 2012; Pipan et al. 2012). This view of spring habitats as windows into a different

48 environment is particularly true in non-karst areas, where the lack of caves prevents human

49 exploration of the subterranean realm and springs are often the only way to access groundwater

50 organisms (Manenti & Pezzoli 2019). Springs are interfaces between groundwater and surface

51 freshwaters

52 with a great variety of connectivity, permeability and biodiversity (Gibert et al. 1997; Kresic 2010;

53 Springer & Stevens 2009). The interplay of both subterranean and epigean habitat features that

54 characterises springs (Alfaro & Wallace 1994; Cantonati et al. 2006), makes challenging to define

55 the spatial boundaries of these ecotones (Gibert et al. 1997).

56 In some cases the transition between surface and subterranean habitats, may also be abrupt; indeed,

57 the magnitude of this transition strongly depends on the morphology of the spring and can be

58 mutable with daylight. Some springs represent an abrupt shift from the subterranean environment

59 to the surface, whereas others, like the natural emitting caves (such as caves from which

60 subterranean streams flow outside) of artificial draining galleries (such as galleries built to collect

61 groundwater), represent extended ecotonal environments (Balland 1992; White 2019). The border

62 between the subterranean and surface environment can be particularly distinct during daytime,

63   when it is strictly demarcated by the sun. Aside from sunlight, the differences that distinguish

64   subterranean and surface environments in a spring, even across the few meters or centimetres that

65   may characterize a spring with a sudden interface (Fig. 1), include the availability of trophic

66   resources, the density of potential predators, and microclimate conditions (Barzaghi et al. 2017;

67   MacAvoy et al. 2016; Manenti et al. 2013; Von Fumetti & Nagel 2011).

68   Because springs are border habitats, it can be difficult for biological studies to consider springs in

69   their entirety; this difficulty has limited the potential for insights from springs to drive stronger

70   advances in different fields of research. For example, studies that focus on springs often only

71   consider a surface perspective and neglect the role played by groundwater (Manenti & Pezzoli

72   2019), whereas in karst areas, scientists studying the subterranean environment see springs as

73   "access points" that can be used to sample the groundwater fauna living in different subterranean,

74   underwater environments, such as the phreatic zone of karst aquifers (Malard et al. 2002). This

75   latter view reflects the scarce consideration that is often given to springs and may limit a more

76   general understanding of the ecological role of border habitats. As some studies have already

77   suggested, transition zones are important for regulating ecosystem processes and the flow of

78   organic matterand organisms between surface (epigean) and underground (hypogean) habitats

79   (Moseley 2009; Plenet & Gibert 1995; Prous et al. 2004). With this opinion paper based on a

80   systematic review of the recent scientific literature, we aim to stimulate a change in the conception

81   of and in the approach to springs by studies dealing with stygobionts and groundwater fauna.

82   Particularly we want to underline that springs have the potential to reveal general patterns related

83   to the zoology of stygobionts.

84   Stygobionts are obligate groundwater-dwellers; the etymology of the word "stygobiont" reflects

85   the fact that these species, and stygofauna more broadly, are "of the River Styx." These organisms

86   have evolved adaptations specific to the underground freshwater habitats in which they spend their

87   entire life cycle (Trajano & De Carvalho 2017). Stygobionts often exhibit morphological features

88   associated with their underground habitat. These characteristics, such as blindness and

89   depigmentation, are commonly referred to as troglomorphisms (Pipan & Culver 2012; Romero

90   2009), and they limit stygobionts' ability to exploit surface environments. However, at night, the

91   constraints are generally less clear and surface borders (i.e., springs) may become more permeable

92   by stygofauna; for example, some samplings of springs' fauna have reported during night the

93   occurrence of organisms considered to be strict stygobionts (Bressi et al. 1999; Fišer 2019;

94   Manenti & Barzaghi 2020; Manenti & Barzaghi In press), as it has been observed also for the

95   twilight zones of terrestrial caves (Mammola & Isaia 2018). One such case is that of *Stygobromus*

96   amphipods, which are believed to regularly leave hypotelminorheic habitats to feed (Culver et al.

97   2006; Culver & Pipan 2014). Nevertheless, these findings are often viewed as exceptions or

98   accidental events, and the use of springs is rarely mentioned as a trait of stygobiont biology.

99   Observations of surface animals in caves have been similarly overlooked in the past (Sket 2008)

100 and improperly seen as accidental; such "accidental" observations have recently been described

101 for both groundwater and terrestrial subterranean habitats (Ficetola et al. 2018; Lunghi et al. 2014a;

102 Manenti 2014).

103 Stygobionts are the main focus of subterranean biology and are usually studied using two distinct

104 approaches. The first approach includes intense taxonomic investigations focused on the discovery

105 and description of new taxa. The second approach views caves as powerful natural laboratories for

106 evolutionary, ecological and behavioural studies on their inhabitants (Culver & Pipan 2014; Culver

107 & Pipan 2019). The idea of caves as natural laboratories, first postulated by the speleologist

108 Édouard-Alfred Martel (1894), has been espoused for more than one hundred years of subterranean

109 studies (Poulson & White 1969) and recently updated and broadened in the current context of

110 biological research (Mammola 2019; Mammola et al. 2020) . However, of the relatively large

111 number of caves that were effectively used as laboratories during last century (Vandel 1964), few

112 remain active. In addition, the outcomes of studies on stygofauna in these caves is rarely compared

113 to insights obtained from studies of surface freshwater organisms. This is partially due to the

114 characteristic features of stygobionts: they are often difficult to sample in deep subterranean

115 environments and springs can be seen as useful sampling points for them, using a combination of

116 different methods depending on the spring morphology and hydrology (Malard et al. 2002).

117 Particularly in springs are recommended the sampling of drifting stygobionts during spates,

118 samplings of the spring benthic layer, and bed sediments and also the use of artificial substrates

119 and baited traps (Malard et al. 2002).

120 Moreover, due to their long life cycles and low fertility, stygobionts are difficult to raise in an

121 experimental settings. Would including springs and other surface/underground border habitats in

122 studies on subterranean biology increase the understanding of how the constraints of the hypogean

123 environment affect the phenotypic responses and genetic makeup of stygobionts? The rationale of

124 this paper takes origin from this question and is to suggest that a substantial inclusion of springs

125 (and other border habitats between underground and surface) in studies on subterranean biology,

126 can increase the understanding of principles governing exploitation and adaptation to hypogean

127 environments.

128 In this paper, we investigate the perspectives of modern researchers on considering springs not

129 only as simple sampling points, but also as core stygobiont habitats that can broaden the insights

130 obtained from biological studies of underground environments. We specifically performed a

131 systematic review of the recent scientific literature to understand i) the relevance of previous

132   observations of typical stygobionts in springs; ii) if these observations vary according with study

133   discipline and the year of publication; and iii) if these observations are more common than

134   observations of epigean animals (i.e. aquatic surface species) in caves. By demonstrating that

135   typical stygofauna are observed in springs more commonly than usually thought, we propose that,

136   at least in some cases, the exploitation of border habitats be considered a non-negligible aspect of

137   stygofauna ecology.

138   **Survey methodology**

139   To avoid bias, many scientific fields have largely started to favour the use of systematic evidence

140   reviews (Acreman et al. 2020). We therefore performed a systematic review to find focused data

141   that addressed our three aims (Table 1). For this review, we followed the Preferred Reporting Items

142   for Systematic Reviews (PRISMA) guidelines (Page & Moher 2017), and we searched the Web of

143   Science database for peer-reviewed papers on both stygofauna and fauna living in spring habitats

144   (Fig. 2). The Web of Science database contains metadata for peer-reviewed scientific articles

145   published since 1965. We used two search strings designed to find all articles in the database that

146   might contain observations of fauna in both caves and springs. Our search was conducted in May

147   2020 from Milano, Italy, using the keywords "groundwater fauna" (GF) and "spring fauna

148   freshwater" (SFF) and searching them by topics. For the search we used a ASUS K501 PC and the

149   Google Chrome browser, after having emptied its cache box.

150   One of us (BP) initially screened the articles that met our search criteria by discarding articles that

151   were not clearly related to our study aims. She rejected articles about botany, palaeontology,

152   geology, and all their associated subdisciplines (paleoecology, stratigraphy, geomorphology, etc.),

153   as well as articles about subterranean environments or groundwater that did not mention animals.

154   The articles found using the key words "spring fauna freshwater" were more difficult to screen;

155   for the most part, the authors of these articles did not specify if their study species were part of

156   stygofauna or not. She therefore discarded these articles only if they were not related to our study

157   aims (e.g., papers about estuaries, palaeontology, or related topics) or if the authors provided clear

158   evidence that the study species were not cave-dwellers.

159   She additionally discarded several articles that dealt strictly with agricultural sciences,

160   biogeochemical cycles, the impacts of various pollutants (crude oils, perchlorate, etc.) on

161   groundwater, or other environments strongly connected to groundwater (i.e., all surface water

162   environments), but did not mention the finding of stygofauna or epigean fauna. She discarded also

163   articles that addressed single species or taxa that are not stygofauna or typical spring fauna and

164   have no hypogean representatives (e.g., Rechulicz 2011 treated *Pseudorasbora parva* and Vilenica

165   et al. 2016 treated mayflies). Articles concerning terrestrial environments, estuaries, swamps,

166   mangroves, streams, rivers, lakes, and all saltwater environments, were similarly discarded (see

167   Table 1 for more detailed information on the article selection procedure). After this first screening,

168   she performed a second selection procedure in which she removed any articles that were

169   unavailable or were written in a language other than English.

170   From the papers she collected the information listed in Table 1, including the typology of the study,

171   distinguishing between ecology, taxonomy behaviour, conservation and fauna assessment and

172   considering that the same paper could belong to multiple categories. Moreover, she assessed the

173   country and the continent of origin of the data provided by the papers.

174   *Statistical analyses*

175   To assess the relationships between features of the selected documents and the occurrence of

176   stygofauna in springs, we built a series of generalized linear models (GLMs) with binomial error

177   distributions.

178   First, we assessed if the fact that a paper reported the occurrence of stygobionts in springs, the

179   occurrence of surface fauna in groundwaters, or the contemporary occurrence of a stygobiont in

180   both groundwaters and springs, was related to the paper's field of study. Reported occurrences

181   were used as the dependent variable, and the study disciplines (ecology, taxonomy, faunal

182   assessment, and conservation) were used as fixed factors (Equation 1). We similarly built GLMs

183   with the same dependent variable but with publication year and the search term as independent

184   variables (Equation 2).

185

186    $Stygofauna\ found\ in\ springs\ OR\ Surface\ fauna\ underground\ OR$

187    $Occurrence\ of\ the\ same\ taxa\ in\ Same\ taxa\ both\ in\ springs\ and\ groundwaters \sim Ecology$ $+\ Taxonomy\ +\ Fauna.assessment + Conservation\ \ + Behaviour, family = binomial.$

188   (eqn 1)

189

190            $Stygofauna\ found\ in\ springs\ OR\ Surface\ fauna\ underground\ OR$

191        $Occurrence\ of\ the\ same\ taxa\ in\ Same\ taxa\ both\ in\ springs\ and\ groundwaters \sim$

192            $Year\ of\ publication + as.factor(\ key\ research\ term), family = binomial.$

193   (eqn 2)

194   Second, using only the papers selected with the GF search term we built GLMs to test two distinct

195   hypotheses: (1) that the number of mentions of stygobiont species in springs is higher than the

196   number of mentions of surface species in groundwater; and (2) that missing information is different

197   among the two situations. Both hypotheses were tested only for papers selected with the keyword

198   "groundwater fauna" to avoid biases associated with the fact that studies found using the keyword

199   "spring fauna freshwater" may not have sampled underground habitats. For the first test (Equation

200   3), we used the number of species mentioned by each paper as a dependent variable, including

201   both stygofauna found in springs and surface fauna found underground. The type of observation

202   (stygofauna in springs vs. surface fauna in groundwater) was used as a fixed factor.

203

204        $Number\ of\ species\ mentioned \sim Typology\ of\ observation, family = nbinom2.$

205   (eqn 3)

206   For the second test (Equation 4), we defined the dependent variable as whether it was possible to

207   assess the number of species mentioned in a study, including both types of observations

208   (stygofauna in springs and surface fauna in groundwater). The type of observation was used as a

209   fixed factor, as before.

210

211        $NAns\ occurrence \sim Typology\ of\ observation, family = nbinom2.$

212   (eqn 4)

213

214   Third, we also built a series of GLMs to test if the fact that a paper reported the occurrence of

215   stygobionts in springs, the occurrence of surface fauna in groundwaters, or the contemporary

216   occurrence of a stygobiont in both groundwaters and springs, was related to the continent of

217   sampling of the springs.

218   For all models we checked models assumptions by verifying the absence of multicollinearity issues

219   though VIF calculation and plotting residuals versus fitted values, versus each covariate; To avoid

220   overdispersion bias in the models used for the second step of analysis, we built both models using

221   a type 2 negative binomial error distribution in the package glmmTMB (Brooks et al. 2017).

222   We used a likelihood ratio test to assess the significance of all the fixed factors included in each

223   GLM (Bolker et al. 2008). All analyses were performed in the R 3.6.3 environment.

224

Results

226 We retrieved 824 potentially relevant papers after removing duplicate articles. After removing

227 articles based on the first selection criteria described above, there were 415 potentially relevant

228 documents. After the second selection procedure, we obtained 342 papers: 275 derived from the

229 search term "groundwater fauna" (GF) and 67 from the search term "spring fauna freshwater"

230 (SFF). Many papers found using the "groundwater fauna" search did not specify the sampling site

231 for the taxa considered, and many papers found using the "spring fauna freshwater" search did not

232 clearly identify if they sampled stygofauna or not. Overall, 57 papers (representing 19% of the

233 papers with information on sampling habitat) reported the occurrence of stygofauna in springs, 37

234 (11.7%) reported the occurrence of typical surface fauna underground, and 33 (11%) reported the

235 same taxa in both springs and groundwater (Table 2). With respect to our search terms, 20% of

236 papers dealing with GF and 16% of papers dealing with SFF described the occurrence of

237 stygobionts in spring habitats.

238 There were 45,375 mentions of species across all papers we retrieved. Of these, 138 were

239 stygobionts observed/sampled in springs and 46 were surface species observed in subterranean

240 habitats.

241 The study disciplines covered by the papers were mainly ecology (196 papers) and faunistic

242 assessments (177 papers). Four papers were behavioural studies, and 24 papers addressed

243 conservation issues. There were 194 papers that encompassed multiple ad hoc definitions that we

244 established in the methods.

245 Our first analysis revealed that faunal assessments are significantly more likely to report the

246 occurrence of surface fauna in groundwater, whereas taxonomic studies are more likely to report

247 the occurrence of the same taxon in both environments (Table 3). We did not detect any

248   relationship between the discipline of a paper (ecology, taxonomy, etc.) and the reported

249   occurrence of stygofauna in springs. However, we did detect a positive relationship between the

250   year of publication and the reports of stygofauna in springs (χ^2=4.53, P=0.03). Papers selected

251   using the SFF search term were significantly less likely to report the occurrence of surface taxa in

252   groundwaters (χ^2=4.09, P=0.04).

253   Most data of the papers came from Europe (59.76%) and Oceania (27.9 %); we detected no

254   significant relationships between the continent of sampling and the occurrence of stygobionts in

255   springs, the occurrence of surface fauna in groundwaters, and the contemporary occurrence of a

256   stygobiont in both groundwaters and springs.

257   GLMs performed on papers selected using the GF search term revealed that the number of

258   mentions of stygobiont species in springs is higher than the number of mentions of surface fauna

259   underground (χ^2=4.19, P=0.04). However, there is also less information available on whether

260   stygofauna have been observed in springs compared to whether surface species have been recorded

261   in groundwaters (χ^2= 14.08, P<0.001).

262   **Discussion and perspectives**
263   Our systematic review revealed that there are more papers about stygofauna available on Web of

264   Science than there are papers addressing fauna and springs. Because the word "spring" is a

265   homograph with multiple meanings, our initial search retrieved many papers that were ultimately

266   discarded because they did not discuss fauna and spring habitats. Preliminary literature searches

267   performed using synonyms of "spring" and/or terms that define specific spring habitats, such as

268   "sources" or "seepage," resulted in fewer papers. Most of these papers were already included in

269   our analysis; however, the few that were not could be used in future study with a larger set of

270   papers. Someone could disagree as it is likely that our research missed some papers (as an example

271   we are sure that we missed at least 15 papers on aquatic cave-dwelling salamanders and 10 papers

272   on the taxonomy of strictly subterranean planarians) and that further keywords should have been

273   added, for example: stygob*, ecoton*, hypogean, subterranean etc, but they would have increased

274   the number of papers dealing with stygofauna without significant increase in the number of papers

275   related to spring fauna. The large difference in the number of papers obtained with the two search

276   terms, GF and SFF, underscores the fact that the fauna of spring habitats have received much less

277   attention not only than the inhabitants of lakes and streams/rivers, as already pointed out by

278   previous studies (Cantonati et al. 2011), but also than stygofauna.

279   We limited our review to articles archived in Web of Science; this approach was more conservative

280   because it included only relatively recent papers published in indexed, high-impact journals that

281   perform selective peer review. An analogous review could be performed using the Google Scholar

282   database or a more exhaustive search of grey literature in online and physical repositories. It is

283   possible that the older, descriptive papers archived in these databases may have reported

284   stygofauna in springs, but it is also possible that some form of bias could arise from using older

285   literature that has not been rigorously peer reviewed. The effects of database selection should

286   therefore be investigated in the future.

287   Using both GF and SFF as search terms, we found papers that mentioned the occurrence of

288   stygofauna in springs, of typical surface fauna in groundwaters, and of the same taxa in both

289   environments. The number of papers that reported stygofauna in springs, as well as the number of

290   stygobiont species that were documented in springs, represented only a fraction of the total papers

291   and documented species but were nevertheless non-negligible. This pattern was not linked to any

292   specific field of study; though taxonomic studies were non-significantly more likely to report

293   stygofauna in springs.

294   Springs have been recognized as relevant habitats for studying stygobionts since the beginning of

295   the 19th century. Most subterranean biologists devoted at least some of their studies to spring

296   habitats (Culver et al. 2012; Culver et al. 2014; Vandel 1920), and Albert Vandel, the founder of

297   the "Laboratoire Souterrain de Moulis" (France), one of the most popular subterranean laboratories

298   in the world (Botosaneanu 1980), stated in 1920 that a systematic study of spring habitats could

299   furnish important insights for solving some of the evolutionary questions posed by cave-dwelling

300   animals (Vandel 1920). However, this concept appears only in Vandel's conclusions and is not

301   further developed; the idea that springs are just sampling points in non-karst areas largely prevails

302   throughout the rest of the paper (Vandel 1920).

303   Stygobionts are known to colonize the mixed assemblages of organisms residing in springs via

304   emigration and drift from groundwaters (Malard et al. 2009; Malard et al. 2002; Manenti &

305   Barzaghi In press). Typical stygobionts may be more or less permanently detected outside the

306   spring outlet, where they can exploit different microhabitats (Malard et al. 2002; Mathieu et al.

307   1994). This is especially true when there is a stable supply of immigrants from karst groundwater

308   (Mathieu et al. 1999). Our results revealed that there is a positive significant correlation with the

309   reporting of typical stygobionts in springs and the year of publication; this means that, with respect

310   to older studies, researchers are paying more attention when reporting data on sample collection

311   habitats, regardless of their study discipline (ecology, taxonomy, conservation, or faunal

312   assessment). However, in the papers that we collected, the occurrence of stygobionts in springs

313   was often reported as either an effect of the sampling method or an occasional finding. None of

314   the papers assessed patterns in the use of springs by stygobionts. This is true also for some papers

315   that we missed with our search but that are well known in spring literature. As an example (Rouch

316   1986) defined "the hemorrhage" the flow of stygofauna pushed out from aquifers during high

317   discharge periods through springs, erroneously considering this only as a passive mechanism. Also

318   the case of the olm (*Proteus anguinus*) is challenging with both mentions of cases of passive drift

319   of individuals from groundwaters (Aljančič 2019) and hypotheses of active exploitations of spring

320   habitats (Bressi et al. 1999). In more recent times, some papers were devoted to spring discharge

321   and the passive presence of stygobionts being flushed from "conductive" or "capacitive" aquifers

322   (Di Lorenzo et al. 2005); other large-scale ecological surveys of springs demonstrated that in

323   mountain areas, where species richness of stygobionts is usually poor due to the effect of

324   Quaternary Galciations, their occurrence seems low or occasional in springs (Stoch et al. 2011),

325   suggesting that the geographical location of springs matters and could be more in depth considered

326   in future systematic reviews dealing with springs. Springs are also being studied with recent and

327   'modern' approaches like DNA metabarcoding techniques and eDNA that can allow to detect the

328   presence of stygobionts in springs (West et al. 2020; White et al. 2020; Yonezawa et al. 2020) and

329   be used in the future to assess the patterns that determine this occurrence.

330   The occurrence of a stygobiont species, or a species that is strictly linked to a hypogean

331   groundwater habitat for its life cycle, in an epigean spring habitat, underlines a contradiction that

332   might reflect the human conceptual limit of understanding borders. The human perception of limits

333   and boundaries may be biased, as humans may recognize or emphasize abrupt distinctions when

334   they do not exist (Pirni 2016; Sturz & Bodily 2016). Our results demonstrate that, at least for some

335   stygobionts, border habitats and adjacent areas are an important part of the range and biology of

336   stygofauna, and a proper consideration of these habitats in subterranean biology studies could

337   provide larger perspectives. For example, stygobiont populations or individuals that exploit springs

338   more or less permanently are likely exposed to different constraints and advantages than

339   populations or individuals that exploit deeper aquifers. Selective pressures may therefore act

340   differently, at least for the individuals living in springs or at the interface between subterranean

341   and epigean habitats.

342   For example, different species and/or populations of the genus *Niphargus*, which shows typical

343   features of stygobionts including depigmentation and the absence of eyes, have the unique ability

344   to detect light (Fišer et al. 2016). This ability has been associated with the need to distinguish the

345   border between surface and subterranean environments and avoid risky surface habitats (Fišer et

346   al. 2016) where UV rays may be dangerous for a depigmented animal. However, surface habitats

347   may also be advantageous by furnishing higher trophic resources and, at night, they are not

348   exposed to UV light. Several studies have reported *Niphargus* amphipods in border habitats (Fiser

349   et al. 2007; Manenti & Pezzoli 2019; Marković et al. 2018). Is light perception the same between

350   individuals from borders and individuals from deeper aquifers? Are there evolutionary adaptations

351   for exploiting not only deep subterranean habitats but also border habitats at the interface with the

352   surface? These questions are applicable to all stygobionts that are recurrently found in springs.

353   Considering border habitats in addition to deeper subterranean environments therefore has the

354   potential to double the insights obtained from studies of stygobionts. These insights could be used

355   not only to disentangle evolution from the adaptations to the selective pressures of groundwater

356   habitats but also to characterize the physiological responses stimulated by the interaction with

357   different environmental conditions.

358   Our results further demonstrate that stygofauna are reported in springs more frequently than

359   surface fauna are reported in groundwater, in terms of both number of papers and overall numbers

360   of species. In recent years, a growing body of literature has shown that even the occurrence of

361   surface species in caves is often not accidental (Lunghi et al. 2014b; Lunghi et al. 2017), a finding

362   that has important implications for the communities of shallow subterranean habitats (Kozel et al.

363   2019; Lunghi 2018; Lunghi et al. 2020; Salvidio et al. 2020; Silva et al. 2020). If stygofauna occur

364   in springs and adjacent microhabitats more commonly than surface fauna occur underground, it is

365   likely that, at least for some stygobionts, the use of the surface environment is not accidental.

366   Further systematic reviews and analyses of the literature on spring fauna could be performed to

367   investigate the countries where the largest number of studies on springs were carried out, the most

368   studied taxa and the most studied functional traits.

369   A parallel can be made between springs and marine caves which can further support the idea that

370   springs are just an ecotone that should also be studied from an ecological viewpoint (Romero, *in*

371   *litteris*). For example, there are sea fish species that enter and exit marine caves playing a

372   significant role in those environments' ecology. That is the case with the cardinal fish *Apogon*

373   *imberbis*. This is a small-sized fish distributed along the eastern Atlantic coast from Morocco to

374   the Gulf of Guinea, including the Azores. It can be found as solitary or forming schools and is

375   common in small crevices to marine caves, where they can be found in large densities. They show

376   no troglomorphisms, yet they play a significant role in transferring organic material to these marine

377   caves as mysid crustaceans do (Romero, *in litteris*). Like bats and Dolichopoda cave crikets

378   (Mammola & Isaia 2018), they tend to stay in the shelters during the day and leave the caves at

379   night, presumably for feeding (Bussotti et al. 2003).

380   The occurrence of stygobionts in springs could affect both the dynamics of boundary habitats and,

381   at the level of the whole stygobiont population, the intrinsic traits of the species. There are several

382   different perspectives for how a stronger conceptual inclusion of springs in subterranean research

383   may provide additional insights on subterranean biology. First, springs may favour intraspecific

384   variation that could be assessed by comparative experimental studies, which would benefit studies

385   of intraspecific dynamics between boundaries and deep areas. Second, springs can inform studies

386 of the processes that promote adaptation to and colonization of border habitats, as research on

387 springs could be used to distinguish possible phenotypic plasticity from local adaptations. Third,

388 given the view of springs as useful laboratories, devoting space and infrastructure at the entrance

389 to subterranean environments could provide important experimental opportunities.

Conclusions

391 Even if the transitional and ecotonal role of springs is known and studied since several decades,

392 and the term Groundwater Dependent Ecosystems applied to springs (Fattorini et al. 2020; Rohde

393 et al. 2017) allows to study the connected network of surface and subterranean ecosystems

394 following the 'holistic' approach suggested by (Linke et al. 2019), these concepts are rarely

395 translated in ecological and evolutionary studies dealing with groundwater animals. The results of

396 our systematic review broadly suggest that springs and other boundaries with surface environments

397 should be considered and investigated as part of subterranean habitats and of the biology of at least

398 some stygobionts. Studies of groundwater environments and stygobiont biology that do not

399 consider springs may furnish only a limited perspective on subterranean environments, because

400 they could exclude *a priori* a potential source of selective pressures for groundwater-dwelling

401 animals. The study of groundwater-adapted organisms in subterranean aquifers has the potential

402 to reveal new insights in several scientific fields (Pipan & Culver 2013; Reboleira et al. 2011), but

403 the study of the boundaries of groundwater environments, such as springs, is not only equally

404 important, but even necessary to understand the zoology ecology and evolution of groundwater

405 fauna.

406

407

408

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625

Table 1

Table 1. Search terms and inclusion/exclusion criteria used to describe published evidence of stygobionts in springs and to answer specific questions.

1 **Table 1**

Categories	Restrictions applied
Number of species mentioned	If clearly stated for all taxa considered in the study
Stygofauna found in springs	If clearly stated that the species found in springs are stygobites
Number of stygofaunal species in springs	If the number of stygobite species found in springs is clearly stated for all taxa considered in the study
Surface fauna found underground	If clearly stated that the species found underground are of epigean origin
Number of surface species found underground	If the number of epigean species found underground is clearly stated for all taxa considered in the study
Species found both in caves and springs	If clearly stated that the species found both in caves and springs are epigean or stygobites
Number of species in both (caves and springs)	If the number of stygobites or epigean species found in both is clearly stated for all taxa
Ecology	Yes/no, depending on whether the paper provides original ecological information (habitat of occurrence, environmental drivers etc..)
Taxonomy	Yes/no, depending on whether the paper provides original taxonomic data
Behavior	Yes/no, depending on whether the paper tests/reports original behavioral information/observations
Conservation	Yes/no, depending on whether the paper explores original conservation/restoration problems or actions
Faunal assessment	Yes/no, depending on whether the paper is mainly devoted to assess faunal composition of spring/groundwater habitat

2 Table 1. Search terms and inclusion/exclusion criteria used to describe published evidence of
3 stygobionts in springs and to answer specific questions.

Table 2

Table 2. Number of papers reporting observations of stygofauna in springs, of surface fauna in groundwaters, and of the same taxa in both environments. Papers are divided based on the key words used for the systematic review: GF, groundwater fauna; SFF, spring freshwater fauna.

1   **Table 2**

		Total	GF	SFF
Stygofauna in springs	YES	57	49	8
	NO	235	195	40
	Information missing	50	31	19
Surface fauna underground	YES	37	34	3
	NO	278	220	58
	Information missing	27	21	6
Same taxa both in springs and groundwaters	YES	33	30	3
	NO	266	214	52
	Information missing	43	31	12

4   Table 2. Number of papers reporting observations of stygofauna in springs, of surface fauna in
5   groundwaters, and of the same taxa in both environments. Papers are divided based on the key
6   words used for the systematic review: GF, groundwater fauna; SFF, spring freshwater fauna.

Table 3

Table 3. The reported occurrence of stygofauna in springs, of surface fauna in groundwaters, and of the same taxa in both habitats shown as a function of study discipline. Relationships were assessed using generalized linear models (GLMs) followed by a likelihood ratio test. Significant relationships are reported in bold.

1 **Table 3**

	Research discipline	Estimate	SE	χ^2	P
	Ecology	0.27	0.34	0.64	0.42
	Taxonomy	0.75	0.39	3.67	0.06
Stygofauna in springs	Faunal assessment	0.24	0.35	0.48	0.49
	Conservation	0.67	0.53	1.50	0.22
	Behavior	-13.84	834.76	0.98	0.32
	Ecology	0.02	0.41	0.00	0.96
	Taxonomy	0.27	0.50	0.29	0.59
Surface fauna underground	Faunal assessment	1.09	0.45	6.62	**0.01**
	Conservation	0.51	0.59	0.68	0.41
	Behavior	1.30	1.20	0.94	0.33
	Ecology	0.09	0.43	0.04	0.84
	Taxonomy	0.91	0.45	3.96	**0.04**
Same taxa both in springs and groundwaters	Faunal assessment	0.06	0.44	0.02	0.89
	Conservation	0.77	0.60	1.45	0.23
	Behavior	-13.15	839.90	0.51	0.48

2 Table 3. The reported occurrence of stygofauna in springs, of surface fauna in groundwaters, and
3 of the same taxa in both habitats shown as a function of study discipline. Relationships were
4 assessed using generalized linear models (GLMs) followed by a likelihood ratio test. Significant
5 relationships are reported in bold.

Figure 1

Figure 1

Figure 1. Diagram of a spring showing differences between surface and groundwater habitats during day (A) and night (B). During night the border between surface waters and groundwaters softens and stygobionts can move outside interacting with surface invertebrates and vertebrates including predators (here represented by a diurnal predator fish and a nocturnal predator salamander larva). White silhouettes represent stygobionts, black silhouettes represent potential predators (fish and salamanders), and brown silhouettes surface aquatic invertebrates. Drawing is modified from Andrea Melotto and Benedetta Barzaghi (unpublished).
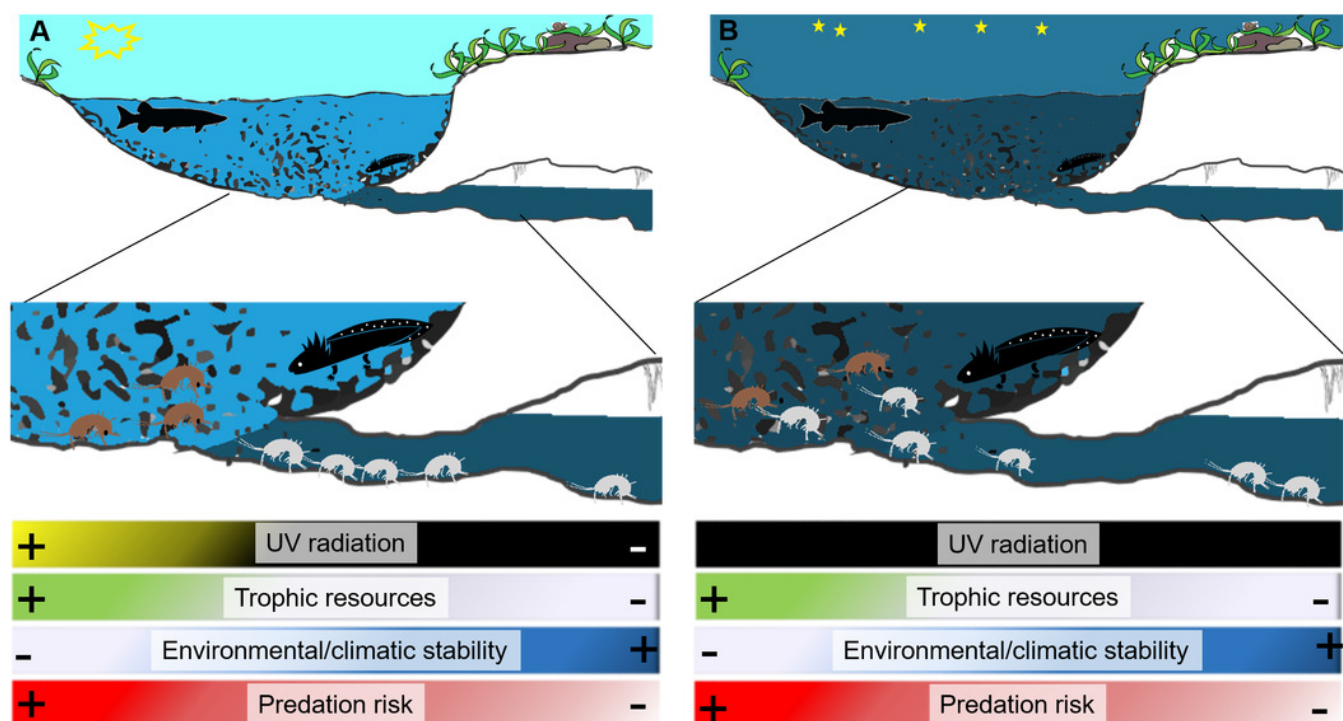
Figure 2

Figure 2

Figure 2 PRISMA 2020 flow diagram for the systematic reviews which included search of Web of Science database only.