Animal Conservation

Loss of heat acclimation capacity could leave subterranean specialists highly sensitive to climate change

SCHOLARONE™
Manuscripts

Loss of heat acclimation capacity could leave subterranean specialists highly sensitive to climate change

Short title: Acclimation capacity in subterranean specialists

Susana Pallarés[1,2], Raquel Colado[2,3], María Botella-Cruz[3], Aitor Montes[4,5], Pau Balart-García[6], David T. Bilton[1,7], Andrés Millán[3], Ignacio Ribera[6†], David Sánchez-Fernández[2,3]

[1]Marine Biology and Ecology Research Centre, School of Biological and Marine Sciences, University of Plymouth, Plymouth, UK

[2]Instituto de Ciencias Ambientales, Universidad de Castilla-La Mancha, Toledo, Spain

[3]Departamento de Ecología e Hidrología, Universidad de Murcia, Murcia, Spain

[4]Basque Society for Biology Conservation, Guipúzcoa, Spain

[5]Cuevas de Oñati-Arrikrutz, Guipúzcoa, Spain

[6]Institut de Biologia Evolutiva (CSIC-Universitat Pompeu Fabra), Barcelona, Spain

[7]Department of Zoology, University of Johannesburg, Johannesburg, South Africa

Corresponding author:

Susana Pallarés (susana.pallares@plymouth.ac.uk)

Marine Biology and Ecology Research Centre, School of Biological and Marine Sciences, University of Plymouth, Drake Circus, Plymouth, PL4 8AA, UK

Tel: +44 1752 584608

† Deceased April 15, 2020

1

Abstract

Physiological traits are key in determining the vulnerability of narrow range, highly specialised animals to climate change. It is generally predicted that species from more stable environments possess lower thermal tolerance breadths and thermal plasticity than those from more variable habitats – the so-called 'climatic variability hypothesis'. However, evolutionary trade-offs between thermal breadth and its plasticity are also seen in some taxa, and the evolution of thermal physiology remains poorly understood. Subterranean environments are excellent systems for exploring these issues, being characterized by stable climatic conditions, with environmental variability increasing predictably from deep to shallow habitats. Acclimation capacity will be fundamental in determining the sensitivity of subterranean species to climate change, since they have poor dispersal capacity and limited possibility to exploit thermally different microhabitats in the uniform cave environment. We assessed critical thermal maximum (CT_{max}) and short-term heat acclimation capacity in three related beetles (Leiodidae: Leptodirini) with differing degrees of specialisation to the subterranean environment (deep, shallow and facultatively subterranean, respectively) and therefore exposed to contrasting thermal variability in nature. Only the facultative subterranean species showed any acclimatory capacity, also having the highest CT_{max} across the taxa studied. However, this species might experience the highest thermal stress in its habitat under climate change. The studied subterranean specialists will be poorly able to cope physiologically with temperature increase, but in contrast exposed to lower magnitude and rate of warming. Our results fit the climatic variability hypothesis, suggesting that adaptation to cave conditions has selected against the retention of acclimation mechanisms. We show that the pathways that determine vulnerability of subterranean species to climate change depend on their degree of specialisation to deep subterranean environments. This information, combined with evaluation of exposure to climatic changes at their present locations, is fundamental in identifying species or populations at greatest risk.

1

26   **Keywords:** critical thermal maximum, physiological plasticity, thermal tolerance, climate

27   variability hypothesis, subterranean environment, conservation, cave beetles, climate change

2

28 **Introduction**

29 It is broadly recognised that physiological data may contribute directly to conservation in the

30 face of climate change and advance the field of conservation physiology (Wikelski & Cooke,

31 2006; Cooke & O'Connor, 2010; Cooke *et al*., 2013; Evans, 2015). Indeed, accurate assessments

32 of species vulnerability to climate change require integrated measurements of its different

33 determinants, including exposure (the magnitude and change of climatic change) and factors of

34 organisms' sensitivity at the individual level, such as the physiological tolerance to

35 environmental change (Williams *et al*., 2008; Foden *et al*., 2019). In this context, the acclimation

36 capacity of organisms determines their ability to track changing environmental conditions and is

37 of fundamental importance, especially for species that are unable to respond to climate change

38 by other means (e.g. range shifts, behavioural adjustments or evolutionary adaption) (Stillman,

39 2003; Seebacher, White, & Franklin, 2015). Thermal acclimation occurs when individuals adjust

40 their physiology to compensate for the effects of temperature variation, and may ultimately

41 extend critical thermal limits for performance and survival (e.g. Da Silva, Riginos & Wilson,

42 2019).

43 Despite multiple hypotheses proposed to understand how thermal plasticity varies across taxa

44 (see Rohr *et al*. (2018) for a recent review), empirical studies suggest that acclimation responses

45 are highly context-dependent. According to the climatic variability hypothesis (CVH) (Stevens,

46 1989), species from more stable environments are predicted to have lower thermal tolerance (i.e.

47 narrower tolerance breadths and less acclimation capacity) than those from more variable

48 habitats (e.g. Feder, 1978; Tomanek, 2008; Magozzi & Calosi, 2015; Shah, Funk, & Ghalambor,

49 2017; Markle & Kozak, 2018), but such a pattern has not always been supported (Seebacher *et*

50 *al*., 2015). Furthermore, evolutionary trade-offs may constrain the evolution of the different

51 components of thermal tolerance, so that species that have evolved the greatest thermal limits

1

52 (particularly upper ones) may have done so at the expense of the plasticity of such limits and

53 therefore show limited acclimation capacity (e.g. Stillman, 2003).

54 Testing between such alternatives is challenging, given the multiple intrinsic and extrinsic

55 factors that affect the range of temperature actually experienced by organisms. For the same

56 reason, teasing apart the relative importance of the different drivers of vulnerability to climate

57 change can be challenging. Climatically stable and isolated systems are excellent models to

58 minimize confounding effects and focus on physiology in a climate change context. This is the

59 case with subterranean habitats, where environmental conditions are more stable and

60 homogeneous than at the surface, limiting the possibility of behavioral adjustments of their

61 inhabitants through differential microhabitat use (Sánchez-Fernández et al., 2018). The dispersal

62 potential is also extremely reduced for most subterranean specialists (Juan & Emerson, 2010;

63 Rizzo et al., 2017). Moreover, low genetic variability (Juan et al., 2010), low reproductive rates

64 and long life cycles (Voituron et al., 2010) may constrain rapid evolutionary change in such taxa

65 (Sánchez-Fernández et al., 2016; Mammola et al., 2019b). Therefore, as with species in many

66 other isolated ecosystems (e.g. mountain summits or islands), they rely strongly on physiological

67 adjustments to cope with environmental changes, even though the magnitude and rate of such

68 changes in subterranean habitats is moderate compared to the surface (Domínguez-Villar et al.,

69 2015; Pipan et al., 2018). Deep subterranean habitats typically experience very stable climates

70 (but see Trontelj, Borko, & Delić, 2019) and within caves, such stability decreases markedly

71 from inner to outer sections (Cigna, 2002; Badino, 2010; Pipan et al., 2010), presenting an ideal

72 setting in which to explore the relationship between thermal tolerance and climatic variability

73 and test macrophysiological hypotheses (Gaston et al., 2009; Sánchez-Fernández et al., 2018;

74 Mammola et al., 2019b).

75 The rarity of many subterranean taxa (Sánchez-Fernández et al., 2018; Trontelj et al., 2019) and

76 difficulties of access for sampling (Raschmanová et al., 2018; Castaño-Sánchez, Hose, &

2

77 Reboleira, 2020; Mammola, *et al.*, 2019a), have limited research on subterranean species to date.

78 In terms of thermal tolerance, most of the data on subterranean organisms come from studies

79 conducted on a handful of species and have generally shown they have a high thermal

80 sensitivity: upper lethal limits are usually not higher than 23ºC (e.g. Issartel *et al.*, 2005; Rizzo *et*

81 *al.*, 2015; Pallarés *et al.*, 2019) and studies based on molecular biomarkers indicate that thermal

82 stress onsets at lower temperatures (Mermillod-Blondin *et al.*, 2013; Pallarés *et al.*, 2020).

83 Several studies have also suggested that the degree of specialisation to the subterranean

84 environment is negatively correlated with thermal tolerance breadth (Lencioni, Bernabò &

85 Latella, 2010; Bernabò *et al.*, 2011; Novak *et al.*, 2014; Raschmanová *et al.*, 2018; Mammola *et*

86 *al.*, 2019c), broadly supporting the CVH. Whether physiological plasticity is reduced in the

87 process of specialisation to subterranean life and how it could affect species sensitivity to climate

88 change remains an open question, however, since most studies have ignored acclimation capacity

89 (but see Rizzo *et al.*, 2015; Pallarés *et al.*, 2019, 2020). In addition, such studies have often

90 involved disparate, unrelated species, sometimes from localities with very different climates,

91 making meaningful comparisons difficult. Efforts to increase our knowledge on the thermal

92 tolerance of subterranean fauna are needed, as they represent an important and typically endemic

93 component of the global animal biodiversity, but still largely neglected in conservation

94 programs.

95 In Leptodirini cave beetles (Coleoptera, Leiodidae), one of the most extreme modifications

96 associated with underground colonisation is a reduction in the number of larval instars in the life

97 cycle, likely as an adaptation to a resource-poor environment (Cieslak, Fresneda, & Ribera,

98 2014a, b). Species that can live in deep forest litter or the upper layers of fractured soil (MSS,

99 *Milieu Souterrain Superficiel* (Juberthie, Delay, & Bouillon, 1980a,b, 1981; Mammola *et al.*,

100 2016)), i.e., not necessarily confined in caves, typically have the standard life cycle of

101 Coleoptera, with three larval instars (Minelli & Fusco, 2013). Deep or shallow subterranean

3

102   specialists (true troglobionts *sensu* Sket (2008)) may show a reduction to two or -in the most

103   extreme specialisation- one larval instar. Therefore, the number of larval instars in these species,

104   clearly associated with the degree of subterranean specialisation, can be used as an indirect proxy

105   for the degree of thermal stability species experienced in their evolutionary history. Here we

106   assess critical thermal maximum (CT_{max}) and short-term heat acclimation capacity in three

107   related Leptodirini taxa from areas with similar climatic histories but with life cycles of one, two

108   and three larval instars respectively, reflecting different degrees of specialisation to the

109   subterranean environment and hence exposed to contrasting thermal variability. We explore

110   whether support for the CVH in subterranean taxa (Latella, Bernabò & Lencioni, 2008; Lencioni

111   *et al.*, 2010; Novak *et al.*, 2014; Raschmanová *et al.*, 2018; Mammola *et al.*, 2019c) also holds

112   for thermal plasticity. Under the CVH, both CT_{max} and acclimation capacity are expected to

113   decrease with increasing subterranean specialisation. However, if trade-offs between thermal

114   limits and plasticity have shaped the evolution of thermal tolerance in these taxa, such a

115   relationship with subterranean specialisation might not be so evident for acclimation capacity

116   (i.e., species living in the more variable habitats could tolerate higher temperatures but show

117   little thermal plasticity). Extending these general hypotheses to the subterranean environment can

118   provide important insights into the evolution of thermal physiology associated with the process

119   of underground specialisation, as well as relevant information for the conservation of specialist

120   animal taxa with limited dispersal capabilities.

121   **Material and methods**

122   *Study species and collection*

123   The study species belong to monophyletic lineages of the beetle tribe Leptodirini; the Pyrenean

124   (*Speonomites crypticola* and *Bathysciola rugosa*) and Cantabrian clades (*Speonomidius crotchi*)

125   (Salgado, Blas, & Fresneda, 2008; Ribera *et al.*, 2010). Rearing experiments revealed that *S.*

4

126   *crotchi* has a life cycle of two larval instars (Jeannel, 1911; Glaçon, 1955), whilst *S. crypticola*

127   and *B. rugosa* were inferred to have one and three larval instars, respectively, based on robust

128   ancestral state reconstructions (Cieslak *et al.*, 2014b). Two of the species are obligate cave

129   inhabitants only found in deep subterranean habitats (*S. crypticola*) or in both deep and shallow

130   subterranean environments (*S. crotchi*), whilst *B. rugosa* is a facultative subterranean species,

131   most commonly found in deep forest litter, always in dark conditions (Salgado *et al.*, 2008).

132   Live adults were collected in different sites within the same geographical region (Northern

133   Spain), with broadly similar mean annual surface temperatures (Table 1), which are known to

134   closely approximate the constant temperatures recorded inside caves (Moore & Sullivan, 1964;

135   Smithson, 1991; Badino, 2004, 2010). Therefore, all species live under similar average climatic

136   conditions but occupy habitats with different daily and seasonal climatic variability. The two

137   subterranean species can be assumed to be exposed to an annual variation of a few degrees ($\leq 4º$

138   C) around mean annual surface temperatures, which is the typical thermal range in caves

139   (Badino, 2010; Mammola & Isaia, 2016). The facultative subterranean species can be assumed to

140   be exposed to a maximum annual variation between $8 - 24.4º$ C (minimum temperature of the

141   coldest month and maximum temperature of the warmest month at the surface in its locality,

142   respectively, obtained from Worldclim v. 1.4 database (http://www.worldclim.org; see Table 1).

143   Projected temperatures for 2070 under the Representative Concentration Pathway (RCP) 4.5 are

144   also shown in Table 1.

145   Specimens of *S. crypticola* and *S. crotchi* were collected by hand in the caves indicated in Table

146   1, whilst specimens of *B. rugosa* were collected in forest litter near the entrances of Cave Orobe.

147   The specimens were transported to the laboratory in a portable fridge with substratum from the

148   cave and moss to retain humidity (ca. 90% RH). In the laboratory, they were maintained for two

149   days prior to experiments in closed plastic containers (10 x 15 x 10 cm) with a plaster layer (1

150   cm), in controlled-temperature incubators (Radiber ERF-360, Radiber S.A, Barcelona, Spain) at

5

151 the approximate mean temperature of their habitats (*S. crypticola* 11ºC; *S. crotchi* 9ºC; *B. rugosa*

152 12ºC), i.e. control temperatures hereafter. Maintenance of high humidity (> 90% RH) throughout

153 the experiments was ensured by adding wetted tissue paper to experimental containers daily and

154 placing trays with water inside the incubators. Temperature and humidity inside the containers

155 were monitored with HOBO MX2301 dataloggers and remained quite constant, with very small

156 variations (±0.5ºC and ±10% RH). Food was provided *ad libitum* with freshly frozen *Drosophila*

157 *melanogaster*.

158   *Upper thermal limits and acclimation capacity*

159 Specimens were acclimated at either a control temperature (see above) or at 20ºC for 10 days (N

160 = 10-13 individuals per treatment), with all other conditions (humidity, food etc.) as described

161 before. These acclimation temperatures were chosen since all subterranean leiodids studied to

162 date can survive relatively long-term exposure (>7 days) at 20ºC, but this is also close to their

163 upper lethal limits (Rizzo *et al*., 2015; Pallarés *et al*., 2019, 2020), and it is well established that

164 insects typically undergo acclimation in response to stressful sublethal temperatures (e.g.

165 Lachenicht *et al*., 2010; Nyamukondiwa & Terblanche, 2010; Terblanche *et al*., 2011; Sgrò,

166 Terblanche, & Hoffmann, 2016). After acclimation, we estimated heat coma temperature (HCT)

167 for each individual. This was defined as the temperature at paralysis prior to death, preceded by

168 spasmodic movements of legs and antennae, and is a typical response used to assess critical

169 thermal maximum (CT_{max}) in insects (see Vannier, 1994; Lutterschmidt & Hutchison, 1997;

170 Chown & Nicolson, 2004). HCT was measured using a dynamic, ramping method

171 (Lutterschmidt & Hutchison, 1997; Terblanche *et al*., 2007), with a heating rate of 1ºC min^{-1} and

172 infrared thermography. CT_{max} measured with such a fast ramping rate tend to be overestimated

173 (Terblanche *et al*., 2007) and cannot be used to directly extrapolate actual thermal limits, but this

174 method provides a robust comparative estimate of the relative thermal tolerance amongst related

175 species and is the most commonly used in thermal tolerance assays on ectotherms (Bennett *et al*.,

6

176 2018). Furthermore, infrared thermography has proven to be a highly sensitive technique to

177 measure CT_{max} in insects (Botella-Cruz *et al*., 2016; Carbonell *et al*., 2016; Gallego *et al*., 2016;

178 Gallego, Verdú, & Lobo, 2018). Specimens were dried on blotting paper and glued dorsally on a

179 ceramic plate using nontoxic glue (ErichKrause) to prevent escape during the trial. The plate was

180 then placed in a programmable BINDER MK53 incubator (BINDER GmbH, Tuttlingen,

181 Germany) in which the temperature was increased 1ºC min^{-1}, starting at the corresponding

182 acclimation temperature. The incubator was coupled with an infrared thermographic camera

183 (FLIR SC305) to record body temperature of the specimens, from which HCT were obtained.

184 High quality images were also recorded with a video camera (Sony DCR-DVD110E, Sony Co.,

185 Tokyo, Japan) to determine the moment of paralysis (cessation of movement of legs and

186 antennae) of each individual. Thermal images were analysed with the software ThermaCAM

187 Researcher Professional 2.10 (FLIR Advanced Thermal Solutions; ATS; Croissy-Beaubourg,

188 France).

189 *Statistical analyses*

190 We performed a two-way ANOVA with HCT as the response variable and species, acclimation

191 temperature (a factor with two levels: control temperature (see above) or 20ºC) and their

192 interaction as predictors, to compare HCT and short-term acclimation capacity across species

193 and assess whether these traits are related to the degree of subterranean specialisation. Pairwise

194 comparisons were made with Bonferroni-adjusted post-hoc tests. Significance level was set at

195 0.05. Data conformed to a normal distribution so no transformation was required. Normality and

196 homoscedasticity assumptions of model residuals were also validated by graphical inspection

197 (Zuur *et al*., 2009). All statistical analyses were conducted in R v.3.6.1 (R Core Team, 2019)

198 using default packages and *phia* (De Rosario-Martinez, 2015).

199 **Results**

7

200   Heat coma temperatures (HCTs) ranged from 34.6 – 38ºC across the studied species and

201   acclimation treatments (Figure 1). HCTs differed significantly amongst species (ANOVA $F_{2, 52}$ =

202   21.963, P < 0.001), being lower in the one-larval instar, deep subterranean species (*S. crypticola*)

203   than in the other two, less specialised taxa investigated (all Bonferroni corrected Ps < 0.001,

204   Table 2). There was also a significant effect of acclimation temperature on HCT (ANOVA $F_{1, 52}$

205   = 18.357, P < 0.001), but species differed significantly in their response to acclimation (ANOVA

206   species x temperature interaction $F_{2, 52}$ = 3.564, P = 0.035).

207   The deep subterranean specialist, *S. crypticola*, had a significantly lower HCT than the two-

208   instar, shallow subterranean *S. crotchi* when acclimated at control temperatures, and lower than

209   either of the other two species following acclimation at 20ºC (all Bonferroni corrected Ps <

210   0.001, Table 2; Fig.1). Only the species with the least specialisation to subterranean

211   environments, *B. rugosa* (three larval instars), showed acclimation capacity. In this species, HCT

212   significantly increased after exposure at 20ºC (Bonferroni corrected P < 0.001, Table 2; Fig. 1),

213   with a difference of 1.2ºC between the mean HCT of the control and 20ºC treatment. The more

214   specialised subterranean taxa lacked acclimation ability because no differences were found in

215   HCT between acclimation treatments (Bonferroni corrected Ps > 0.05, Table 2; Fig. 1).

Discussion

217   Among the three studied subterranean species, the most specialised to the subterranean

218   environment and hence living under the more stable climatic conditions, *S. crypticola*, showed

219   the lowest HCT values. Such finding in relation to the upper thermal limit fits the general

220   predictions of the CVH, as seen in other subterranean groups for both lower (Latella *et al*., 2008;

221   Lencioni *et al*., 2010; Novak *et al*., 2014) and upper thermal limits (Raschmanová *et al*., 2018;

222   Mammola *et al*., 2019c). The main novelty of our study is that the CVH is tested by comparing

223   not only thermal limits, but also thermal plasticity, something largely ignored for subterranean

8

224 taxa to date. Again, in agreement with the CVH, the more specialised subterranean taxa (*S.*

225 *crypticola* and *S. crotchi,* both obligate subterranean) lacked acclimation ability at the

226 acclimation temperatures and heating rates tested here; whilst *B. rugosa*, the facultative

227 subterranean species exposed to more fluctuating daily and seasonal temperatures, significantly

228 increased HCT after acclimation at the higher temperature. As well as a positive acclimation

229 response, *B. rugosa* showed the highest HCT values across the three species after acclimation at

230 20ºC. Therefore, there is not apparent evolutionary trade-off between CT_{max} and its short-term

231 plasticity in these species, as reported in a clade of aquatic beetles (Calosi, Bilton & Spicer,

232 2008), but in contrast to patterns seen in other taxa such as intertidal porcellanid crabs (Stillman,

233 2003). As suggested by Calosi *et al*. (2008), the mechanisms and constraints underlying thermal

234 limits and acclimation capacity appear to be taxon and context specific.

235 A lack of thermal acclimation capacity has also been reported for other subterranean groups,

236 such as groundwater crustaceans (Di Lorenzo & Galassi, 2017), and previous work on

237 Leptodirini suggests that the physiological mechanisms to cope with heat stress are rather

238 inefficient in specialised subterranean species compared with their less specialised relatives. For

239 example, Bernabò *et al.* (2011) showed that the intensity of the heat shock response was

240 negatively correlated with the degree of adaptation to the cave environment. Pallarés *et al*.

241 (2020) found that an obligate subterranean species of the clade studied here failed to acclimate

242 when exposed to a fixed stressful temperature. Such inability to acclimate was coupled with

243 oxidative stress and shifts in the activity of key enzymes at sublethal temperatures. The

244 physiological mechanisms underlying thermal acclimation capacity in ectotherms are

245 energetically costly processes (Krebs & Loeschcke, 1994; Monaghan, Metcalfe, & Torres, 2009;

246 Tomanek, 2010) that could have been selected against during the evolution of specialisation to

247 deep subterranean habitats. In these environments, with limited resources and highly stable

248 climatic conditions, both temporally and spatially (Badino, 2010; Howarth & Moldovan, 2018),

9

249   traits that do not affect fitness could be loss or reduced either by neutral evolution or indirect

250   natural selection favouring energetic economy (Culver & White, 2005, Ribera *et al*., 2018).

251   Extreme modifications of thermal physiology have been found in organisms inhabiting other

252   highly stable habitats, such as the absence of a heat shock response in Antarctic notothenioid fish

253   (Somero, 2005). The greatest modifications of thermal physiology in subterranean taxa, resulting

254   in lower thermal limits and poor acclimation capacity, may be expected in those lineages that

255   colonised subterranean habitats longer ago during their evolutionary history. This is the case of

256   Western Mediterranean Leptodirini, which were apparently already adapted to subterranean life

257   by the Early-Mid Oligocene, some 34-28 MYA (Ribera *et al*., 2010; Cieslak, *et al*., 2014b).

258   Accordingly, other specialised deep subterranean species of these beetles are also likely to lack

259   acclimation capacity and have similar CT_{max} than the one-larval instar species studied here (*S.*

260   *crypticola*). In contrast, deep subterranean species in lineages that colonised subterranean

261   habitats more recently may retain greater heat tolerance and acclimation capacity from their less

262   specialised or surface-dwelling ancestors, as previously suggested (Pallarés *et al*., 2019). Further

263   experimental data on thermal limits and acclimation for several lineages that have independently

264   colonised the subterranean environment would be invaluable in testing this evolutionary

265   hypothesis. Unfortunately, the difficulties in accessing subterranean habitats, of collecting

266   sufficient number of specimens for experimental studies and maintaining and rearing

267   subterranean organisms in the laboratory (Raschmanová *et al*., 2018; Castaño-Sánchez *et al*.,

268   2019; Mammola *et al*., 2019a) impose serious limitations when conducting comparative studies

269   on cave-dwelling species. Therefore, despite its relatively modest taxonomic coverage, our study

270   represents an important milestone in understanding physiological evolution of subterranean

271   invertebrates.

272   Overall vulnerability to climate change in subterranean species will depend mainly on their

273   thermal sensitivity and the magnitude and rate of climate change in their habitats, as range shifts

10

274 through dispersal will be largely limited. Both aspects (sensitivity and exposure to climate

275 change) will in turn be determined by the degree of subterranean specialisation, but in opposite

276 directions. Here we show that subterranean species have higher thermal sensitivity than surface

277 ones, something that has been generally assumed but rarely tested. The HCT of the three species

278 studied are markedly lower than the average value of CT_{max} (45.5 ± 1.0ºC, N = 40) recorded

279 experimentally in a range of arthropods at the same heating rate (Bennett *et al.*, 2018). We also

280 show that thermal sensitivity increases with subterranean specialisation. In deep subterranean

281 Leptodirini, experiments under relatively long-term exposure, which may better represent natural

282 conditions, have shown that heat injury at the biochemical level occurs at 20ºC (Pallarés *et al.*,

283 2020) and survival is not possible above 23ºC (Rizzo *et al.*, 2015). Such upper lethal limits seem

284 to be highly conservative across deep subterranean specialist species of this clade, irrespective of

285 the current and historical temperature of their habitats (Rizzo *et al.*, 2015). Likewise, the lack of

286 acclimation observed here in *S. crypticola* and *S. crotchi* appears to be mirrored in related

287 species with similar specificity to subterranean habitats (as discussed above). In contrast,

288 changes in the underground climate will be delayed compared to those experienced at the surface

289 (Fejér & Moldovan, 2013; Domínguez-Villar *et al.*, 2015) and will occur primarily in the cave

290 sections closest to the surface and in superficial subterranean habitats (Culver & Pipan, 2014),

291 such as small cavities in the uppermost karst layers, deep soil and litter strata, surface cracks and

292 fissures (Badino, 2004; Mammola, Goodacre, & Isaia, 2018). In a proximate climate change

293 scenario, the projected annual mean surface temperature apparently will not reach

294 physiologically stressful levels for the two obligate subterranean species studied here (Table 1).

295 However, this cannot be generalized, as some coastal or southern populations of specialist cave

296 invertebrates in the Mediterranean Basin are currently living at temperatures close to their lethal

297 limits (e.g. Sánchez-Fernández *et al.*, 2016; Pallarés *et al.*, 2019) and the same could occur in the

298 tropics (Zeh *et al.*, 2012). As the possibilities for behavioural thermoregulation through

11

299   microhabitat selection are extremely reduced in the deep subterranean environment, and

300   considering the limited capacity to physiologically adjust to rising temperature observed here,

301   these habitats may become physiological and evolutionary traps for some of their obligate

302   inhabitants (Mammola *et al*., 2019b). Compared to species confined to caves, those occupying

303   forest litter or the MSS, like *B. rugosa,* may cope better with warming, because they are able to

304   increase thermal limits via acclimation and exploit a wider range of microhabitats. In this case,

305   the magnitude and rate of warming could be the key determinant of their vulnerability to climate

306   change, as these species are more exposed to the thermal fluctuations of the surface, where

307   maximum temperatures could reach physiologically stressful values in a proximate future (e.g.

308   28.5ºC in the localities where *B. rugosa* was collected).

309   In summary, our results stress the need to account for physiological constraints such as thermal

310   sensitivity and acclimation capacity if we are to accurately forecast the impacts of global change

311   on specialised invertebrates with low dispersal ability. This information, combined with

312   evaluation of exposure to climatic changes in occupied locations, can greatly assist in identifying

313   species or populations at higher risk. We show that in the case of the subterranean environment,

314   the relative importance of factors determining overall vulnerability may differ depending on the

315   degree of habitat specialisation. As many authors have already urged (e.g. Sánchez-Fernández *et*

316   *al*., 2018; Mammola *et al*., 2019a,b; Castaño-Sánchez *et al.,* 2020), it is necessary to increase

317   research, monitoring and conservation efforts on this unknown, fragile and valuable component

318   of global biodiversity.

Acknowledgments

12

327   **References**

328   Badino, G. (2004). Cave temperatures and global climatic change. *Int. J. Speleol.* **33**, 103–114.

329   Badino, G. (2010). Underground meteorology. What's the weather underground? *Acta Carsol.*

330   **39**, 427-448.

331   Bennett, J.M., Calosi, P., Clusella-Trullas, S., Martínez, B., Sunday, J., Algar, A., Araújo, M.B.,

332   Hawkins, B.A., Keith, S., Kühn, I., Rahbek, C., Rodríguez, L., Singer, A., Villalobos, F.,

333   Angel Olalla-Tárraga, M. & Morales-Castilla, I. (2018). GlobTherm, a global database on

334   thermal tolerances for aquatic and terrestrial organisms. *Sci. Data* **5**, 180022.

335   Bernabò, P., Latella, L., Jousson, O., & Lencioni, V. (2011). Cold stenothermal cave-dwelling

336   beetles do have an HSP70 heat shock response. *J. Therm. Biol.* **36**, 206–208.

337   Botella-Cruz, M., Carbonell, J. A., Pallarés, S., Millán, A., & Velasco, J. (2016). Plasticity of

338   thermal limits in the aquatic saline beetle *Enochrus politus* (Küster 1849) (Coleoptera:

339   Hydrophilidae) under changing environmental conditions. *Limnetica* **35**, 131–142.

340   Calosi, P., Bilton, D. T., & Spicer, J. I. (2008). Thermal tolerance, acclimatory capacity and

341   vulnerability to global climate change. *Biol. Lett.* **4**, 99–102.

342   Carbonell, J. A., Millán, A., Green, A. J., Céspedes, V., Coccia, C., & Velasco, J. (2016). What

343   traits underpin the successful establishment and spread of the invasive water bug

344   *Trichocorixa verticalis verticalis*? *Hydrobiologia* **768**, 273–286. (doi:10.1007/s10750-015-

345   2556-y)

13

346   Castaño-Sánchez, A., Hose, G. C., & Reboleira, A. S. P. S. (2020). Ecotoxicological effects of

347   anthropogenic stressors in subterranean organisms: A review. *Chemosphere* **244**, 125422.

348   Chown, S. L., & Nicolson, S. W. (2004). *Insect Physiological Ecology: Mechanisms and*

349   *Patterns*. Oxford: Oxford University Press.

350   Cieslak, A., Fresneda, J., & Ribera, I. (2014a). Developmental constraints in cave beetles. *Biol.*

351   *Lett.* **10**, 20140712.

352   Cieslak, A., Fresneda, J., & Ribera, I. (2014b). Life-history specialization was not an

353   evolutionary dead-end in Pyrenean cave beetles. *Proc. R. Soc. B* **281**, 20132978.

354   Cigna, A. A. (2002). Modern trend(s) in cave monitoring. *Acta Carsol.* **31**, 35–54.

355   Cooke, S. J., & O'Connor, C. M. (2010). Making conservation physiology relevant to policy

356   makers and conservation practitioners. *Cons. Lett.* **3**, 159-166.

357   Cooke, S. J., Sack, L., Franklin, C. E., Farrell, A. P., Beardall, J., Wikelski, M., & Chown, S. L.

358   (2013). What is conservation physiology? Perspectives on an increasingly integrated and

359   essential science. *Conserv. Physiol.* **1**, cot001.

360   Covington, M.D., & Perne, M. (2015). Consider a cylindrical cave: A physicist's view of cave

361   and karst science. *Acta Carsol*. **44**, 363–380.

362   Culver, D.C. & Pipan, T. (2014). *Shallow Subterranean Habitats. Ecology, Evolution, and*

363   *Conservation*. Oxford: Oxford University Press.

364   Culver, D.C., & White, W.B. (2005). *Encyclopedia of caves*. Amsterdam: Elsevier.

365   Da Silva, C.R.B., Riginos, C., & Wilson, R.S. (2019). An intertidal fish shows thermal

366   acclimation despite living in a rapidly fluctuating environment. *J. Comp. Physiol. B* **189**,

367   385-398.

368   De Rosario-Martinez, H. (2015). phia: Post-Hoc Interaction Analysis. R package version 0.2-1.

14

369   https://CRAN.R-project.org/package=phia

370 Di Lorenzo, T., & Galassi, D.M.P. (2017). Effect of temperature rising on the stygobitic

371   crustacean species *Diacyclops belgicus*: Does global warming affect groundwater

372   populations? *Water* **9**, 951.

373 Domínguez-Villar, D., Lojen, S., Krklec, K., Baker, A., & Fairchild, I. J. (2015). Is global

374   warming affecting cave temperatures? Experimental and model data from a paradigmatic

375   case study. *Clim. Dyn.* **45**, 569–581.

376 Evans, T. G., Diamond, S. E., & Kelly, M. W. (2015). Mechanistic species distribution modelling

377   as a link between physiology and conservation. *Conserv. Physiol.* **3**, cov056.

378 Feder, M. E. (1978). Environmental variability and thermal acclimation in neotropical and

379   temperate zone salamanders. *Physiol. Zool*. **51**, 7–16.

380 Fejér, A., & Moldovan, O. (2013). Population size and dispersal patterns for a *Drimeotus*

381   (Coleoptera, Leiodidae, Leptodirini) cave population. *Subt. Biol*. **11**, 31–44.

382   (doi:10.3897/subtbiol.11.4974)

383 Foden, W.B., Young, B.E., Akçakaya, H.R., Garcia, R.A., Hoffmann, A. A., Stein, B.A., *et al*.

384   (2019) .Climate change vulnerability assessment of species. *Wiley Interdiscip. Rev. Clim.*

385   *Change* **10**, e551.

386 Gallego, B., Verdú, J. R., & Lobo, J. M. (2018). Comparative thermoregulation between different

387   species of dung beetles (Coleoptera: Geotrupinae). *J. Therm. Biol.* **74**, 84–91.

388 Gallego, B., Verdú, J. R., Carrascal, L. M., & Lobo, J. M. (2016). A protocol for analysing

389   thermal stress in insects using infrared thermography. *J. Therm. Biol.* **56**, 113–121.

390 Gaston, K. J., Chown, S. L., Calosi, P., Bernardo, J., Bilton, D. T., Clarke, A., Clusella-Trullas,

391   S., Ghalambor, C. K., Konarzewski, M., Peck, L. S., Porter, W. P., Pörtner, H. O., Rezende,

15

392   E. L., Schulte, P. M., Spicer, J. I., Stillman, J. H., Terblanche, J. S., & van Kleunen, M.

393   (2009). Macrophysiology: A Conceptual Reunification. *Amer. Nat*. **174**, 595–612.

394   Glaçon, S. (1955). Remarques sur la morphologie et la biologie de quelques larves de

395   Bathysciinae cavernicoles. *CR Acad. Sci. Paris* **240**, 679-681.

396   Howarth, F. G., & Moldovan, O. T. (2018). Where Cave Animals Live. In *Cave Ecology:* 23–37.

397   Moldovan, O.T., Kovác, L., & Halse, S. (Eds.) Cham: Springer.

398   Issartel, J., Hervant, F., Voituron, Y., Renault, D., & Vernon, P. (2005). Behavioural, ventilatory

399   and respiratory responses of epigean and hypogean crustaceans to different temperatures.

400   Comp. Biochem. Phys. A **141**, 1-7.

401   Jeannel, R. (1911). Rèvision des Bathysciinae. *Arch. Zool. Exp. Gen. Paris Ser 5*. **7**, 1–641.

402   Juan, C., & Emerson, B.C. (2010). Evolution underground: shedding light on the diversification

403   of subterranean insects. *J. Biol.* **9**, 17.

404   Juan, C., Guzik, M.T., Jaume, D. & Cooper, S.J.B. (2010). Evolution in caves: Darwin's 'wrecks

405   of ancient life' in the molecular era. *Mol. Ecol.* **19**, 3865–3880.

406   Juberthie, C., Delay, B., & Bouillon, M. (1980a). Extension du milieu souterrain superficiel en

407   zone non-calcaire: description d'un nouveau milieu et de son peuplement par les coleopteres

408   troglobies. In *Evolution des coleopteres souterrains et endoges*. *Mem. Biospeol*. **7**, 19–52.

409   Juberthie, C., Delay, B., & Bouillon, M. (1980b). Sur l'existence d'un milieu souterrain

410   superficiel en zone non calcaire. *CR Acad. Sci. Paris* **290**, 49–52.

411   Juberthie, C., Delay, B., & Bouillon, M. (1981). Sur l'existence du milieu souterrain superficiel

412   en zone calcaire. In *Les entrees d'energie dans le karst et communications libres*. *Mem.*

413   *Biospeol*. **8**, 77–93.

414   Krebs, R. A., & Loeschcke, V. (1994). Costs and benefits of activation of the heat-shock response

16

in *Drosophila melanogaster*. *Funct. Ecol.* **8**, 730–737.

Lachenicht, M. W., Clusella-Trullas, S., Boardman, L., Le Roux, C., & Terblanche, J. S. (2010). Effects of acclimation temperature on thermal tolerance, locomotion performance and respiratory metabolism in *Acheta domesticus* L. (Orthoptera: Gryllidae). *J. Insect Physiol.* **56**, 822–830.

Latella, L., Bernabò, P., & Lencioni, V. (2008). Distribution pattern and thermal tolerance in two cave-dwelling Leptodirinae (Coleoptera, Cholevidae). *Subterr. Biol.* **6**, 81–86.

Lencioni, V., Bernabò, P., & Latella, L. (2010). Cold resistance in two species of cave-dwelling beetles (Coleoptera: Cholevidae). *J. Therm. Biol*. **35**, 354-359.

Lutterschmidt, W. I., & Hutchison, V. H. (1997). The critical thermal maximum: data to support the onset of spasms as the definitive end point. *Can. J. Zool.* **75**, 1553–1560.

Magozzi, S., & Calosi, P. (2015). Integrating metabolic performance, thermal tolerance, and plasticity enables for more accurate predictions on species vulnerability to acute and chronic effects of global warming. *Glob. Chang. Biol.* **21**, 181–194.

Mammola, S., & Isaia, M. (2016). The ecological niche of a specialized subterranean spider. *Invertebr. Biol*. **135**, 20–30.

Mammola, S., Cardoso, P., Culver, D. C., Deharveng, L., Ferreira, R. L., Fišer, C., Galassi, D. M. P., Griebler, C., Halse, S., Humphreys, W. F., Isaia, M., Malard, F., Martinez, A., Moldovan, O. T., Niemiller, M. L., Pavlek, M., Ana Sofia P, Souza-Silva, M., Teeling, E. C., Judson Wynne, J., & Zagmajster, M. (2019a). Scientists' Warning on the Conservation of Subterranean Ecosystems. *BioScience* **69**, 641–650

Mammola, S., Giachino, P. M., Piano, E., Jones, A., Barberis, M., Badino, G., & Isaia, M. (2016). Ecology and sampling techniques of an understudied subterranean habitat: the Milieu Souterrain Superficiel (MSS). *Naturwissenschaften* **103**, 88.

17

439   Mammola, S., Goodacre, S. L., & Isaia, M. (2018). Climate change may drive cave spiders to

440   extinction. *Ecography* **41**, 233–243.

441   Mammola, S., Piano, E., Cardoso, P., Vernon, P., Domínguez-Villar, D., Culver, D. C., Pipan, T.,

442   & Isaia, M. (2019b). Climate change going deep: The effects of global climatic alterations

443   on cave ecosystems. *Anthr. Rev*. **6**, 98–116.

444   Mammola, S., Piano, E., Malard, F., Vernon, P., & Isaia, M. (2019c). Extending Janzen's

445   hypothesis to temperate regions: A test using subterranean ecosystems. *Funct. Ecol.* **33**,

446   1638–1650.

447   Markle, T. M., & Kozak, K. H. (2018). Low acclimation capacity of narrow-ranging thermal

448   specialists exposes susceptibility to global climate change. *Ecol. Evol.* **8**, 4644–4656.

449   Mermillod-Blondin, F., Lefour, C., Lalouette, L., Renault, D., Malard, F., Simon, L., & Douady,

450   C. J. (2013). Thermal tolerance breadths among groundwater crustaceans living in a

451   thermally constant environment. *J. Exp. Biol.* **216**, 1683-1694.

452   Minelli, A., & Fusco, G. (2013). Arthropod post-embryonic development. In *Arthropod Biology

453   and Evolution*: 91–122. Minelli, A., Boxshall, G., & Fusco, G. (Eds.) Berlin: Springer.

454   Monaghan, P., Metcalfe, N. B., & Torres, R. (2009). Oxidative stress as a mediator of life history

455   trade-offs: mechanisms, measurements and interpretation. *Ecol. Lett.* **12**, 75–92.

456   Moore, G. W., & Sullivan, G. N. (1964). Out of phase seasonal temperature fluctuations in

457   Cathedral Cave, Kentucky. *Geol. Soc. Am*. **76**, 313

458   Novak, T., Šajna, N., Antolinc, E., Lipovšek, S., Devetak, D., & Janžekovič, F. (2014). Cold

459   tolerance in terrestrial invertebrates inhabiting subterranean habitats. *Int. J. Speleol*. **43**, 3.

460   Nyamukondiwa, C., & Terblanche, J. S. (2010). Within-generation variation of critical thermal

461   limits in adult Mediterranean and Natal fruit flies *Ceratitis capitata* and *Ceratitis ros*a:

18

462   thermal history affects short-term responses to temperature. *Physiol. Entomol*. **35**, 255–264.

463 Pallarés, S., Colado, R., Pérez-Fernández, T., Wesener, T., Ribera, I., & Sánchez-Fernández, D.

464   (2019). Heat tolerance and acclimation capacity in subterranean arthropods living under

465   common and stable thermal conditions. *Ecol. Evol.* **9**, 13731–13739.

466 Pallarés, S., Sanchez-Hernandez, J.C., Colado, R., Balart-García, P., Comas, J. & Sánchez-

467   Fernández, D. (2020). Beyond survival experiments: using biomarkers of oxidative stress

468   and neurotoxicity to assess vulnerability of subterranean fauna to climate change. *Conserv.*

469   *Physiol.* **8**, coaa067.

470 Pipan, T., López, H., Oromí, P., Polak, S., & Culver, D. C. (2010). Temperature variation and the

471   presence of troglobionts in terrestrial shallow subterranean habitats. *J. Nat. Hist*. **45**, 257–

472   273.

473 Pipan, T., Petrič, M., Šebela, S. & Culver, D.C. (2018). Analyzing climate change and surface–

474   subsurface interactions using the Postojna Planina Cave System (Slovenia) as a model

475   system. *Reg. Environ. Change* **19**, 379–389.

476 Pipan, T., Petrič, M., Šebela, S., & Culver, D.C. (2018). Analyzing climate change and surface–

477   subsurface interactions using the Postojna Planina Cave System (Slovenia) as a model

478   system. *Reg. Environ. Change* **19**, 379–389.

479 R Core Team 2019. R: A language and environment for statistical computing. R Foundation for

480   Statistical Computing, Vienna, Austria. URL https://www.R-project.org/.

481 Raschmanová, N., Šustr, V., Kováč, Ľ., Parimuchová, A., & Devetter, M. (2018). Testing the

482   climatic variability hypothesis in edaphic and subterranean Collembola (Hexapoda). *J.*

483   *Therm. Biol*. **78**, 391–400.

484 Ribera, I., Cieslak, A., Faille, A., & Fresneda, J. (2018). Historical and ecological factors

485   determining cave diversity. In *Cave Ecology*: 229-252. Moldovan, O.T., Kovác, L., & Halse,

19

486   S. (Eds.) Cham: Springer.

487   Ribera, I., Fresneda, J., Bucur, R., Izquierdo, A., Vogler, A. P., Salgado, J. M., & Cieslak, A.

488   (2010). Ancient origin of a Western Mediterranean radiation of subterranean beetles. *BMC*

489   *Evol. Biol.* **10**, 29.

490   Rizzo, V., Sánchez-Fernández, D., Alonso, R., Pastor, J., & Ribera, I. (2017). Substratum

491   karstificability, dispersal and genetic structure in a strictly subterranean beetle. *J.*

492   *Biogeogr.* **44**, 2527-2538.

493   Rizzo, V., Sánchez-Fernández, D., Fresneda, J., Cieslak, A., & Ribera, I. (2015). Lack of

494   evolutionary adjustment to ambient temperature in highly specialized cave beetles. *BMC*

495   *Evol. Biol.* **15**, 10.

496   Rohr, J. R., Civitello, D. J., Cohen, J. M., Roznik, E. A., Sinervo, B., & Dell, A. I. (2018). The

497   complex drivers of thermal acclimation and breadth in ectotherms. *Ecol. Lett.* **21**, 1425–

498   1439.

499   Salgado, J. M., Blas, M., & Fresneda, J. (2008). *Fauna Ibérica, Vol. 31, Coleoptera, Cholevidae*.

500   Madrid: MNCN, CSIC.

501   Sánchez-Fernández, D., Rizzo, V., Bourdeau, C., Cieslak, A., Comas, J., Faille, A., Fresneda, J.,

502   Lleopart, E., Millán, A., Montes, A., Pallarés, S., & Ribera, I. (2018). The deep subterranean

503   environment as a potential model system in ecological, biogeographical and evolutionary

504   research. *Subt. Biol.* **25**, 1.

505   Sánchez-Fernández, D., Rizzo, V., Cieslak, A., Faille, A., Fresneda, J., & Ribera, I. (2016).

506   Thermal niche estimators and the capability of poor dispersal species to cope with climate

507   change. *Sci. Rep.* **6**, 23381.

508   Šebela, S., Turk, J., & Pipan, T. (2015). Cave micro-climate and tourism: Towards 200 years

509   (1819–2015) at Postojnska jama (Slovenia). *Cave & Karst Science* **42**, 78–85.

20

510   Seebacher, F., White, C. R., & Franklin, C. E. (2015). Physiological plasticity increases resilience

511   of ectothermic animals to climate change. *Nat. Clim. Change* **5**, 61-66.

512   Sgrò, C. M., Terblanche, J. S., & Hoffmann, A. A. (2016). What can plasticity contribute to

513   insect responses to climate change? *Annu. Rev. Entomol.* **61**, 433–451.

514   Shah, A. A., Funk, W. C., & Ghalambor, C. K. (2017). Thermal acclimation ability varies in

515   temperate and tropical aquatic insects from different elevations. *Integr. Comp. Biol.* **57**,

516   977–987.

517   Sket, B. (2008). Can we agree on an ecological classification of subterranean animals? *J. Nat.*

518   *Hist.* **42**, 1549–1563.

519   Smithson, P. A. (1991). Inter-relationships between cave and outside air temperatures. *Theor.*

520   *Appl. Climatol.* **44**, 65–73.

521   Somero, G. N. (2005). Linking biogeography to physiology: Evolutionary and acclimatory

522   adjustments of thermal limits. *Front. Zool.* **2**, 1.

523   Stevens, G. C. (1989). The latitudinal gradient in geographical range: how so many species

524   coexist in the tropics. *Amer. Naturalist* **133**, 240–256.

525   Stillman, J. H. (2003). Acclimation capacity underlies susceptibility to climate change. *Science*

526   **301**, 65.

527   Terblanche, J. S., Deere, J. A., Clusella-Trullas, S., Janion, C., & Chown, S. L. (2007). Critical

528   thermal limits depend on methodological context. *Proc. Biol. Sci.* **274**, 2935–2942.

529   Terblanche, J. S., Hoffmann, A. A., Mitchell, K. A., Rako, L., le Roux, P. C., & Chown, S. L.

530   (2011). Ecologically relevant measures of tolerance to potentially lethal temperatures. *J.*

531   *Exp. Biol.* **214**, 3713–3725.

532   Tomanek, L. (2008). The importance of physiological limits in determining biogeographical

21

533   range shifts due to global climate change: the heat-shock response. *Physiol. Biochem. Zool.*

534   **81**, 709–717.

535   Tomanek, L. (2010). Variation in the heat shock response and its implication for predicting the

536   effect of global climate change on species' biogeographical distribution ranges and

537   metabolic costs. *J. Exp. Biol.* **213**, 971–979.

538   Trontelj, P., Borko, Š., & Delić, T. (2019). Testing the uniqueness of deep terrestrial life. *Sci.*

539   *Rep.* **9**, 15188.

540   Vannier, G. (1994). The thermobiological limits of some freezing intolerant insects: the

541   supercooling and thermostupor points. *Acta Oecol.* **15**, 31–42.

542   Voituron, Y., De Fraipont, M., Issartel, J., Guillaume, O. & Clobert, J. (2010). Extreme lifespan

543   of the human fish (*Proteus anguinus*): A challenge for ageing mechanisms. *Biol. Lett.* **7**,

544   105–107.

545   Wikelski, M., & Cooke, S.J. (2006). Conservation physiology. *Trends Ecol. Evol*. 21, 38-46.

546   Williams, S.E., Shoo, L.P., Isaac, J.L., Hoffmann, A.A., & Langham, G. (2008). Towards an

547   integrated framework for assessing the vulnerability of species to climate change. *PLoS Biol.*

548   **6**, e325.

549   Zeh, J.A., Bonilla, M.M., Su, E.J., Padua, M.V., Anderson, R.V., Kaur, D., Yang, D. & Zeh,

550   D.W. (2012). Degrees of disruption: projected temperature increase has catastrophic

551   consequences for reproduction in a tropical ectotherm. *Glob. Chang. Biol.* **18**, 1833-1842.

552   Zuur, A., Ieno, E., Walker, N., Saveliev, A., & Smith, G. (2009). Mixed effects models and

553   extensions in ecology with R. New York: Springer

22

Tables

Table 1. Information on collection sites and their current and projected temperature.

Species	No instars	Locality	Province	Mean annual Temperature (ºC)[a]		Annual Temperature range (ºC)[a,b]	
				Current	2070	Current	2070
Speonomites crypticola	1	Cave Forat Negre	Lleida	10.4	11.8	-2 – 23.1	-0.8 – 28.0
Speonomidius crotchi	2	Cave Arrikrutz	Guipuzcoa	11.1	14.9	3.4 – 24.6	5.2 - 28.4
Bathysciola rugosa	3	Cave Orobe	Navarra	12	13.6	8 – 24.4	26.8 – 28.5

[a]Source: Worldclim v. 1.4 database (https://www.worldclim.org/), 30 second spatial resolution.

Future projected temperatures in 2070 were obtained for the Representative Concentration

Pathway 4.5 and by averaging 17 different Global Circulation Models.

[b]Minimum temperature of the coldest month – Maximum temperature of the warmest month

23

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

Table 2. Results of post-hoc tests (with Bonferroni-adjusted P-values) to compare heat coma temperature i) among species, ii) between acclimation treatments (C: control vs 20ºC) within each species and iii) among species within each acclimation treatment.

	Value	df	Sum of Sq	F	P
B. rugosa - S. crotchi	-0.088	1	0.073	0.220	1
B. rugosa - S. crypticola	1.047	1	8.698	26.125	<0.001
S. crotchi - S. crypticola	1.135	1	13.206	39.668	<0.001
Residuals		52	17.312		
B. rugosa (C - 20)	-1.336	1	6.643	19.953	<0.001
S. crotchi (C - 20)	-0.446	1	1.294	3.886	0.162
S. crypticola (C - 20)	-0.360	1	0.548	1.646	0.615
Residuals		52	17.312		
C (*B. rugosa - S. crotchi*)	-0.532	1	1.400	4.206	0.272
C (*B. rugosa - S. crypticola*)	0.560	1	1.327	3.985	0.307
C (*S. crotchi - S. crypticola*)	1.091	1	6.335	19.030	<0.001
20 (*B. rugosa - S. crotchi*)	0.356	1	0.577	1.732	1
20 (*B. rugosa - S. crypticola*)	1.534	1	8.784	26.385	<0.001
20 (*S. crotchi - S. crypticola*)	1.178	1	6.871	20.638	<0.001
Residuals		52	17.312		

df: degrees of freedom, Sum of Sq: sum of squares

24

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

Figure legends

Figure 1. Mean ± s.e.m heat coma temperatures measured after acclimation at a control temperature (*S. crypticola* 11ºC; *S. crotchi* 9ºC; *B. rugosa* 12ºC) or 20ºC. Significant differences between acclimation treatments within each species (P<0.05 in post-hoc tests) are indicated with asterisks.

25

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

Figures

26