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1 Modified nest architecture and increased bioturbation in response to rise in  
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3

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15

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24 CONFLICT OF INTEREST

25 We have no conflicts of interest to disclose.

26

27 AUTHOR CONTRIBUTIONS:

28 Fátima GARCÍA IBARRA, Pascal JOUQUET and Thibaud MONNIN conceived the ideas,  
29 designed the methodology and led the writing of the manuscript; Fátima GARCÍA IBARRA and  
30 Angélique BULTELLE carried out the experiment; Fátima GARCÍA IBARRA and Nicolas  
31 BOTTINELLI analysed the CT Scan images; Fátima GARCÍA IBARRA analysed the data; all  
32 authors contributed critically to the drafts and gave final approval for publication.

33

34 DATA AVAILABILITY STATEMENT:

35 Data are available upon request on DataSuds repository: <https://doi.org/10.23708/ZJTSDI> (Garcia  
36 Ibarra, et.al., 2023).

37

38 ABSTRACT

39 1. Ants are important bioturbators that actively produce biopores and move soil particles.  
40 They could be particularly affected by global warming as they are ectotherms.  
41 Nevertheless, they can indirectly regulate their temperature, through changes in their  
42 circadian cycles and the architecture of their nests (e.g., digging deep nests or using  
43 insulating materials). Nest architecture has been considered an expanded functional trait of  
44 ant colonies and thus sensitive to environmental changes such as increasing temperatures.  
45 This work aimed to study the nest architecture of ants as a functional trait and its effects on  
46 soil bioturbation. We hypothesized that, when exposed to increased surface temperatures,  
47 ants would increase their excavation activities, build deeper nests, and alter the layout of

48 chambers to maintain their preferred temperature and humidity, thus enhancing soil  
49 porosity.

50 2. We allowed 17 young *Lasius niger* ant colonies to excavate nests in soil columns exposed  
51 to three surface temperatures (mild, n=5; medium, n=6; and high, n=6) for 100 days. We  
52 measured the amount of soil excavated weekly, and took X-ray scans of the soil column on  
53 days 7, 14, 28, and 88 to characterise the three-dimensional structure of the nests (depth,  
54 shape, volume of chambers and tunnels). We then collected the colonies and measured their  
55 growth during the experiment, and the size and weight of workers.

56 3. Ants reacted to surface temperature. Colonies exposed to medium and high temperatures  
57 excavated larger and deeper nests than those exposed to mild temperature. Nests excavated  
58 under high and medium temperatures had the same maximal depth, but chambers were  
59 located deeper in the former, which were further characterised by the refiling of some of the  
60 upper chambers. Colonies grew well in all treatment, although less under mild temperature.  
61 They produced normal-sized workers despite differences in surface temperature. Overall,  
62 these results suggest ants exposed to higher temperatures live in deeper chambers.

63 4. This study shows that surface temperature affects ant nest architecture, confirming its status  
64 as extended phenotype and highlighting its flexibility over time, which has in turn  
65 consequences on soil porosity.

66

## 67 INTRODUCTION

68 Global warming may particularly affect ectotherms including ants, since they largely depend on  
69 external temperatures to regulate their own body temperature (Jørgensen et al., 2022). For instance,  
70 increasing temperatures accelerate ant development (S. Porter & Tschinkel, 1993), yielding smaller  
71 adults (Verberk et al., 2021), and increase their metabolism, i.e. their movements become faster  
72 (Hulbert et al., 2008) and their food and oxygen consumption increase (Coenen-Stass et al., 1980).  
73 Ants can thermoregulate to some extent, but this is an energy-consuming process that depends on

74 many factors such as population size, moisture and thermal conductivity of the material, and nest  
75 size (Coenen-Stass et al., 1980; Kadochová & Frouz, 2013).

76 Most of the studies about temperature regulation in ant nests have focused on mounds or ant  
77 hills (above-ground structures usually built with soil or organic materials). For example, the mound  
78 nests of fire ants (*Solenopsis spp.*) are asymmetrical and their architecture changes seasonally to  
79 increase the surface directly exposed to the sun (Vogt et al., 2008). Overheating during day and loss  
80 of temperature during night are also avoided in the nests of *Acromyrmex heyeri* because of the  
81 lower thermal diffusivity of their mounds that are thatched with plant fragments (Bollazzi & Roces,  
82 2010). *Atta vollenweideri* nests are thermoregulated with a wind-ventilation system consisting of an  
83 outflow of air through central tunnels coupled to an inflow through peripheral tunnels (Kleineidam  
84 et al., 2001). *Formica polyctena* underground nests are covered with a dome of pine needles whose  
85 decay, produced by the aerobic metabolism of microorganisms, warms the nest (Coenen-Stass et al.,  
86 1980). In addition to the above mentioned thermoregulation mechanisms, ants exposed to increased  
87 temperature may alter their circadian rhythm and forage for food at cooler times of the day (Lei et  
88 al., 2021), or even relocate their nest to cooler microenvironments (Penick & Tschinkel, 2008).  
89 Nevertheless, these mechanisms might cause negative interactions with other species or be  
90 detrimental to the development of the colony (Adler & Gordon, 2003; Penick et al., 2017).

91 Another simple way to deal with raising temperatures may be to modify the underground  
92 activities, e.g., excavate deeper nests and/or reshape tunnels and chamber connectivity as previously  
93 documented in ants (Sankovitz & Purcell, 2021), earthworms (Gerard, 1977; Perel, 1977), termites  
94 (Korb & Linsenmair, 1998) and other macroarthropods like beetles and flies (Villani & Wright,  
95 1990). Such modifications of underground activities in response to environmental factors would  
96 affect soil bioturbation, i.e., the dispersal and reorganization of soil particles and aggregates through  
97 the activity of animals (Meysman et al., 2006; Bottinelli et al., 2015).

98 Soil bioturbation is a crucial process in soil formation and ecosystem functioning  
99 (Wilkinson et al., 2009). Termites, earthworms, and ants are usually considered the most important

100 bioturbators, especially regarding their ability to produce soil biopores and biogenic structures  
101 (Paton et al., 1995; Lavelle et al., 1997; Jouquet et al., 2006). Ants are likely to play a key role in  
102 the dynamics of nutrients and water in the soils (Cammeraat & Risch, 2008; Benckiser, 2010; Finér  
103 et al., 2013; Farji-Brener & Werenkraut, 2017; Sousa et al., 2021). Indeed, several ant species with  
104 populous colonies build large underground nests, which are likely to increase soil macroporosity  
105 (Frouz & Jilková, 2008) (e.g., *Atta* (Moreira et al., 2004) and *Acromyrmex* leafcutters (Verza et al.,  
106 2020), *Formica* wood ants (Mikheyev & Tschinkel, 2004), *Aphaenogaster* (Richards, 2009) and  
107 *Pogonomyrmex* seed harvesters (Tschinkel, 2004)). In addition, less populous but highly abundant  
108 species such as *Lasius niger* or *Prenolepis imparis* can also be important to soil processes (Rasse &  
109 Deneubourg, 2001; Tschinkel, 2003). Moreover, a meta-analysis showed that excavation activities  
110 of ants in soils increase soil fertility through an augmentation in nutrient and cation contents  
111 independently on their feeding type, the latitude or type of vegetation, which increases the  
112 performance and fitness of plants (Farji-Brener & Werenkraut 2017). Although the importance of  
113 ant bioturbation is well established (Tschinkel, 2021; Viles et al., 2021), the structure and properties  
114 of the biopores they produce remain relatively understudied especially compared to those produced  
115 by earthworms (e.g., Cheik et al., 2021; Pham et al., 2023).

116 Nest architecture has been studied by excavating colonies, sometimes by casting nests with  
117 liquid plaster or aluminium (Tschinkel, 2010) prior to their excavation. This has shown that  
118 underground nests of most ants have the same basic structure, consisting of chambers where brood  
119 and workers are typically located, and that are connected by narrower tunnels (Mikheyev &  
120 Tschinkel, 2004). The depths of the nests vary from few centimetres to several metres depending on  
121 species and environmental conditions (Tschinkel, 2004; Tschinkel, 2021). Due to the technical  
122 impossibility of studying how underground nest structure develops through time in the field, most  
123 of the works exploring ant nests construction or evolution have used a two-dimensional approach,  
124 in which ants dig their nests in soil a few cm thick placed between two glass plates (but see Minter  
125 et al., 2012; Pinter-Wollman, 2015). An example is the study carried on by Sankovitz and Purcell

126 (2021) where *Formica podzolica* colony fragments collected at two different elevations were reared  
127 in a 2D setup under two temperature regimes in a full factorial experiment, and differences between  
128 nests were analysed. Nest architecture differed between the two temperature treatments, with  
129 maximal complexity matching the original temperature of the colonies (i.e., ants from higher sites  
130 built more complex nests under cool temperatures, whereas ants from lower sites had the opposite  
131 response). These results are interesting yet should be treated with caution for the artificial approach  
132 of the experimentation (i.e. only a small number of workers without brood nor the queen, two-  
133 dimensional setup, and no temperature gradient in the soil).

134         During the last decades, X-ray computed tomography (X-ray CT) has been used as a non-  
135 destructive method to investigate the effect of invertebrates like earthworms, termites, and beetles  
136 on soil properties (Perna et al., 2008; Booth et al., 2020; Cheik et al., 2021). This study uses X-ray  
137 CT to analyse the effects of increased surface temperatures (and different gradients across the soil)  
138 on the nests excavated by complete young *L. niger* colonies. X-ray CT being non-destructive, also  
139 allows repeated measures of colonies over time enabling the study of the temporal nest architecture  
140 dynamics.

141         We hypothesized that ants react to increased surface temperatures by modifying the  
142 architecture of their nest (digging a deeper nest and/or altering the layout of chambers) ensuring  
143 suitable abiotic conditions (i.e. temperature, humidity) for the colony. For instance, higher  
144 temperature accelerates development and results in the production of smaller adults in insects.  
145 Producing smaller workers may be detrimental to colony success hence we expect workers to  
146 excavate deeper nests with deeper chambers providing adequate temperature for brood to develop in  
147 normal-sized workers. As a by-product of this increased excavation activity, we also expect soil  
148 porosity to increase.

149

150 MATERIALS AND METHODS

151 *Study species*

152 *L. niger* (L.) is one of the most common species of ants in European urban areas. They are typically  
153 found in habitats such as open areas with scattered plant cover, as gardens, meadows, and roadsides  
154 (Czechowski et al., 2012), and they can change significantly the chemical and bacterial dynamics of  
155 soils (Holec & Frouz, 2006). In addition, their claustral independent colony foundation facilitates  
156 the rearing of new colonies, and the adult colonies are easy to maintain in the laboratory.

157 *Collection of colonies and rearing*

158 We collected 51 fertilized *L. niger* queens immediately after their mating flight in Paris in July  
159 2021. This species uses claustral colony foundation to found new colonies. After the mating flight  
160 the queen excavates a small nest and starts rearing the first generation of workers using her  
161 metabolic reserves only, without ever leaving the safety of the nest. We set up the queens to found  
162 colonies in the laboratory, in a windowless climatized room, by keeping ants in glass test tubes (10  
163 cm length x 0.7 cm diam.) with a water reserve held by wet cotton. Once the queens had produced  
164 cocoons, 18 queens were randomly selected for the experiment. The growth of their colonies was  
165 boosted by providing each of them with the cocoons produced by two other queens. Note that this  
166 procedure is not unnatural as *L. niger* colonies raid neighbouring colonies to steal and adopt their  
167 brood as a mean to increase colony growth (e.g. Pollock & Rissing 1989, Sommer & Hölldobler  
168 1995). Workers started hatching after three months. Colonies were then removed from the tubes and  
169 installed in 12.5 cm length x 12.5 cm width x 5.5 cm height clear plastic boxes. They were fed with  
170 a mix of equal parts of honey and apple puree, fresh mealworms, and a balanced protein gel twice a  
171 week, with water provided *ad libitum* by a test tube filled with water and plugged with cotton. The  
172 experiment started seven months later (February 2022). At that time, colonies contained in average  
173  $106 \pm 27$  (mean $\pm$ SD) workers, the queen and brood.

174

175 *Experimental design*



176 Twenty-one polyvinyl chloride (PVC) tubes (30 cm height x 20 cm diameter) had the bottom closed  
177 with a nylon cloth (mesh size = 150  $\mu\text{m}$ ) to retain soil but allow water to pass through. They were  
178 filled to a height of 24 cm with air-dried soil previously sampled in the topsoil layer (0-20 cm) of  
179 the park of the French National Research Institute for Sustainable Development (IRD) campus in  
180 Bondy (7.5 Km Northeast of Paris), France. The soil had a neutral pH ( $\text{pH}_{\text{H}_2\text{O}} = 7.2$ ) and was sandy  
181 (70% sand, 15% silt, and 15% clay) with 4.5% organic carbon. It was sieved at 2 mm to discard  
182 large size particles and roots, and then manually compacted to reach the bulk density found in the  
183 field ( $1.08 \text{ g cm}^{-3}$ ), stepwise in 3 cm layers to reach 24 cm height. The soil column was afterward  
184 watered to reach 70% of the field capacity ( $0.14 \text{ g H}_2\text{O g}^{-1} \text{ soil}$ ) following Weil & Brady (2016).

185 To avoid border effects affecting the shape of the excavated nest, e.g. tunnels following the  
186 side of the PVC tubes, a plastic lid with a central opening was placed on the soil surface. The side  
187 of the lid was glued to the side of the PVC tubes to force ants to dig through the lid central opening  
188 only, i.e. in the centre of the soil surface. The lid had numerous perforations (10 mm diameter each)  
189 totalling  $\frac{1}{4}$  of its surface, to allow watering of soil and gaseous exchanges between the soil and the  
190 atmosphere. The lid was underlined by a nylon fabric that prevented ants from digging through the  
191 perforations. A short plastic tube connected the central opening of the lid to a tall plastic petri dish  
192 (8.5 cm diameter x 5 cm height) whose bottom had been drilled. The petri dish allowed providing  
193 food and water to the colony as well as collecting the excavated soil for weighing (Fig S1).

194 The 18 colonies were randomly assigned to high ( $T_{\text{high}}$ ), medium ( $T_{\text{medium}}$ ), and mild  
195 temperature ( $T_{\text{mild}}$ ) treatments ( $n = 6$  for each treatment). The surface of the soil columns was heated  
196 using 40 W UV lamps placed 20 or 40 cm above the soil surface ( $T_{\text{high}}$  and  $T_{\text{medium}}$ , respectively) or  
197 fluorescent lights 50 cm above the soil surface ( $T_{\text{mild}}$ ). Soil temperature was monitored in two  
198 columns per treatment using two iButton temperature sensors on the surface and at  $\sim 10$  cm depth.  
199 To avoid any effect of the sensors on the nest structure, they were placed on the edge of the column.  
200 None of the ant nests reached an iButton during the experiment. This protocol was successful in  
201 producing three treatments with significantly different temperatures. Specifically, daytime average

202 temperatures (i.e., while the lamps were on) on the soil surface were  $22.2 \pm 1.1$ ,  $35.9 \pm 5.8$  and  $47.9$   
203  $\pm 10.7^\circ\text{C}$  for  $T_{\text{mild}}$ ,  $T_{\text{medium}}$ , and  $T_{\text{high}}$  treatments, respectively (mean  $\pm$  SD,  $F_{2,7497} = 83$ ,  $P < 0.001$ ,  
204  $T_{\text{mild}}$  vs  $T_{\text{medium}}$ ,  $P < 0.001$ ,  $T_{\text{mild}}$  vs  $T_{\text{high}}$ ,  $P < 0.001$ ,  $T_{\text{medium}}$  vs  $T_{\text{high}}$ ,  $P < 0.001$ ), and night-time average  
205 temperatures (when the lamps were off) were  $21.5 \pm 1.1$ ,  $24.8 \pm 5.4$  and  $28.4 \pm 11.1^\circ\text{C}$  ( $F_{2,7527} = 584.5$ ,  
206  $P < 0.001$ ,  $T_{\text{mild}}$  vs  $T_{\text{medium}}$ ,  $P < 0.001$ ,  $T_{\text{mild}}$  vs  $T_{\text{high}}$ ,  $P < 0.001$ ,  $T_{\text{medium}}$  vs  $T_{\text{high}}$ ,  $P < 0.001$ ). As expected,  
207 this difference markedly declined in the soil, with a mid-depth daytime temperature of  $22.2 \pm 1.1$ ,  
208  $25.5 \pm 2.1$ , and  $26.2 \pm 2.4^\circ\text{C}$  for  $T_{\text{mild}}$ ,  $T_{\text{medium}}$ , and  $T_{\text{high}}$  treatments, respectively ( $F_{2,2469} = 567.4$ ,  $P <$   
209  $0.001$ ,  $T_{\text{mild}}$  vs  $T_{\text{medium}}$ ,  $P < 0.001$ ,  $T_{\text{mild}}$  vs  $T_{\text{high}}$ ,  $P < 0.001$ ,  $T_{\text{medium}}$  vs  $T_{\text{high}}$ ,  $P < 0.001$ ), and night-time  
210 temperature of  $21.8 \pm 1.0$ ,  $23.8 \pm 2.1$  and  $24.6 \pm 2.4^\circ\text{C}$  ( $F_{2,2487} = 270.1$ ,  $P < 0.001$ ,  $T_{\text{mild}}$  vs  $T_{\text{medium}}$ ,  
211  $P < 0.001$ ,  $T_{\text{mild}}$  vs  $T_{\text{high}}$ ,  $P < 0.001$ ,  $T_{\text{medium}}$  vs  $T_{\text{high}}$ ,  $P < 0.001$ ). Because one colony died during the  
212 experiment, the number of replicates was  $n = 5$  for  $T_{\text{mild}}$ , and  $n = 6$  for  $T_{\text{medium}}$ , and  $T_{\text{high}}$ . The impact  
213 of ants on water evaporation was also monitored using soil columns without ants as control ( $n = 1$   
214 per treatment). All colonies were kept with a light/dark 12h/12h schedule for 100 days.

215           Ants were put in the plastic box connected to the top of the soil column four days before  
216 turning on the lamps, to allow them to start digging under the same conditions and not under the  
217 stress of excess heat. The start of the experiment was marked by the lighting of the lamps.

218

### 219 *Soil bioturbation by ants*

220 To assess the underground excavation activity, the amount of soil excavated by ants and deposited  
221 on the ground was collected. This assessment, may underestimate the underground activity as it has  
222 been reported that ants refill chambers and tunnels according to their space necessities taking the  
223 soil previously deposited out of the nest (Römer & Roces, 2015). However, such remodelling of the  
224 nest in our experiment may be revealed by the tomography if refilled spaces differ in density from  
225 the surrounding soil. The excavated soil was collected weekly, dried at  $80^\circ\text{C}$  for 48h and weighted.  
226 Soil columns were also weekly weighed for measuring water loss and watered to maintain soil

227 moisture at 70% of the field capacity to allow the development of ants, without promoting the  
228 development of anoxic bacteria. The amount of evaporated water (i.e. the added water to keep 70%  
229 field capacity), was calculated as follows:

$$\text{Calculated column weight} = \text{Previous week column weight} - (\text{Excavated soil} \times 70\% \text{ field capacity})$$

$$H_2O_{\text{evaporated}} = \text{Calculated column weight} - \text{real weight}$$

230

231 On days 7, 14, 28, and 88 of the experiment, soil columns were scanned using a medical  
232 Computed X-ray Tomograph (IQon - Spectral CT, Philips) at the Pitié-Salpêtrière Hospital in Paris  
233 to acquire a set of 0.8 mm thick images with a pixel size of 0.45 mm. The X-ray beam was operated  
234 at 58 mA and 120 kV. Images (16-bit DICOM format, 512 x 512 pixels) were obtained and  
235 subsequently transformed into 8-bit TIFF format and rendered isotropic of 0.45 mm. Images were  
236 processed and quantified with ImageJ software version 1.53s (Schindelin et al., 2012) and  
237 visualized with Avizo software version 2021.2 (ThermoScientific).

238 Once the images were pre-processed, the soil volume was defined using the ROI manager  
239 tool and its volume was measured using the Volume Fraction tool on ImageJ. The level of grey of  
240 the histograms was bimodal, hence the Otsu automatic thresholding method was applied before  
241 segmentation. The excavated nests on the other hand, were selected by applying a 5-pixel 3D ball  
242 opening, 8-connection fill holes, 10-pixel 3D ball opening, and a 17-pixels 3D disc erosion and  
243 removing small spots 3D (100 pixels) on Avizo, after confirming that these parameters were the  
244 most suitable for all the images. Nest volume was calculated with Volume Fraction as well, and  
245 MorphoLibJ plugin was used to further describe their i) shape: sphericity index (i.e., the ratio of the  
246 squared volume over the surface area, such that ratio of a ball equals one), Euler number (an  
247 indicator of topology corresponding to the number of objects minus the number of holes in the  
248 object), number of pores; ii) position: maximal depth, ellipse elevation (orientation of the inertia  
249 ellipse in degrees); and iii) diameter: the maximal radius of the inscribed ball.

250 Chambers and tunnels (Figs 1, S2, S3, S4), have different shapes (i.e., bulbous for the  
251 former, and tubular for the latter). To evaluate them independently, we used filter tools on Avizo,  
252 which allowed us to select them precisely. Chambers were separated from the nests by using the  
253 Avizo Label analysis tool in mode 3D and Analysis filter tool, until the selected volume best fitted  
254 most of our observations (filter:  $\text{Breadth } 3d < 7$  and  $\text{Breadth } 3d / \text{Length } 3d < 0.8$ , where breadth  
255 (width) and length are geometrical descriptors of the chambers). Tunnels were obtained by  
256 subtracting the chambers from the nests. The mean thickness (diameter of the greatest sphere that  
257 fits within) of both tunnels (1.68 to 3.67 mm) and chambers (4.41 to 7.61 mm) were calculated with  
258 ImageJ BoneJ plugin.

259 During the image processing, we noticed a third level of grey on some images (Fig S5),  
260 intermediate between those corresponding to the soil (higher density) and the nests (density equal to  
261 zero). It represented structures less dense than the soil and herein suspected to correspond to refilled  
262 parts of the nests i.e. denoting nest remodelling. Prior to segmentation, a 3D non-local means filter  
263 of three pixels was applied to reduce noise and scatter. Avizo manual thresholding (110, 160) and a  
264 manual 3D ball opening were used to ensure the desired objects were segmented (Fig S2 to S5).  
265 Volume was measured with the Volume Fraction tool on ImageJ. All volumes (i.e. nests, chambers,  
266 tunnels, and refilled chambers) were transformed into percentages (structure volume / soil volume  
267 x100).

268 To assess the distribution of soil excavated along the entire column, the pore area was  
269 measured by counting voxels per slice with 2D Analyse particles tool on ImageJ and multiplying by  
270 voxel size (0.45 mm). Next, this porosity was transformed in percentage (pore area / soil area x  
271 100). To analyse the nest structure, a skeleton was obtained using the Avizo AutoSkeleton tool, and  
272 the 3D Centroid path tortuosity (that considers tortuosity as a path formed by the centroids of the  
273 objects on each plan, compared to the distance between its ends along the Z-axis) was applied to  
274 measure tortuosity and coordination number (number of branches connected to each node).

275

276 *Growth of colonies*

277 At the end of the experiment, the soil columns were opened, and the colonies recollected, to  
278 measure the effects of the temperature treatments on colony growth, worker size and weight. The  
279 number of workers was counted to measure colony growth throughout the duration of the  
280 experiment. Moreover, 10 workers per colony were placed in 70% ethanol and their morphological  
281 traits were measured. Head length and Weber's length (the diagonal length of the mesosoma, from  
282 anterior edge of the pronotum to the posterior corner of the metapleuron, Weber, 1938), were  
283 measured and summed as indicator of the individual size. In addition, 10 other workers per colony  
284 were chill killed at -20°C for 12 hours, dried in a stove at 40°C for 48 hours, and weighed using a  
285 Sartorius Cubis microbalance.

286

287 *Statistical analysis*

288 Data were analysed using R (version 4.0.0) software. Differences among treatments for water  
289 evaporation, soil excavated, number, size and weight of ants were analysed using one-way Anova  
290 and Tukey tests, after verifying the normality (Shapiro-Wilk test, rstatix package (Kassambara,  
291 2021)) and homogeneity of variances (Levene's test, rstatix package) of the data. Data from the  
292 tomography analyses were analysed using a PCA (factoextra package (Kassambara & Mundt,  
293 2020)) to determine which traits of the nests contributed the most to total variance. Depth (D),  
294 volume of nests ( $V_{\text{nest}}$ ), chambers ( $V_{\text{Ch}}$ ), tunnels ( $V_{\text{Tu}}$ ) and refilled chambers ( $V_{\text{Re}}$ ), coordination  
295 number, and tortuosity were considered suitable for further description and analysed using two-way  
296 (treatment, time) mixed Anova (rstatix package) after verifying the absence of significant outliers,  
297 normality, homogeneity of variances, sphericity of variance (Maulchy's test) and homogeneity of  
298 covariances (Box's M test), to assess the effect of treatment and time. Differences among groups  
299 were analysed with one-way Anova (when an interaction between main factors was present) and  
300 Bonferroni adjusted pairwise comparisons.

301

## 302 RESULTS

### 303 *Soil bioturbation*

304 Excavation activity was similar across treatments during the first half of the experiment. It was  
305 highest at the onset of the experiment (time 0) and then gradually decreased. A late increase in  
306 excavation (after day 50) occurred for  $T_{\text{medium}}$ , but not for  $T_{\text{high}}$  and  $T_{\text{mild}}$  (Fig 2A). The total amount  
307 of soil excavated (Fig 2B) was significantly higher at  $T_{\text{medium}}$  ( $26.4 \pm 12.1$  g, mean  $\pm$  SD) than at  
308  $T_{\text{mild}}$  ( $12.8 \pm 4.7$  g), with  $T_{\text{high}}$  having intermediate values ( $19.5 \pm 8.3$  g,  $F_{2,14} = 3.06$ ,  $P < 0.05$ ).

309 From the beginning of the experiment, ants produced nests whose maximum depth did not  
310 change with time ( $F_{3,64} = 2.56$ ,  $P > 0.05$ , Figs 3, S2 to S4). No nest reached the bottom of the soil  
311 column, in any of the treatment. However, differences in temperature led to differences in nest  
312 depth, with shallower nests for  $T_{\text{mild}}$  than  $T_{\text{medium}}$  and  $T_{\text{high}}$  ( $13.7 \pm 3.5$  for  $T_{\text{mild}}$  vs.  $17.2 \pm 3.2$  and  
313  $19.3 \pm 2.2$  cm for  $T_{\text{medium}}$  and  $T_{\text{high}}$ , respectively  $F_{2,14} = 7.99$ ,  $P < 0.05$ ,  $T_{\text{mild}}$  vs  $T_{\text{medium}}$ ,  $P < 0.01$ ,  $T_{\text{mild}}$   
314 vs  $T_{\text{high}}$ ,  $P < 0.001$ ,  $T_{\text{medium}}$  vs  $T_{\text{high}}$ ,  $P < 0.05$ ).

315 Porosity profiles showed the vertical distribution of chambers and tunnels (Fig 3). The peak  
316 of soil porosity (% of empty volume compared to soil volume) was located deeper under increased  
317 temperature, ranging from 7.6 to 13.1 and 16.9 cm depths for  $T_{\text{mild}}$ ,  $T_{\text{medium}}$  and  $T_{\text{high}}$ , respectively  
318 (Fig S9).

319 The Principal Component Analysis (PCA) of the variables describing nest architecture (Fig  
320 4, see also Tables S1 and S2) showed that the first axis described 40.9% of the total variance and  
321 corresponded mainly to a gradient of size, with smaller nests towards higher values and bigger nests  
322 toward lower values. This gradient also corresponds to nests development, with younger nests  
323 towards higher values and older nests towards lower values. The second axis described 14.9% of  
324 the whole variance and corresponded to a shape and position gradient.  $T_{\text{medium}}$  and  $T_{\text{high}}$  were better  
325 described by size related variables (i.e. volume of nests, volume of chambers, volume of tunnels,

326 surface area (number of pixels corresponding to the external boundary of the 3D nest)) than by  
327 shape or position related variables (i.e. number of pores, sphericity index). Conversely,  $T_{\text{mild}}$  was  
328 better explained by shape than by size related variables. Nests excavated under  $T_{\text{mild}}$  differed from  
329 those under  $T_{\text{medium}}$  and  $T_{\text{high}}$ . Nests under  $T_{\text{medium}}$  and  $T_{\text{high}}$  were similar at days 7, 14 and 28, but less  
330 so at day 88 where the overlap in characteristics was lower. Within each treatment, nests differed  
331 little between days 7 and 14, and increasingly differed from day 28 onwards, with nests becoming  
332 deeper. This temporal change was less evident under  $T_{\text{mild}}$  than under  $T_{\text{medium}}$  and  $T_{\text{high}}$ , with nest  
333 characteristics overlapping largely over days under  $T_{\text{mild}}$ , but not under  $T_{\text{medium}}$  and  $T_{\text{high}}$ .

334 Nest volume ( $V_{\text{nest}}$ ) was affected by the interaction between the time and the temperature  
335 treatments ( $F_{3,24} = 3.28$ ,  $P < 0.001$ ). There was no difference among treatments until day 88 when  
336 the nests at  $T_{\text{mild}}$  were smaller than those at  $T_{\text{medium}}$  ( $F_{2,14} = 5.20$ ,  $P < 0.05$ , Fig S6). Nests of colonies  
337 at  $T_{\text{high}}$  showed intermediate volumes compared to the other two treatments. Chambers volume  
338 ( $V_{\text{Ch}}$ ) showed the same trend as the nest volume, i.e. it was affected by the interaction between the  
339 time and temperature ( $F_{3,24} = 3.67$ ,  $P < 0.001$ , Fig S6). Only on day 88, chambers in  $T_{\text{medium}}$  were  
340 larger than  $T_{\text{mild}}$  whereas  $T_{\text{high}}$  had intermediate volumes ( $F_{2,14} = 5.71$ ,  $P < 0.05$ , Fig S6). Tunnels  
341 volume ( $V_{\text{Tu}}$ ) increased over time ( $F_{1,26} = 0.26$ ,  $P < 0.001$ ), without influence of the temperature  
342 ( $F_{2,14} = 0.26$ ,  $P > 0.05$ , Fig S6).

343 The volume of refilled chambers ( $V_{\text{Re}}$ ) was significantly larger for  $T_{\text{high}}$  than  $T_{\text{medium}}$  ( $F_{2,14} =$   
344  $8.62$ ,  $P = 0.011$ ) and  $T_{\text{mild}}$  ( $F_{2,61} = 8.62$ ,  $P = 0.006$ ) on day 28 (Fig S7). A similar trend was observed  
345 on day 88 but was not statistically significant. The porosity profiles of the refilled chambers (Fig 5)  
346 showed that they were mostly located in the first cm of the soil columns. The highest peak of  $V_{\text{Re}}$   
347 was observed on day 88 at 3.69, 6.98 and 5.04 cm depth for  $T_{\text{mild}}$ ,  $T_{\text{medium}}$ , and  $T_{\text{high}}$ , respectively.

348 The tortuosity of the nest system was affected neither by the sampling time nor by the  
349 temperature treatments ( $F_{6,42} = 0.344$ ,  $P > 0.05$ ). Coordination number did not present any changes  
350 amongst treatments at any moment of the experiment ( $F_{3,26} = 0.965$ ,  $P > 0.05$ ).

351

352 *Water evaporation*

353 Water evaporation showed an effect of temperature, as soil columns exposed to  $T_{\text{mild}}$  lost less water  
354 ( $185.2 \pm 60.1$  ml) than those exposed to  $T_{\text{medium}}$  ( $261.1 \pm 75.5$  ml) and  $T_{\text{high}}$  ( $293.1 \pm 84.0$  ml) during  
355 the whole experiment ( $F_{2,17} = 134.56$ ,  $P < 0.001$ ). The evaporation rate of  $T_{\text{medium}}$  and  $T_{\text{high}}$   
356 treatments were similar to one another from the start of the experiment until day 79, date after  
357 which  $T_{\text{high}}$  showed higher evaporation than  $T_{\text{medium}}$  ( $F_{2,238} = 3.19$ ,  $P < 0.001$ , Fig S8). The presence  
358 of ants had no effect on water evaporation since no difference occurred between soil columns with  
359 and without ants in any of the treatments ( $F_{2,167} = 0.21$ ,  $P > 0.05$ ), however these results should be  
360 treated with caution due to low number of replicates ( $n=1$ , per treatment).

361

362 *Development of the colonies*

363 The colonies exposed to  $T_{\text{mild}}$  ( $253 \pm 122$  workers, mean  $\pm$  SD) grew less than those under  $T_{\text{medium}}$   
364 and  $T_{\text{high}}$  ( $503 \pm 93$  workers,  $F_{2,14} = 10.45$   $P < 0.05$ , Fig S9, Table S3). Temperature had no effect on  
365 the size of workers ( $1.67 \pm 0.10$  mm, mean  $\pm$  SD,  $F_{2,167} = 0.37$ ,  $P > 0.05$ , Fig S9), but ants exposed  
366 to  $T_{\text{mild}}$  were significantly heavier ( $0.25 \pm 0.08$  mg, mean  $\pm$  SD) than those exposed to  $T_{\text{medium}}$  and  
367  $T_{\text{high}}$  ( $0.18 \pm 0.06$  mg,  $F_{2,167} = 19.67$ ,  $P < 0.001$ , Fig S10).

368

369 DISCUSSION

370 *Soil bioturbation by ants*

371 Ant activity is known to increase with the temperature in the field, i.e., during spring and summer  
372 (Andrew et al., 2013; Nobua-Behrmann et al., 2017). Although this general trend was confirmed by  
373 our experiment, we also found that soil excavation did not increase proportionally to the increase in  
374 temperature (i.e., no difference between  $T_{\text{medium}}$  and  $T_{\text{high}}$ , Fig 2). However, ants seem to be located



375 proportionally deeper in the nests with increasing temperatures (Fig 3). We assume that the  
376 modification of the nest architecture provided suitable conditions of temperature and humidity, so  
377 that the ants did not need to excavate further to survive and develop. Also, the average daytime  
378 surface temperature on  $T_{\text{high}}$  (47.9°C) was above their critical thermal maximum ( $CT_{\text{max}}$ , *i.e.*, the  
379 temperature at which the individuals no longer control their locomotion (Lutterschmidt &  
380 Hutchison, 1997)). At this temperature, ants could be at risk of thermal stress if the duration of  
381 exposure was too long or if they were close to their point of desiccation. The  $CT_{\text{max}}$  of *L. niger*  
382 measured through a stepwise method in our laboratory was 47.5°C, hence workers could be active  
383 outside, *e.g.* for foraging and rejecting excavated soil, but not for too long or solely at night when  
384 the temperatures decreased (Lei et al., 2021).

385         Soil excavation by *L. niger* occurred very rapidly in our experiment. Nests had already  
386 reached their maximal depths at the time of the first X-ray scan at day 7, and they were later  
387 enlarged by excavating chambers. Excavation in all treatments occurred with a phase of intense  
388 digging (between days 1 and 28) followed by a phase of lower activity (between days 28 and 88,  
389 Fig 2A). These results are comparable to the findings of Rasse & Deneubourg (2001) and Toffin et  
390 al. (2009), who described an initial phase of maximal and fast digging activity (amplification  
391 phase), followed by a second phase (saturation phase) when the activity ceases almost completely.  
392 However, our results show that ants also respond to increased temperatures by modifying their  
393 excavation process, since ants exposed to moderately increased temperatures ( $T_{\text{medium}}$ ) intensified  
394 their excavation after being almost inactive (around day 50, Fig 2A) while the others kept their  
395 digging activity low. We could assume that the temperature stimulated the oviposition of the queen,  
396 as previously described for other species like *Solenopsis invicta* (Abril et al., 2008; Asano &  
397 Cassill, 2012). As expected, we also found that ants exposed to room temperature produced the  
398 most superficial nests among the three treatments. Ants exposed to medium and high temperatures  
399 built nests of similar maximal depth. However, chambers were distributed differently along the  
400 depth of the nest. They were located deeper under high temperatures than under medium

401 temperatures (Fig 3), hinting that the higher the temperature on the surface, the deeper the ants were  
402 located. In ant species living belowground, queens, workers, and brood are primarily located in  
403 intermediate and lower chambers inside the nest, while upper chambers are empty, used for  
404 stocking or seasonally used for warming up cocoons (Mikheyev & Tschinkel, 2004; Kadochová &  
405 Frouz, 2013). As such, ants likely continually reshape their nests by refilling the less crowded upper  
406 chambers, leaving the bottom, more populous part of the nest, untouched. In our study, we showed  
407 that changes in surface temperature could significantly influence the dynamics of the nest chambers.  
408 Indeed, we found that ants exposed to higher temperatures responded by filling back some of their  
409 most shallow galleries (Fig 5). Although these structures were small (~2% of the total nest volume),  
410 they were significantly larger for  $T_{\text{medium}}$  and  $T_{\text{high}}$ , in comparison with  $T_{\text{mild}}$ . The underground  
411 movement of soil material carried by ants has been previously reported by Tschinkel and Seal  
412 (2016) who showed that 17% of the soil excavated by the gardening ant, *Trachymyrmex*  
413 *septentrionalis* was deposited underground. Also, Römer & Roces (2014) reported that in a 2D  
414 setup, workers of *Acromyrmex lundii* adjust the size of chambers and tunnels depending on the  
415 number of inhabiting workers and the presence of brood and fungus by either digging or refilling  
416 spaces using pellets of soil previously removed from the nest.

417         Despite changes in soil porosity, no difference in soil water evaporation could be measured  
418 between the columns with ants and those without ants in all three treatments. These results did not  
419 confirm other previously reported findings that suggested that ants decrease (Li et al., 2017) or  
420 increase (Woodell and King, in 1991; Blomqvist *et al.*, 2000) water evaporation from soil. Our  
421 results could be explained by the specific conditions of our experiment. Nests had a single small  
422 opening, and the total porosity due to nests was very low (less than 0.5% of the column volume).  
423 Also, our soil columns were only 24 cm deep, and the differences in soil water evaporation among  
424 treatments (Fig S8) most likely stemmed from the increased temperatures, that intensify the  
425 atmospheric evaporative demand (i.e., drying, or evaporating power of the atmosphere)  
426 (Vicente- Serrano et al., 2020), rather than from the ants' burrowing activities. Soil humidity is an

427 important factor in nest excavation. Ants burrowing activity depends on the grain size and level of  
428 soil moisture (i.e. ants tend not to dig when the soil is fully wet and when it is completely dry  
429 (Monaenkova et al., 2015)). Our results show that ants respond to warming by modifying nest  
430 architecture, presumably setting up chambers at the depth providing adequate temperature for brood  
431 development. We kept humidity constant in our experiment; however, soil humidity may also be  
432 affected by global changes so that ants may have to compromise between nest architecture  
433 optimising one factor or the other.

434

#### 435 *Influence of changes in surface temperature on colony development*

436 This study dealt with the influence of an increase in temperature and ant colony development.  
437 Colonies grew under the three temperature treatments, which suggests that the nests and food  
438 provided adequate conditions for colony development. However, colonies reared under room  
439 temperature ( $T_{\text{mild}}$ , 22.2°C during daytime) produced fewer workers than those reared under higher  
440 temperatures (35.9 and 47.9°C). This may be due to room temperature being lower than the natural  
441 temperature since *L. niger* typically occurs in open environments where the sun heats the soil, often  
442 above this temperature. Colonies produced normal-sized workers under the three temperatures (3.5-  
443 5.0 mm length (Seifert, 2018)), despite ant development being sensitive to temperature (i.e. high  
444 temperatures promote maturity at smaller size in ectotherms, Atkinson, 1994, Verberk et al., 2021).  
445 Given that nest architecture was affected by soil surface temperature, one may hypothesize that ants  
446 modified nest architecture so that it provided adequate abiotic environment (temperature, humidity)  
447 to allow for the development of brood into normal-sized workers. Indeed, ant nests provide  
448 protection against predators but also against environmental hazards (Porter, 1988; Porter &  
449 Tschinkel, 1993), and it is well known that ants place brood of various developmental stages at  
450 varied nest depths to provide it with optimal environment (Penick & Tschinkel, 2008). This  
451 modification of the nest requires additional soil excavation and is energetically costly. Therefore,  
452 species with shallow nests (i.e. more exposed to increased surface temperature) or with small

453 colony size or with foundation of new colonies by a solitary queen (i.e. less capable of supporting  
454 the cost of extra excavation) may be more affected by increased temperature than species with deep  
455 nests, large populations or foundation of new colonies by colony fission (Cronin et al., 2013). For  
456 instance, queens founding new colonies solitarily could excavate deeper nests to provide optimal  
457 conditions to their brood, but this extra energetic expenditure would presumably decrease the  
458 number of workers they would produce, hence negatively affect the incipient colony survival and  
459 growth. Alternatively, they could not adjust nest depth and their brood would develop at a higher  
460 temperature than normal and presumably yield smaller workers with lower foraging efficiency and  
461 lifespan, again presumably negatively affecting the incipient colony survival and growth. In  
462 comparison, queens founding new colony by colony fission would be less affected as the workers  
463 accompanying the queen carry out nest excavation.

464 In our study, increased temperatures seem to allow the growth of the colonies. Overall, the  
465 only negative effect that we could observe in ants exposed to high temperatures ( $T_{\text{medium}}$  and  $T_{\text{high}}$ )  
466 was the reduction of worker body mass compared to those reared under room temperature ( $T_{\text{mild}}$ , Fig  
467 S8). This may result from increased metabolic costs during worker development in warm  
468 environments, as previously reported in ants (Kaspari, 2005). This could decrease workers  
469 longevity and foraging efficiency, for instance by lowering their competitiveness against larger  
470 workers of other colonies or species. Alternatively, it may be that workers reared under  $T_{\text{mild}}$  were  
471 heavier than those exposed to high and medium temperatures because the brood grew slower (as  
472 colonies grew less) and worker stored food resources as fat bodies.

473

#### 474 *Global warming and its consequences on nest and colony*

475 Our study, as well as several others (Jenkins et al., 2011; Verberk et al., 2021; Parr & Bishop, 2022;  
476 Nascimento et al., 2022), support the idea that ants would be affected by the augmentation of global  
477 temperatures. At first glance, ants successfully mitigated the effect of high temperatures by

478 excavating deeper nests as colonies grew well and produced normal-sized workers under high  
479 temperatures. However, our experiment focused on nest architecture did not include the effects of  
480 temperature on worker longevity or foraging (directs effects such as heat stress and desiccation,  
481 indirect effects such as food availability and competition). In addition, it lasted 100 days and longer  
482 exposure to high temperature may reveal accumulated effects, which are important when evaluating  
483 thermal risk (Jørgensen et al., 2021).

484         It has been suggested that ants of temperate and cold environments, such as our model  
485 species *L. niger*, are more resistant to global warming because they already deal with strong  
486 temperature variations between summer and winter (Diamond et al., 2012; Andrew et al., 2013).  
487 Our results support this hypothesis as *L. niger* showed resistance to high temperatures through nest  
488 plasticity. However, heat induced changes in *L. niger*'s nest architecture may modify physical soil  
489 structure as this species is highly abundant in temperate environments (Czerwinski et al., 1971;  
490 Holec & Frouz, 2006) and contributes to the movement of soil particles and the dynamic of water  
491 (Cerdà & Jurgensen, 2008). Moreover, modifications in colony development could have important  
492 ecological consequences due to its prevalence as topsoil predator that feeds on invertebrates, seeds,  
493 and honeydew (Czerwinski et al., 1971), and its dominance in initial stages of ecological succession  
494 (Dauber & Wolters, 2005). Our results need to be confirmed under natural conditions as our setup  
495 could not exactly mimic the natural temperature dynamics. For instance, soil humidity was kept  
496 constant, and temperature rapidly rose and fell (lamps on or off) whereas variation is more gradual  
497 in nature. However, rapid fluctuations in temperature will be increasingly common as global  
498 warming increases the intensity and frequency of heat-waves, which reduced the foraging  
499 efficiency of soil-dwelling ants living in temperate environments (Andrew et al., 2013).

500         Carbon dioxide is expected to rise in the atmosphere as a consequence of climate change.  
501 Its effects on ants are not well understood yet (Parr & Bishop, 2022) and we did not measure it in  
502 our experiment. Nevertheless, it could affect nest architecture. Ants cope with levels of CO<sub>2</sub> through  
503 ventilation systems and moving the brood to shallower chambers where the levels are lower. For

504 example, the leafcutter ants *A. lundii* prefers to excavate soils with lower content of CO<sub>2</sub>, which  
505 could explain why they excavate superficial chambers probably to suit the needs of the fungus  
506 inside and brood the nest (Römer et al., 2018). Nevertheless, these effects seem to depend on the  
507 size of the colony, as experiments with fewer individuals of *Formica pallidefulva* did not show any  
508 difference on excavation (Mikheyev & Tschinkel, 2004). We show that *L. niger* ants respond to  
509 heat by adjusting nest architecture in the laboratory, but global warming is a complex phenomenon  
510 with multiple effects (heat, humidity, gases, etc) that need to be investigated in several ant species  
511 to see how they will respond to these changes.

512

## 513 CONCLUSION

514 Our hypothesis that ants modify the architecture of their nest as a response to temperature was  
515 confirmed, since most architectural features and digging activities were increased by increased  
516 temperatures, probably to provide an adequate environment to the colony. Also, ants were able to  
517 produce normal-sized workers despite the high temperatures. As such, our study supports the idea  
518 that ant nest architecture is a plastic response to environmental changes (i.e., ant nest as an extended  
519 phenotype construction, sensu Jouquet et al., 2006; Richards, 2009; Sankovitz & Purcell, 2021).  
520 Interestingly, bioturbation was not proportional to the increase in temperature. Therefore, we  
521 foresee two different possibilities: a) our experiment reached a temperature threshold above which  
522 the effects on nest architecture and bioturbation are no longer discernible, or b) if exposure to high  
523 temperature continued for longer, additional or stronger effects would be observed. Longer studies  
524 with narrower temperature intervals between treatments would improve our understanding of the  
525 effects of increasing temperatures on ants' bioturbation and nest architecture. Moreover, more  
526 research is also needed to determine how global warming will impact ant bioturbation in different  
527 ecosystems (e.g., in the tropics, in different soil types, in presence of plants) and if this could in turn  
528 affect other ecological processes such as soil organic matter or water dynamics in soils.

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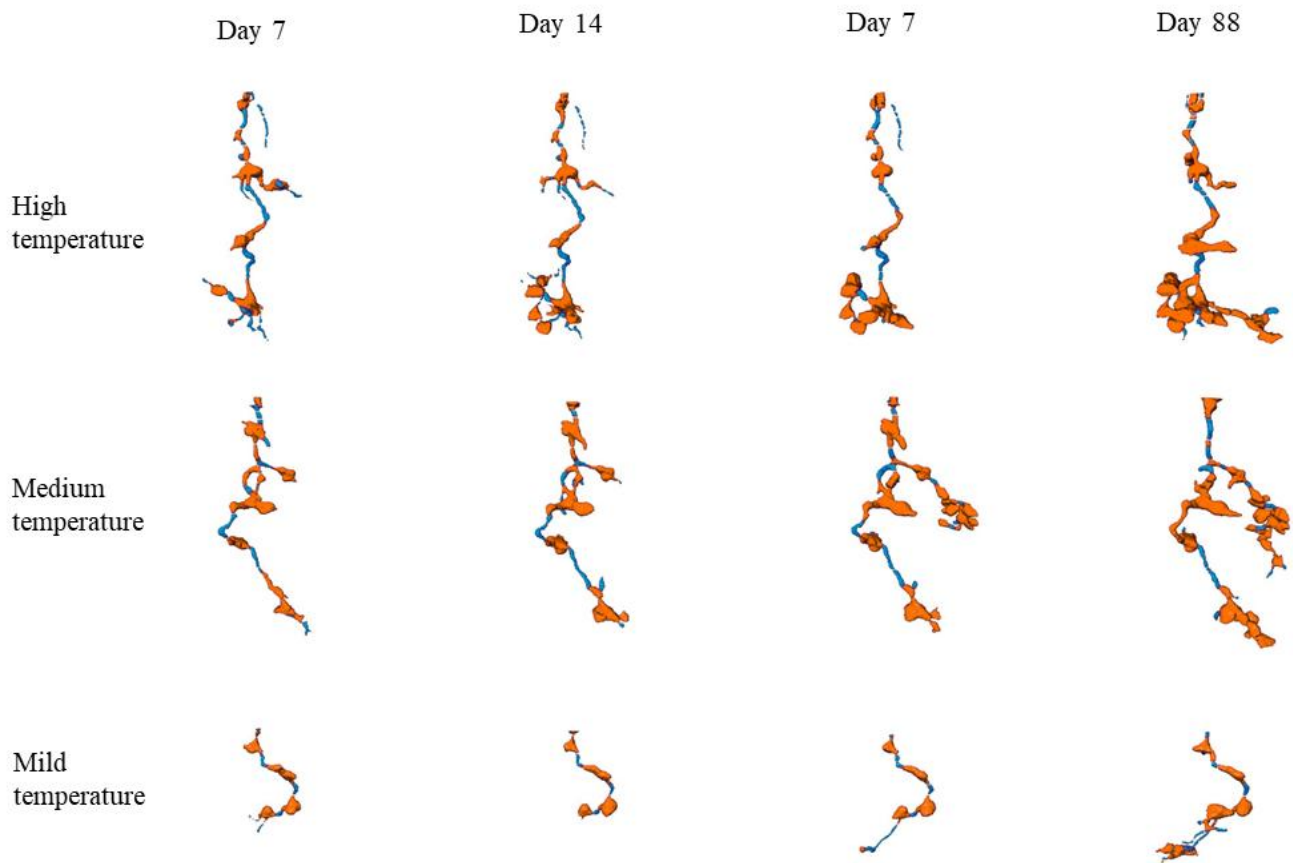


Fig 1. Examples of three nests illustrating the effects of treatment and time on their structure. Chambers (orange) and tunnels (blue) can be distinguished.

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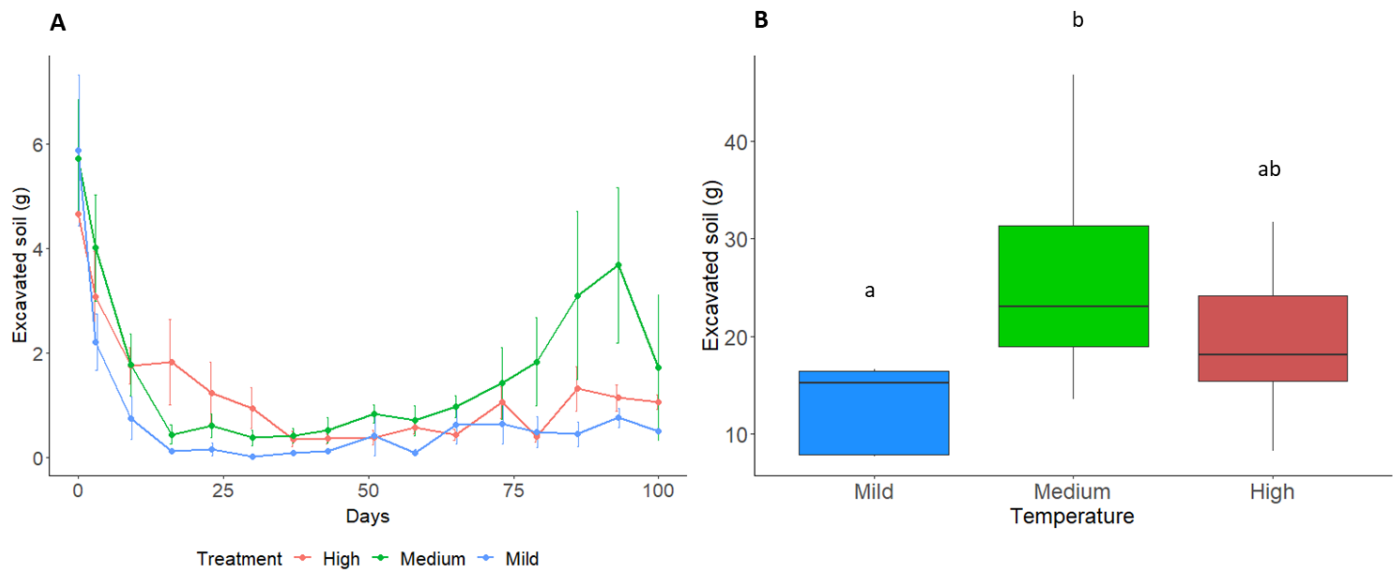


Fig 2. Amount of soil excavated (g) at each time point during the whole experiment (A) and total amount of soil excavated during the experiment (B). Bars in (A) indicate SEM. 778

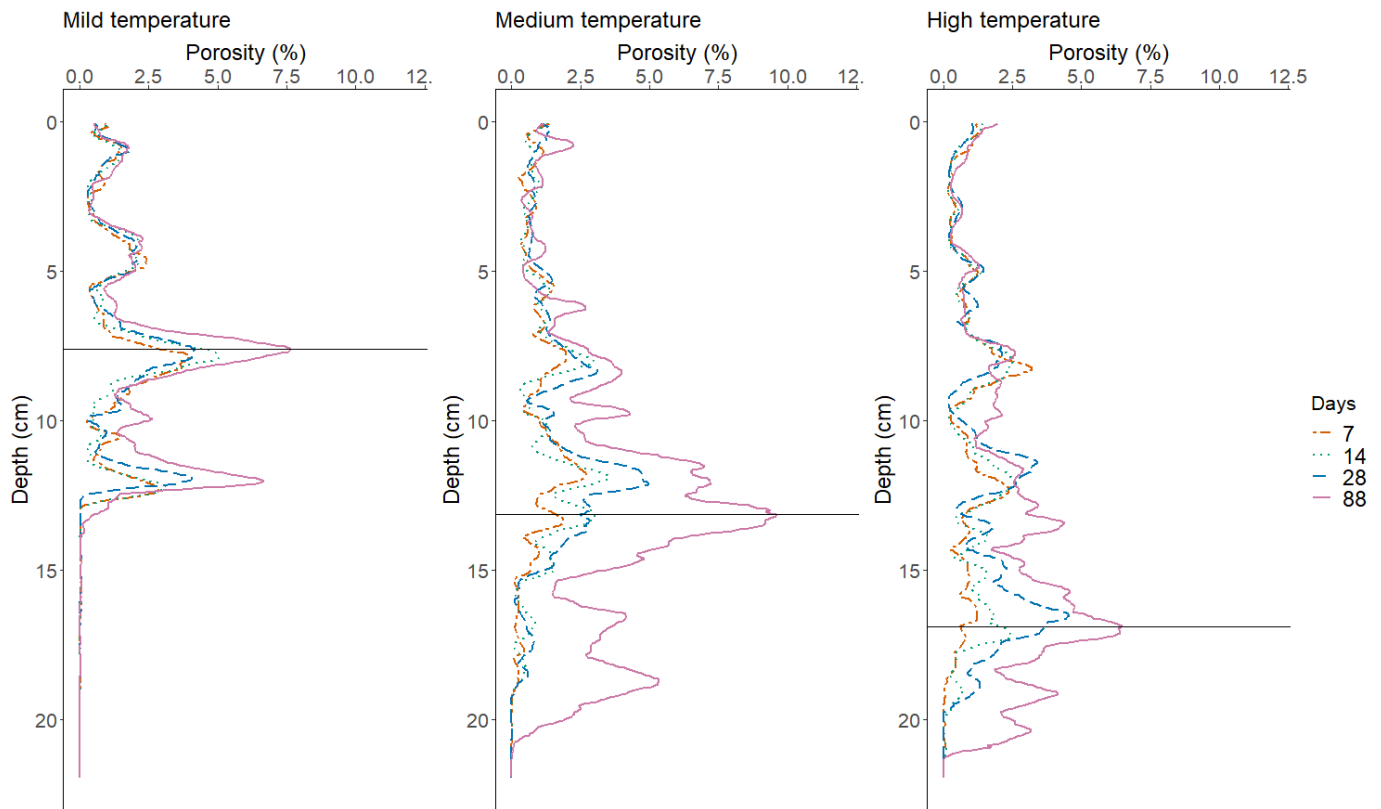


Fig 3. Mean porosity profiles (percentage of the empty volume compared to soil volume) per treatment. Colours and line types correspond to dates. Black horizontal lines indicate the point of maximal porosity on day 88.

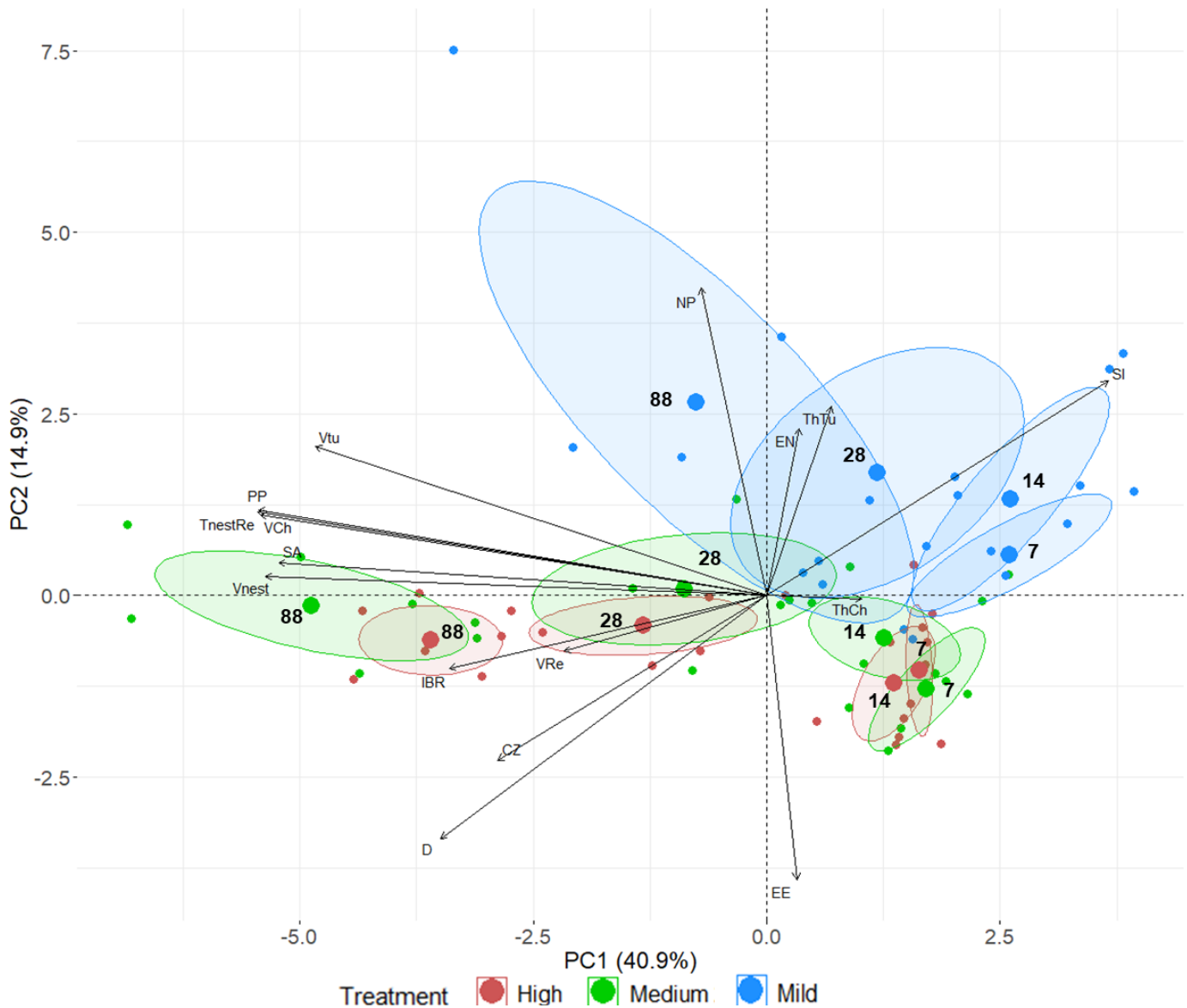


Fig 4. Biplot showing the principal component analysis (PCA) from variables describing the nest architectural features. Variables are sphericity index (SI), largest inscribed ball radius (IBR), ellipse elevation (EE), number of pores (NP), surface area (SA), Euler's number (EN), centroid Z (CZ), maximal nest depth (D), tunnel thickness (Th<sub>Tu</sub>), chamber thickness (Th<sub>Ch</sub>), nest volume (V<sub>nest</sub>), tunnel volume (V<sub>Tu</sub>), chamber volume (V<sub>Ch</sub>), refilled chambers volume (V<sub>Re</sub>), nest volume + refilled chambers volume (T<sub>nestRe</sub>) and porosity percentage (PP). All volumes are expressed in percentage of volumes compared to total column volume. Numbers correspond to the day of tomography. Ellipses correspond to the confidence interval (95%).

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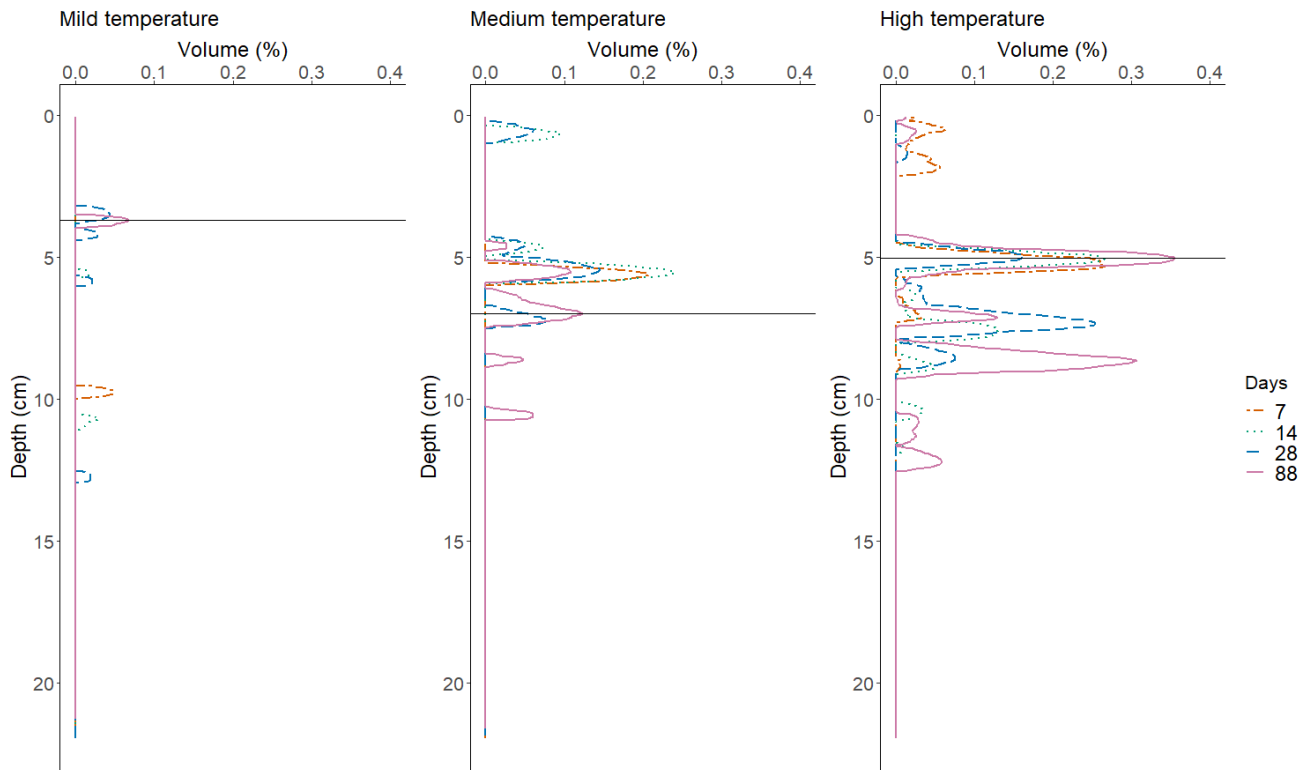


Fig 5. Mean volume profiles of refilled galleries only (percentage of  $V_{Re}$  compared to soil volume) per treatment. Colors and line types correspond to dates. Black horizontal lines indicate the point of maximal refilled volume on day 88.

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