

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

Temnothorax rugatulus ants do not change their nest walls in response to environmental
humidity

Greg T. Chism^{1*}, Wiley Faron², Anna Dornhaus^{3*}

¹ Graduate Interdisciplinary Program in Entomology and Insect Science, University of Arizona,
Tucson, Arizona, United States of America

² Department of Molecular and Cellular Biology, University of Arizona, Tucson, AZ, United
States of America

³ Department of Ecology and Evolutionary Biology, University of Arizona, Tucson, AZ, United
States of America

* Corresponding authors

E-mail: Greg T. Chism gchism@arizona.edu; Anna Dornhaus dornhaus@email.arizona.edu

GC and AD contributed conceptualization, methodology, project administration, supervision,
and original draft preparation. GC and WF contributed data curation, investigation, and
validation. GC contributed formal analysis and visualization. AD contributed funding acquisition
and resources.

28 **Abstract**

29 Animal architectures are interesting biological phenomena that can greatly increase the
30 fitness of the builder and exist in a variety of forms and functions across taxa. Among the most
31 intricate architectures are social insect nests, which may have several functions, one of which is
32 the control of internal microclimate. In social insects, the regulation particularly of humidity in
33 the nest can be crucial for the survival and growth of the brood. Though much is known on how
34 nest *excavating* social insects respond to environmental humidity, little is known about how ants
35 that build on to pre-existing cavities respond. Here we use the rock ant *Temnothorax rugatulus* to
36 determine whether and how colonies respond to environmental humidity by building and
37 changing their nest architectures in pre-existing nest spaces. We specifically test the hypothesis
38 that *T. rugatulus* colonies build different nest walls, e.g. wider or denser ones, in response to
39 lower environmental humidity. We allowed *T. rugatulus* colonies to build nest walls with two
40 substrates across a 0-100% relative humidity gradient. We further compare the porosity - empty
41 volume in built nest walls - of natural *T. rugatulus* nest walls with these artificial building
42 substrates and the substrate compositions of built walls from our experiment. We found that
43 humidity did not influence the nest walls *T. rugatulus* colonies built in our experiment,
44 concluding that regulating humidity is likely not a key function of *T. rugatulus* nest wall
45 architecture. We also found that the porosities of the artificial substrate that was predominantly
46 used by the ants in our experiment were like the porosity of natural *T. rugatulus* nest walls,
47 indicating that ants had constant preferences for particular substrates. Physical nest wall features,
48 including porosity, are therefore unlikely to be flexibly regulated in response to external
49 humidity, but may be adaptations in other ways.

50

51 **Introduction**

52 Ants are some of the most ecologically diverse and evolutionarily successful organisms [1] likely
53 at least in part due to the existence of nests that span in complexity from simple one-chambered
54 spaces to complex spaces with several dozen chambers [2–8]. Nests serve as a stable and
55 defensible space that likely facilitated the development of the social lifestyle and reproductive
56 division of labor, i.e. a separation between reproducing individuals and non-reproducing
57 ‘workers’ [1, 9]. Due to this intimate tie to their nest, social insect colonies have been referred to
58 as a ‘factory within a fortress’ [9].

59
60 Many social insects modify their nests to respond to different environmental conditions to
61 control their microclimate [10]. Regulating humidity for example has been shown to be a key
62 role in the nest architecture in some social insects, such as leaf cutter ants, in which workers plug
63 nest holes to prevent desiccation [11, 12] and mound building termites which construct a
64 sophisticated nest ventilation system that constantly changes throughout the day [13-15]
65 Humidity is particularly relevant to ants since larvae and eggs desiccate in dry conditions [16-
66 18]. Though we know much about how humidity influences social insect colonies such as the
67 above that excavate their nests by removing substrate from the ground, the effect of the abiotic
68 environment on social insect colonies that build nests by adding material, such as walls in rock
69 crevices (additive nest builders), are not well studied.

70
71 *Temnothorax rugatulus* ants are found in pine and juniper zones in northern Mexico, the
72 western United States and southwestern Canada, and have a colony size between 50 to 400 ants
73 [19]. They reside in preexisting structures such as rock crevices and acorns (i.e. arboreal and

74 hypogaeic), thus creating a dark, cool, and humid nest space. Members of the *Temnothorax*
75 genus are additive builders that utilize stones (i.e. what might, at this scale, be called sand grains)
76 and other environmental substrates to produce walls that change their occupied nest space [20-
77 21]. *Temnothorax* colonies choose a smaller-grained substrate when carrying is less energetically
78 expensive (e.g. placed closer to the nest) [22-23], but larger stones are preferred during nest
79 expansion and contraction [24], possibly because of the need for speed in building. *Temnothorax*
80 *rugatulus* colonies both build thicker walls when they have more brood, and build longer, larger
81 area walls in higher environmental humidity [25]. However, in that study, colonies were only
82 offered one grain type. Walls built with two stone sizes that are well mixed produce a higher
83 angle stability and thus are more structurally stable, because smaller grains can fill in the gaps of
84 larger grains [23]. We propose that mixed walls may also regulate nest microclimate more
85 efficiently than single substrate walls by creating a more compact structure.

86

87 In this study we asked whether the ant *Temnothorax rugatulus* produces different nest walls in
88 response to differing environmental humidity. Response to humidity is reasonable since *T.*
89 *rugatulus* colonies live inside crevices found in granite boulders in a desert environment that
90 likely experience occasional high ground temperatures and low environmental humidity. In this
91 study, we had *Temnothorax rugatulus* build nests under different relative humidity levels, and
92 then quantified differences in nest properties. We also tested whether the nest properties scaled
93 with colony size. To confirm whether environmental humidity constitutes a selective force on
94 these ants, we also measured worker and brood mortality under the humidity experienced in this
95 experiment. Brood and workers are vulnerable to desiccation, so we would expect lower
96 environmental humidity to cause more death. We also compared the porosity of *T. rugatulus*

97 walls from our experiment and natural walls collected in the field, which may relate to the degree
98 of moisture saturation that the nest wall may exhibit.

99

100 **Methods**

101 **Colony collections**

102 We collected twenty-two colonies of *Temnothorax rugatulus* from the Santa Catalina Mountains
103 (GPS: 32.395, -110.688), USA, Pima County, Arizona in a pine-forest zone (altitude
104 approximately 2500m) in October 2018. From February to October 2018, we further collected
105 ten separate *T. rugatulus* complete nest walls (e.g. in Fig 1a), which were obtained after the
106 entire colony was removed. We only collected unbroken walls to be certain that all substrate was
107 accounted for.

108

109 **Fig 1. Comparing a natural nest wall of a *Temnothorax rugatulus* colony in the field (a) and**
110 **wall built by a *T. rugatulus* colony experimentally (b).** Notably, the natural nest wall is moist
111 and caked together, where the stone (sand) walls in the lab can easily be pushed apart. In both
112 photos, the outer and inner boundaries represent the built nest wall and inside of the inner
113 boundary is where the queen(s), workers, and brood reside (internal nest area).

114

115 **Colony acclimation period**

116 We first acclimated our experimental colonies in a controlled environment produced in a climate
117 chamber for five days (see Initial housing and care). We determined experimental length through
118 a preliminary building assay, where we allowed the colonies to build nests using experimental

119 building substrates (see description below) for twenty days and found no substantial nest wall
120 changes after 10 days. We therefore set 10 days as the building duration for our future
121 experimental building phases.

122

123 **Initial housing and care**

124 During the acclimation period, we housed the colonies in 17.5cm x 12.5cm x 6cm plastic
125 containers with inside walls coated in ‘insect-a-slip’ (BioQuip product #2871A) to prevent
126 escape. We gave each colony a nest space made of two glass panes (102mm x 76mm) separated
127 by a 1.5-mm-thick strip of cardboard at the back and smaller piece of cardboard at the front [25].
128 On the opposite end of the container, we gave each colony a water-filled 5 ml plastic tube with a
129 cotton ball stopper and fed each colony *ad libitum* weekly with both a 2ml microcentrifuge tube
130 of honey water with a concentration of 1/4 teaspoon per 50ml water, and 1/8 of a fresh-frozen
131 cockroach (approximately 0.075g) (*Nauphoeta cinerea*). During acclimation and between
132 experimental trials, we kept colonies in a climate chamber with a 12:12 h light cycle (8 a.m. to 8
133 p.m.), constant temperature (approximately 20°C) and relative humidity (approximately 20-
134 25%).

135

136 **Experimental timeline**

137 We exposed each colony to one of nine humidity levels (see S1 Table). We allowed the humidity
138 in each container to stabilize for one day, at which point we then provided the experimental
139 building substrates for colonies to build for 10 days. We photographed the colony on days 1, 5,
140 and 10 to determine the average number of workers and brood, but only considered day 10 for
141 calculating nest wall properties. We gave colonies a 10-day rest period in ambient temperature

142 and humidity before the second trial in which we placed the colonies into the humidity three
143 places forward along the gradient (i.e. 55% went to 85%, and 85% went to 1%).

144

145 **Experimental building substrates**

146 We chose two distinct building substrates to allow *T. rugatulus* colonies to modify their nest
147 spaces. We offered 10g of both 1.25mm diameter white aquarium substrate (CaribSea Super
148 Naturals™ white aquarium substrate: substrate I - weight of 100 pieces = 0.529g) and 0.65mm
149 diameter black aquarium substrate (Flourite® black sand: substrate II - weight of 100 pieces =
150 0.035g) as building materials. Though substrate I would cover more area per grain when
151 building, its weight per grain is 15x larger than substrate II, making it harder to transport.

152

153 **Experimental setup**

154 **Experimental nest housing**

155 We placed colonies in new nest sites and containers following the same protocol as during the
156 acclimation period (see Fig 2 for a full set visualization).

157

158 **Fig 2. Experimental set up for each humidity treatment.** Ants are confined to the nest
159 container, which contains a small pile of each building substrate and food and water. A small fan
160 gently circulated air above the saturated salt water to ensure homogenous humidity across the
161 system. While this system was closed (locked airtight), a small hole was cut from the lid
162 allowing access to the nest container to deposit each building substrate, thus temporarily opening
163 the system when needed.

164

165 **Experimental humidity levels**

166 For each experimental trial, we placed the nine individual colonies and their nest containers into
167 larger plastic containers (31.3cm x 23cm x 10.2cm). We created a discrete-step humidity
168 gradient consisting of 9 separate boxes, each with a different, constant, regulated humidity level.
169 Eight of these were achieved by using saturated salt solutions, which produce highly replicable
170 humidity levels at 20°C in a closed, airtight system (Fig 2; S1 Table; Winston and Bates 1960;
171 Greenspan 1977). We used the desiccant phosphorous pentoxide to produce nearly 0% RH
172 (Winston and Bates 1960). We placed each saturated salt solution and desiccant in a plastic
173 container (17.5cm x 12.5cm x 6cm) next to the nest container in the experimental setup (Fig 2).
174 We used a DC current fan (4cm x 4cm x 4cm, 12V, 0.1A) in the top left corner of the
175 experimental setup to circulate air in the closed system, since saturated salt solutions require air
176 circulation for reproducibility (Winston and Bates 1960). This fan was placed in the top left
177 corner above the saturated salt solution, angled horizontally, facing parallel to the length of the
178 box (Fig 2).

179

180 **Substrate, food, and water placement**

181 We inserted substrates, food, and water through a small 3.5cm hole above the nest section, which
182 only temporarily broke the closed, airtight system of the colony box. We placed individual 10.0g
183 piles of each building substrate at the opposite end of the housing nest, such that one substrate
184 type was on the left and the other on the right. On day 7 of the experiment, we provided new 5ml
185 cotton-ball-clogged water tubes, 5ml honey water microcentrifuge tubes, and 1/8 fresh-frozen
186 cockroaches to continue *ad libitum* feeding. We randomized the arrangement of the building
187 substrates such that half of the colonies had the heavier substrate on the left side and the other

188 half on the right side (Fig 2). During the second trial we flipped each colony's substrate
189 placement. We performed this procedure to prevent a side bias from affecting building substrate
190 choice.

191

192 **Data collection**

193 **Environmental data**

194 During each experimental round, we recorded the temperature (°C) and relative humidity (%) of
195 our experimental closed systems every 45 minutes using permanently imbedded U12-012 HOBO
196 data loggers (Onset, Bourne, MA, USA) to ensure stability and reproducibility of each section of
197 the relative humidity gradient (S1 Table for experimentally produced humidity levels).

198

199 **Image capture and analysis**

200 We photographed each colony with an HD camera (Nikon D7000 with 60mm lens). We used the
201 image analysis software *Fiji* [28] to measure the wall length (mm), wall area (mm²), and nest
202 area (mm²) for every colony, which are measurement methods we derived from [25].

203 Additionally, we assigned coordinates to every worker and brood item in the nest. We
204 standardized all measurements and coordinates from *Fiji* using the x-distance between the top
205 left and bottom right of the glass pane as reference points as the known distance of 102mm.

206

207 **Nest wall composition**

208 After each building period, we collected each built wall by gently tilting a colony's housing nest
209 space and extracting the grains while the ants were inside the nest, allowing us to prevent panic.

210 We sieved the nest walls built by each colony using a 1mm colander to separate the two

211 substrates, and then weighed each substrate using a digital scale (Ohaus, USA) to the nearest
212 0.00001g.

213

214 **Nest wall weight and density**

215 We determined wall weight by weighing the substrates each colony used to build their nest walls.

216 We calculated wall volume (mm^3) as the nest area multiplied by 1.5 mm (the height of the

217 provided nest cavity). We then calculated nest wall density as total wall weight (g) / wall volume

218 (mm^3).

219

220 **Building substrate porosity**

221 We used the collected substrates (see colony collections) of real *T. rugatulus* nest walls to

222 compare the porosity between natural nests and our artificial building substrates. We allowed the

223 substrates to dry in open air for seven days before storing them again. Our final sample size was

224 10 measures of porosity for the natural and each experimental substrate. Porosity (Pt) is

225 calculated by determining the void space (Vp) in which water can fill in a substrate and dividing

226 it by the bulk volume (Vt) which is the void and substrate (Vs) volumes: $Vt = Vp + Vs$; $Pt =$

227 $(Vp/Vt) \times 100$.

228

229 **Experimental substrates**

230 We measured the porosity of each artificial substrate by filling a 5 ml tube with 2 ml of each

231 substrate (total volume: Vt). We determined the pore volume by fully saturating the substrate

232 with deionized water injected through a syringe to the 2 ml mark. We took water from a

233 container of water (weighed to the nearest 0.001g) and then determined the volume used (Vp) by

234 subtracting the remaining container weight from the original weight of water. We converted
235 water weight to volume per the 1g/ml standard conversion for pure water. We then calculated
236 porosity for each substrate using the formula: $Pt = (Vp/Vt) \times 100$.

237

238 **Natural nest walls**

239 To compare the porosities of natural and experimentally built nest walls, we measured the
240 porosity of natural wall substrates by filling 5 ml tubes with each natural nest's substrates and
241 then marking where the tube was filled. We again filled the container with deionized water from
242 a container of water (weighed to the nearest 0.001g) to complete saturation at the marked
243 substrate volume line, then we subtracted the remaining container weight from the original
244 weight of water to determine the pore volume (Vp). We removed the substrates from the
245 containers and filled water to the line representing the volume of each substrate and weighed that
246 amount to the nearest 0.001g (Vt). We again converted volume from water weight to volume per
247 the standard 1g/ml conversion for pure water. We then calculated porosity for each substrate
248 using the formula: $Pt = (Vp/Vt) \times 100$.

249

250 **Final data and analyses**

251 We conducted all data wrangling, analyses, and visualizations in the software R (*v4.1.1*) [29] in
252 RStudio (*v1.2.5042*) [30], primarily utilizing the tidyverse language ('tidyverse' *v1.3.1*) [31]. We
253 have made the final data and R script used for this study openly available in a GitHub repository:
254 <https://github.com/Gchism94/HumidityProject>

255

256 **Humidity treatment final data set**

257 We had different final sample sizes for the first (trial 1) and second (trial 2) humidity treatments
258 a colony experienced. We did not include three colonies in our final trial 1 data set due to colony
259 death (trial 1 final N = 19 colonies). Three colonies were not placed into a second treatment since
260 the first relative humidity produced in the first trial was not reliable (trial 2 final N = 16).

261

262 **Humidity treatment analyses**

263 Substrate preference: we used Mann-Whitney U tests to see whether colonies built their walls
264 with a preferred substrate.

265

266 Influence of humidity and colony size on built walls: we took average values for worker and
267 brood number (see image capture and analysis) to reduce measurement error from worker and
268 brood occlusion in nest containers. We used linear mixed effects models using the packages to
269 examine whether relative humidity or colony size influenced nest wall feature, or internal nest
270 area. All linear mixed effects models here and below were conducted using the R package ‘lme4’
271 (*v1.1-27.1*; Bates et al., 2014), where p values were calculated through the R package ‘lmerTest’
272 (*v3.1-3*; Kuznetsova et al., 2017). We found that an order effect was present when considering a
273 colony's first and second trial placement, which we could not separate from the humidity
274 treatment placement due to unequal sample sizes (lower:higher N = 14; higher:lower = 5). We
275 therefore assigned the first and second trial (‘Trial’) as a random effect in our models, and by
276 comparing the variation explained by the fixed effects alone (marginal R^2) and with the random
277 effect included (conditional R^2), we determined the amount of variation that Trial number
278 explained (marginal and conditional R^2 values calculated through the R package ‘MuMIn’
279 *v1.43.17*; Kamil Bartoń 2020).

280

281 Worker and brood mortality and humidity exposure: we first used a linear regression to test
282 whether brood and worker death were correlated. We then calculated worker and brood mortality
283 as the proportion of average workers or brood in trial 2 over the average in trial 1, signifying
284 how many of each died in between trials (10 days). We used binomial family generalized linear
285 models to test whether the relative mortality of workers and brood was affected by the level of
286 relative humidity each colony experienced in trial 1. Finally, we tested whether colony mortality
287 was higher in smaller or larger colonies (average number of brood or workers) by comparing the
288 two linear regressions with an ANOVA: formula = $\log(\text{Trial 2 colony size}) \sim \log(\text{Trial 1 colony}$
289 $\text{size})$; formula = $\log(\text{Trial 2 colony size}) \sim 1 + \text{offset}(\text{Trial 1 colony size})$. The offset function
290 changes the model intercept to 1, where smaller colonies experienced higher mortality with a
291 model intercept smaller than 1 and larger colonies experienced higher mortality with a model
292 intercept greater than 1.

293

294 **Building substrate porosity analysis**

295 We used pairwise Dunn's tests with False Discovery Rate corrected p values [35] to compare the
296 median porosity of our experimental and collected natural nest wall substrates (each N = 10).

297

298 **Post hoc power analyses**

299 We used the R package *simr* (v1.0.6) [36, 37] to calculate post hoc power analyses for each of
300 our linear mixed effects models where we used 0.5 and 0.8 as biologically relevant Cohen's d
301 effect sizes (moderate and high effect sizes) [38]. We first derived the appropriate effect size for
302 each model ('Humidity' fixed effect β coefficient) from the formulas: Cohen's $d = \beta / (\text{sqrt}(N) \times$

303 SE), where N = sample size and SE = standard error of each ‘Humidity’ term. The simr package
304 determines statistical power by (i) simulating new values for the response variable from our
305 models; (ii) refitting our models to the simulated response variable; (iii) applying a likelihood
306 ratio test to the simulated model fit. Statistical power is then determined by the ratio of
307 significant p values over non-significant.

308

309 **Results**

310 **Ants prefer smaller-grained substrate but show no side bias**

311 We used two-sided Wilcoxon tests with a predicted median value of 0.5 to show that ants
312 showed a substrate preference in trial 1 ($W = 183$, $p < 0.001$) and trial 2 ($W = 132$, $p < 0.001$).
313 The predominant substrate that ants used for building was the smaller-grained substrate: in trial
314 1, the median weight of substrate I that colonies used to build walls 0.160g, and 0.516g for
315 substrate II, with the median proportion of substrate II per substrate I being 0.746, while in trial 2
316 the median grams of substrate I used was 0.011, and 0.073 substrate II, with the median
317 proportion of substrate II per substrate I being 0.849.

318

319 **Relative humidity did not influence any measured nest trait**

320 In our experiment, ants did not change any wall characteristics with environmental relative
321 humidity levels (linear mixed effects models: wall weight - $p = 0.772$; Fig 3a, S2 Table; wall
322 length - $p = 0.459$; Fig 3b, S3 Table, wall area - $p = 0.978$; Fig 4c, S4 Table, wall density - $p =$
323 0.653; Fig 4d, S5 Table, wall substrate composition - $p = 0.248$; Fig 4e, S6 Table, internal nest
324 area - $p = 0.215$; Fig 4f, S7 Table). ‘Humidity’ as a fixed effect explained little to none of the

325 variation in the data, while random effects did explain most of the data variation (S2-6 Tables),
326 except for internal nest area (S7 Table). Since we did not find an effect of humidity on any of our
327 measured nest traits, but our sample sizes may be argued to be small, we ran post hoc power
328 analyses to determine the statistical power of each linear mixed effects model. Our models had
329 80%-85.0% power (therefore at least conventional power) [38] to find any effects of 0.5 (mean
330 difference, β , divided by standard deviation) or higher and 98%-100% power to find any effects
331 of 0.8 or higher, indicating that our negative results are likely not a consequence of low power
332 (S8 Table), and we can conclude that if any effects existed they are likely to be small.

333

334 **Fig 3. The built nest wall traits measured show no relationship with external humidity**
335 **levels for trial 1 or 2.** Wall weight (a), length (b), area (c), density (d), proportion of substrate II
336 in nest wall (e), and internal nest area (f). Here, and below, trial 1 data points are circles and trial
337 2 are triangles.

338

339 **No evidence for colony size influencing nest traits**

340 Colonies used in our experiment varied in their demography (note that colony size was
341 calculated by averaging observations of workers and brood from days 1, 5, and 10 of the
342 experiment): the median colony size was 84 workers in trial 1 (range 13-300; 65.3 brood items,
343 range 2-259; 2 queens, range 1-11) and 65.5 workers in trial 2 (range 15-159; 65.7 brood items,
344 range 11 - 190; 2 queens, range 1-11). In our experiment, ant colony size (number of brood or
345 workers) did not influence any wall characteristics (linear mixed effects models: wall weight -
346 brood: $p = 0.275$, workers: $p = 0.517$; Fig 4a, S9 Table, wall length - brood: $p = 0.054$, workers:
347 $p = 0.367$; Fig 4b, S10 Table, wall area - brood: $p = 0.178$, workers: $p = 0.625$; Fig 4c, S11

348 Table, wall density - brood: $p = 0.288$, workers: $p = 0.448$; Fig 4d, S12 Table; wall substrate
349 composition - brood: $p = 0.622$, workers: $p = 0.142$; Fig 4e, S13 Table, internal nest area - brood:
350 $p = 0.488$, workers: $p = 0.730$; Fig 4f, S14 Table). Since we did not find an effect of colony size
351 on any of our measured nest traits, but our sample sizes may be argued to be small, we ran post
352 hoc power analyses to determine the statistical power of each linear mixed effects model. Our
353 models had 64%-81% power to find any effects of 0.5 (mean difference, β , divided by standard
354 deviation) or higher and 99%-100% power to find any effects of 0.8 or higher, indicating that our
355 negative results are likely not a consequence of low power (S15 Table). Notably, in [25] nest
356 wall area increasing with brood number with an effect size (calculated as we did above) of 0.52
357 and our model testing for this relationship had 78% statistical power, so we likely had sufficient
358 power to detect an analogous effect.

359

360 **Fig 4. The built nest wall traits measured show no relationship with the number of brood**
361 **(blue) or workers (red) in a colony for trial 1 or 2.** Wall weight (a), length (b), area (c),
362 density (d), proportion of substrate II in nest wall (e), and internal nest area (f).

363

364 **Colony mortality did not relate to relative humidity, but larger**
365 **colonies had higher mortality**

366 We found that worker and brood mortality was highly correlated ($\beta = 1.042 \pm 0.168$, $p < 0.001$;
367 S1 Fig, S16 Table). We however found no relationship between relative humidity and the
368 proportion of colony member deaths between trials (10-days) in our generalized linear models
369 (Workers: $p = 0.694$; Brood: $p = 0.756$; S2 Fig, S17 Table). We lastly found that the slopes in
370 our linear models predicting colony size in trial 2 from trial 1 were less than 1 (S2 Fig, S18

371 Table) and were significantly different than models with a slope of 1 (ANOVA: Brood: $F = 8.47$,
372 $p = 0.021$; Workers: $F = 6.79$, $p = 0.011$; S19 Table). Slopes smaller than 1 indicate that
373 mortality was lower in larger colonies, but since there was no correlation with humidity, the
374 cause is likely not desiccation from low relative humidity or disease (e.g. fungal infection) from
375 high relative humidity.

376

377 **Colonies preferred the more porous building substrate, which** 378 **resembled natural nest walls**

379 We found that ants prefer a more porous substrate: substrate I, the one with smaller grains and
380 thus lower porosity, was not preferred; and in fact, the walls built in our experiment had similar
381 porosity to natural walls collected in the field ($Z = 154$, $p = 0.077$), as well as being similar to
382 pure substrate II ($Z = 134$, $p = 0.345$) (Fig 5, S20 Table). However, the natural wall substrate was
383 likely not as compact in our porosity assays as in nature, which could possibly change the results.
384 In natural walls, substrate appears tamped down, whereas it was loosely shaken into the test vial
385 for our porosity assays.

386

387 **Fig 5. Comparison of porosity between the artificial and natural *Temnothorax rugatulus***
388 **nest wall substrates.** Bars denote sample medians, boxes represent the first and third quantiles,
389 lines denote the data range. Stars denote significant Dunn's pairwise multiple comparisons tests.

390

391 **Discussion**

392 Our study does not support the hypothesis that environmental humidity affects nest building in
393 *Temnothorax rugatulus*. We found no influence of external humidity on any measured nest
394 property (Fig 3). This might indicate that the nest wall properties are not under selection for
395 maintaining a certain humidity level inside the nest space, or that if they are, they are not plastic
396 (ants do not flexibly adapt them to changing needs for insulation from the environment). We also
397 did not find the effect reported in [25], that nest wall area scales up with brood number in a
398 colony (Fig 4), nor did any other nest property increase with colony size (Fig 4). This
399 inconsistency could stem from different experimental designs – the authors in [25] considered
400 low and high humidity while our study had nine humidity levels - or from the low statistical
401 power of our study. We additionally did not find a relationship between the first relative
402 humidity level that colonies experienced in our study and worker or brood mortality; we did find
403 that larger colonies had overall higher relative mortality (S1-2 Figs). We lastly saw that both our
404 small experimental substrate (substrate II) and the built experimental walls are significantly more
405 porous than the larger experimental substrate but had similar porosity to natural *T. rugatulus*
406 built nest walls. This may be because ants are aiming for porous walls, or it may result from a
407 preference for carrying larger sand grains (which may make the building process more efficient)
408 [23].

409
410 Humidity regulation is only one function of social insect nests; however, the internal humidity of
411 the nest can be so important that social insects build structural modifications towards its strict
412 regulation. Examples of these structures include ventilation turrets and thatched nests in *Atta*
413 leafcutter ants [11, 12, 39, 40] and thicker, reinforced mounds along the east-to-west nest axes of
414 *Macrotermes* termites towards retaining water in the nest [13-15]. Here we show in contrast that

415 environmental humidity does not influence *Temnothorax rugatulus* nest wall structure inside nest
416 cavities, suggesting that humidity regulation is not a function of *Temnothorax* nests. Indeed,
417 intrinsic (genetics) rather than extrinsic (temperature and humidity) factors are shown to be more
418 influential towards the nest architectures that harvester ants build [41]. In addition, our *T.*
419 *rugatulus* colonies selected a less energetically expensive substrate to build with (substrate II),
420 consistent with previous work on wall building substrate choice in *Temnothorax albipennis*
421 colonies [22]. Changing built nest walls in response to humidity might be energetically
422 expensive, which may constrain the flexibility that *T. rugatulus* has in regulating in-nest
423 humidity through wall composition. Additionally, in the desert, external humidity and
424 temperature can change quickly and thus plastic adjustment of nest walls may not be possible,
425 leading to ants building a nest wall structure that is suitable at all levels of humidity.
426 Alternatively, humidity may not be regulated in *T. rugatulus* nests, but instead are
427 physiologically resistant to varying environmental humidity. Instead, *T. rugatulus* colonies may
428 consider other purposes such as nest defense or regulating worker interactions through changing
429 nest densities.
430
431 The innate internal humidity of nest cavities may be an important consideration for *Temnothorax*
432 nest site selection. *Temnothorax* ants demonstrate extensive decision-making in house-hunting
433 [42-46], which relates several properties in potential nest cavities. For example, emigrating
434 colonies determine nest size through interactions with other exploring nest mates (quorum
435 sensing) [47]. Also, nest cavities that have smaller nest entrances [44, 48] are more sought after
436 by *Temnothorax* ants for properties such as less light invasion [48]. Additionally, rock-dwelling
437 *Temnothorax albipennis* colonies have been shown to remove substrate from new nest cavities in

438 relation to worker density in the nest (i.e. nest ‘molting’) [27], posing an alternative mechanism
439 that may also regulate in-nest humidity. Therefore, either innate nest cavity properties, or
440 alternative mechanisms to nest wall building, may produce a desirable humidity inside of the
441 nest space (i.e. rock crevices), where *Temnothorax* ants do not need to build nest walls towards
442 its regulation.

443

444 Our study is also the first to consider the porosity of the natural and artificial substrates used by
445 *Temnothorax* ants for wall building. Porosity, i.e. the amount of void space between the packed
446 substrate, may influence nest properties in a variety of ways, including thermoregulation and
447 moisture retention, as well as costs of building per volume of wall, none of which has been well
448 studied so far in *Temnothorax* ants. In addition, the soil available to *Temnothorax* ants in nature
449 likely exhibits a variety of properties that don’t exist in grains of sand, such as the ability to
450 retain moisture in extremely small soil and cellulose grain sizes. The natural nest walls of
451 *Temnothorax rugatulus* ants can be very densely packed which translated to very slow water
452 penetration in our porosity assays when compared to the virtually instantaneous water
453 penetration of our experimental walls. The mud-brick-like natural *T. rugatulus* nest walls (GC
454 personal observation, but also see Fig 1) may therefore trap humidity differently than loosely
455 packed stone walls. A separate experiment would test this by providing substrates with a variety
456 of weights, types, and sizes could allow rock dwelling ants to select material that produces more
457 compact nest walls than previously possible in both ours and past studies [22, 23, 25].

458 Alternatively, *Temnothorax* ants may just build with what is available and produce walls with a
459 random mix of substrates that are more energetically efficient to build and those that retain more
460 moisture. Notably, colonies of the ponerine ant *Rhytidoponera metallica* also dwell in rocks and

461 build nest modifications from environmental substrates [49, 50], and other rock dwelling ants
462 differ in their nest size preference [50]. We suggest that exploring the traits of substrates that
463 rock-dwelling ants build nest walls with will provide greater insight to the purpose of these nest
464 walls.

465

466

467

468

469

470

471

472

473

474

475

476

477

478

479

480

481

482

483

484 **Acknowledgements**

485 We thank Dr. Nicholas DiRienzo for his insights and help with the photo analysis.

486

487 **References**

- 488 1. Wilson EO, Kinne O. Success and dominance in ecosystems: the case of the social
489 insects. Oldendorf/Luhe: Ecology Institute; 1990. Available From: [https://www.int-](https://www.int-res.com/articles/eebooks/eebook02.pdf)
490 [res.com/articles/eebooks/eebook02.pdf](https://www.int-res.com/articles/eebooks/eebook02.pdf)
- 491 2. Jeanne RL. The adaptiveness of social wasp nest architecture. Q Rev Biol. 1975
492 Sep;50(3):267-87. doi: [10.1086/408564](https://doi.org/10.1086/408564)
- 493 3. Seeley TD, Seeley RH, Akrotanakul P. Colony defense strategies of the honeybees in
494 Thailand. Ecol Monogr. 1982 Mar;52(1):43-63. doi: [10.2307/2937344](https://doi.org/10.2307/2937344)
- 495 4. Tschinkel WR. Seasonal life history and nest architecture of a winter-active ant,
496 *Prenolepis imparis*. Insectes Soc. 1987 Sep;34(3):143-64. doi: [10.1007/BF02224081](https://doi.org/10.1007/BF02224081)
- 497 5. London KB, Jeanne RL. The interaction between mode of colony founding, nest
498 architecture and ant defense in polistine wasps. Ethol Ecol Evol. 2000 Mar;12(1):13-25.
499 doi: [10.1080/03949370.2000.9728440](https://doi.org/10.1080/03949370.2000.9728440)
- 500 6. Tschinkel WR. The nest architecture of the ant, *Camponotus socius*. J Insect Sci. 2005
501 Jan;5(1):9. doi: [10.1093/jis/5.1.9](https://doi.org/10.1093/jis/5.1.9)
- 502 7. Tschinkel WR. The nest architecture of three species of north Florida *Aphaenogaster*
503 ants. J Insect Sci. 2011 Jan;11(1):105. doi: [10.1673/031.011.10501](https://doi.org/10.1673/031.011.10501)
- 504 8. Pinter-Wollman N. Nest architecture shapes the collective behaviour of harvester ants.
505 Biol Lett. 2015 Oct;11(10):20150695. doi: [10.1098/rsbl.2015.0695](https://doi.org/10.1098/rsbl.2015.0695)

- 506 9. Wilson EO. The ergonomics of caste in the social insects. *Am Nat.* 1968
507 Jan;102(923):41-66. doi: [10.1086/282522](https://doi.org/10.1086/282522)
- 508 10. Hansell M. *Animal architecture*. 1st edition. Oxford University Press on Demand; 2005.
509 doi: [10.1093/acprof:oso/9780198507529.001.0001](https://doi.org/10.1093/acprof:oso/9780198507529.001.0001)
- 510 11. Bollazzi M, Roces F. To build or not to build: circulating dry air organizes collective
511 building for climate control in the leaf-cutting ant *Acromyrmex ambiguus*. *Anim Behav.*
512 2007 Nov;74(5):1349-1355. doi: [10.1016/j.anbehav.2007.02.021](https://doi.org/10.1016/j.anbehav.2007.02.021)
- 513 12. Bollazzi M, Roces F. Leaf-cutting ant workers (*Acromyrmex heyeri*) trade off nest
514 thermoregulation for humidity control. *J Ethol.* 2010 May;28(2):399-403. doi:
515 [10.1007/s10164-010-0207-3](https://doi.org/10.1007/s10164-010-0207-3)
- 516 13. Noirot C, Darlington JP. Termite nests: architecture, regulation and defence. *Termites:*
517 *evolution, sociality, symbioses, ecology*. Dordrecht: Springer; 2000:121-139. doi:
518 [10.1007/978-94-017-3223-9_6](https://doi.org/10.1007/978-94-017-3223-9_6)
- 519 14. Korb J. Thermoregulation and ventilation of termite mounds. *Naturwissenschaften.* 2003
520 May;90(5):212-9. doi: [10.1007/s00114-002-0401-4](https://doi.org/10.1007/s00114-002-0401-4)
- 521 15. Korb J. Termite mound architecture, from function to construction. *Biology of termites: a*
522 *modern synthesis*. Dordrecht: Springer; 2010:349-373. doi: [10.1007/978-90-481-3977-](https://doi.org/10.1007/978-90-481-3977-4_13)
523 [4_13](https://doi.org/10.1007/978-90-481-3977-4_13)
- 524 16. Brian MV, Hibble J, Stradling DJ. Ant pattern and density in a southern English heath.
525 *The J Anim Ecol.* 1965 Oct;1:545-55. doi: [10.2307/2448](https://doi.org/10.2307/2448)
- 526 17. Coenen-staß D. Investigations on the water balance in the red wood ant, *Formica*
527 *polyctena* (Hymenoptera, Formicidae): workers, their larvae and pupae. *Comp Biochem*
528 *Physiol A Physiol.* 1986 Jan;83(1):141-7. doi: [10.1016/0300-9629\(86\)90101-5](https://doi.org/10.1016/0300-9629(86)90101-5)

- 529 18. North RD. Transpiration and humidity preference in a temperate wood ant *Formica rufa*
530 L. *J Insect Physiol.* 1991 Jan;37(4):279-86. doi: [10.1016/0022-1910\(91\)90062-5](https://doi.org/10.1016/0022-1910(91)90062-5)
- 531 19. Bengston SE. Life-history and behavioral trait covariation across 3 years in *Temnothorax*
532 ants. *Behav Ecol.* 2018 Nov;29(6):1494-1501. doi: [10.1093/beheco/ary101](https://doi.org/10.1093/beheco/ary101)
- 533 20. Franks NR, Wilby A, Silverman BW, Tofts C. Self-organizing nest construction in ants:
534 sophisticated building by blind bulldozing. *Anim Behav.* 1992 Aug;44:357-75. doi:
535 [10.1016/0003-3472\(92\)90041-7](https://doi.org/10.1016/0003-3472(92)90041-7)
- 536 21. Franks NR, Deneubourg JL. Self-organizing nest construction in ants: individual worker
537 behaviour and the nest's dynamics. *Anim Behav.* 1997 Oct;54(4):779-96. doi:
538 [10.1016/0003-3472\(92\)90041-7](https://doi.org/10.1016/0003-3472(92)90041-7)
- 539 22. Aleksiev AS, Longdon B, Christmas MJ, Sendova-Franks, AB, Franks NR. Individual
540 choice of building material for nest construction by worker ants and the collective
541 outcome for their colony. *Anim Behav.* 2007 Sep;74(3):559-566. doi:
542 [h10.1016/j.anbehav.2006.12.019](https://doi.org/10.1016/j.anbehav.2006.12.019)
- 543 23. Aleksiev AS, Sendova-Franks AB, Franks NR. The selection of building material for
544 wall construction by ants. *Anim Behav.* 2007 May;73(5), 779-788. doi:
545 [10.1016/j.anbehav.2006.06.014](https://doi.org/10.1016/j.anbehav.2006.06.014)
- 546 24. Aleksiev AS, Sendova-Franks AB, Franks NR. Nest 'moulting' in the ant *Temnothorax*
547 *albipennis*. *Anim Behav.* 2007 Sep;74(3), 567-575. doi: [10.1016/j.anbehav.2006.12.023](https://doi.org/10.1016/j.anbehav.2006.12.023)
- 548 25. DiRienzo N, Dornhaus A. *Temnothorax rugatulus* ant colonies consistently vary in nest
549 structure across time and context. *PLoS One.* 2017 Jun;12(6):e0177598. doi:
550 [10.1371/journal.pone.0177598](https://doi.org/10.1371/journal.pone.0177598)

- 551 26. Winston PW, Bates DH. Saturated solutions for the control of humidity in biological
552 research. *Ecology*. 1960 Jan;41(1):232-7. doi: [10.2307/1931961](https://doi.org/10.2307/1931961)
- 553 27. Greenspan L. Humidity fixed points of binary saturated aqueous solutions. *J Res Natl Bur*
554 *Stand A Phys Chem*. 1977 Jan;81(1):89. doi: [10.6028/jres.081A.011](https://doi.org/10.6028/jres.081A.011)
- 555 28. Schindelin J, Arganda-Carreras I, Frise E, Kaynig V, Longair M, Pietzsch T, et al. Fiji: an
556 open-source platform for biological-image analysis. *Nat Methods*. 2012 Jul;9(7):676-82.
557 doi: [10.1038/nmeth.2019](https://doi.org/10.1038/nmeth.2019)
- 558 29. Team RC. R: A language and environment for statistical computing. Available From:
559 URL <https://www.R-project.org/>.
- 560 30. Allaire J. RStudio: integrated development environment for R. Boston, MA.
561 2012;770(394):165-71. Available From: <http://www.rstudio.com/>.
- 562 31. Wickham H, Averick M, Bryan J, Chang W, McGowan LD, François R, et al. Welcome
563 to the Tidyverse. *J Open Source Softw*. 2019 Nov 21;4(43):1686. doi:
564 [10.21105/joss.01686](https://doi.org/10.21105/joss.01686)
- 565 32. Bates D, Mächler M, Bolker B, Walker S. Fitting linear mixed-effects models using
566 lme4. arXiv:1406.5823 [Preprint]. 2014 [cited 2022 May 18]. Available from:
567 [10.18637/jss.v067.i01](https://doi.org/10.18637/jss.v067.i01)
- 568 33. Kuznetsova A, Brockhoff PB, Christensen RH. lmerTest package: tests in linear mixed
569 effects models. *J Stat Softw*. 2017 Dec;82:1-26. doi: [10.18637/jss.v082.i13](https://doi.org/10.18637/jss.v082.i13)
- 570 34. Bartoń K. MuMIn: Multi-Model Inference. R package version 1.43.17. 2022. Available
571 From: <https://CRAN.R-project.org/package=MuMIn>

- 572 35. Benjamini Y, Hochberg Y. Controlling the false discovery rate: a practical and powerful
573 approach to multiple testing. *J R Stat Soc Series B Stat Methodol.* 1995 Jan;57(1):289-
574 300. doi: [10.1111/j.2517-6161.1995.tb02031.x](https://doi.org/10.1111/j.2517-6161.1995.tb02031.x)
- 575 36. Champely S, Ekstrom C, Dalgaard P, Gill J, Weibelzahl S, Anandkumar A, et al. pwr:
576 Basic Functions for Power Analysis. R package version 1.3-0. 2017. Available From:
577 <https://CRAN.R-project.org/package=pwr>
- 578 37. Green P, MacLeod CJ. SIMR: an R package for power analysis of generalized linear
579 mixed models by simulation. *Methods Ecol Evol.* 2016 Apr;7(4):493-8. doi:
580 [10.1111/2041-210X.12504](https://doi.org/10.1111/2041-210X.12504)
- 581 38. Cohen J. Statistical power analysis for the behavioral sciences. 2nd ed. New York:
582 Routledge; 2013. doi: [10.4324/9780203771587](https://doi.org/10.4324/9780203771587)
- 583 39. Roces F, Kleineidam C. Humidity preference for fungus culturing by workers of the leaf-
584 cutting ant *Atta sexdens rubropilosa*. *Insectes Soc.* 2000 Nov;47(4):348-50. doi:
585 [10.1007/PL00001728](https://doi.org/10.1007/PL00001728)
- 586 40. Halboth F, Roces F. The construction of ventilation turrets in *Atta vollenweideri* leaf-
587 cutting ants: Carbon dioxide levels in the nest tunnels, but not airflow or air humidity,
588 influence turret structure. *PLoS One.* 2017 Nov;12(11):e0188162. doi:
589 [10.1371/journal.pone.0188162](https://doi.org/10.1371/journal.pone.0188162)
- 590 41. O’Fallon S, Lowell ES, Daniels D, Pinter-Wollman N. Harvester ant nest architecture is
591 more strongly affected by intrinsic than extrinsic factors. *Behav Ecol.* 2022
592 May;33(3):644-53. doi: [10.1093/beheco/arac026](https://doi.org/10.1093/beheco/arac026)

- 593 42. Dornhaus A, Franks NR, Hawkins RM, Shere HN. Ants move to improve: colonies of
594 *Leptothorax albigenis* emigrate whenever they find a superior nest site. *Anim Behav.*
595 2004 May;67(5):959-63. doi: [10.1016/j.anbehav.2003.09.004](https://doi.org/10.1016/j.anbehav.2003.09.004)
- 596 43. Franks NR, Pratt SC, Mallon EB, Britton NF, Sumpter DJ. Information flow, opinion
597 polling and collective intelligence in house-hunting social insects. *Proc R Soc Lond B*
598 *Biol Sci.* 2002 Nov;357(1427):1567-83. doi: [10.1098/rstb.2002.1066](https://doi.org/10.1098/rstb.2002.1066)
- 599 44. Franks NR, Dornhaus A, Metherell BG, Nelson TR, Lanfear SA, Symes WS. Not
600 everything that counts can be counted: ants use multiple metrics for a single nest trait.
601 *Proc R Soc Lond B Biol Sci.* 2006 Jan;273(1583):165-9. doi: [10.1098/rspb.2005.3312](https://doi.org/10.1098/rspb.2005.3312)
- 602 45. Sasaki T, Pratt SC. Ants learn to rely on more informative attributes during decision-
603 making. *Biol Lett.* 2013 Dec;9(6):20130667. doi: [10.1098/rsbl.2013.0667](https://doi.org/10.1098/rsbl.2013.0667)
- 604 46. Sasaki T, Colling B, Sonnenschein A, Boggess MM, Pratt SC. Flexibility of collective
605 decision making during house hunting in *Temnothorax* ants. *Behav Ecol Sociobiol.* 2015
606 May;69(5):707-14. doi: [10.1007/s00265-015-1882-4](https://doi.org/10.1007/s00265-015-1882-4)
- 607 47. Pratt SC. Quorum sensing by encounter rates in the ant *Temnothorax albigenis*. *Behav*
608 *Ecol.* 2005 Mar;16(2):488-96. doi: [10.1093/beheco/ari020](https://doi.org/10.1093/beheco/ari020)
- 609 48. Pratt SC, Pierce NE. The cavity-dwelling ant *Leptothorax curvispinosus* uses nest
610 geometry to discriminate between potential homes. *Anim Behav.* 2001 Aug;62(2):281-7.
611 doi: [10.1006/anbe.2001.1777](https://doi.org/10.1006/anbe.2001.1777)
- 612 49. Robinson WA. Selection and sharing of sheltered nest sites by ants (Hymenoptera:
613 Formicidae) in grasslands of the Australian Capital Territory. *Aust J Entomol.* 2008
614 Aug;47(3):174-83. doi: [10.1111/j.1440-6055.2008.00632.x](https://doi.org/10.1111/j.1440-6055.2008.00632.x)

615 50. Thomas ML. Nest site selection and longevity in the ponerine ant *Rhytidoponera*
616 *metallica* (Hymenoptera, Formicidae). *Insectes Soc.* 2002 May;49(2):147-52. doi:
617 [10.1007/s00040-002-8294-y](https://doi.org/10.1007/s00040-002-8294-y)

618

619 **Supporting information**

620 **S1 Fig. Percentage of brood and worker death in each colony after trial 1 shows no**
621 **relationship with external humidity levels.**

622 **S2 Fig. Relatively fewer brood and workers died in larger colonies during the experiment.**

623 Points are the average brood or workers, black lines denote a slope of 1 and red lines are slopes
624 derived from linear models (e.g., formula: $\log(\text{BroodTrial2}) \sim \log(\text{BroodTrial1})$).

625 **S1 Table. Predicted and empirical levels of relative humidity (%) produced from saturated**
626 **salt solutions.** Predicted relative humidity levels (mean \pm standard deviation) are in 20 - 25°C
627 and empirical in 20.40°C \pm 0.18°C. Note that magnesium acetate was not used in trial 2 because
628 it produced an inconsistent RH % in trial 1.

629 **S2 Table. Relationship between built nest wall weight (g) and relative humidity (%).** Linear
630 mixed effects model: $\text{WallWt} \sim \text{Humidity} + (1 \mid \text{Trial})$

631 **S3 Table. Relationship between built nest wall length (mm) and relative humidity (%) for**
632 **each experimental trial.** Linear mixed effects model: $\text{Length} \sim \text{Humidity} + (1 \mid \text{Trial})$

633 **S4 Table. Relationship between built nest wall area (mm²) and relative humidity (%) for**
634 **each experimental trial.** Linear mixed effects model: $\text{Area} \sim \text{Humidity} + (1 \mid \text{Trial})$

635 **S5 Table. Relationship between built nest wall density (g/mm³) and relative humidity (%)**
636 **for each experimental trial.** Linear mixed effects model: $\text{Density} \sim \text{Humidity} + (1 \mid \text{Trial})$

637 **S6 Table. Relationship between the wall substrate composition (proportion of substrate II**
638 **in build walls) and relative humidity (%) for each experimental trial.** Linear mixed effects
639 model: PropIIWall ~ Humidity + (1 | Trial)

640 **S7 Table. Relationship between the internal nest area (mm²) and relative humidity (%) for**
641 **each experimental trial.** Linear mixed effects model: Nest.Area ~ Humidity + (1 | Trial)

642 **S8 Table. Statistical power analyses of linear mixed effects models assessing the effect of**
643 **humidity on nest properties.** Power analyses considered a moderate (Cohen's d = 0.5) and high
644 (Cohen's d = 0.8) effect size. The direction of the effect was taken from the corresponding linear
645 mixed effects model.

646 **S9 Table. Relationship between built nest wall weight (g) and colony size (number of brood**
647 **and workers).** Linear mixed effects model: CollWallWt ~ Number.Colony + (1 | Trial)

648 **S10 Table. Relationship between built nest wall length (mm) and colony size (number of**
649 **brood and workers).** Linear mixed effects model: Length ~ Number.Colony + (1 | Trial)

650 **S11 Table. Relationship between built nest wall area (mm²) and colony size (number of**
651 **brood and workers).** Linear mixed effects model: Area ~ Number.Colony + (1 | Trial)

652 **S12 Table. Relationship between built nest wall density (g/mm³) and colony size (number**
653 **of brood and workers).** Linear mixed effects model: Density ~ Number.Colony + (1 | Trial)

654 **S13 Table. Relationship between built nest wall composition (proportion of substrate II)**
655 **and colony size (number of brood and workers).** Linear mixed effects model: PropIIWall ~
656 Number.Colony + (1 | Trial)

657 **S14 Table. Relationship between internal nest area (mm²) and colony size (number of**
658 **brood and workers).** Linear mixed effects model: Nest.Area ~ Number.Colony + (1 | Trial)

659 **S15 Table. Statistical power of our linear mixed effects models assessing the effect of colony**

660 **size on nest properties.** Power analyses considered a moderate (Cohen's $d = 0.5$) and high

661 (Cohen's $d = 0.8$) effect size. The direction of the effect was taken from the corresponding linear

662 mixed effects model.

663 **S16 Table. Relationship between brood and worker mortality (%) after trial 1.** Linear

664 regression: Brood.Death ~ Worker.Death

665 **S17 Table. Relationship between the percentage of worker and brood death in a colony (%)**

666 **and relative humidity (%).** The percent death was taken after the first experimental trial.

667 Generalized linear model: worker or brood death ~ Humidity, family = Binomial

668 **S18 Table. Relationship between the log of average brood or workers in trials 1 and 2.**

669 Linear regression: Model 1 = Formula: $\log(\text{avg.worker Trial 2}) \sim \log(\text{avg.worker Trial 1})$; Model

670 2 = Formula: $\log(\text{avg.brood Trial2}) \sim 1 + \text{offset}(\log(\text{avg.brood Trial1}))$

671 **S19 Table. Comparing linear models from Table S18 to models with an intercept of 1.**

672 Model comparison: ANOVA(Model 1 ~ Model 2); Model 1 = Formula: $\log(\text{avg.worker Trial 2})$

673 $\sim \log(\text{avg.worker Trial 1})$; Model 2 = Formula: $\log(\text{avg.brood Trial2}) \sim 1 + \text{offset}(\log(\text{avg.brood}$

674 Trial1))

675 **S20 Table. Comparing the artificial and natural wall substrates porosities (%).** Dunn's

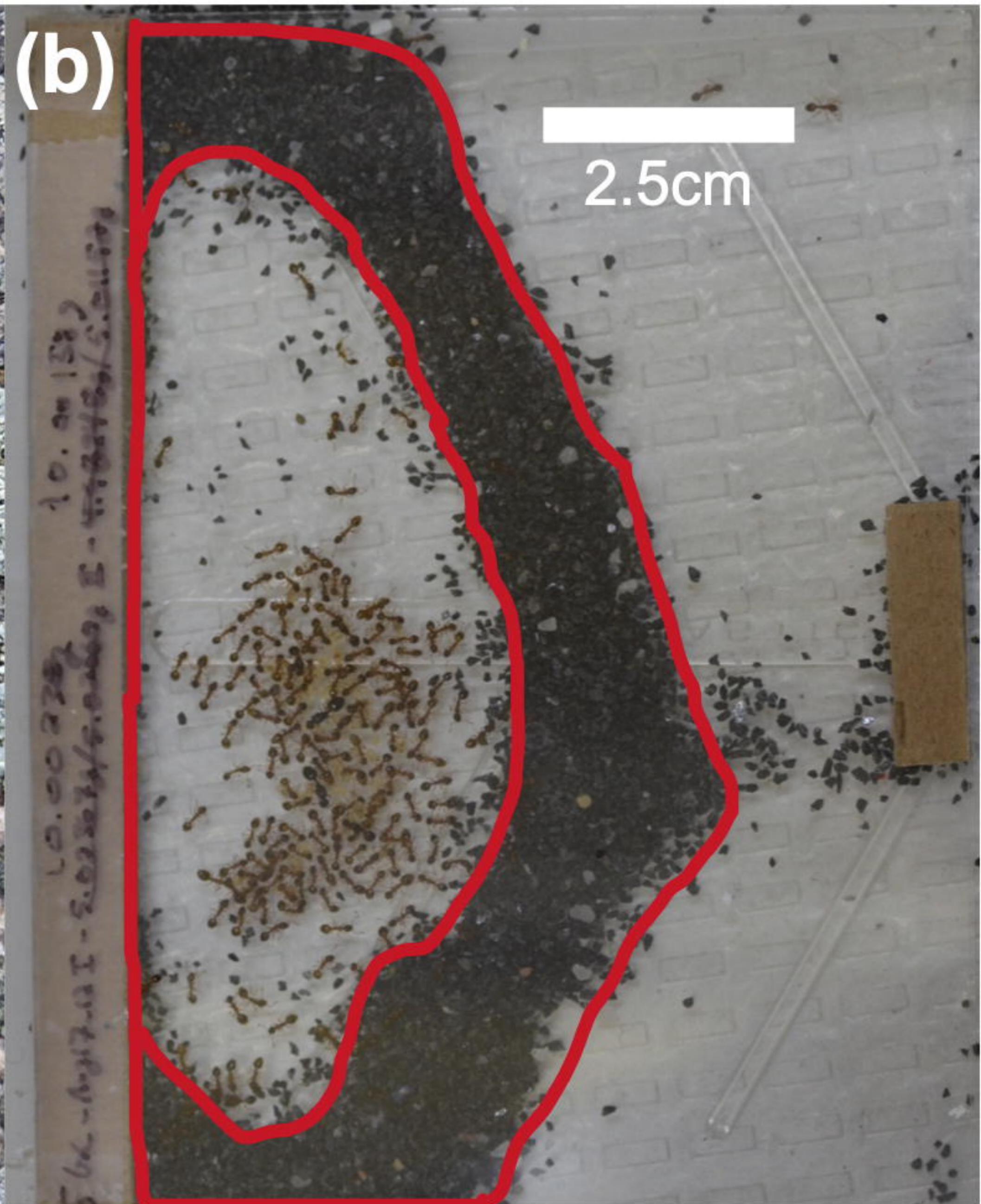
676 pairwise tests for wall substrate types: Porosity ~ SubstrateType

677

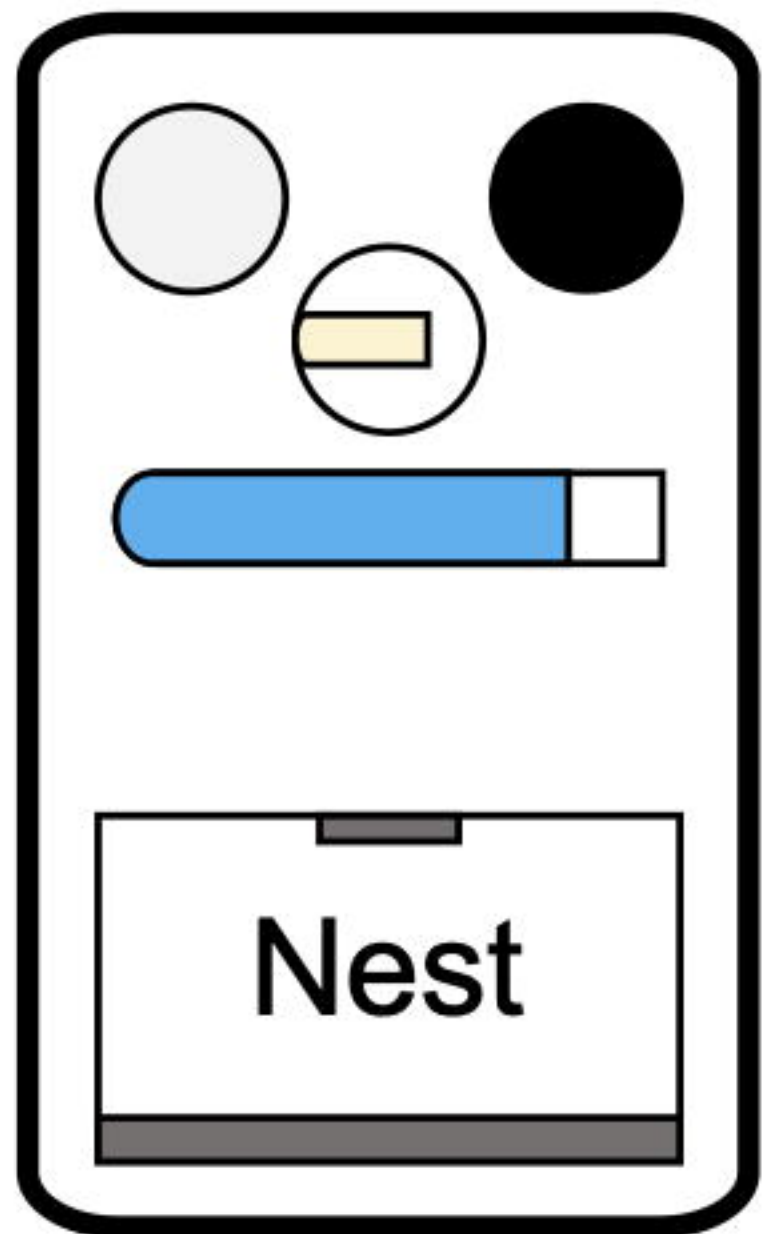
(a)



(b)



Sub I Sub II



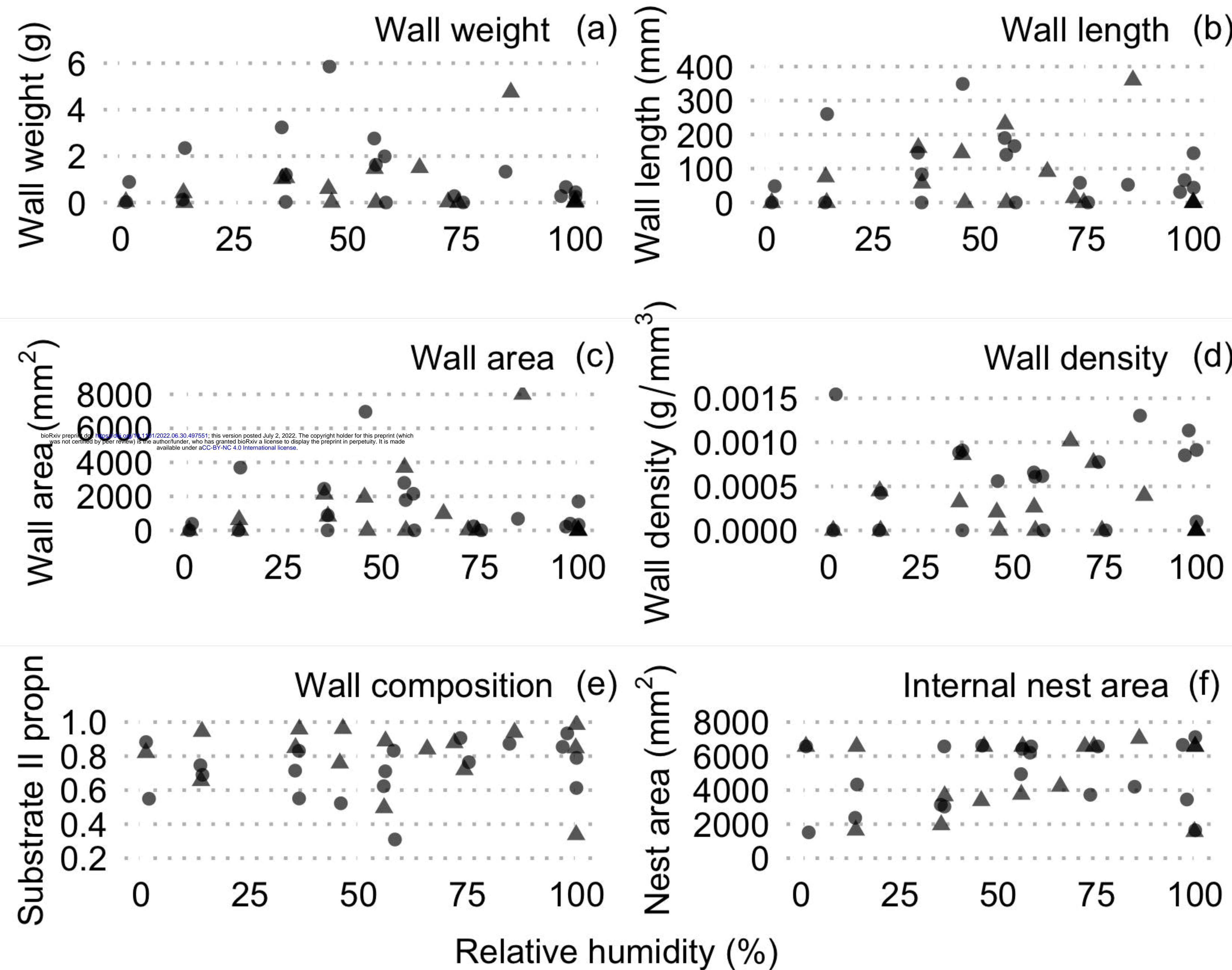
Nest container

Fan



Saturated
salt bath

Trial ● 1 ▲ 2



Trial

● 1

▲ 2

Colony member

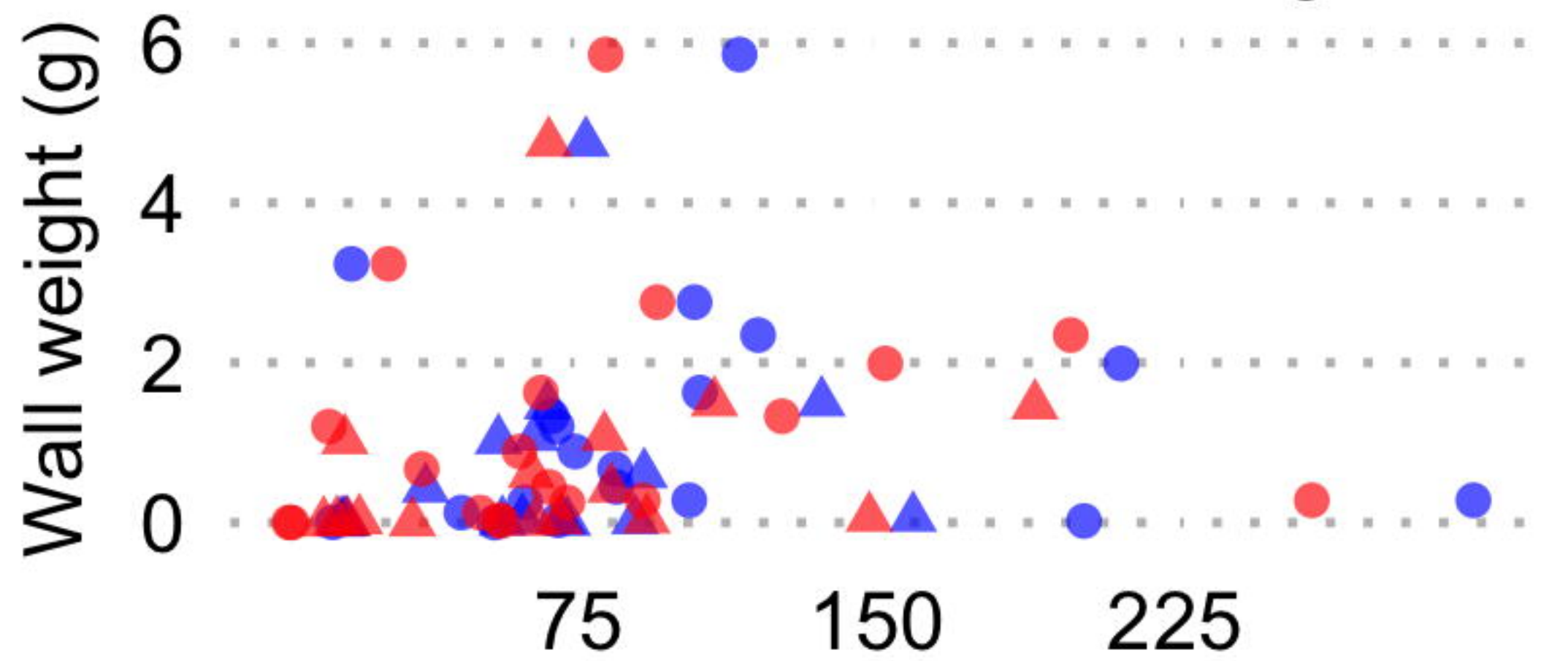
● Brood

Workers

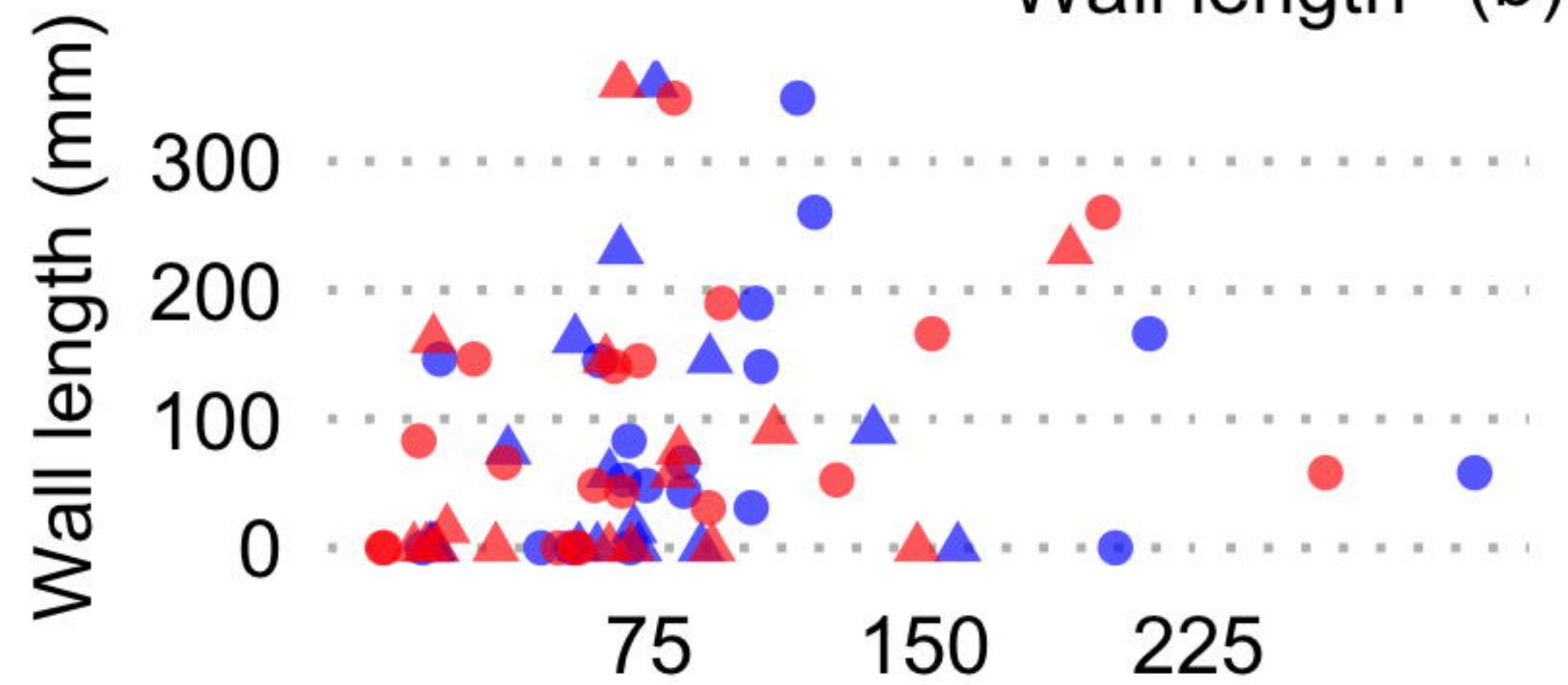
●

Workers

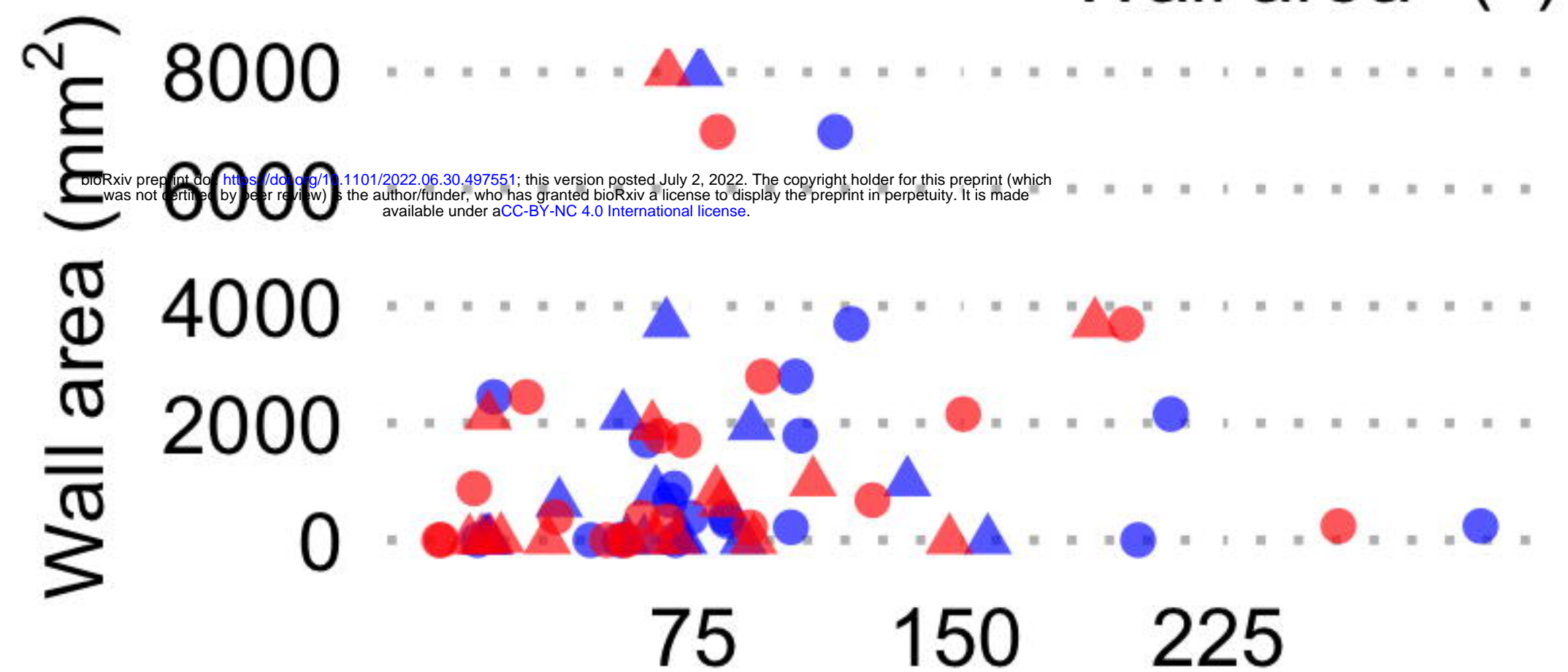
Wall weight (a)



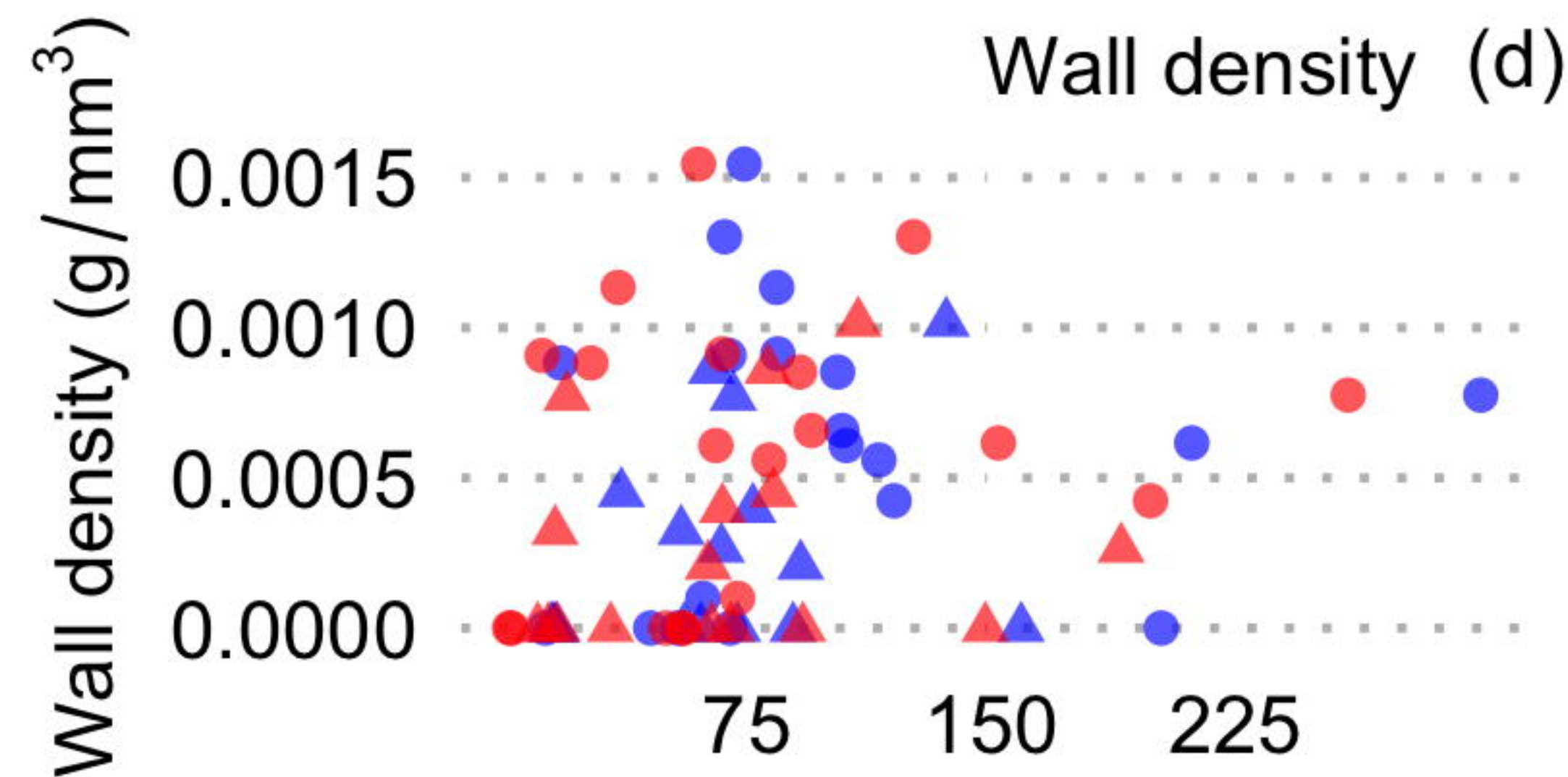
Wall length (b)



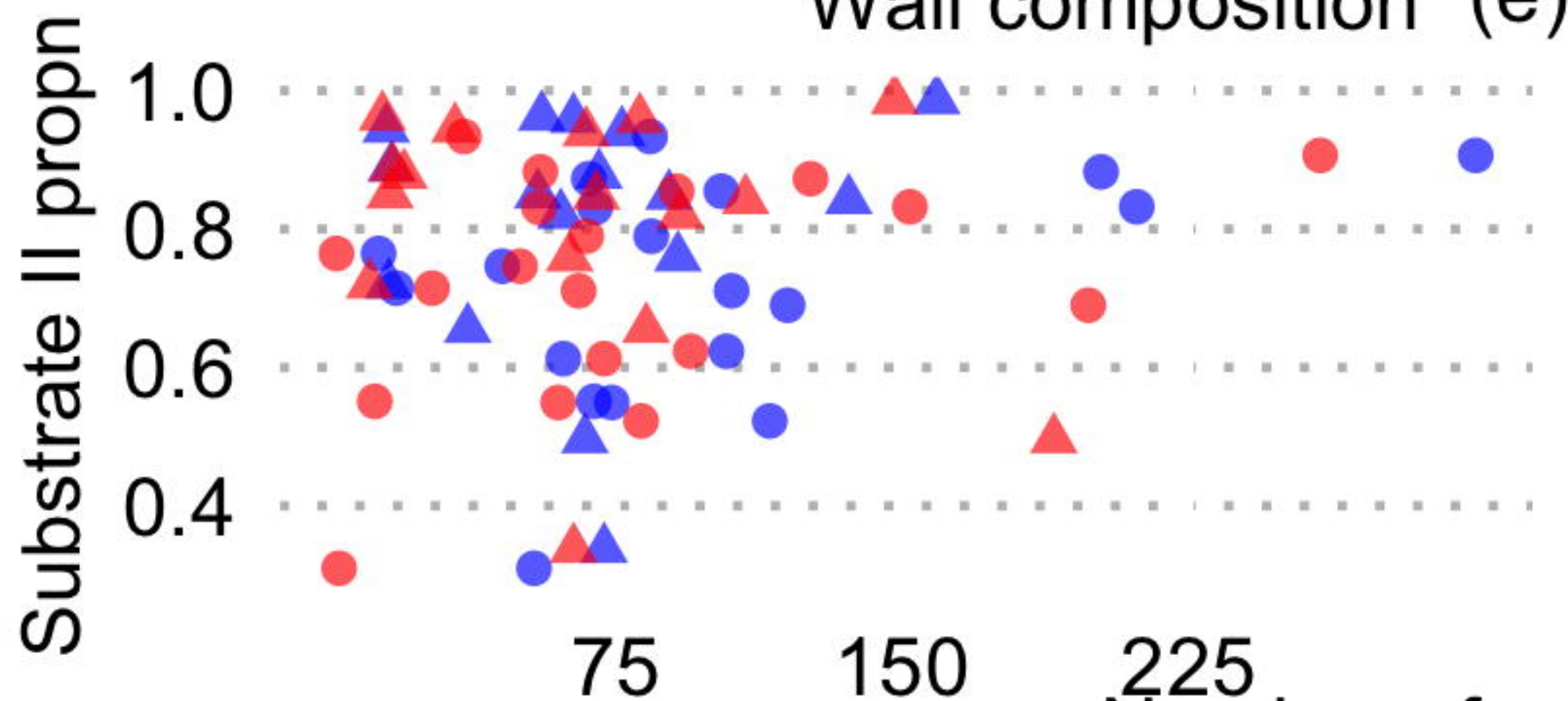
Wall area (c)



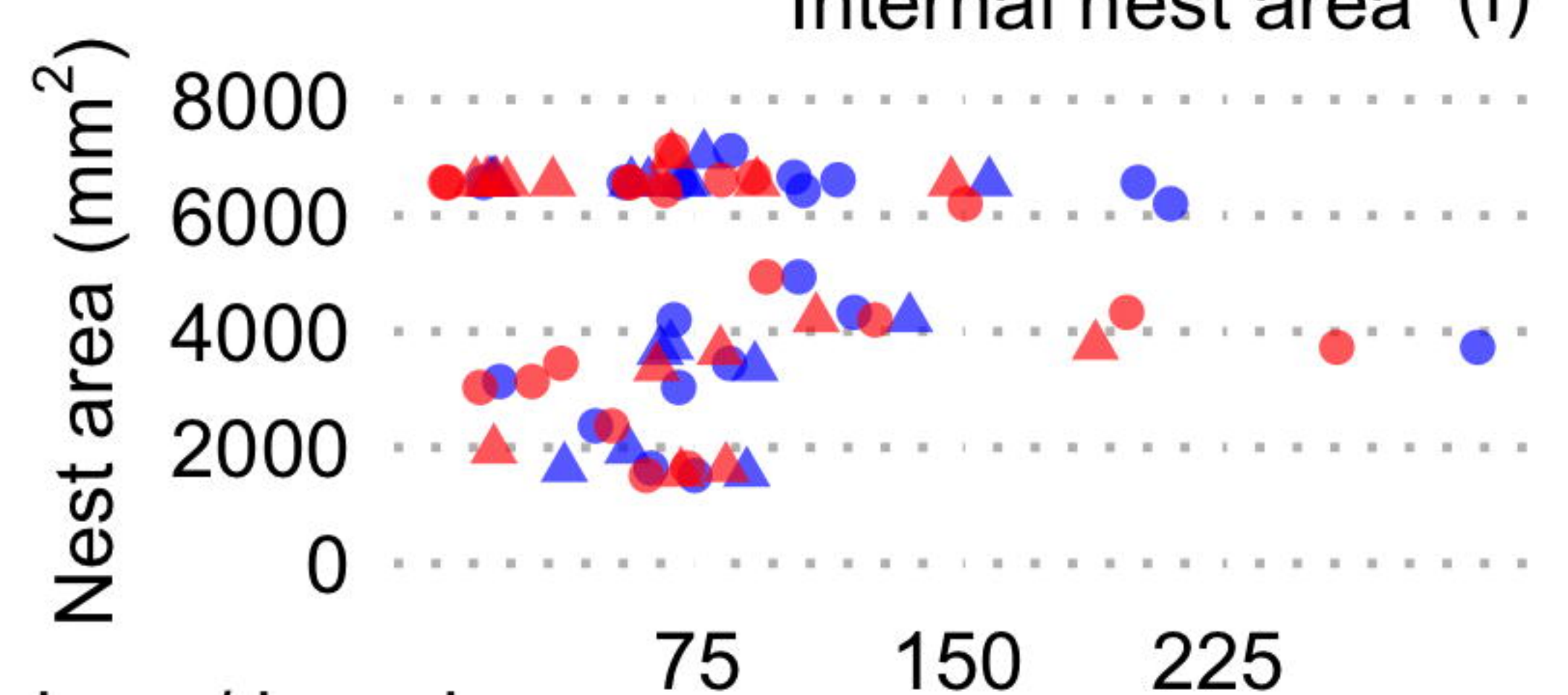
Wall density (d)



Wall composition (e)



Internal nest area (f)



Number of workers / brood

Porosity comparison

