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1 Spring colonies of the ant *Temnothorax nylanderi* tolerate cadmium better than winter colonies, in
2 both a city and a forest habitat

3

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14 University).

15 **Conflicts of interest**

16 The authors declare that they have no conflict of interest.

17 **Availability of data and materials**

18 Data are provided with the manuscript in the supplementary information section.

19 **Ethical approval**

20 All applicable international, national, and/or institutional guidelines for the care and use of animals
21 were followed.

22 **Authors' contribution**

23 LJ designed the study, collected colonies, reared them, performed the experiments and statistical
24 analyses, and wrote the manuscript. MM co-wrote the manuscript. CD contributed to statistical
25 analyses and co-wrote the manuscript. All authors read and approved the final manuscript.

26

27 **Key words: trace metal, colony size, diapause, spring, winter**

28 **Abstract**

29 A recent study showed that, in the ant *Temnothorax nylanderi*, city colonies are more tolerant to
30 cadmium than forest colonies. However, because of annual variation in biological factors (e.g. body
31 size, anti-stress protein production or trace metal accumulation rate), trace metal tolerance may vary
32 over the year. We aimed at testing whether tolerance to cadmium of colonies of *T. nylanderi* differs
33 between two different seasons within the same year (winter and spring). We also assessed whether
34 the better cadmium tolerance of city colonies was constant over these two different time points. We
35 collected colonies at the end of their hibernation period (winter colonies) and several weeks after
36 (spring colonies) from two different habitats (forest and city) to assess whether response to cadmium
37 was consistent regardless of the environment. We exposed colonies to a cadmium or a control
38 treatment for 61 days. We compared tolerance to cadmium between spring/winter and city/forest
39 colonies by measuring several life history traits. We found that spring colonies tolerates cadmium
40 better than winter colonies, and that city colonies have a higher tolerance to cadmium but only in
41 spring. Although further studies with replicated pairs of city/forest habitats and different years will
42 be necessary to confirm those results, our study suggests that tolerance to trace metals can fluctuate
43 along the yearly cycle.

44 **Introduction**

45 Pollution is one of the main human-induced rapid environmental changes. The nature of pollutants is
46 diverse: physical such as light or noise, or chemical such as plastic and trace metals. While physical
47 pollutants are more or less spatially localized, chemical pollutants are more diffuse and found in all
48 ecosystems, even in those that were once considered as pristine (Lenoir et al., 2016). Pollution is not
49 evenly distributed throughout the world, and some locations are more contaminated than others.
50 Trace metal pollution is important in active and disused mining or industrial sites and also in cities
51 (Foti et al., 2017; Roelofs et al., 2009). Some trace metals such as zinc are essential for life, but they
52 can also have many deleterious effects on organisms at high concentration. For example, they
53 increase oxidative damages (Sevcikova et al., 2011), impair embryonic development, and increase

54 mortality rates (Gomot, 1998; Hutchinson and Sprague, 1986). Some populations of organisms living
55 in heavily-contaminated sites have evolved better resistance to trace metal pollution (Jacquier et al.,
56 2021a; Mouneyrac et al., 2003; Roelofs et al., 2009), for example through higher expression or
57 turnover of metallothionein (a protein involved in trace-metals detoxification processes, Mouneyrac
58 et al., 2003; Roelofs et al., 2009) or higher quantity of metal-rich granules that store heavy metals
59 (Wallace et al., 1998).

60 Most of the studies investigating the response to trace metal exposure are carried out at a specific
61 period of the year (Gomot, 1998; Malakar et al., 2009; Vesela and Vijverberg, 2007). However,
62 organisms react differently to environmental changes and stress depending on the season (Bujan et
63 al., 2020; Morgan and Morgan, 1993; Rabitsch, 1997). Especially, trace metal accumulation in
64 organisms can differ among seasons (Cossa et al., 1980; Fialkowski et al., 2003; Morgan and Morgan,
65 1993; Rabitsch, 1997). For example, by sampling 30-50 workers from three different colonies every
66 month for one year, Rabitsch (1997) showed that concentration of trace metals in the body of the
67 workers of the red wood ant *Formica pratensis* is lower during spring and autumn (when the workers
68 are the heavier), for five different collection sites (at different distances from a smelter site). In a
69 mussel species, *Mytilus edulis*, the accumulation rate of trace metals was higher during spring
70 because of reproductive activities, and larger individuals had lower trace metal concentration, in four
71 different sites (Cossa et al., 1980). Therefore, variation in trace metal concentration can be impacted
72 by the yearly seasonal variation of body mass. Trace metal concentration in the environment is also
73 season-dependent, for example because of yearly seasonal variations in human activities or climate
74 conditions (Kulshrestha et al., 2009; Li and Zhang, 2010). This may impact further trace-metal
75 tolerance in organisms, for example by changing their trace metal accumulation rate or by impacting
76 the expression of trace-metal tolerance genes. Metallothionein concentration can also vary across
77 seasons independently of trace metal concentration (Gorbi et al., 2005; Oaten et al., 2017).
78 Moreover, physiological processes accompanying the course of seasonal changes trigger the
79 secretion of anti-stress proteins, including chaperones or metallothionein (Denlinger et al., 2001;
80 Robert et al., 2016; Yocum, 2001). For example, winter diapause triggers the secretion of anti-stress
81 factors, such as heat shock protein (Yocum, 2001), immune protein (Robert et al., 2016), anti-
82 oxydative protein (such as glutaredoxin, Popović et al., 2015) and/or metallothionein (Popović et al.,
83 2015), which may ultimately impact the way individuals cope with stress related to trace metals.

84 Ants are good candidates to monitor the impacts of trace metals on wildlife (Grześ, 2010a). In ants,
85 foragers are wingless and forage locally, and colonies are long-lived and sedentary, favoring the
86 bioaccumulation of trace metals (Grześ, 2010a). Ants are eusocial organisms: they live in colonies
87 where individuals perform tasks according to their caste (worker or queen), resulting in caste-specific

88 contamination risks (Grześ, 2010a). However, ants seem to be relatively tolerant to trace metals, as
89 they are able to maintain colonies in highly contaminated sites (Grześ, 2010a). The accumulation of
90 trace metals in their body depends on several factors, such as caste, diet or season. For example,
91 foragers accumulate more trace metals than queens or pupae in the field (Markert, 2008) and
92 workers are less sensitive to zinc than larvae in *Myrmica rubra* in the laboratory (Grześ, 2010b).
93 Accumulation also varies greatly among species because of different diets with honeydew-based
94 regimes being much more subject to trace metal intake (Starý and Kubizňáková, 1987). The
95 bioaccumulation of trace metals in some temperate ant species varies across seasons, with lower
96 concentration during spring and autumn (Rabitsch, 1997), which may be due to individual mass
97 variation as workers have more fat tissues during spring and autumn, when they forage actively.
98 However, the simple measure of trace metal contents in individual body does not entirely reflect
99 trace metal tolerance. There is still a lack of studies investigating variations of trace metal tolerance
100 in ants depending on the annual season, especially after winter diapause (but see Rabitsch, 1997).
101 Overwintering can be a particularly important phase of the colony life cycle as winter temperature
102 can induce diapause and affect subsequent larval development (Gill et al., 2017; Kipyatkov et al.,
103 1997).

104 In this paper, we compared colony tolerance to cadmium between city and forest populations
105 collected just after the hibernation period (winter) and during the growing season (spring) in the ant
106 *Temnothorax nylanderi*. This species is an interesting model because colonies nest in twigs and
107 acorns above ground and are thus directly exposed to winter climatic conditions (Herbers, 1989).
108 Interestingly, a previous study carried out just after the hibernation period showed that city colonies
109 of *T. nylanderi* are more tolerant to cadmium than their forest counterparts under common garden
110 laboratory conditions (Jacquier et al., 2021a), regarding larval emergence rate, larval mortality rate
111 and body size of emerging workers. Cadmium is a trace metal with toxic effects on organisms, as it
112 binds to the mitochondria leading to cell proliferation, apoptosis or production of Reactive Oxygen
113 Species (ROS) , interferes with DNA repair mechanisms, and interferes with other essential metals
114 such as calcium, zinc or iron (Martelli et al., 2006; Rani et al., 2014). Organisms exposed to cadmium
115 undergo decreased hatching rate, extended or stopped development, or reduced body size (Cervera
116 et al., 2004; Gomot, 1998; Malakar et al., 2009). Cadmium is found in high concentrations in active or
117 disused mine sites, but also in cities, where it can be ten times more concentrated than in forests
118 because of traffic and industrial activities (Foti et al., 2017). The better tolerance to cadmium of city
119 colonies in *T. nylanderi* was found in three different locations in France (Jacquier et al., 2021a).
120 However, this study was performed at one specific time of the year, at the end of winter, when
121 colonies were just coming out of winter diapause, and larvae and workers had just finished diapause

122 (Jacquier et al., 2021a). The aim of our study was to test whether (i) the response to cadmium was
123 constant regardless of the time point at which the study is conducted and (ii) the response to
124 cadmium over different time points was constant between two different habitats, i.e. city and forest.
125 On one hand, we could expect spring colonies to tolerate cadmium better than winter colonies as
126 hibernation decreases the fat content and overall body mass of workers and larvae in winter
127 colonies. On the other hand, we could expect winter colonies to tolerate cadmium better as winter
128 diapause triggers the secretion of anti-stress proteins (for example, metallothionein or heat shock
129 proteins) that may enhance trace metal tolerance (Denlinger et al., 2001; King and MacRae, 2015).
130 Moreover, because of their chronic exposition to stress (e.g. heat, trace metal, human presence), city
131 populations often have higher anti-stress gene expression (Cassone et al., 2014; Watson et al., 2017)
132 that could be even more expressed after hibernation leading to a better cadmium tolerance than
133 forest colonies in winter (as found in Jacquier et al 2021a).

134 Whatever the underlying mechanism, city colonies are therefore expected to have a higher cadmium
135 tolerance than forest colonies, especially in winter colonies at the end of hibernation. In order to
136 assess colony tolerance to cadmium, we measured the impact of cadmium on different life history
137 traits depending on the season: worker mortality rate, larval mortality rate, larval paused rate and
138 size of laboratory-born workers.

139

140 **Material and Methods**

141 ***Colony collection and rearing***

142 The small acorn ant *Temnothorax nylanderi* is a common species in Europe, found in both city and
143 rural habitats. Colonies consist of few hundreds individuals and nest in pieces of dead wood on the
144 ground (twigs, acorns, chestnuts, etc.), making them easy to collect. Collection sites were a city park
145 of Paris (Jardins Ecologiques, 48°50'59.684"N 2° 21' 40.385"E) and a forest 50km away from Paris
146 (Chantilly, 49°10'59.8"N 2°28'43.6"E). These two sites have deciduous trees (mainly ash, hazelnut and
147 oak), and the ground is covered with ivy and leaf litter. The city park is about 1 hectare, and it is
148 surrounded by unsuitable areas for nesting. The forest is approximately 6300 hectares. The soil of the
149 city wood is more contaminated with trace metals, such as cadmium, than the soil of the forest (Foti
150 et al., 2017).

151

152 We quantified the response of city and forest colonies to cadmium at two different times of the year:
153 just after the winter diapause (winter colonies), and three months after colonies had come out of

154 diapause (spring colonies) and we therefore collected colonies at two different times of the year. The
155 first one occurred between 8th and 16th March 2020 (113 ‘winter colonies’) and the second one
156 between 1st and 4th June 2020 (129 ‘spring colonies’). Different colonies were collected for March
157 and June. Colonies were brought back to the laboratory and put in 11.5x11.5x5.5cm plastic boxes.
158 Boxes were then kept in a climatic chamber (reference CTS TP10/600) at 22-27°C 12-12 light cycles
159 under 70% humidity. The lid of the boxes was pierced for air circulation. Artificial nests were made of
160 two microscope glasses separated by a 2mm thin moss chamber. Artificial nests were covered by a
161 dark plastic sheet to keep colonies in the dark. Colonies were provided ad libitum water in a small
162 tube plugged with cotton. We discarded 54 colonies with no queen, 16 colonies with more than one
163 queen, and 4 colonies with pale-yellow cestode-infected workers. We kept 70 winter colonies (37 city
164 colonies, 33 forest colonies) and 85 spring colonies (46 city colonies, 39 forest colonies). We let
165 colonies acclimatize to the laboratory conditions for about three weeks.

166 Before the start of the experiments, we removed all eggs and larvae from the colonies except for the
167 second instar larvae, which correspond to an early developmental stage. We counted workers
168 (‘colony size’, ranging from 11 to 225 in winter colonies and from 6 to 150 in spring colonies) and
169 second instar larvae (ranging from 4 to 33 in winter colonies and from 2 to 29 in spring colonies).

170

171 ***Experimental design***

172 We started the experiment on April 6th 2020 for winter colonies, and on June 24th 2020 for spring
173 colonies. In the climatic chamber, we exposed colonies to two different treatments for 61 days:
174 control or cadmium. Spring colonies were smaller than winter colonies (48 workers in average
175 against 99 workers in average, $F_{145,146}=73.21$, $P<10^{-13}$). For a given season, colony size distribution was
176 the same between city and forest habitats ($F_{143,146}=0.17$, $P=0.68$). We evenly assigned colonies of a
177 given season to control or cadmium treatment so that the colony size distribution was the same
178 between the two treatments within each season (anova, $F_{145,146}=0.26$, $P=0.60$). We removed every
179 newly laid egg every week. Sixty-one days correspond to the maximal duration of larvae
180 development from second-instar to a newly emerged worker, at 22-27°C, so any larva that would
181 have escaped the egg removal protocol could not have reached adulthood before the end of the
182 experiment. Colonies were fed every other day with a mixture of yoghurt, dried crickets, diluted
183 honey and vitamins, with 100µg of cadmium per g of food (cadmium treatment) or without cadmium
184 (control treatment). Food was provided in excess. Cadmium concentration was set to this value as
185 (Jacquier et al., 2021a) found that it led to approximately 50% worker mortality after a 2 months
186 exposure.

187

188 ***Response variables***

189 We counted dead workers every week and removed corpses from the boxes. We computed the
190 “worker mortality rate” as the ratio between the total number of dead workers and the initial
191 number of workers in each colony. We also counted the remaining number of larvae every other day.
192 The “larval mortality rate” was computed as the ratio between the total number of dead larvae
193 (larvae missing at the end of the experiment, but that had not developed into adult) and the initial
194 number of larvae. The “emergence rate” was computed as the ratio between the total number of
195 emerged adults within a colony (workers, males and gynes) at the end of the experiment and the
196 initial number of larvae. As observed in Jacquier et al (2021a), some larvae were still alive at the end
197 of the experiment but apparently did not grow during the entire course of the experiment and had
198 apparently paused their development. The “larval pause rate” was therefore computed as the ratio
199 between the number of paused larvae and the number of initial larvae.

200 We measured newly born workers (subsequently called “lab-workers”), which are easily recognizable
201 by their typical pale orange color. We collected lab-workers, froze them, and stored them in 90°
202 ethanol. At the end of the experiment, they were beheaded, and their head were stuck on double-
203 side tape. Heads were photographed using a stereomicroscope (Zeiss Discovery.V12). Head width
204 was measured as the eye-to-eye (posterior-side) length, a good proxy of worker size (Araujo and
205 Tschinkel, 2010). Measures were performed with ImageJ (<https://imagej.nih.gov/ij/>; Abràmoff et al.,
206 2004). Some males and gynes emerged during the experiment, but only in winter colonies. Sexu-
207 als were probably not produced in spring colonies because they only develop from larvae that
208 underwent winter temperature (Vargo and Passera, 1992). In the present study, we only left young
209 stage 2 larvae in colonies, so the larger and older larvae that had overwintered were removed from
210 spring colonies. Therefore, we chose to not present results regarding the size of males and gynes.

211 ***Statistical analyses***

212 We used R v3.6.2 (Team, 2018) for all subsequent statistical analyses.

213 Six queens died over the course of the experiment (three cadmium, one control for forest winter
214 colonies, and one control, one cadmium for forest spring colonies). These colonies were removed
215 from subsequent analyses. We used models assessing the effects of treatment (control or cadmium),
216 habitat (city or forest) and season (winter or spring) and their second and first-order interactions on
217 four response variables (worker mortality rate, larval mortality rate, larval paused rate and head
218 width of lab-workers). We also included colony size as a covariate and the interaction between

219 colony size and treatment, as colony sizes differed between spring and winter colonies. We expected
220 a differential effect of treatment depending on the season and habitat, that is a Treatment : Habitat :
221 Season second-order interaction.

222 Two distinct models were used for statistical analyses. 1/ Regarding colony-level variables (worker
223 mortality rate, larval mortality rate, emergence rate, larval pause rate), we used generalized linear
224 models (GLMs, Venables and Ripley, 2013) with quasibinomial distribution. Indeed these variables
225 were based on pairs of counts (e.g. worker mortality rate was input as the number of dead workers
226 and the number of surviving workers). The quasibinomial distribution was used to incorporate over-
227 dispersion. 2/ Regarding the individual-level variable (head width of lab workers), we used mixed
228 models with colony as a random factor to control for pseudoreplication as several measurements
229 were made within each colony. In both cases, the complete initial models contained all independent
230 variables (treatment, habitat, season) and their interactions (second-order and first-order), as well as
231 the independent variable colony size and its first-order interaction with treatment. We obtained a
232 Minimum adequate model (MAM) using a backward stepwise procedure. We first tested the second-
233 order interaction, then first-order interactions, and finally simple variables. When the P-value was
234 higher than 0.05, the tested independent variable or interaction was removed from the model, until
235 no variable or interaction could be removed (i.e. the MAM had been obtained). Final P-values were
236 obtained by adding or removing an dependent variable from this MAM and comparing models using
237 a log likelihood ratio test. When a first-order interaction was found, we used the emmeans package
238 on the MAM to test for the effect of each independent variable separately. Analyses were performed
239 using the lme4 package (Bates et al., 2007).

240 We visually checked for homoscedasticity of data and normal distribution of the model residuals for
241 mixed models using plot functions. No transformation of data was required.

242

243 **Results**

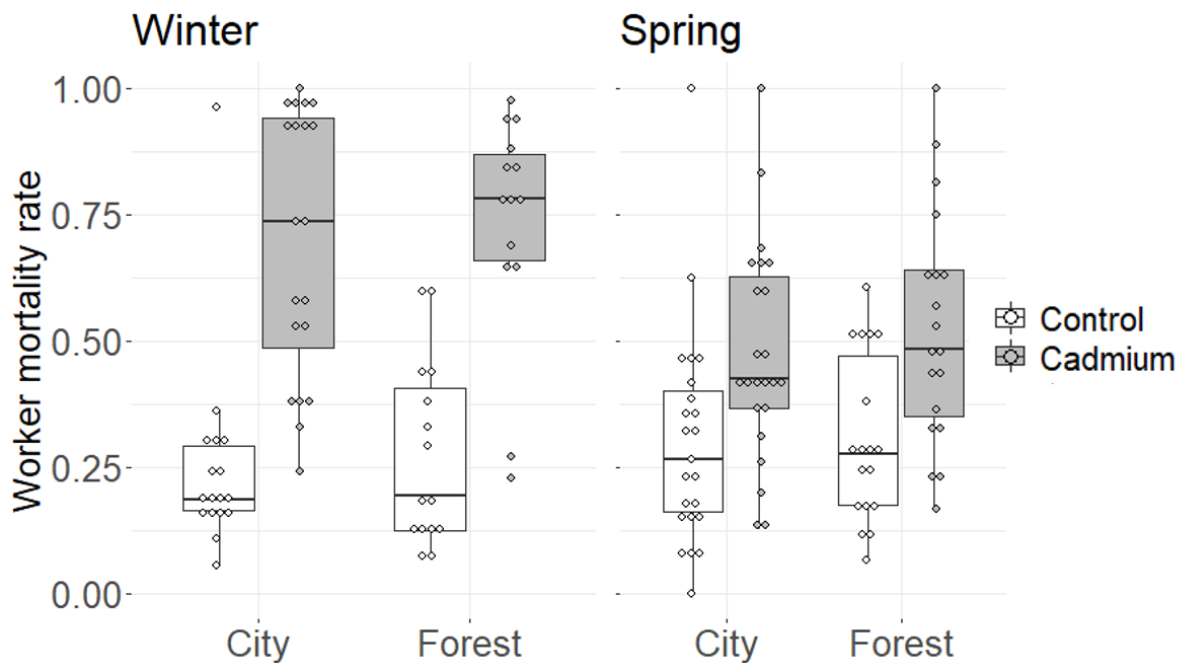
244 Out of the 66 winter colonies and 83 remaining spring colonies, we collected a total of 367 workers,
245 one male and zero gyne for spring colonies, and 437 workers, 146 males and 24 gynes for winter
246 colonies. Gynes were produced by control forest colonies only. Due to this lack of males and gynes in
247 spring colonies, we could not perform statistical analyses regarding males and gynes. Details about
248 the numbers of colonies, lab-workers and males for each season, habitat and treatment are given in
249 Table S1.

250 *Worker mortality rate*

251 As expected, worker mortality significantly increased in colonies treated with cadmium ($z=-9.58$,
 252 $P<0.0001$ Table 1, Fig 1). The effect was stronger for winter colonies than for spring colonies
 253 whatever the habitat (no Treatment : Season : Habitat interaction, $F_{145,141}=0.21$ $P=0.93$; significant
 254 Treatment : Season interaction $F_{147,145}=17.82$ $P<10^{-6}$, Table 1, Fig 1). Worker mortality decreased with
 255 colony size in the control, but not under cadmium (Colony size : Treatment interaction $F_{140,139}=14.31$
 256 $P=0.00022$, Table 1, Fig 2). Worker mortality rate under cadmium was high whatever the colony size,
 257 so that the higher mortality rate of winter colonies under cadmium is not due to their higher mean
 258 colony size.

259

260



261

262 **Fig 1** Comparison of worker mortality rate between spring and winter colonies from city and forest.
 263 For each box, median and quartiles are shown, as well as colony-level data points. White: control
 264 treatment, grey: cadmium treatment

265

266 *Larval mortality rate*

267 The effect of cadmium on larval mortality rate significantly differed between seasons and habitats
 268 (significant Treatment : Habitat : Season interaction, $F_{141,140}=5.90$ $P=0.016$, Table 2). We first tested
 269 for a season-specific effect of cadmium within each habitat. Regarding city colonies, cadmium
 270 increased the larval mortality rate more in winter than in spring (significant Treatment : Season

271 interaction, $F_{77,78}=7.20$ $P=0.0089$, Fig 3). Regarding forest colonies, this effect was reversed, i.e.
272 cadmium increased the larval mortality rate more in spring than in winter (significant Treatment :
273 Season interaction, $F_{60,61}=5.32$, $P=0.024$, Fig 3).

274 We then tested for a habitat-specific effect of cadmium within each season. Regarding winter
275 colonies, cadmium increased larval mortality rate (treatment effect, $F_{64,65}=38.97$ $P<10^{-7}$, Table 2, Fig
276 3) in a similar way in the two habitats (no significant Treatment : Habitat interaction $F_{64,62}=0.17$,
277 $P=0.84$, Table 2 Fig 3). Regarding spring colonies, cadmium also increased larval mortality rate
278 (Treatment effect, $z=4.75$, $P<0.0001$ Table 2, Fig 3), but city colonies tolerated cadmium better than
279 forest colonies (significant Treatment : Habitat interaction, $F_{79,78}=7.40$ $P=0.0080$, Table 2, Fig 3).
280 Colony size did not impact larval mortality rate (no significant Treatment : Colony size interaction, no
281 significant Colony size effect, Table 2).

282

283 *Larval pause rate*

284 Some larvae did not grow during the entire course of the experiment. This allowed us to compute a
285 larval pause rate, that should not be confused with winter diapause. The effect of cadmium on larval
286 pause rate significantly differed between seasons and habitats (significant Treatment : Habitat :
287 Season interaction, $F_{141,140}=5.61$ $P=0.019$, Table 2). We tested for a season-specific effect of cadmium
288 within each habitat. City colonies increased their larval pause rate in response to cadmium more in
289 winter than in spring (significant Treatment : Season interaction, $F_{77,78}=12.80$ $P=0.00060$). Forest
290 colonies increased their larval pause rate when treated with cadmium in winter, but they decreased
291 it in spring (significant Treatment : Season interaction, $F_{60,61}=41.85$ $P<10^{-7}$).

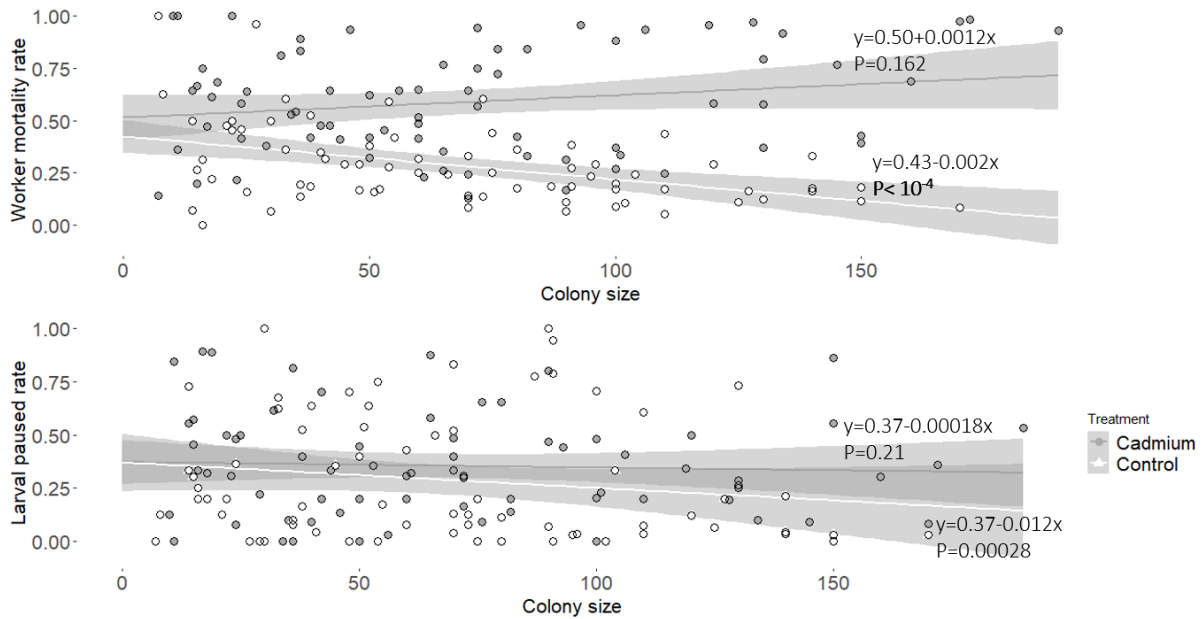
292 We tested for a habitat-specific effect of cadmium within each season. In winter colonies, the larval
293 pause rate was significantly higher under cadmium (Treatment effect, $F_{64,63}=52.76$ $P<10^{-9}$, Table 2, Fig
294 3) in a similar way in city and forest colonies (no Treatment : Habitat interaction, $F_{62,61}=0.58$ $P=0.45$,
295 Table 2, Fig 3). Interestingly, in spring colonies, city and forest colonies responded in an opposite
296 direction to cadmium, with higher larval pause rate in city colonies under cadmium, but lower pause
297 rate in forest colonies under cadmium (significant Treatment : Habitat interaction $F_{79,78}=7.99$
298 $P=0.0059$ Table 2, Fig 3). Under control, larval pause rate decreased with colony size. Under
299 cadmium, larval pause rate was high whatever the colony size (significant Treatment : Colony size
300 interaction, $F_{140,139}=4.56$ $P=0.034$, Table 2, Fig 2).

301

302 *Emergence rate*

303 Cadmium significantly decreased the emergence rate of larvae (Treatment effect, $z=-10.03$ $P<0.0001$)
 304 with a stronger effect in winter colonies (Treatment : Season interaction, $F_{143,144}=35.42$ $P<10^{-7}$ Table
 305 2, Fig 3) whatever the habitats (no significant Treatment : Habitat : Season interaction, $F_{143,140}=0.13$
 306 $P=0.94$, Table 2, Fig 3). Emergence rate slightly decreased with colony size whatever the treatment
 307 (significant colony size effect, $F_{143,144}=19.17$ $P<10^{-4}$ Table 2).

308



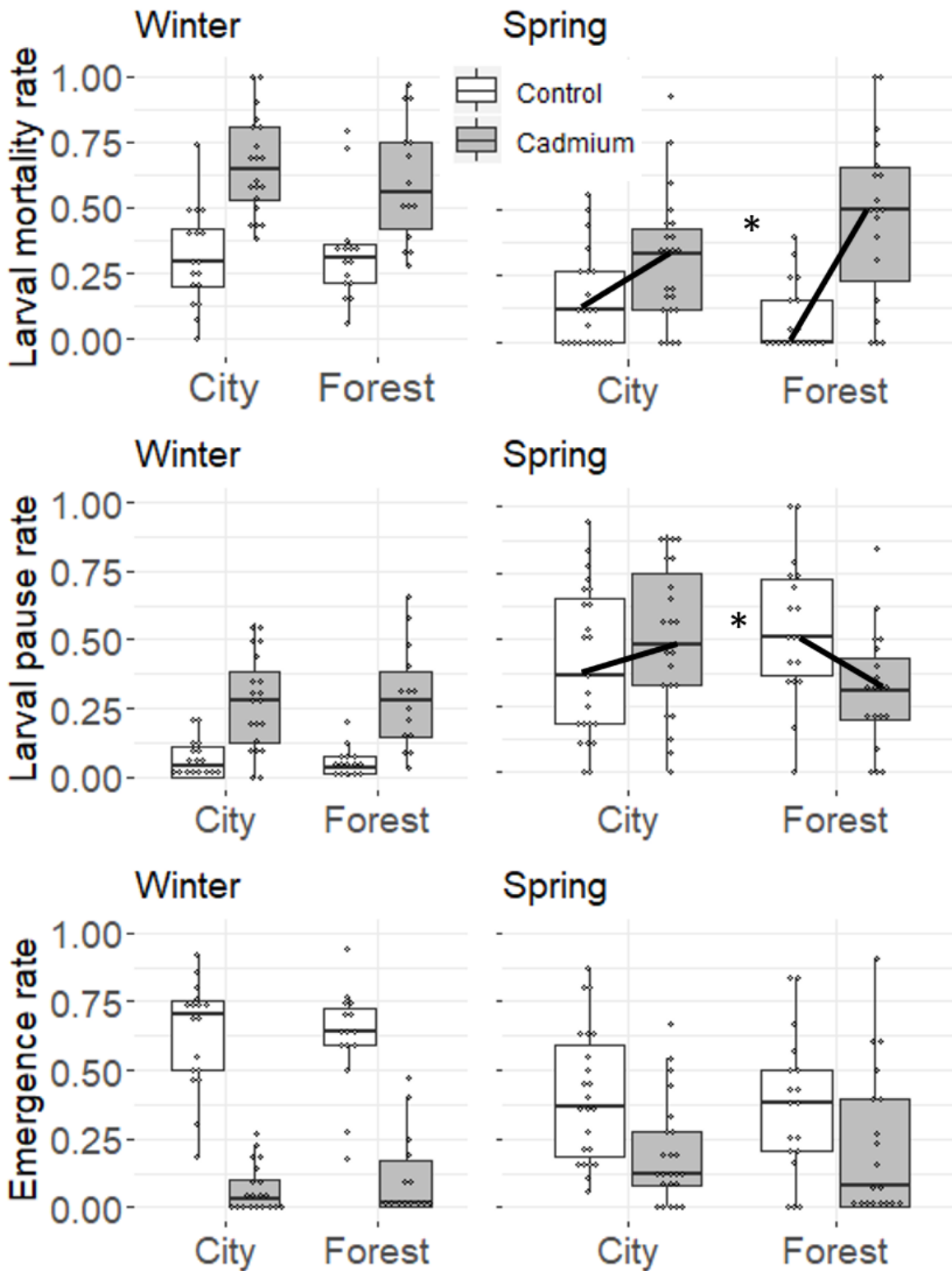
309

310 Fig 2: Worker mortality rate and larval pause rate depending on colony size and treatment (grey
 311 circles: cadmium, white circles: control). For each graph, line equation is provided as $y = \text{intercept} +$
 312 $\text{slope} \cdot x$. Ninety-five percent confidence intervals are shown as grey zones. P-values correspond to
 313 the colony size effect within each treatment.

314

315

316



317

318 **Fig 3** Comparison of larvae mortality rate, larval paused development rate and emergence rate
 319 between spring and winter colonies from city and forest. For each box, median and quartiles are
 320 shown, as well as colony-level data points. White: control treatment, grey: cadmium treatment.
 321 Significant Treatment : Habitat interactions are shown by * and depicted by black lines. All variables
 322 for which a significant Treatment : Habitat first-order interaction was found were also subject to a

323 significant Treatment : Habitat : Season second-order interaction (not shown for clarity, but visible by
324 comparing the left 'Winter' and right 'Spring' panels).

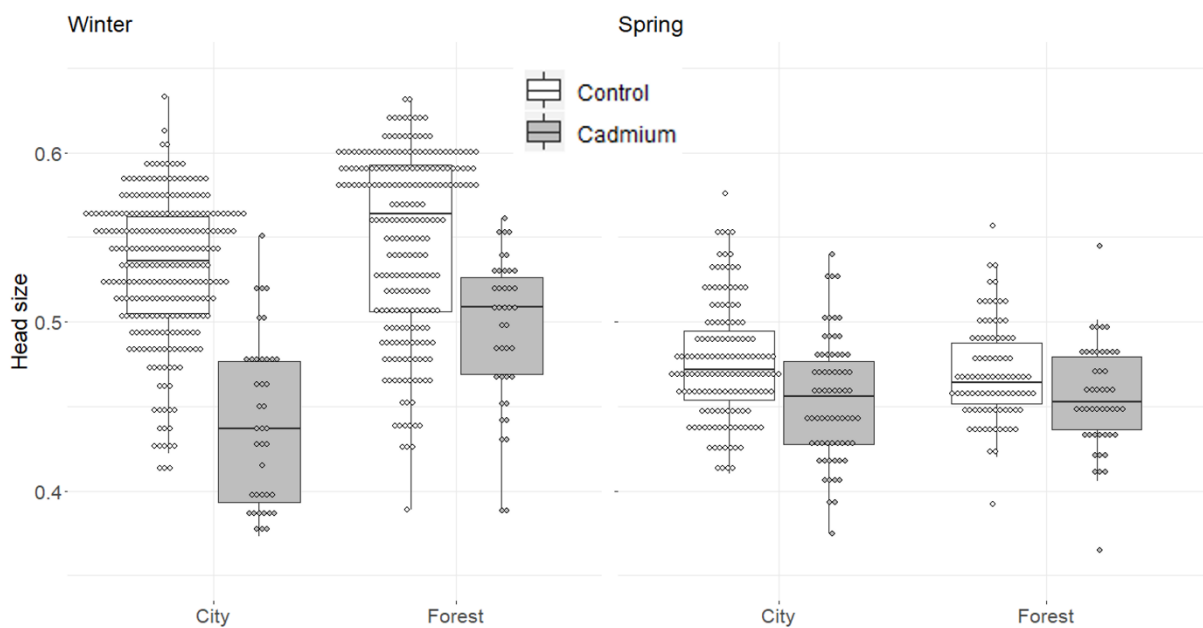
325

326

327 *Size of lab-workers*

328 The size of lab-workers decreased under cadmium ($t_{130}=8.84$ $P<0.0001$, Table 1, Fig 3) but the effect
329 of cadmium was less pronounced in spring colonies than in winter colonies (significant Treatment :
330 Season, $\chi_1=24.11$ $P<10^{-6}$). Moreover, the effect of cadmium was more pronounced during winter in
331 city colonies than in forest colonies, but this differential effect faded in spring colonies (marginally
332 significant Treatment : Season : Habitat, graphical tendencies , marginally significant Treatment :
333 Habitat interaction, $\chi_1=3.42$ $P=0.064$, Table 1, Fig 4). Colony size had no effect on the size of lab-
334 workers ($\chi_1=2.43$ $P=0.12$, Table 1).

335



336

337 **Fig 4** Comparison of lab-workers head size (mm) between spring and winter colonies from city and
338 forest. For each box, median and quartiles are shown, as well as individual-level data points. White:
339 control treatment, grey: cadmium treatment

340

341

342

343

344

345

346

| | Worker mortality rate | Size of lab-workers |
|------------------------------|----------------------------------------------------------------------|--------------------------------------------------------------|
| Treatment : Habitat : Season | $F_{145,141}=0.21$ P=0.93 | $X_2=4.81$ P=0.090 |
| Treatment : Habitat | $F_{145,143}=0.35$ P=0.70 | $X_1=3.42$ P=0.064 |
| Habitat : Season | $F_{145,143}=0.40$ P=0.67 | $X_1=7.37$ P=0.0066 |
| Treatment : Season | $F_{147,145}=17.82$ P<10⁻⁶ | $X_1=24.11$ P<10⁻⁶ |
| Treatment | z=-9.58 P<0.0001 | t₁₃₀=8.84 P<0.001 |
| Habitat | $F_{145,144}=0.70$ P=0.40 | t₁₀₀=2.28 P=0.02 |
| Season | z=5.84 P<0.0001 | t₁₂₃=6.23 P<0.001 |
| Colony size : Treatment | $F_{140,139}=14.31$ P=0.00022 | $X_1=1.83$ P=0.17 |
| Colony size | NA | $X_1=2.43$ P=0.12 |

347 Table 1: Effects of the different independent variables (season, treatment, habitat, colony size) and
348 their interactions on the worker-related variables, i.e. worker mortality rate and size of lab-workers.
349 When a first-order interaction was significant, we used the emmeans package to assess the separate
350 effects of the variables involved in the interaction. In that case, the t ratio is presented. NA means
351 that there was no biological meaning in testing the effect because of a significant higher-level
352 interaction (first-order). Significant P-values are shown in bold.

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| | Larval mortality rate | | Emergence rate | Larval pause rate | |
|----------------------------|----------------------------------------------------------------|--------------------------------------------------|-----------------------------------------------------------------|---------------------------------------------------------------|--------------------------------------------------|
| | Winter | Spring | | Winter | Spring |
| T : H : S | F_{141,140}=5.90 P=0.016 | | F _{143,140} =0.13 P=0.94 | F_{141,140}=5.61 P=0.019 | |
| Treatment : Habitat | F _{64,62} =0.17, P=0.84 | F_{79,78}=7.40 P=0.0080 | F _{143,142} =0.35 P=0.55 | F _{62,61} =0.58 P=0.45 | F_{79,78}=7.99 P=0.0059 |
| Habitat : Season | NA | | F _{143,142} =0.011 P=0.91 | NA | |
| Treatment : Season | NA | | F_{143,144}=35.42 P<10⁻⁷ | NA | |
| Treatment | F_{64,65}=38.97, P<10⁻⁷ | z=4.75 P<0.0001 | z=-10.03 P<0.0001 | F_{64,63}=52.76 P<10⁻⁹ | z=1.87 P=0.061 |
| Habitat | F _{64,63} =0.072 P=0.78 | z=0.24 P=0.81 | F _{143,144} =1.11 P=0.29 | F _{63,64} =0.044 P=0.83 | z=2.01 P=0.040 |
| Season | NA | | z=-2.38 P=0.017 | NA | |
| Colony size : Treatment | F _{140,139} =1.66 P=0.20 | | F _{140,139} =1.43 P=0.55 | F_{140,139}=4.56 P=0.034 | |
| Colony size | F _{140,141} =0.41 P=0.52 | | F_{143,144}=19.17 P<10⁻⁴ | NA | |

359 Table 2: Effects of the different independent variables (season, treatment, habitat, colony size) as
360 well as their interactions on the larvae-related response variables (larval mortality rate, emergence
361 rate and larval paused rate). When a significant Treatment : Habitat : Season (T : H : S) interaction
362 was found, the subsequent statistical analyses are shown for data splitted by season (left side of the
363 column: winter, right side: spring). When a significant first-order interaction involving a discrete
364 variables (Treatment, Season, Habitat) was found, we used the emmeans package to assess the
365 separate effects of the variables involved in the interaction. In that case, the z ratio is presented. NA
366 means that there was no biological meaning in testing the effect because of a significant higher-level
367 interaction (first-order or second-order). Significant P-values are shown in bold.

368

369 **Discussion**

370 As expected, high cadmium concentration had a negative effect on worker and larval mortality rates,
371 emergence rate of lab-workers and head size of lab-workers, as described in other studies performed
372 in the laboratory on invertebrates (snail: Gomot, 1998; ants: Jacquier et al., 2021a). This negative
373 effect was found for both forest and city habitats, as well as for both winter and spring seasons. First,
374 we showed that spring colonies tolerate cadmium better than winter colonies, with lower increase in
375 worker mortality rate, lower decrease in worker emergence rate and lower reduction of lab-worker
376 size when exposed to cadmium. This suggests that the putative secretion of anti-stress factors during
377 cold winter (e.g. heat shock proteins or detoxification enzymes, see Robert et al., 2016; Yocum, 2001)
378 does not improve robustness against cadmium in winter. Second, in contrast with what we expected
379 based on other studies investigating tolerance to trace metal in city populations (Andrew et al., 2019;
380 Jacquier et al., 2021a; Mireji et al., 2008) we did not find any differential response to cadmium
381 between city and forest colonies in winter colonies. We found a higher tolerance to cadmium of city
382 colonies, but only in spring colonies regarding larval mortality rate. We also found a differential
383 response to cadmium in spring colonies regarding larval pause rate, which increased under cadmium
384 exposure in city colonies but decreased in forest colonies. Spring colonies also had a higher larval
385 pause rate than winter colonies, under both control and cadmium.

386 Many ecotoxicological studies report seasonal variation in trace metal accumulation in organisms
387 (Cossa et al., 1980; Morgan and Morgan, 1993; Rabitsch, 1997). For example, Cossa et al., 1980
388 showed that in the mussel *Mytilus edulis*, the trace metal accumulation rate increased in all the
389 assessed sites from spring to summer (estuary and gulf). Morgan and Morgan (1993) monitored
390 cadmium and lead concentrations in two earthworms species every months for 13 months and found
391 that cadmium concentration was at its lowest and the lead concentration at its highest in summer
392 (July), which corresponds to the diapause period of one the two studied species (the other one does
393 not go through diapause). (Rabitsch, 1997) showed that in worker ants of *Formica pratensis*, trace
394 metal accumulation varied consistently over eight months and in five sampling sites, with the lowest
395 metal concentration found in spring and autumn workers. Such variation can be linked with annual
396 variation in the environmental availability of trace metals. Hence, higher trace metal accumulation in
397 a given period could just reflect a higher trace metal concentration in the environment, but not
398 necessarily a better tolerance to these trace metals. In our study, the lower worker mortality rate in
399 spring could be due to a heavier body mass of workers. Many studies showed a positive link between
400 mean body size and trace metal tolerance because of lower metabolism rate per unit in larger
401 individuals (Durou et al., 2005; Grześ, 2010b; Vesela and Vijverberg, 2007). Ant body mass varies
402 across seasons, with body mass reaching its maximum during spring or autumn, as workers forage

403 actively and accumulate fat reserves, whereas towards the end of hibernation, workers have
404 depleted fat reserves (Blanchard et al., 2000; Rabitsch, 1997; Tschinkel, 1993). Because of the lower
405 worker mortality rate, more of them are available to rear spring larvae, which could explain the
406 higher emergence rate and larger emerging lab-workers. To our knowledge, our study is the first to
407 address how tolerance to a trace metal differs between two points along the yearly cycle specifically
408 in terms of life history traits, while other studies only assessed bio-accumulation.

409 The better cadmium tolerance of spring colonies could be caused by differences in colony size
410 between spring and winter colonies, as colony size impacts stress buffering in ants (Crall et al., 2019;
411 Kaspari and Vargo, 1995; Naug, 2009). However, we did not find any effect of colony size on larval
412 mortality rate nor on lab-worker size. Emergence rate decreased with colony size in our study, but
413 treatment affected colonies similarly whatever their size. As the emergence rate in winter colonies
414 under control condition was higher than in spring colonies, it is unlikely that lower spring colony size
415 could explain the better tolerance against cadmium in spring. Interestingly, we found that the effect
416 of colony size differed between control and cadmium treatments for worker mortality rate and larval
417 pause rate. Worker mortality rate decreased with colony size under control but increased with
418 colony size under cadmium. This suggests a limit to colony stress-buffering under very stressful
419 conditions. It also suggests that winter colonies had a higher worker mortality rate than spring
420 colonies under cadmium because they were larger. Larval pause rate decreased with colony size
421 under control, but barely varied with colony size under cadmium. Therefore, it is unlikely that the
422 higher larval pause rate under cadmium in spring colonies is due to their lower colony size.

423 The larval pause rate varied depending on both season and habitat. This rate was much higher in
424 spring colonies than in winter colonies, even in control treatment, but it was also the only measured
425 trait that showed opposite patterns of response to cadmium between city and forest spring colonies.
426 The paused larval development observed in our experiment may correspond to what is called
427 diapause, i.e. “a state of arrested growth or development and prolonged hypometabolism associated
428 with various stressful challenges” (Popović et al., 2015). In some ant species, diapause is controlled
429 by the social environment. In *Myrmica rubra*, worker control over initiation or termination of larval
430 diapause depends on their physiological state: spring workers can trigger diapause termination
431 whereas autumn workers cannot (Brian, 1955). In contrast, in *Leptothorax acervorum* (closer to the
432 *Temnothorax* genus than *Myrmica*, Prebus, 2017), larvae seem to control queen diapause (Kipyatkov
433 et al., 1997). In our case, we can hypothesize that spring city larvae initiate or maintain their
434 developmental pause in response to stress caused by cadmium, and that spring forest larvae lose this
435 ability because stress is too high. Winter colonies may not have this problem as they just ended
436 winter diapause, and they may therefore keep the ability to pause the development of larvae

437 efficiently in both city and forest habitats. Another hypothesis is that city and forest workers differ in
438 their ability to respond to larval stimuli; because city colonies are exposed to higher cadmium
439 concentration than forest colonies in nature (2.45mg.kg⁻¹ in city wood soil vs 0.30mg.kg⁻¹ in forest
440 soils, see Foti et al 2017), city workers could maintain control over larval pause despite high levels of
441 cadmium, but forest spring workers could become incapacitated by cadmium stress. A cross-
442 fostering experiment would be useful to test these two alternative hypotheses. However, it is
443 important to note that other stressors than trace metals could differ between rural and city sites and
444 be responsible of the observed difference, but we did not specifically test them in this study. Overall,
445 the higher rate of paused larvae in city colonies could be an adaptive response to cadmium; paused
446 larvae avoid feeding on cadmium and they may resume their development once cadmium exposure
447 is over, as shown in other studies (Aránguiz-Acuña and Serra, 2016; Oskina et al., 2019). This may also
448 explain the differential larval mortality rate in spring colonies, with lower mortality in city colonies
449 because of more paused larvae. However, all those hypotheses remained highly speculative and
450 would deserve further investigations to confirm or infirm this.

451 We expected city colonies to tolerate cadmium better, whatever the season. In spring, we found that
452 city colonies tolerate cadmium better than forest colonies, with a lower larval mortality rate, as
453 found in a previous study (Jacquier et al., 2021a). However, we did not find a better cadmium
454 tolerance of city colonies regarding emergence rate and lab-worker size, in contrast with Jacquier et
455 al. (2021a). We even found that forest colonies were marginally more tolerant than city colonies as
456 their lab-worker size decreased less when exposed to cadmium. We found a differential response to
457 cadmium for the larval pause rate, with more paused larvae in city colonies but less paused larvae in
458 forest colonies when treated with cadmium. Regarding winter colonies, in contrast with Jacquier et al
459 (2021a), we found that forest and city winter colonies did not exhibit any differential response to
460 cadmium whatever the life history traits measured. One possible explanation is that 2019/2020
461 winter was unusually warm, i.e. 2°C warmer relative to 2017/2018 and 2018/2019 winters
462 (November 2017 to March 2018, [https://www.infoclimat.fr/stations-meteo/analyses-
463 mensuelles.php](https://www.infoclimat.fr/stations-meteo/analyses-mensuelles.php)). Winter temperature impacts individual and colony survival. For example, in the ant
464 *Lasius niger*, warm winters enhance worker survival (Haatanen et al., 2015), as found in other insect
465 species (Takeda et al., 2010). On the other hand, warm winters decrease winter survival in the boreal
466 wood ant *Formica aquilonia* and other insect species (Radchuk et al., 2013; Sgolastra et al., 2011;
467 Stuhldreher et al., 2014), maybe because metabolism remains high (Hahn and Denlinger, 2011;
468 Sinclair, 2015). In the *Temnothorax* genus, colonies are directly exposed to weather conditions during
469 winter because they nest above-ground (Herbers, 1989). A warm winter may have disturbed

470 physiological processes in city and/or forest colonies, leading to low cadmium tolerance and a lack of
471 city/forest differential response.

472 To conclude, our study suggests that the seasonal context of studies is of high importance, even
473 though additional replicates would be needed to generalize our findings, both with different sites
474 and with different years. We found that, for one specific area (Paris) and one specific year (2020-
475 2021), spring colonies tolerate cadmium better than winter colonies. We also found that differential
476 tolerance to trace metal between city and forest ant colonies is not detectable during winter, but it is
477 detectable in spring. The absence of differential response to cadmium during winter 2020 could be
478 due to a warmer winter, as (Jacquier et al., 2021a, 2021b) found a differential response to cadmium
479 when working on the exact same populations but during colder winters or under cold laboratory
480 conditions (2°C colder in mean from November 2017 to March 2018, (Jacquier et al., 2021a, 2021b).
481 Seasonality impacts some life history traits, in our case the response to a trace metal, but more
482 studies are needed as we only used two time points (two different seasons) within the same year and
483 only one location for each habitat. Stochasticity could thus also be responsible for the observed
484 differences, unless the pattern is proven to repeat itself over the years in future studies.
485 Nevertheless, experiments assessing responses to environmental changes should avoid focusing on a
486 single time point but instead incorporate the whole yearly life-cycle of the model organism.
487 Repeating the experiment at different times of the year could highlight season-dependent response
488 to a given stressor.

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