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1           **Disentangling the roles of social and individual effects on cadmium**  
2                           **tolerance in the ant *Temnothorax nylanderi*.**

3  
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20  
21   **Abstract**

22   Urbanization brings new pressures for individuals. Among them, trace elements such as  
23   cadmium are important stressors. A recent study highlights a weaker negative effect of  
24   cadmium on city colonies relative to their forest counterparts in the ant *Temnothorax*  
25   *nylanderi*. Here, we aim to test whether this better tolerance of city colonies in this species  
26   results from (1) a better ability of workers to rear larvae despite stressful conditions and/or (2)  
27   a better ability of larvae to develop properly despite stressful conditions. We performed a  
28   cross-fostering experiment of workers and larvae from city and forest colonies, under  
29   common garden in the laboratory. Colonies were fed using cadmium-enriched or cadmium-  
30   free food for two months, and we measured four life-history traits. As expected, cadmium had  
31   a negative impact on all traits. Unexpectedly, we did not observe a better tolerance of city  
32   colonies to cadmium contrary to our previous study, preventing us from disentangling the  
33   respective contributions of workers and larvae to cadmium tolerance. Interestingly, forest

34 colonies seem to be of better quality in our laboratory conditions. Finally, colony size  
35 increased adult survival, but only in the absence of cadmium, suggesting that social buffering  
36 could collapse under strong external disturbances.

37

38 **Key-words:** trace element, body size, colony size, common garden, cross-fostering,  
39 urbanization.

40

## 41 **Introduction**

42

43 Cities are natural laboratories for investigating evolutionary changes in an urbanizing world  
44 (Alberti, Marzluff, & Hunt, 2017). Urbanization and human-induced environmental changes  
45 are forcing organisms to face many new pressures (Grimm *et al.*, 2008), including changes in  
46 impervious land cover and increases in temperature (including the ‘urban heat island’ -  
47 (Levermore *et al.*, 2018; Diamond & Martin, 2020). Pollution is also a major component of  
48 urbanization (be it sound, chemicals, light or others; e.g. Westby and Medley, 2020),  
49 especially trace metals which are persistent pollutants that bioaccumulate in the food chain.  
50 For example, cadmium is a highly toxic trace metal to various species (e.g. increasing  
51 mortality rate, decreasing hatching rate or impeding development, Gomot, 1998 (snails);  
52 Järup and Åkesson, 2009 (human); Malakar *et al.*, 2009 (grasshopper)) that can be found in  
53 concentrations ten times higher in cities than in rural soils (Foti *et al.*, 2017). These growing  
54 pressures lead to changes in various individuals traits (physiology, morphology, phenology,  
55 behavior), both plastically (e.g. changing in behavior (Ditchkoff, Saalfeld, & Gibson, 2006) or  
56 morphology (Prosser, Hudson, & Thompson, 2006)) and evolutionarily (e.g. changes in  
57 phenology (Chick *et al.*, 2019) or physiology (Partecke, Schwabl, & Gwinner, 2006) - see  
58 also Johnson and Munshi-South, 2017; reviewed in Alberti *et al.*, 2017). For instance, urban  
59 populations exhibit earlier timing of reproduction (plants: Jochner and Menzel, 2015; ants:  
60 Chick *et al.*, 2019), lower corticosterone stress response (birds: Partecke *et al.*, 2006), higher  
61 thermal tolerance (ants: Diamond *et al.*, 2017), decreased migratory behavior and  
62 aggressiveness (birds, respectively: Evans *et al.*, 2012; Hasegawa *et al.*, 2014, and smaller  
63 size (Daphnia: Brans *et al.*, 2017; spider: Dahirel *et al.*, 2019).

64 Although many organisms face these pressures alone, individuals living in groups  
65 benefit from a filter provided by their society against external disturbances. The social  
66 environment is a key component to be considered when studying the response of social  
67 species to external pressures, as it may buffer external disturbances (Straub *et al.*, 2015). For

68 example in social insects, colony size plays a role in buffering the effects of temperature, with  
69 larger colonies usually having less mortality or stronger trait stability (cold: Heinze et al.,  
70 1996; warm: Molet et al., 2017). This social buffering is found for various pressures, such as  
71 exposure to parasites (Scharf *et al.*, 2012), seasonality (Kaspari & Vargo, 1995), starvation  
72 (Heinze *et al.*, 1996; Modlmeier, Foitzik, & Scharf, 2013) and pesticides (Crall *et al.*, 2019).  
73 In addition to workers, the presence of larvae can also improve colony resistance to stress in  
74 harsh periods, such as under starvation (Rueppell & Kirkman, 2005; Modlmeier *et al.*, 2013).  
75 Furthermore, the social environment highly modulates brood development in insect societies  
76 such as in ants (Linksvayer, 2006; Wills *et al.*, 2018) or bees (Linksvayer *et al.*, 2011), and  
77 therefore can influence the phenotypes produced within colonies. Cross-fostering  
78 experiments, mixing worker and larval origins, are particularly useful to partition the effects  
79 of the social environment, that is assessing the relative contribution of adults and larvae. For  
80 example, crossfostering has been used in some ants species to assess the relative contributions  
81 of genetic and social effects on individual and colony traits (Linksvayer, 2006), the influence  
82 of social structure on brood development (Purcell & Chapuisat, 2012), the effects of  
83 matrilines and patriline on immune defense (Armitage *et al.*, 2011) and the consequences of  
84 the rearing environment on individual and colonial personalities (Keiser *et al.*, 2015).  
85 However, as far as we are aware, it has never been used to investigate the relative effect of the  
86 social environment and larvae themselves on the ability to tolerate stressful conditions such as  
87 pollution.

88 In this study, we used a cross-fostering experiment while rearing colonies under  
89 common laboratory conditions in the ant *Temnothorax nylander* in the presence or absence of  
90 a stressor (cadmium). A previous study showed that city colonies were less negatively  
91 affected by cadmium than forest ones for two traits: the size of produced workers and the  
92 survival of larvae (Jacquier *et al.*, 2021a). In the present study, we used the same laboratory  
93 rearing setup and we measured the same traits (i.e. body size at emergence and survival) as  
94 well as the development duration. Moreover, in contrast with Jacquier *et al.* (2021a) who only  
95 assessed worker traits, we also measured sexual production and size of sexuals. Size of  
96 sexuals is an important trait linked with fitness in ants as larger sexuals have greater mating  
97 success and colony founding success in some species (Abell *et al.*, 1999; Wagner & Gordon,  
98 1999; Wiernasz *et al.*, 2001; Wiernasz & Cole, 2003; Fjerdingstad & Keller, 2004). By  
99 performing a cross-fostering, our study allows us to test the relative contribution of the origin  
100 of workers (forest or cities) and of the origin of larvae (forest or cities) on the life history  
101 traits measured in presence and absence of cadmium.

102

## 103 **Material and methods**

104

### 105 *Ants rearing*

106 *Temnothorax nylanderi* is a tiny ant, nesting in acorns or twigs, widespread in western and  
107 central Europe and living in small colonies (a few dozen workers), and common in urban and  
108 forest habitats (Foitzik, Strätz, & Heinze, 2003). We collected colonies at two sites where a  
109 differential response to cadmium has been documented (Jacquier *et al.*, 2021a). We collected  
110 103 city colonies in an urban park in Paris (Parc des Buttes-Chaumont, 48°52'47.1"N;  
111 2°23'45.6"E) and 109 forest colonies in a forest located 50km away from Paris (Chantilly  
112 forest, 49°10'59.8"N; 2°28'43.6"E). Colonies were harvested in April 2019 and then installed  
113 in the laboratory. The artificial nest consisted of two microscope slides (75 x 26 x 1 mm)  
114 separated by a 1-mm auto-adhesive layer of plastic foam with 3 chambers. The top slide was  
115 covered with dark paper to protect the colony from the light. The nest was placed in a plastic  
116 box (11.5 x 11.5 x 5.5cm) for foraging. We discarded 52 colonies with more than one queen  
117 or with no queen (28 colonies in city, 24 in forest), as well as 18 colonies (3 in city, 15 in  
118 forest) with cestode-infected workers (that display typical pale-yellow coloration (Scharf *et*  
119 *al.*, 2012)). We finally retained 72 queenright colonies for city habitats and 70 colonies for  
120 forest habitats. Workers and larvae in each colony were counted. Colony size ranged from 3  
121 to 256 workers and brood from 8 to 96 larvae. Colonies were acclimated for seven days in the  
122 laboratory before the experiment began. Using a microscope, we only kept second instar  
123 larvae (i.e. larvae under 2mm long with hairs mainly on the front, Plateaux, 1970), so that all  
124 larvae would be exposed to cadmium at the same time and for the majority of their  
125 development duration.

126

### 127 *Experimental design*

128 Workers and larvae had two different origins (city or forest), resulting in four different  
129 crosses: city workers with city larvae from another colony (CC, 36 colonies), forest workers  
130 with forest larvae from another colony (FF, 36 colonies), city workers with forest larvae (CF,  
131 35 colonies) and forest workers with city larvae (FC, 35 colonies). Colonies were matched by  
132 pairs for brood exchange, depending on the number of workers and larvae. Each colony  
133 received all the second-instar brood from its paired colony. Larvae of the two paired colonies  
134 were exchanged simultaneously, larvae remained isolated for a maximum of 15 minutes  
135 without workers. Such brood exchanges have already been performed in other *Temnothorax*

136 species (Linksvayer, 2006, 2007), and the brood is accepted by workers without  
137 discrimination.

138 Each colony was then exposed to a feeding treatment with two levels: control or  
139 cadmium. Colonies were evenly assigned to control or cadmium treatment depending on their  
140 colony size, in order to obtain a similar distribution of colony sizes within treatment levels.  
141 Control colonies were fed three times a week with a mixture of diluted honey, soya yogurt  
142 and dried crickets. Cadmium colonies were fed with the same mixture containing 100µg/g  
143 cadmium (Jacquier *et al.*, 2021a). The concentration used in the food corresponds to the LC50  
144 (the concentration that triggers 50% mortality over a given period of time), a physiologically  
145 but not ecologically relevant dose. Indeed, this concentration is about fifty times higher than  
146 the typical cadmium concentration measured in urban soils. However, in the field, recurrent  
147 exposure to pollutants can lead to bioaccumulation and cadmium concentration can reach 102  
148 mg/kg in the ant *Formica polyctena*, for a soil concentration of 2 mg.kg<sup>-1</sup> (Starý &  
149 Kubizňáková, 1987). Therefore, the concentration that we used was high but still in the upper  
150 range of real field values (Jacquier *et al.*, 2021a). We used 15 “CC”, “FC” and “FF” colonies,  
151 and 16 colonies “CF” with control food. We assigned more colonies to cadmium treatment in  
152 order to compensate for the potential risk of mortality, so we used 21 “CC” and “FF”  
153 colonies, 19 “CF” colonies and 20 “FC” colonies with cadmium food.

154 Colonies were reared in a climatic chamber (reference CTS TP10/600) at 22-27°C –  
155 12h : 12h cycle with natural photoperiod for 64 days, so that larvae could emerge as adults  
156 (Molet *et al.*, 2017). Workers that emerged in the laboratory (called “laboratory-born  
157 workers”) were identified based on their pale color and collected once they started to move.  
158 They were anesthetized by cooling down and stored in 96% ethanol for morphological  
159 measurements (see below). Newly-laid eggs were removed every week to prevent the  
160 hatching of additional larvae in order to keep the initial number of larvae constant and not to  
161 interfere with the experimental larvae that originate from crosses. To do so, we removed the  
162 top microscope slide from the nest, took out the eggs, and then let the colony move to a new  
163 nest positioned just in front of the old one. This way, we did not need to put a microscope  
164 slide back on the nest, which could have crushed some workers.

165 Adult workers born in the field were removed from the colonies when they died and  
166 counted to compute the worker mortality rate. The emergence rate was computed as the ratio  
167 between the number of larvae that developed to adulthood over the number of larvae initially  
168 given to the colony. For each produced worker, gyne and male, we also measured the

169 development duration (computed as the number of days from the beginning of the experiment  
170 to emergence) as well as the head width (see below).

171

### 172 *Size measurement*

173 For morphological measurements, heads were separated from bodies. Heads were stuck on  
174 double-sided tape and photographed under a Discovery V12 Zeiss stereomicroscope  
175 connected to a computer via a Zeiss AxioCam ICc 5 video camera and measured with ImageJ  
176 1.8 (Abràmoff, Magalhães, & Ram, 2004). Head width is a standard measurement of body  
177 size in ants (Tschinkel, Mikheyev, & Storz, 2003).

178

### 179 *Statistical analyses*

180 We investigated the effects of worker origin (forest/city), larval origin (forest/city), treatment  
181 (cadmium/control) and the initial colony size on the adult mortality rate, the emergence rate,  
182 the development duration of produced individuals (sexuals and workers) and the size of  
183 produced individuals. We included a second-order interaction between feeding treatment,  
184 larval origin and worker origin, that allowed us to test whether the effect of treatment differed  
185 across each crossing combination. We also included the interaction between the feeding  
186 treatment and the colony size to investigate whether larger colonies were less affected by  
187 cadmium than smaller ones.

188 All statistical analyses were carried out with R v3.6.1 ([www.r-project.org](http://www.r-project.org)). All plots  
189 were generated using *ggplot2* (Wickham, 2009). First, we assessed the effect of predictor  
190 variables on colony-level dependent variables (adult worker mortality rate, emergence rate,  
191 and the head width of produced individuals) using generalized linear models (GLMs). Adult  
192 worker mortality and emergence were analyzed using quasibinomial distribution. Finally, we  
193 assessed the effect of predictor variables on the development duration and the head width of  
194 emerged individuals, two individual-level dependent variables, using linear mixed models  
195 (LMMs, package *nlme*, (Pinheiro *et al.*, 2016)), with colony as random factor to take into  
196 account pseudo-replication. All models are summarized in Table 1.

197 For each analysis, the minimum adequate model was selected using a backward  
198 stepwise approach where explicative variables were removed one by one from a full model  
199 based on a log likelihood ratio test. Fisher or  $\text{Chi}^2$  tests (according to the type of model used)  
200 and associated *P*-values for each predictive variable were obtained to obtain the *P*-values for  
201 each predictor variable by comparing the minimum adequate model with a model excluding  
202 or including the variable of interest (according to whether the variable was present in or

203 absent from the minimal adequate model respectively). We first tested the significance level  
204 of the second-order interaction, then first-order interactions. Normality of the residuals and  
205 homogeneity of variances were checked visually following (Pinheiro & Bates, 2000); no  
206 transformation of the data was necessary.

207

#### 208 *Data availability statement*

209 The dataset and R scripts are available on Zenodo (10.5281/zenodo.5105734).

210

## 211 **Results**

212

213 Six queens died during the experiment (one ‘FF’, ‘FC’ and ‘CC’ colony; three ‘CC’ colonies)  
214 and their colonies were excluded from data analyses. Our final dataset consisted of 136  
215 queenright colonies. We collected a total of 1 688 workers, 1 092 males, one intercaste  
216 (worker phenotype with two ocelli, Okada et al., 2013) and 134 gynes. As we collected gynes  
217 from only 15 colonies (13 control colonies: four ‘FF’ colonies, three ‘CC’, ‘CF’ and ‘FC’  
218 colonies; two cadmium colonies: one ‘CF’ and one ‘FF’ colony), we did not include gynec  
219 production in our dataset because of a lack of statistical power, with only five gynes under  
220 cadmium treatment.

221       Whatever the variables measured, we did not find a significant second-order  
222 interaction (Treatment : Worker origin : Larva origin, Table 1), i.e. there was no differential  
223 sensitivity to cadmium depending on the crossing. Moreover, contrary to our expectation,  
224 there was no better cadmium tolerance in city colonies: we did not find any interaction  
225 between Treatment and Worker origin (at the exception of male development duration, see  
226 below) or Treatment : Larval origin interaction (Table 1). Those interactions were therefore  
227 removed from the model and in the following results, we only present the main effects of  
228 worker origin, larval origin, colony size and treatment, as well as the interaction between  
229 colony size and treatment (detailed statistics are presented in Table 1).

230

#### 231 *Adult mortality rate*

232 Cadmium increased adult mortality rate by 148% (Treatment effect,  $F_{131:133} = 92.43$ ,  $P <$   
233  $0.001$ ; figure 1a). The origin of workers (city or forest) significantly affected the mortality  
234 rate ( $F_{131:132} = 7.72$ ,  $P = 0.0060$ ) with city workers (‘CC’ and ‘CF’) having a 14% higher  
235 mortality rate than forest workers (origin of workers effect,  $F_{131:132} = 7.72$ ,  $P = 0.0060$ ; figure  
236 1a). Not surprisingly, there was no effect of the origin of larvae (Table 1). The mortality rate



237 marginally differed depending on the treatment in interaction with the colony size (Treatment  
238 x Colony size interaction,  $F_{131:132} = 3.67$ ,  $P = 0.057$ ). The mortality rate increased with the  
239 initial colony size under cadmium, while it decreased with the initial colony size in the control  
240 (figure 2).

241

#### 242 *Emergence rate and development duration*

243 Cadmium decreased emergence rate by 50% (Treatment effect,  $F_{131:132} = 107.93$ ,  $P < 0.001$ ;  
244 figure 1b). The origin of workers had no effect on emergence rate ( $F_{129:131} = 0.55$ ,  $P = 0.60$ ;  
245 figure 1b) whereas the origin of larvae significantly affected the emergence rate (origin of  
246 larvae effect,  $F_{131:132} = 7.54$ ,  $P = 0.0070$ , figure 1b) with forest larvae having a 17% higher  
247 emergence rate than city larvae. There was no effect of colony size on emergence rate (Table  
248 1).

249 The development of produced individuals was 6% longer under cadmium (Treatment  
250 effect, workers:  $\chi^2_1 = 7.14$ ,  $P = 0.007$ ; figure 1c - males:  $\chi^2_1 = 64.69$ ,  $P < 0.001$ ; figure 1d).  
251 The origin of workers had no effect on worker development duration whereas the origin of  
252 larvae had a significant effect (origin of larvae effect,  $\chi^2_1 = 4.22$ ,  $P = 0.039$ ; Table 1). Forest  
253 larvae took 3.1% less time to develop into adult workers than city larvae (figure 1c). We  
254 found no effect of colony size on the development duration of larvae (Table 1). Regarding  
255 males, we found a marginally significant Treatment : Worker origin interaction ( $\chi^2_1 = 3.82$ ,  $P$   
256  $= 0.051$ ), with longer development under cadmium for males, but not under control. The  
257 origin of larvae had no effect on the development duration of males (Table 1). Development  
258 duration increased with the initial colony size (Colony size effect, workers:  $\chi^2_1 = 6.22$ ,  $P =$   
259  $0.012$ ; males:  $\chi^2_1 = 5.07$ ,  $P = 0.024$ ).

260

#### 261 *Size (head width) of produced individuals*

262 Cadmium reduced the size of the produced workers and males by 14% and 16% respectively  
263 (Treatment effect, workers:  $\chi^2_1 = 157.97$ ,  $P < 0.001$ ; figure 3a - males:  $\chi^2_1 = 156.24$ ,  $P <$   
264  $0.001$ ; figure 3b). Neither worker origin nor larvae origin had an effect on worker head width  
265 (Table1). Colony size had no effect on worker head width (Table 1). Regarding males, forest  
266 workers produced marginally larger males relative to city workers (+2.3%, effect of worker  
267 origin,  $\chi^2_1 = 3.57$ ,  $P = 0.059$ ; figure 3b). Larvae origin and colony size had no effect on male  
268 head width (Table 1).

269

270

## 271 **Discussion**

272

273 We aimed at disentangling the implications of workers (the social environment) and larvae in  
274 the better cadmium tolerance of larvae previously observed in city colonies by Jacquier et al.  
275 (2021a). Just like other studies (den Besten *et al.*, 1989; Gomot, 1998; Cervera *et al.*, 2004;  
276 Demenesku *et al.*, 2014; Jacquier *et al.*, 2021a), we found that cadmium had a negative  
277 impact on all life history traits measured, i.e. it increased worker mortality rate (+148%),  
278 reduced emergence rate (-50%), extended development duration (+6%) and reduced the size  
279 of individuals, for both workers (-14%) and males (-16%). The intensity of the effect on  
280 workers was of the same order of magnitude as in Jacquier et al. (2021a). However, in  
281 contrast with Jacquier *et al.* (2021a), the effect of cadmium was similar in city and forest  
282 colonies regarding emergence rate and worker head width, preventing us from disentangling  
283 the effect of workers and larvae on the expected differential cadmium tolerance. This absence  
284 of differential response between habitats could result from annual random variation and the  
285 fact that a comparison between only two sites did not allow us to detect a difference in our  
286 study. However, our sample size in terms of number of colonies was large, excluding a lack  
287 of statistical power. In addition, the differential response between the two habitats was  
288 observed for five replicated comparisons (Jacquier *et al.*, 2021a,b) making chance an unlikely  
289 explanation. Also, as the same persons performed the experiments, with the same laboratory  
290 set up (even though in our experiment larvae were manipulated which was not the case in  
291 Jacquier et al (2021a) – see below), we think that the observed difference is a true biological  
292 phenomenon.

293 We propose three non-mutually exclusive hypotheses that could explain the  
294 unexpected absence of a differential cadmium tolerance between forest and city colonies. A  
295 first explanation is a disruptive effect of cross-fostering. This experimental method is  
296 commonly used to split the relative contributions of different social aspects on measured traits  
297 in ants (Linksvayer, 2006; Armitage *et al.*, 2011; Purcell & Chapuisat, 2012). Therefore, adult  
298 workers had to rear larvae from foreign colonies. Because workers are unrelated to these  
299 larvae, they may invest less in rearing them. However, Foitzik et al., (2007) showed that in  
300 *Temnothorax nylanderi* there was little to no discrimination of non-nestmates, maybe because  
301 colonies and populations are weakly differentiated genetically and thus could have similar  
302 cuticular hydrocarbons, therefore impeding worker discrimination based on this cue. Besides,  
303 as in other successful cross-fostering experiments (Linksvayer, 2006; Armitage *et al.*, 2011;  
304 Purcell & Chapuisat, 2012), larvae were largely accepted by workers at the beginning of our

305 experiment (more than 90% of the brood survived after the larval exchange between  
306 colonies), so workers are probably not able to detect that larvae originated from another  
307 colony. A second hypothesis is a within year variation of cadmium tolerance. We collected  
308 colonies in April, whereas (Jacquier *et al.*, 2021a) collected them in February. In some  
309 species, trace metals show a seasonal pattern of accumulation, correlated with seasonal  
310 variation in detoxification enzymes or proteins (Fialkowski *et al.*, 2003; Gorbi, Baldini, &  
311 Regoli, 2005). Recent data suggest that the better cadmium tolerance of city colonies depends  
312 on both hibernation temperature and the period of the year at which the experiment was  
313 conducted (Jacquier *et al.*, 2021b; Jacquier *et al.*, *under review*). In this study, we collected  
314 colonies later (April) than in (Jacquier *et al.*, 2021a), in order to obtain more sexuals. This  
315 may have impeded further differential cadmium tolerance between city and forest colonies, as  
316 Jacquier *et al.* (*under review*) showed that colonies collected several weeks after hibernation  
317 (and therefore with eggs that were laid after the hibernation period, i.e. early June here) do not  
318 have differential emergence rates in response to cadmium. Further studies are needed to  
319 investigate the possible effect of within-year variation in environmental factors on cadmium  
320 tolerance between the two habitats, since urban and forest ants have a slightly shifted  
321 phenology (Chick *et al.*, 2019). A last hypothesis stands in inter-annual variation in cadmium  
322 tolerance. In Jacquier *et al.* (2021a), urban colonies tolerated cadmium better than forest  
323 colonies, but the level of differential tolerance varied among sites and years. In this study, we  
324 only used one study site, therefore we lack replication and the observed results could be due  
325 to natural variation in cadmium tolerance at this site for this particular year. Moreover,  
326 Jacquier *et al.* (2021b) suggested that cadmium tolerance could also depend on environmental  
327 factors such as hibernation temperature. Colonies may have experienced special climatic  
328 conditions the year of our experiment, and therefore we found results that contrast with  
329 previous findings.

330 Our study quantified the effect of cadmium on male production, and therefore the  
331 potential consequences on the reproductive success of colonies. In *T. nylanderi*, brood  
332 production is highly synchronized (Kipyatkov, 1993), but males develop faster than workers  
333 (our study and (Jacquier *et al.*, 2021a). As we started our study later (April vs February –  
334 March for Jacquier *et al.* 2021a) and winter was milder, colonies were probably more  
335 advanced in the process of male production. The relative lack of gyne production is not  
336 surprising, given that resource allocation can be redirected towards workers and males after a  
337 large worker loss (29% on average for control colonies, 72% for cadmium colonies at the end  
338 of the experiment) in this species (Foitzik & Heinze, 2000; Foitzik *et al.*, 2003; Honorio,

339 Doums, & Molet, 2020). Forest workers reared males that were 1.7% larger than those reared  
340 by city workers. Thus, when forest workers reared larvae, they produced larger males  
341 whatever the origin of larvae and whatever the treatment. Larger males have greater mating  
342 success and sperm production in ants (Abell *et al.*, 1999; Wiernasz *et al.*, 2001; Brown &  
343 Keller, 2006), therefore forest colonies may have a higher fitness than city colonies whatever  
344 the treatment, suggesting a better general condition. As adult city workers unexpectedly faced  
345 a higher mortality (in contrast with Jacquier *et al.* (2021a), a hypothesis is that city workers  
346 may have undergone higher stress levels, maybe leading to less brood care.

347 Different studies documented phenotypic divergence between city and rural insect  
348 populations. For example, body size is sometimes higher (Weller & Ganzhorn, 2004; y  
349 Gomez & Van Dyck, 2012; Lowe, Wilder, & Hochuli, 2014; Merckx *et al.*, 2018; Theodorou  
350 *et al.*, 2021) and sometimes lower (Merckx *et al.*, 2018; Eggenberger *et al.*, 2019) in city  
351 populations depending on the species. Interestingly, in *Temnothorax* species, previous studies  
352 found no differences in worker size across urban and rural populations (Yilmaz *et al.*, 2019;  
353 Jacquier *et al.*, 2021a). Emergence rate (grasshoppers: Gomez and Van Dyck, 2012;  
354 damselfly: Tüzün *et al.*, 2017) and development duration in the field also differs between  
355 urban and rural populations depending on the species (mosquitoes: Li *et al.*, 2014, damselfly:  
356 Tüzün *et al.*, 2017). In this study, we found a limited effect of the origin of larvae,  
357 independently of the feeding treatment, and no effect of the social environment, except for  
358 male size. Forest larvae had a 17% higher emergence rate and a 3.1% shorter development  
359 duration but a 2.3% larger size at emergence when they developed into workers, therefore  
360 appearing to be of better quality (even though worker size does not seem to be of primary  
361 value for colony fitness in this species (Colin *et al.*, 2017; Honorio *et al.*, 2020, 2021 - but see  
362 Modlmeier *et al.*, 2013). Our study does not allow to further substantiate the processes  
363 involved, but two potential proximate mechanisms can be proposed. First, city colonies may  
364 have had a poor physiological and metabolic state at the beginning of the experiment because  
365 they faced multiple stressors related to their urban environment prior to the experiment.  
366 Therefore, city queens could lay lower quality eggs, with larvae that develop slower and  
367 undergo lower emergence rate. Differences in egg provisioning through maternal or epigenetic  
368 effects can generate differences in development and emergence rate between larvae. For  
369 instance, juvenile hormone and ecdysteroid are transmitted to brood and influence  
370 development and emergence rate in ants (Schwander *et al.*, 2008; Cahan, Graves, & Brent,  
371 2011; Libbrecht *et al.*, 2013) - see also (LeBoeuf *et al.*, 2016, 2018). Second, the differences  
372 between city and forest colonies could be genetically determined, as a recent study found a

373 few discriminating loci (Khimoun *et al.*, 2020). However, this study also highlighted a lack of  
374 genetic differentiation between urban and forest populations.

375 Another important social trait is colony size. Stress tolerance increases with colony  
376 size in many eusocial species (Naug, 2009; Scharf *et al.*, 2012; Modlmeier *et al.*, 2013; Crall  
377 *et al.*, 2019). Here, we found no buffering effect of colony size with the exception of a  
378 contrasting effect on worker mortality rate (positive relationship under control treatment, but  
379 negative under cadmium) and a positive relationship with the development duration of  
380 workers and males. This is surprising, as other studies on *T. nylanderi* showed that larger  
381 colonies better resist starvation (Modlmeier *et al.*, 2013), parasites (Scharf *et al.*, 2012) or  
382 high temperatures (Molet *et al.*, 2017). (Jacquier *et al.*, 2021a) did not find any buffering  
383 effect of colony size on worker mortality, whatever the treatment. This suggests that the  
384 buffering effect of colony size in *Temnothorax nylanderi* is quite unstable and may depend on  
385 the measured traits and on the time at which the experiment is conducted. The absence of  
386 buffering effect of colony size under cadmium in our study could be explained by a higher  
387 sensitivity of large colonies to disturbances. Having more individuals could enhance the  
388 exposures to various stressors (pathogens, parasites, pollutions, etc.) and their transmission to  
389 nestmates. For example, in honey bees, larger colonies could be more sensitive to sublethal  
390 effects of pesticides and pathogens and undergo colony collapse disorder (Barron, 2015).  
391 When the disturbance is too high, larger colonies could be more destabilized and collapse.  
392 Thus, social organisms could be more exposed and vulnerable to environmental stressors than  
393 solitary organisms. Finally, the positive relationship between colony size and the development  
394 duration of workers and males is consistent with the loss of per-capita productivity as a  
395 function of colony size, found in many ant species (reviewed in Kramer *et al.*, 2014).

396 To conclude, we did not find any difference in cadmium tolerance between forest and  
397 city colonies, so we were not able to assess the relative contributions of social environment and  
398 larval intrinsic factors. Replicating this study with colonies collected at the end of winter (the  
399 period at which a differential response to cadmium was found between forest and city  
400 colonies) would be necessary to assess it. However, interestingly, we found phenotypic  
401 differences between forest and city colonies, independently of the treatment, with forest  
402 larvae having a higher emergence rate and turning into larger workers. These differences were  
403 independent of the social environment, i.e. workers, contrasting with previous studies on other  
404 social insect species (Pankiw, Tarpay, & Page, 2002; Linksvayer, 2006, 2007; Linksvayer *et*  
405 *al.*, 2011; Armitage *et al.*, 2011). However, regarding males, we found a slight effect of the  
406 social environment, suggesting a higher social control on the phenotype of sexuals than on

407 that of workers. We suggest that more attention should be paid to the role of the social  
408 environment in phenotypic divergence. Since our studies only involved two sites and one  
409 species, the generalization of our results will require the assessment of additional locations  
410 and species.

411

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415

#### 416 **Author's contributions**

417 LJ and RH designed the study, collected and reared ants, performed the experiment and  
418 statistical analyses, and wrote the manuscript. CD designed the study, contributed to statistical  
419 analyses and wrote the manuscript. MM designed the study, wrote the manuscript and  
420 supervised the project. All authors read and approved the final manuscript.

421

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425

#### 426 **Compliance with ethical standards**

427

#### 428 **Conflict of interest**

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

430

#### 431 **Ethical approval**

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

434

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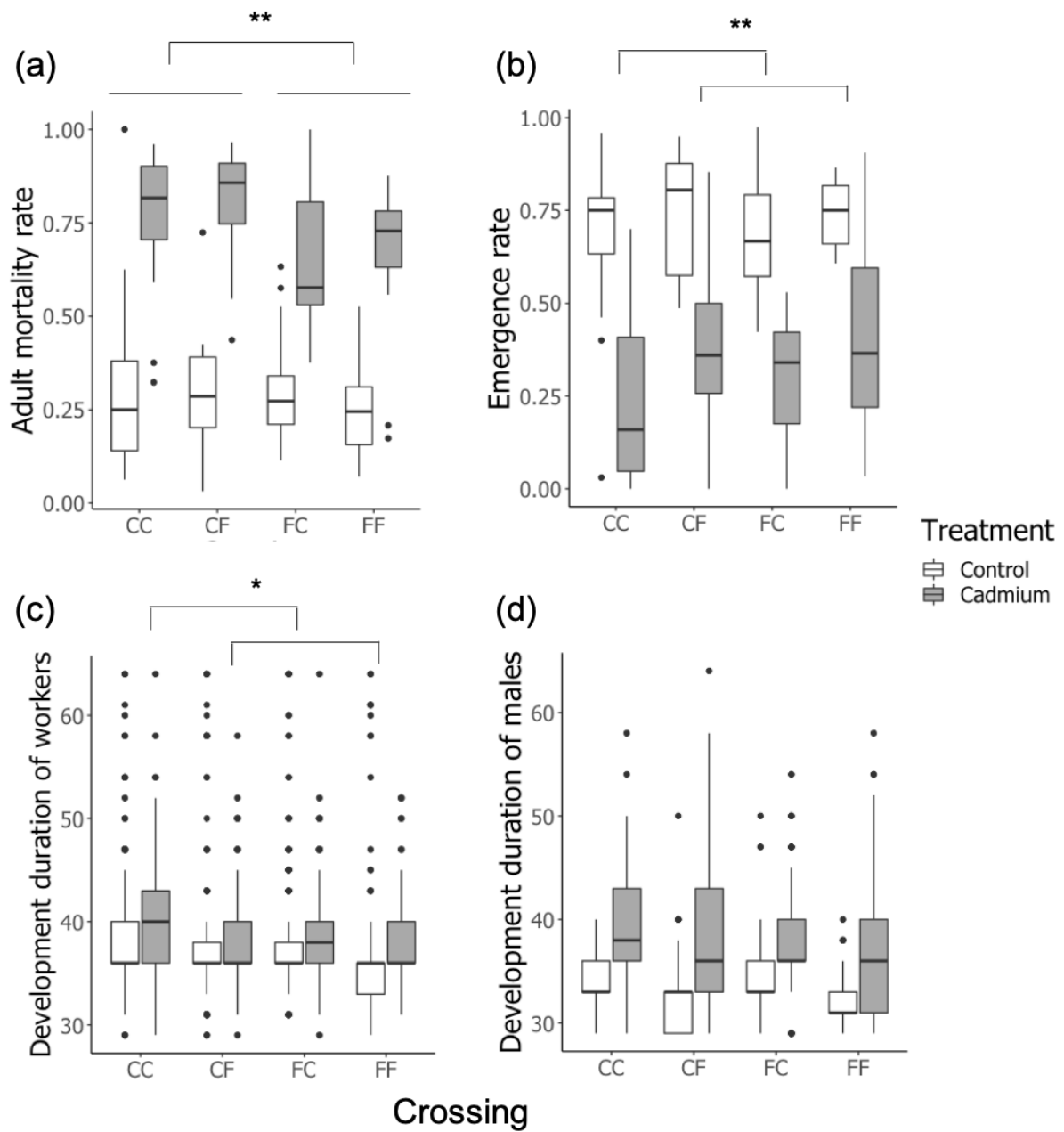
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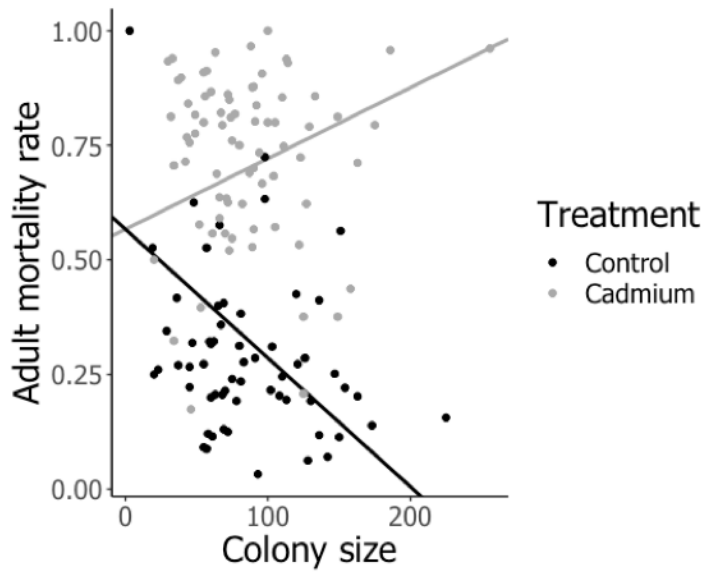
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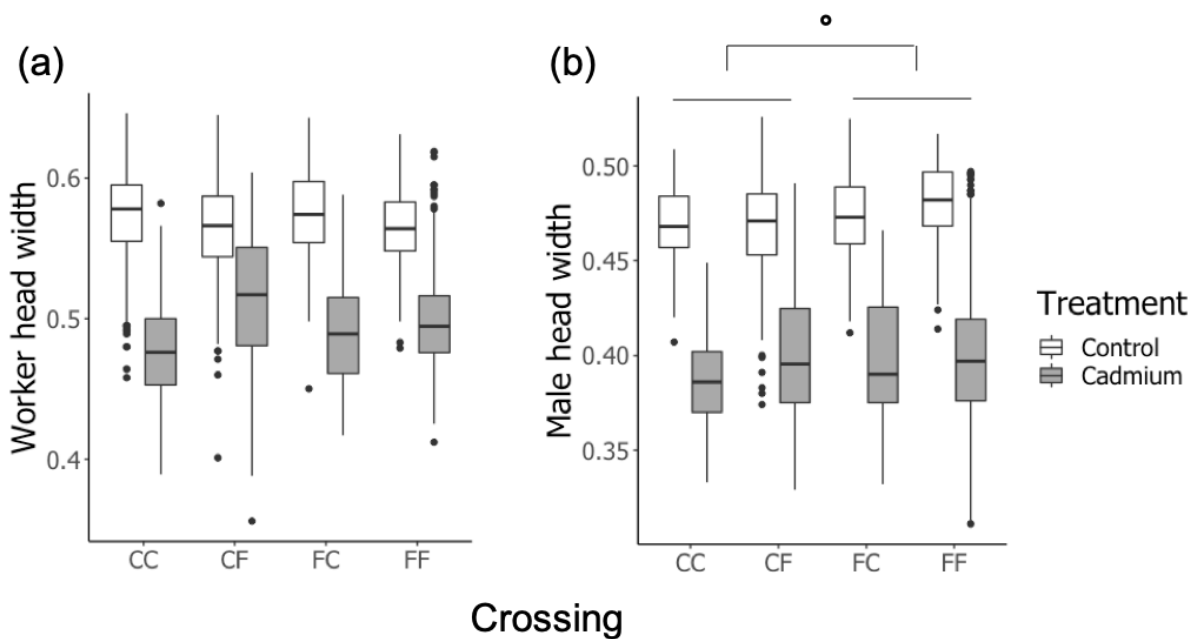
**Figures & table**



**Figure 1:** Boxplots comparing the effects of the worker and larval origins (crossing) and feeding treatment on (a) the adult worker mortality rate, (b) the larval emergence rate, the development duration of laboratory-born (c) workers and (d) males. Boxes show median, quartiles and extremes. Significance is indicated by \*\* $p < 0.01$  and \* $p < 0.05$ . Feeding had a significant effect on all variables (not shown in the figure for clarity). Statistics are presented in Table 1.



**Figure 2:** Correlation between the initial colony size and the adult worker mortality rate. When the initial colony size increased, the adult mortality increased for cadmium treatment and decreased for control treatment (black line: control, estimate =  $-2.81e^{-3} \pm 0.43e^{-3}$ , grey line: cadmium, estimate =  $1.54e^{-3} \pm 0.42e^{-3}$ ).



**Figure 3:** Boxplots comparing the effects of the worker and larval origins (crossing) and treatment on the head width (mm) of laboratory-born (a) workers and (b) males. Boxes show median, quartiles and extremes. Trend ( $0.05 < p < 0.06$ ) is indicated by °. Treatment had a significant effect on all variables (not shown in the figure for clarity). Statistics are presented in Table 1.

**Table 1:** Models and statistics for the different response variables. “Minimum model” means that the predictor was retained in the minimum model selected. A mixed effect model with colony as random factor was used for the development duration and head width of produced individuals, in order to control for the origin of workers. Significant effects are in bold.

Predictors	Response variables					
	Adult mortality rate	Emergence rate	Worker development duration	Male development duration	Worker head width	Male head width
Treatment	<b>Minimum model</b> $F_{131:133} = 92.43$ , $P < 0.001$	<b>Minimum model</b> $F_{131:132} = 107.93$ , $P < 0.001$	<b>Minimum model</b> $\chi^2_1 = 7.14$ , $P = 0.0075$	<b>Minimum model</b> $\chi^2_1 = 64.69$ , $P < 0.001$	<b>Minimum model</b> $\chi^2_1 = 157.97$ , $P < 0.001$	<b>Minimum model</b> $\chi^2_1 = 156.24$ , $P < 0.001$
Worker origin	<b>Minimum model</b> $F_{131:132} = 7.72$ , $P = 0.0060$	$F_{130:131} = 0.81$ , $P = 0.37$	$\chi^2_1 = 0.46$ , $P = 0.50$	$\chi^2_1 = 0.56$ , $P = 0.45$	$\chi^2_1 = 0.57$ , $P = 0.45$	<b>Minimum model</b> $\chi^2_1 = 3.57$ , $P = 0.059$
Worker origin - Treatment	$F_{130:131} = 2.96$ , $p = 0.087$	$F_{129:131} = 0.60$ , $P = 0.55$	$\chi^2_2 = 2.00$ , $P = 0.37$	<b>Minimum model</b> $\chi^2_1 = 3.82$ , $P = 0.05$	$\chi^2_2 = 0.62$ , $P = 0.73$	$\chi^2_1 = 0.027$ , $P = 0.87$
Colony size	<b>Minimum model</b> $F_{131:133} = 2.77$ , $P = 0.066$	$F_{130:131} = 1.65$ , $P = 0.20$	<b>Minimum model</b> $\chi^2_1 = 6.22$ , $P = 0.012$	<b>Minimum model</b> $\chi^2_1 = 5.61$ , $P = 0.017$	$\chi^2_1 = 1.01$ , $P = 0.31$	$\chi^2_1 = 0.22$ , $P = 0.64$
Colony size - Treatment	<b>Minimum model</b> , $F_{131:132} = 3.67$ , $P = 0.057$	$F_{129:131} = 2.28$ , $P = 0.10$	$\chi^2_1 = 0.01$ , $P = 0.91$	$\chi^2_1 = 0.75$ , $P = 0.38$	$\chi^2_2 = 3.59$ , $P = 0.16$	$\chi^2_2 = 0.24$ , $P = 0.89$
Larva origin	$F_{131,130} = 0.0054$ $P = 0.94$	<b>Minimum model</b> $F_{131:132} = 7.54$ , $P = 0.007$	<b>Minimum model</b> $\chi^2_1 = 4.22$ , $P = 0.039$	$\chi^2_1 = 2.53$ , $P = 0.11$	$\chi^2_1 = 0.41$ , $P = 0.52$	$\chi^2_1 = 0.07$ , $P = 0.80$
Larva origin - Treatment	$F_{131,129} = 0.14$ $P = 0.86$	$F_{130:131} = 0.91$ , $P = 0.34$	$\chi^2_1 = 1.21$ , $P = 0.27$	$\chi^2_2 = 2.9$ , $P = 0.23$	$\chi^2_2 = 2.57$ , $P = 0.28$	$\chi^2_2 = 0.30$ , $P = 0.86$
Second-order interaction worker origin - larva origin - Treatment	$F_{131,126} = 0.82$ $P = 0.53$	$F_{126:131} = 0.45$ , $P = 0.81$	$\chi^2_5 = 3.70$ , $P = 0.59$	$\chi^2_4 = 4.38$ , $P = 0.36$	$\chi^2_6 = 6.01$ , $P = 0.42$	$\chi^2_5 = 2.78$ , $P = 0.73$