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

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Abstract

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

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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 patrilines 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 nylanderi* 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

Material and methods

103

104

Ants rearing

105

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

126

Experimental design

127

Workers and larvae had two different origins (city or forest), resulting in four different crosses: city workers with city larvae from another colony (CC, 36 colonies), forest workers with forest larvae from another colony (FF, 36 colonies), city workers with forest larvae (CF, 35 colonies) and forest workers with city larvae (FC, 35 colonies). Colonies were matched by pairs for brood exchange, depending on the number of workers and larvae. Each colony received all the second-instar brood from its paired colony. Larvae of the two paired colonies were exchanged simultaneously, larvae remained isolated for a maximum of 15 minutes 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 LC_{50}
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^{-1} (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). 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 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 gyne
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

Discussion

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

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

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

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Author's contributions

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

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Conflict of interest

The authors declare that they have no conflict of interest.

Ethical approval

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

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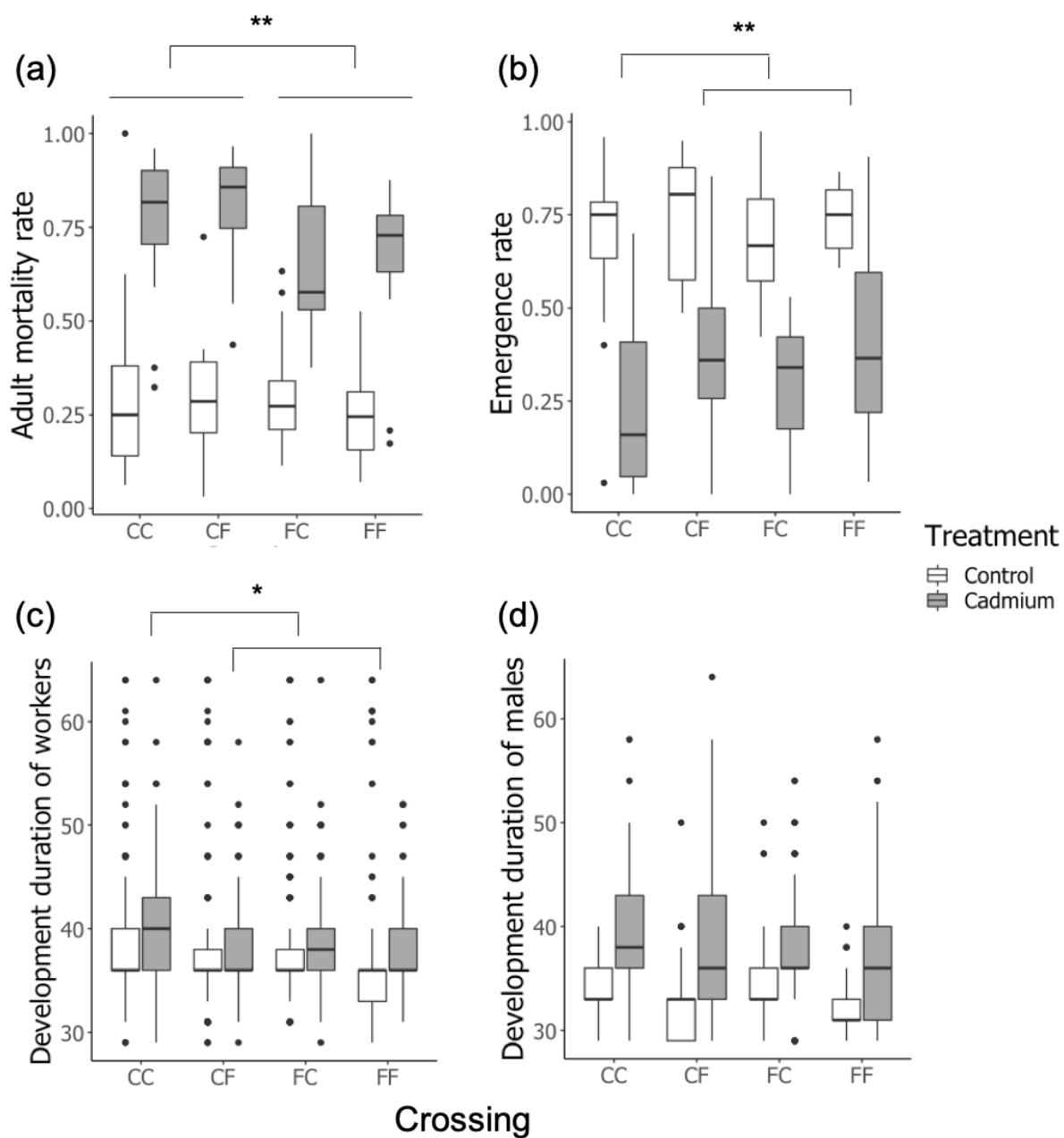
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Figures & table

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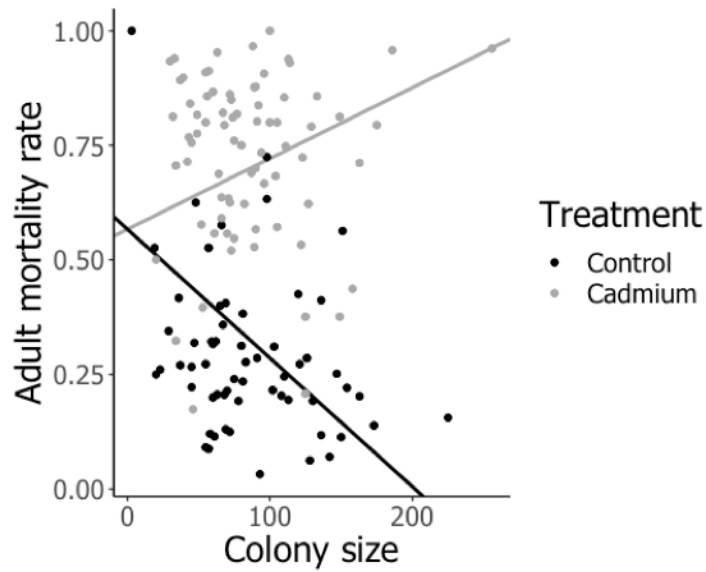
Figure 2: Correlation between the initial colony size and the adult worker mortality rate. When the initial colony size increased, the adult mortality increased for cadmium treatment and decreased for control treatment (black line: control, estimate = $-2.81e^{-3} \pm 0.43e^{-3}$, grey line: cadmium,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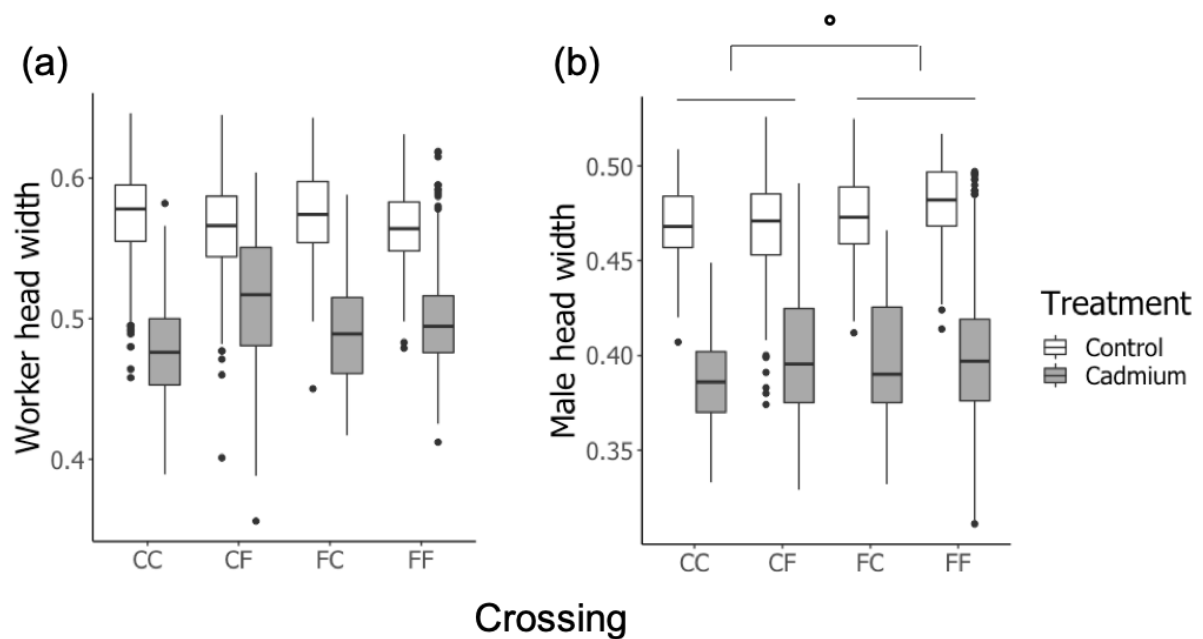

Figure 3: Boxplots comparing the effects of the worker and larval origins (crossing) and treatment on the head width (mm) of laboratory-born (a) workers and (b) males. Boxes show median, quartiles and extremes. Trend ($0.05 < p < 0.06$) is indicated by °. Treatment had a significant effect on all variables (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.

Response variables

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