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

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Declarations

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Conflicts of interest

The authors declare that they have no conflict of interest.

Availability of data and materials

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

Ethical approval

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

Authors' contribution

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

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

Abstract

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

Introduction

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

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

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

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

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

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

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

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

Material and Methods

Colony collection and rearing

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

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

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

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

Experimental design

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

Response variables

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

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

Statistical analyses

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

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

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

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

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

Results

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

Worker mortality rate

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

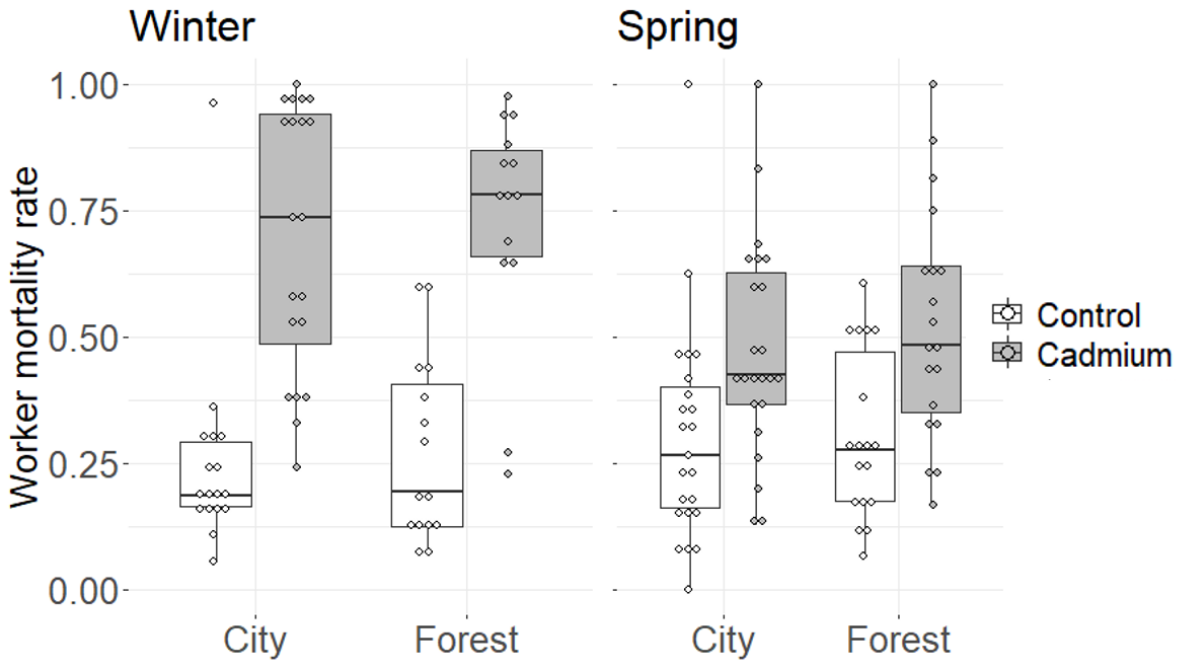


Fig 1 Comparison of worker mortality rate between spring and winter colonies from city and forest. For each box, median and quartiles are shown, as well as colony-level data points. White: control treatment, grey: cadmium treatment

Larval mortality rate

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

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

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

Larval pause rate

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

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

Emergence rate

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

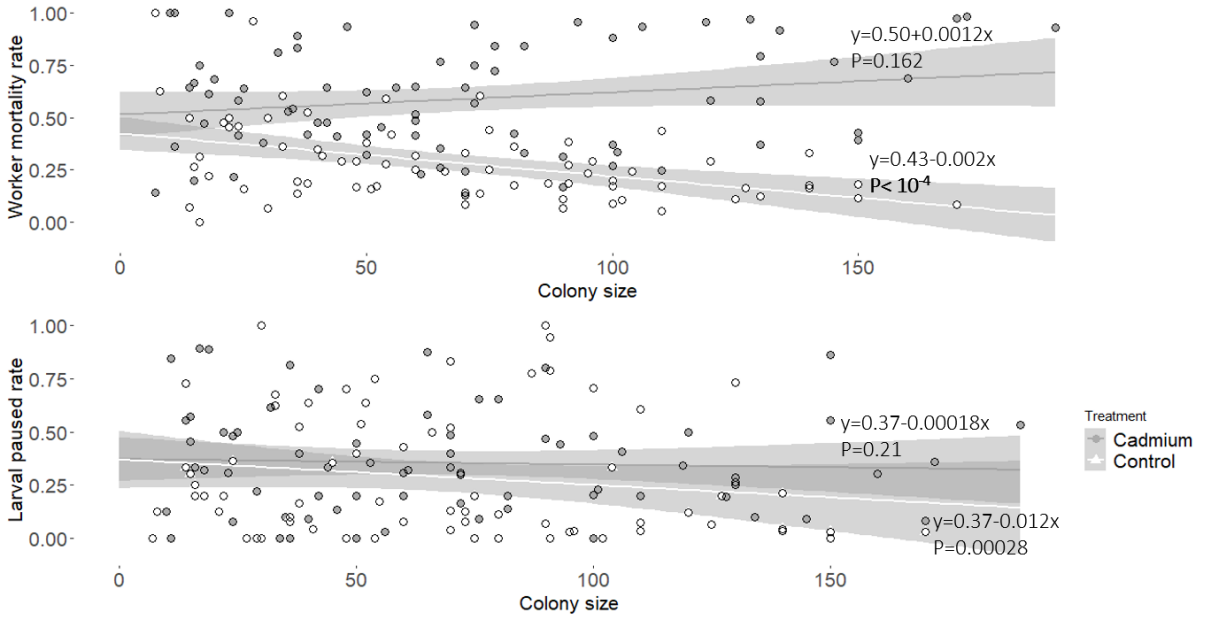
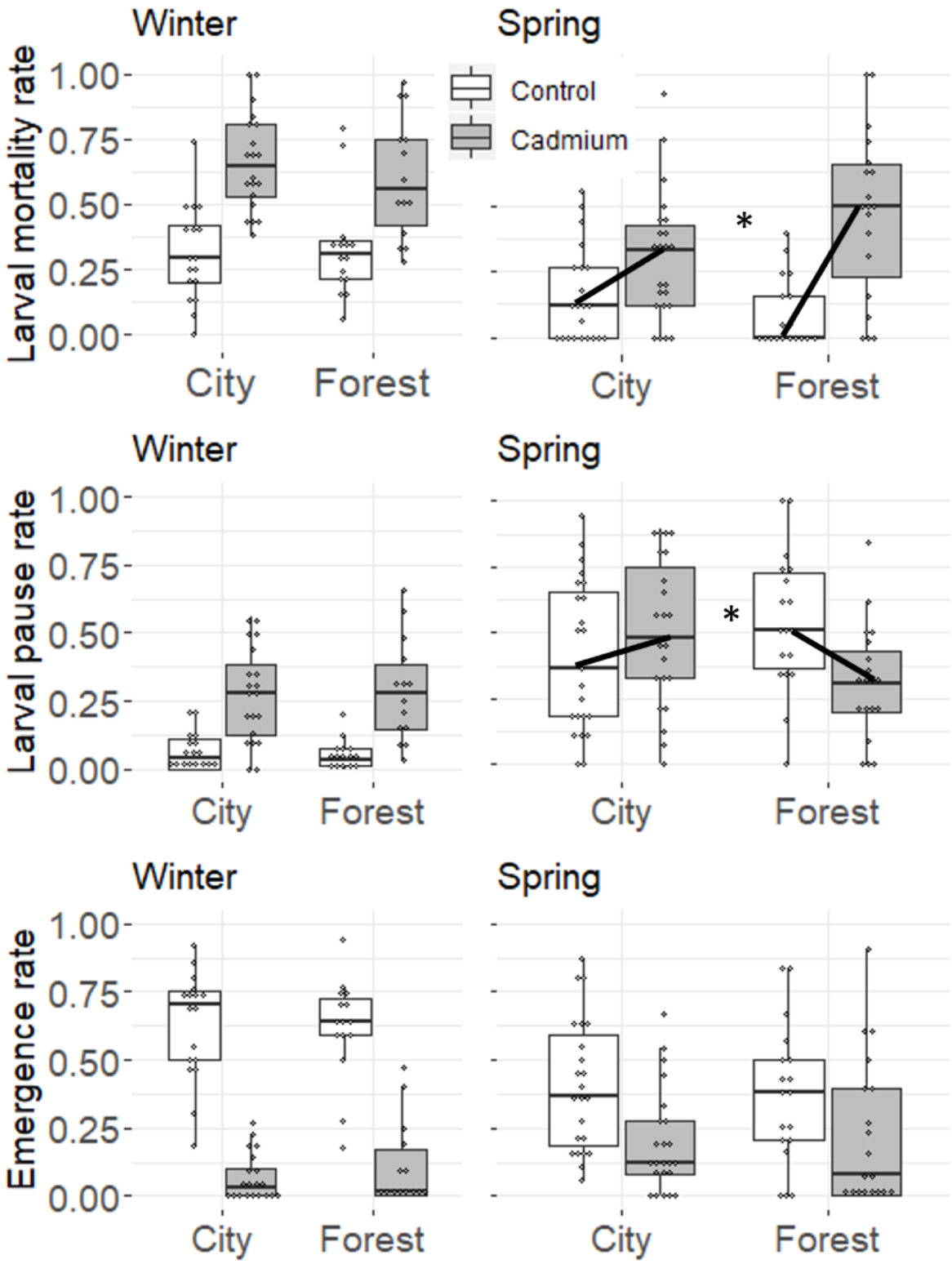


Fig 2: Worker mortality rate and larval pause rate depending on colony size and treatment (grey circles: cadmium, white circles: control). For each graph, line equation is provided as y = intercept + slope · x. Ninety-five percent confidence intervals are shown as grey zones. P-values correspond to the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



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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

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

Size of lab-workers

The size of lab-workers decreased under cadmium (t_{130}=8.84 P<0.0001, Table 1, Fig 3) but the effect of cadmium was less pronounced in spring colonies than in winter colonies (significant Treatment : Season, χ_1=24.11 P<10^{-6}). Moreover, the effect of cadmium was more pronounced during winter in city colonies than in forest colonies, but this differential effect faded in spring colonies (marginally significant Treatment : Season : Habitat, graphical tendencies , marginally significant Treatment : Habitat interaction, χ_1=3.42 P=0.064, Table 1, Fig 4). Colony size had no effect on the size of lab-workers (χ_1=2.43 P=0.12, Table 1).

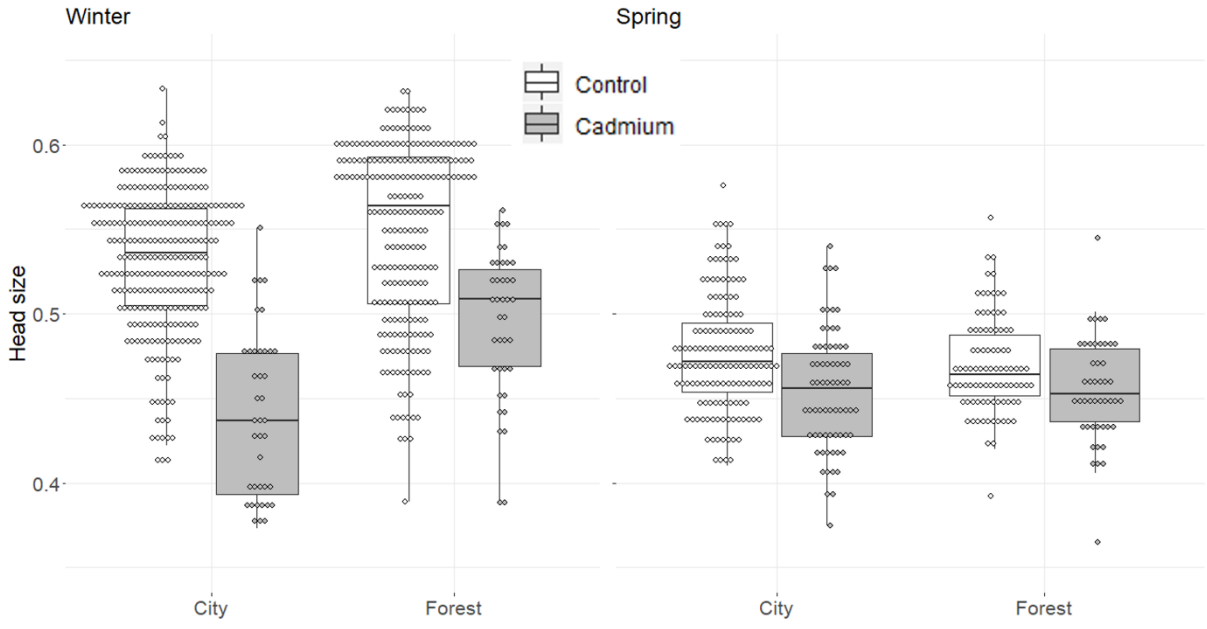

Fig 4 Comparison of lab-workers head size (mm) between spring and winter colonies from city and forest. For each box, median and quartiles are shown, as well as individual-level data points. White: control treatment, grey: cadmium treatment

	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**	**t_{130}=8.84** **P<0.001**
Habitat	$F_{145,144}$=0.70 P=0.40	**t_{100}=2.28** **P=0.02**
Season	**z=5.84** **P<0.0001**	**t_{123}=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

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

	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	

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

Discussion

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

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

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

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

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

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

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