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Intraspecific variability in herbivore response to elemental defences is caused by the metal itself

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Authors Contributions

DG, SM and CB conceived and design the study. DG performed the experiments. DG and SM wrote the manuscript with considerable help from CB.

Abstract

Some plants are able to accumulate on their leaves metals taken from the soil, using this as a defence against herbivorous arthropods. However, herbivore response to metal accumulation in plants is known to be variable. While some species and taxonomic groups are less affected than others, hormetic effects have also been observed in spider-mites, herbivorous crop pests. Still, knowledge on the range and causes of intraspecific variation in the response of herbivores to metal accumulation is lacking. Here, using two species of spider-mites, *Tetranychus urticae* and *T. evansi*, we tested the variation of 17 populations in response to cadmium-accumulating tomato plants and the drivers of such variation. We observed a non-linear, hormetic response of mites to plants with cadmium in some, but not all, populations. The same pattern was recaptured in artificial diets with different concentrations of cadmium but not in artificial diets with sugars, which change in the plant in response to cadmium. This indicates that herbivores on metal-accumulating plants respond to metals, not to the variations in leaf carbohydrates. Therefore, metals exert different effects on herbivores according to the amount accumulated, but independently of other plant traits. This knowledge is key to the understanding of the mechanisms underlying herbivore responses to metal-based plant defences and to pesticides containing heavy metals. Additionally, our findings draw attention to the need of considering intraspecific variation and non-linearities when studying the effects of metals and other contaminants on herbivorous arthropods.

Keywords: metal-accumulation; hormesis; plant-herbivore interactions; spider mites; cadmium;

38 **Introduction**

39 Some plants accumulate metals translocated from the soil into their shoots, up to levels that
40 are toxic to most organisms, a process termed hyper-accumulation (Baker 1987). The
41 elemental defence hypothesis suggests that this process may serve as a defence against
42 herbivores (Martens and Boyd 1994). Indeed, herbivores induce less damage and have
43 reduced performance on metal-accumulating plants than on non-accumulating plants under
44 laboratory conditions (Martens and Boyd 1994; Boyd and Moar 1999; Freeman et al. 2006;
45 Quinn et al. 2010; Kazemi-Dinan et al. 2014). In nature, metal-accumulating plants also
46 suffer less herbivory than neighbouring plants (Freeman et al. 2007; Noret et al. 2007; Galeas
47 et al. 2008; Mohiley et al. 2020).

48 Herbivore response to metal-accumulation in plants is variable, which affects the
49 effectiveness of elemental defenses. For example, it varies according to the feeding type of
50 herbivores (Hanson et al. 2003; Jhee et al. 2005; Konopka et al. 2013) and between
51 taxonomic groups (Vesk and Reichman 2009; Jaffe et al. 2018). Some species are even
52 extremely tolerant to metals being considered specialists of metal-accumulating plants (Wall
53 and Boyd 2006; Boyd et al. 2007). Despite the clear evidence of interspecific variation in
54 herbivore responses to metal-accumulation, data on intraspecific variation lags behind. An
55 exception is that of *Plutella xylostella*, a pest of cruciferous crops, for which a population
56 collected in selenium-rich environments was shown to be more tolerant to metal-
57 accumulation than other populations, suggesting local adaptation of the populations exposed
58 to this stress (Freeman et al. 2006). Whether intraspecific variability in the response of
59 herbivores to metal accumulation is common and the causes and consequences of such
60 variability remain, yet to be determined. Addressing this issue may provide clues to the
61 mechanisms underlying this process, as well as evidence for the ability of herbivores to adapt

to metal toxicity, not only accumulated from the soils but also present in many pesticides (Gimeno-García et al. 1996).

Metal-accumulating plants may be better defended against herbivores due to the direct harmful effects of metals on herbivores (Boyd and Moar 1999; Cheruiyot et al. 2013; Konopka et al. 2013). However, many physiological and metabolomic changes occur in the plant due to metal toxicity (Barcelò and Poschenrieder 1990; Dalcorsio et al. 2013) and these may have indirect negative effects on herbivores as well. For example, the response of plants to metal toxicity affects its redox balance, leading to changes in enzymatic activities and the accumulation of soluble sugars in plant tissues (Mishra et al. 2014), which in turn may affect herbivore performance (Scheirs et al. 2006; Godinho et al. 2018). Some authors pin-pointed the direct effect of metals on herbivores when these were fed with artificial diets containing metals (Boyd and Moar 1999; Cheruiyot et al. 2013; Konopka et al. 2013). However, most studies do not identify physiological changes in metal-accumulating plants that may affect herbivore performance as well (but see Boyd and Moar 1999; Konopka *et al.* 2013), and do not compare the response of herbivores to individual factors, to the response of herbivores to metal accumulation in plants.

Most studies addressing the response of herbivores to metal accumulation in plants in the laboratory consider a very simple landscape of metal exposure, in which metals are present or absent, or at most assuming a linear response to one or two concentrations of each contaminant (Martens and Boyd 1994; Boyd and Moar 1999; Hanson et al. 2003; Jhee et al. 2005; Freeman et al. 2006, 2007). However, metal accumulation in plants is quite variable between individuals and between leaves, despite having the same bioavailable concentrations in the soil (Lombi et al. 2000; Assunção et al. 2003; Mohiley et al. 2020), which creates heterogeneous landscapes for herbivores at small scales. This heterogeneity may help maintain inter- and intraspecific variability in the response of herbivores. Moreover, it may

lead to non-linear responses. For example, hormetic responses, where mild doses of a compound enhance the performance of organisms whereas higher doses affect them negatively (Calabrese 1999), are associated with unstable and heterogeneous environments (Forbes 2000; Costantini et al. 2010). For herbivorous arthropods, hormesis may be advantageous to cope with plant heterogeneity in metal accumulation. In turn, non-linear responses of herbivores to metal accumulation may have important consequences for plant communities as well. If mild amounts of metal accumulated in the shoots lead to higher herbivore reproductive performances, this may, in turn, reduce plant fitness. Thus, plants may be selected to accumulate more metals or to not accumulate metals at all, becoming excluders (Boyd 2012). Still, whether hormetic responses to metal accumulation are common among and between herbivore species is unknown. Since detection of non-linear responses requires several concentrations to be used, hormesis might have been missed in some studies.

One example of hormetic responses to metal-accumulation is that of two species of spider-mites, *Tetranychus urticae* and *T. evansi*, infesting cadmium-accumulating tomato plants (*Solanum lycopersicum*). Both herbivorous arthropods are important crop pests, with *T. urticae* causing worldwide losses in many cultures (Attia et al. 2013) and *T. evansi* being an invasive species in the Mediterranean basin, with a huge impact on solanaceous crops, such as tomato (Boubou et al. 2012). A hormetic response was observed in laboratory populations of both species, with their performance increasing on tomato plants with mild cadmium concentrations and decreasing on plants exposed to higher concentrations (Godinho et al. 2018). This hormetic pattern may be caused either by a direct effect of cadmium on spider mites, or by cadmium-induced metabolic changes in the plant (Gallego et al. 2012). In fact, accumulation of cadmium may lead to a hormetic response of plants, with their growth stimulated (Carvalho et al. 2020; Calabrese and Agathokleous 2021), and the response of herbivores may be a reflection of that. However, in this system, cadmium accumulation did

not affect the measured plant traits (Specific leaf area; Root/shoot; Carbon/Nitrogen content; NDVI), except for the amount of soluble sugars accumulated in the leaves, which also co-varied with spider-mite performance (Godinho et al. 2018). Despite no evidence for hormesis in the plant, the hormetic pattern observed in the herbivores might be caused by changes in the amount of sugars accumulated in plant leaves, rather than by a direct effect of cadmium. To clarify this issue and to assess intraspecific variability in the response to metal accumulation, we studied the effect of cadmium accumulation in tomato plants on several field populations of both species of spider mites. Additionally, we exposed a sub-set of those populations to artificial diets with different concentrations of cadmium or soluble sugars. We then tested if the hormetic pattern found when feeding on tomato plants exposed to cadmium was recaptured in the response to the metal, to the soluble sugars or both.

Material and methods

Study organisms

Tomato plants (*Solanum lycopersicum*, cv. Moneymaker) were sown in a climate chamber (25°C, photoperiod 16/8 h light/darkness), in a soil/vermiculite mixture (4:1) and watered with different cadmium chloride concentrations (0, 0.25, 0.5, 0.75 or 1.5 mM) as described by Godinho et al. 2018. Within these concentrations, growth and physiology of the plants was not affected (Godinho et al. 2018). Additionally, cadmium accumulation increased linearly with cadmium supply in the soil, up to levels above the hyperaccumulation threshold, inducing a hormetic response on two species of herbivorous spider mites (Godinho et al. 2018).

Spider-mite populations were maintained in cages containing leaves of tomato plants, in a climate chamber (25 °C, photoperiod 16/8 h light/darkness). Here, we used: (a) a population

of *Tetranychus evansi*, collected in Portugal in 2013 (TeQL), for which the response to cadmium accumulation on tomato is known (Godinho *et al.* 2018); (b) 14 natural populations of *T. evansi* and of *T. urticae*, 7 of each species, collected on tomato plants in 2017 and (c) an outbred population of each species created in the laboratory by merging natural populations (Godinho *et al.* 2020; Table S1).

For the experiments, groups of adult females were isolated on separate leaves and allowed to lay eggs for 48 h. Fourteen days later, mated adult females resulting from these cohorts were used.

The animals used in this research are not vertebrates or invertebrates with ethical and care limitations.

Herbivore performance on plants exposed to cadmium

To measure the response curve of mite populations to cadmium-accumulating tomato plants, spider mite females were individually placed (N = 3-5 per plant) for three days on discs (Ø 16 mm) from the 3rd oldest leaf of plants (N= 7-10 per cadmium concentration, plants randomized across populations in blocks of 3-4 plants per cadmium concentration). Daily survival and oviposition were recorded.

Herbivore performance on artificial diets

To unravel the cause of spider-mite responses to cadmium-accumulating tomato plants, we exposed a subset of the spider-mite populations to artificial diets (Table S1), created with 0.445 mL Schneider medium, 0.545 ml of a given solution and 0.01 ml of food-colouring dye (Zélé *et al.* 2019). These solutions contained either cadmium chloride or glucose, at different concentrations (0, 50, 150, 300, 500, 750 mg/kg and 0, 5, 8, 10, 15, 20, 25 mmol, respectively), covering the range of concentrations accumulated in tomato plants amended with up to 1.5 mM of cadmium chloride (Godinho *et al.* 2018).

To feed these artificial diets to the mites, parafilm squares (1 cm²) were placed in a metallic microplate well connected to a vacuum pump, creating a parafilm bubble. 30 µL of artificial diet were then micropipetted into the bubble, which was then closed with tape (Zélé et al. 2019). Each bubble was placed in a Petri dish with 30 females of a given population. The Petri dish was closed with a fine fabric mesh. 24h later, the females that had fed on the artificial diet were distinguished via blue coloration in their gut due to the dye. 10 of these females were collected from each petri dish (6 petri dishes, thus 60 females / population / treatment – in blocks of 2 petri dishes per concentration) and isolated individually on tomato leaf-discs (Ø 16 mm). 24h later, the number of eggs was registered.

Statistical analysis

Dose-response curves were modelled using the “mselect” function of drc package in R, which compares the fit of the data to different dose-response curves (Ritz et al. 2015). However, our data did not fit any of the models with 4 or 5 parameters (including the hormetic effect or not, Tables S2 and S3). Therefore, we tested spider-mite responses using general linear models.

To compare the response to cadmium accumulation by tomato plants among spider-mite populations, we performed a general linear mixed model with a gaussian distribution, using population as fixed factor, concentration of cadmium as covariate, block and plant as random factors (Bates et al. 2019). To compare the performance of different spider-mite populations on artificial diets, we performed similar models with cadmium or glucose concentration as covariates, block and petri dish as random factors.

To test if the response of each population was hormetic, we performed, independently for each population, two general linear mixed models with a gaussian distribution, with concentration of cadmium as a covariate, and block and plant or petri dish (for the artificial diets) as a random factor. In one model we included only a linear term for cadmium

concentration and in the other, we included a quadratic term as well. We then assessed if the model with the quadratic term explained better the variance in our data using the anova function and comparing the Akaike Information Criterion (AIC) of the models. If the model with the quadratic term had a better fit to our data (i.e. had a lower AIC) the response was considered hormetic. Polynomial regressions have been previously used to assess hormetic and non-linear responses of arthropods to chemical compounds such as pesticides (Stevens et al. 1999; Yu et al. 2010).

Given the gaussian distribution of the models, we assessed the normality of the residuals and if needed we performed a boxcox transformation to the data using the package MASS in R (Brian et al. 2013).

Results

Herbivore performance on plants exposed to cadmium

The oviposition rate of spider mites was significantly affected by the cadmium concentration the plants were exposed to ($F_1 = 185.15$, $P < 0.001$). This effect differed significantly across populations (cadmium * population: $F_{16} = 2.46$, $P = 0.001$, Figs. 1, 2 and S1).

A hormetic response to cadmium accumulation was observed for some populations of both species (Figs. 1a-c, 2a-b and S1a-b). For those, including a quadratic term for the concentration of cadmium in the model increased the model fit, as compared to a model with a linear term (Table S4). The exception was for TeOUT, for which the model with a quadratic term did not have a better fit but a model with a cubic term did (Fig 1c, Table 1). For the remaining populations (Figs. 1d-f, 2c-e and S1 c-f) the model with a quadratic term for cadmium concentration did not have a higher fit to our data as compared to a model with only a linear term for the concentration of cadmium (Table 1).

Herbivore performance on artificial diets

Effect of cadmium

The oviposition rate of spider mites was significantly affected by cadmium concentration on the artificial diet ($F_1 = 795.65$, $P < 0.001$). This effect differed significantly across populations (cadmium * population: $F_{10} = 4.32$, $P < 0.001$, Fig. 1 and Fig. 2).

For some populations (Figs. 1g-i and 2f-h) including a quadratic term for the concentration of cadmium increased the fit of the model explaining their oviposition rate when feeding on artificial diets (Table 2). For the remaining populations (Figs. 1j-l and 2i-j) including a quadratic term for the concentration of cadmium did not increase the model fit (Table 2).

Effect of glucose

The oviposition rate of all spider-mite populations was not affected by the concentration of glucose on the artificial diet ($F_1 = 1.17$, $P = 0.28$, Fig. 3).

Discussion

Here we show that the effect of the elemental defence on a herbivore species is not universal. Indeed, populations of two spider-mite species differed in their response to cadmium accumulation by tomato plants. Moreover, we show that hormesis, previously found in this response (Godinho et al. 2018), maybe a common phenomenon, as it was found in several populations of both species, independently of their haplotype (for *T. evansi*) or colour morph (for *T. urticae*, (Godinho et al. 2020)). Finally, we show that the observed patterns are explained by the response of the herbivores to the metal itself, not to metabolic changes in the plant, associated with metal accumulation.

The direct effect of metals on herbivores has been shown before by exposing them to artificial diets with such contaminants (Behmer et al. 2005; Jhee et al. 2006; Cheruiyot et al.

2013; Stolpe and Müller 2016). However, such studies did not compare the performance of herbivores on artificial diets to that on metal-accumulating plants, nor were indirect effects through changes in plant physiology identified and tested. As such, whether the direct effect of metals is the main driver of the response of herbivores to metal accumulation in plants was still to be demonstrated. Here, we show that the presence or absence of an hormetic response to cadmium accumulation by tomato plants is remarkably recaptured, for most spider-mite populations, when using an artificial diet with different cadmium concentrations. These results demonstrate that the direct effect of the metal is indeed the main driver of the response of herbivores, rather than physiological changes in the plant in carbohydrate content. In previous studies, the range of metal concentrations used on entire plants was restricted, limiting the comparisons between the response on artificial diets with that on plants (Boyd and Moar 1999; Konopka et al. 2013). Thus, similarities in the pattern associated with the direct response to metals and the response to metal accumulation in plants may have been missed.

Glucose did not affect the performance of any spider-mite population, despite the range of concentrations on artificial diets being wider than that found in tomato leaves (Godinho *et al.*, 2018). This is at odds with earlier findings, in which spider-mite performance correlated with the amount of sugars in the plant (Joutei et al. 2000; Ximénez-Embún et al. 2016, 2017; Godinho et al. 2018). However, such studies were done on entire plants. Our results suggest that sugars are not the direct cause of such changes in herbivore performance. Indeed, the performance of spider mites on plants stressed by abiotic factors may be correlated with other changes in the plant, such as nitrogen content (Joutei et al. 2000) and proline (non-essential protein), a known reproductive stimulant in arthropods (Ximénez-Embún et al. 2016). Although herbivores may respond to similar physiological changes in plants stressed by metal toxicity, that were not measured in our system, the fact that we recapture the pattern observed

255 on plants using artificial diets with metals suggests that the direct effect of the metal is
256 stronger than the effects stemming from alterations in plant physiology. If this is the case, the
257 elemental defence is a strong selective pressure on herbivores regardless of within and
258 between plant species variability in physiological responses. On the one hand, metal
259 contamination is expected to lead to similar herbivore responses across plant species, since
260 the effect of a given concentration of metal accumulated may be the same across metal-
261 accumulating plant species. On the other hand, plant variability for the amount of metal
262 accumulated may create a heterogeneous landscape for herbivores, which may depend on the
263 limits of their tolerance to metal toxicity and on the presence or absence of hormetic
264 responses. In turn, the intraspecific variability for the response of herbivores to metal
265 accumulation is also possibly a mediator of the relationship between the amount of metal
266 accumulated and how much a plant is eaten.

267 The fact that the presence or absence of hormesis is explained by a direct effect of the metal
268 on spider mites suggests that populations have been exposed to different amounts of
269 cadmium in their recent evolutionary history. Indeed, hormesis may be a compensatory
270 response to abiotic stress (Calabrese 1999), and so, populations previously exposed to
271 cadmium or other contaminants may be adapted to such stress, not presenting the hormetic
272 response. Additionally, hormesis may be advantageous in populations growing in
273 heterogeneous environments (Forbes 2000), whereas in more homogeneous environments
274 with no cadmium or with high amounts of cadmium such response would not be selected for.
275 Alternatively, is possible that hormesis results from a negative effect on parasites or diseases
276 if those have a lower threshold for metal toxicity than their hosts (Forbes 2000). Thus,
277 differences in the microbiome may explain the presence or absence of hormesis in spider-
278 mite populations. Independently of the mechanism, this intraspecific variability in the

response of herbivores to metal accumulation in plants reveals the potential for distinct routes of adaptation to metal toxicity.

In sum, metals have a direct effect on herbivores, independently of the metabolic changes occurring in the plants. However, the response of a given herbivore species to metal accumulation is not binary (i.e. affected / not affected). Instead, there is intraspecific variation for how herbivores are affected by the elemental defences, which may have important consequences for the ecology and evolution of herbivores and their host plants. Additionally, given that many pesticides contain heavy metals such as cadmium, unravelling the range and causes of intraspecific variability in the response to heavy metals will provide key knowledge for the understanding of adaptation of herbivorous crop pests to pesticides. Therefore, our study underscores the importance of including intraspecific variability and non-linear responses in studies focusing on how metals, and other pollutants, may affect herbivorous arthropods.

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Declarations

The authors declare no conflict of interests.

Data is deposited in Figshare (<https://figshare.com/s/e43238fe8cd2834084a7>), DOI will be provided upon acceptance of the manuscript.

The animals used in this research are not vertebrates or invertebrates with ethical and care limitations.

No human subjects were involved in this study.

Authors Contributions

DG, SM and CB conceived and design the study. DG performed the experiments. DG and SM wrote the manuscript with considerable help from CB.

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Figure 1 Average oviposition rate ($\pm$ s.e.) of *T. evansi* populations on plants exposed to different concentrations of cadmium: a) to f); and artificial diets containing different concentrations of cadmium: g) to l). On tests on plants (N = 7 to 10 per concentration), 3 to 5 spider mites were used; tests on artificial diets were done with 10 spider mites per Petri dish (N = 6 per concentration).

Figure 2 Average oviposition rate ($\pm$ s.e.) of *T. urticae* populations on plants exposed to different concentrations of cadmium: a) to e); and artificial diets containing different concentrations of cadmium: f) to j). On tests on plants (N = 7 to 10 per concentration), 3 to 5 spider mites were used; tests on artificial diets were done with 10 spider mites per Petri dish (N = 6 per concentration).

Figure 3 Average oviposition rate ($\pm$ s.e.) of spider-mite populations on artificial diets containing different concentrations of glucose. 10 spider mites tested per Petri dish (N = 6 per concentration).

Table 1. Comparison of the models explaining the oviposition rate of each spider-mite population when feeding on entire plants exposed to different cadmium concentrations. Shown are general linear mixed models with cadmium concentration as covariate, including a linear term for this factor (m1), a quadratic term (m2) and, for Te OUT, a cubic term (m3). * comparison between m1 and m3. Significant differences between models are represented in bold.

Population	model	AIC	anova (m1, m2)
Te QL	m1	375	Chisq = 10.78 ; P = 0.001
	m2	366	
Te 6M1	m1	468	Chisq = 3.83 ; P = 0.05
	m2	466	
Te OUT	m1	153	Chisq = 0.41 ; P = 0.52
	m2	154	
	m3	151	*(Chisq = 5.71 ; P = 0.05)
Te VIT	m1	380	Chisq = 0.08 ; P = 0.78
	m2	381	
Te QG	m1	373	Chisq = 2.90 ; P = 0.09
	m2	373	
Te PBS	m1	297	Chisq = 0.51 ; P = 0.47
	m2	298	
Te CG	m1	481	Chisq = 3.73 ; P = 0.05
	m2	479	
Te ER	m1	415	Chisq = 0.38 ; P = 0.53
	m2	416	
Te VC	m1	470	Chisq = 0.78 ; P = 0.38
	m2	471	
Tu OUT	m1	1145	Chisq = 11.97 ; P < 0.001
	m2	1135	
Tu DEF	m1	432	Chisq = 2.76 ; P = 0.05
	m2	431	
Tu MON	m1	435	Chisq = 0.003 ; P = 0.95
	m2	437	
Tu ALP	m1	416	Chisq = 0.63 ; P = 0.43
	m2	417	
Tu HFM	m1	182	Chisq = 0.04 ; P = 0.85
	m2	183	
Tu 6M2	m1	229	Chisq = 6.74 ; P < 0.001
	m2	224	
Tu LIM	m1	360	Chisq = 0.47 ; P = 0.49
	m2	361	
Tu LNEC	m1	-90	Chisq = 0.05 ; P = 0.82
	m2	-89	

Table 2. Comparison of the models explaining the oviposition rate of each spider-mite population when feeding on artificial diets with different cadmium concentrations. Shown are general linear mixed models with cadmium concentration as covariate, including a linear term for this factor (m1), a quadratic term (m2) and, for Te OUT, a cubic term (m3). * comparison between m1 and m3. Significant differences between models are represented in bold.

Population	model	AIC	anova (m1, m2)
Te QL	m1	1094	Chisq = 3.28 ; P = 0.05
	m2	1091	
Te 6M1	m1	1504	Chisq = 7.26 ; P = 0.007
	m2	1499	
Te OUT	m1	793	Chisq = 1.15 ; P = 0.28
	m2	794	
	m3	780	*(Chisq = 16.85 ; P < 0.001)
Te VIT	m1	650	Chisq = 1.65 ; P = 0.2
	m2	650	
Te QG	m1	864	Chisq = 1.33 ; P = 0.25
	m2	864	
Te PBS	m1	625	Chisq = 0.87 ; P = 0.35
	m2	626	
Tu OUT	m1	1499	Chisq = 6.53 ; P = 0.01
	m2	1495	
Tu DEF	m1	639	Chisq = 4.16 ; P = 0.04
	m2	637	
Tu MON	m1	1449	Chisq = 8.85 ; P = 0.003
	m2	1442	
Tu ALP	m1	668	Chisq = 3.13 ; P = 0.09
	m2	668	
Tu HFM	m1	266	Chisq = 2.96 ; P = 0.09
	m2	265

Supplementary Files

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