



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

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## 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 *Tetranychus evansi*, we tested the variation in 17 populations in response to cadmium-accumulating tomato plants and the drivers of such variation. We observed a nonlinear, 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 studied 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 nonlinearities when studying the effects of metals and other contaminants on herbivorous arthropods.

**Keywords** Metal accumulation · Hormesis · Plant-herbivore interactions · Spider mites · Cadmium

## Introduction

Plants can grow in heavy metal contaminated soils either by limiting metal translocation in the roots, or by storing metals translocated from the soil into their shoots, up to levels that are toxic to most organisms, a process termed hyper-accumulation (Baker 1987). The elemental defence hypothesis suggests that this process may serve as a defence against herbivores (Martens and Boyd 1994). Indeed, herbivores induce less damage and have reduced performance on metal-accumulating plants than on non-accumulating plants grown on soils with metals under laboratory conditions (Martens and Boyd 1994; Boyd and Moar 1999; Freeman et al. 2006; Quinn et al. 2010; Kazemi-Dinan et al. 2014). In contaminated soils in nature, species or populations of plants that accumulate metal also suffer less herbivory than their non-accumulating counterparts (Freeman et al. 2007; Noret et al. 2007; Galeas et al. 2008; Mohiley et al. 2020).

Herbivore response to metal accumulation in plants is variable, which affects the effectiveness of elemental

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defences. For example, it varies according to the feeding type of herbivores (Hanson et al. 2003; Jhee et al. 2005; Konopka et al. 2013) and between taxonomic groups (Vesk and Reichman 2009; Jaffe et al. 2018). Some species are even extremely tolerant to metals, being considered specialists of metal-accumulating plants (Wall and Boyd 2006; Boyd et al. 2007). Despite the clear evidence of interspecific variation in herbivore responses to metal accumulation, data on intraspecific variation lag behind. An exception is *Plutella xylostella*, a pest of cruciferous crops, for which a population collected in selenium-rich environments was shown to be more tolerant to metal accumulation than other populations, suggesting local adaptation of the population exposed to this stress (Freeman et al. 2006). Still, the prevalence, causes and consequences of such intraspecific variability remain to be determined. Addressing this issue may provide clues to the mechanisms underlying this process, as well as evidence for the ability of herbivores to adapt to metal toxicity, not only caused by metals accumulated from the soils but also by those 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. This has been shown using artificial diets containing such metals (Boyd and Moar 1999; Cheruiyot et al. 2013; Konopka et al. 2013). However, the metals accumulated may also cause physiological and metabolomic changes in the plant (Barcelò and Poschenrieder 1990; Dalcorsio et al. 2013) and these may have indirect negative effects on herbivores as well, but empirical evidence disentangling the two possibilities is lacking.

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 even for the same bioavailable concentrations in the soil (Lombi et al. 2000; Assunção et al. 2003; Mohiley et al. 2020), which creates heterogeneous environments for herbivores at small scales. This heterogeneity may help maintaining inter- and intraspecific variability in the response of herbivores. Moreover, it may lead to nonlinear responses. For example, hormetic responses, where mild doses of a compound enhance the performance of organisms whereas higher doses affect them negatively (Calabrese 1999), allow organisms to adjust their homeostatic balance, thereby maintaining their fitness in changing environments (Forbes 2000; Costantini et al. 2010). In herbivorous arthropods, hormetic responses are commonly associated with toxic compounds such as pesticides, having

enormous consequences for agricultural ecosystems by potentially affecting population dynamics not only of the target crop pests but also of natural enemies and pollinators (Guedes and Cutler 2014; Cutler et al. 2022; Iltis et al. 2022). Similarly, hormesis may be advantageous for herbivorous arthropods to cope with plant heterogeneity in metal accumulation. Still, whether hormetic responses to metal accumulation are common among and between herbivore species is unknown.

One example of hormetic responses to metal accumulation is that of two species of spider mites, *T. 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. Despite no evidence for hormesis in the plant in this system, the hormetic pattern observed in the herbivores might be caused by the observed changes in the amount of sugars accumulated in plant leaves, rather than by a direct effect of cadmium (Godinho et al. 2018). 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 subset 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 in Godinho et al.

2018). Briefly, two weeks after sowing, plants were watered with 60 mL of a solution with a given cadmium concentration twice a week for 2.5 weeks (i.e. 5 additions) and one additional time per week with tap water to compensate for micronutrient deficiencies (Godinho et al. 2018). The chosen concentrations were within the physiological limits of tomato plants (Godinho et al. 2018), thus reflecting conditions favourable for plant growth in soils contaminated with cadmium. Within these concentrations, cadmium accumulation increased linearly with cadmium supply in the soil, up to levels above the hyper-accumulation threshold, and we were able to recapture the hormetic response observed in 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 *T. evansi*, collected in Portugal in 2013 (TeQL; Table S1), 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 different fields, in 2017 (Godinho et al. 2020; Table S1), 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 detached leaves in separated boxes 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 accumulation on tomato plants (circa 4.5 weeks old, two days after the last cadmium supply), spider mite females were individually placed for three days on discs (Ø 16 mm) made from the 3rd oldest leaf of plants ( $N=7-10$  per cadmium concentration). Each plant was used to form leaf discs for mites of several populations (3–5 mites per population), and each population was tested on leaf discs from different plants (3–4 plants per cadmium concentration per experimental block). Daily survival and the number of eggs laid were recorded for each female on a leaf disc using a stereo microscope (Leica S9).

### 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 of cadmium chloride or glucose, and 0.01 ml of food-colouring dye (Zélé et al. 2019). The final solutions contained either cadmium chloride or glucose, at different concentrations (0, 50, 150, 300, 500, 750 mg/kg for cadmium chloride and 0, 5, 8, 10, 15, 20, 25 mmol for glucose), covering the range of concentrations accumulated in tomato plants exposed to up to 1.5 mM of cadmium chloride (Godinho et al. 2018).

To feed these artificial diets to the mites, parafilm squares (1 cm<sup>2</sup>) were placed in a metallic microplate well connected to a vacuum pump, creating a parafilm bubble. 30 µL of artificial diet was 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. 24 h later, using a stereo microscope (Leica S9), the females that had fed on the artificial diet were distinguished via blue colouration in their gut due to the dye. Ten 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). 24 h later, the number of eggs laid was counted using a stereo microscope (Leica S9).

### Statistical analysis

Concentration response curves were modelled using the “mselect” function of the 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 generalized linear models.

To compare the response to cadmium accumulation by tomato plants among spider mite populations, we fitted our data with a generalized 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 fitted 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 fitted, independently for each population, two generalized 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 a Chi-squared test and comparing the Akaike Information

Criterion (AIC) of the models (using the ANOVA function in *R*). 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 nonlinear responses of arthropods to chemical compounds such as pesticides (Stevens et al. 1999; Yu et al. 2010; Iltis et al. 2022).

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 1). 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, e, f, 2c, d, e and S1 c, d, e, 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:  $F_{10} = 4.32$ ,  $P < 0.001$ , Figs. 1 and 2).

For some populations (Figs. 1g, h, i and 2f, g, 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, k, 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 ( $F_1 = 1.17$ ,  $P = 0.282$ , Fig. S2).

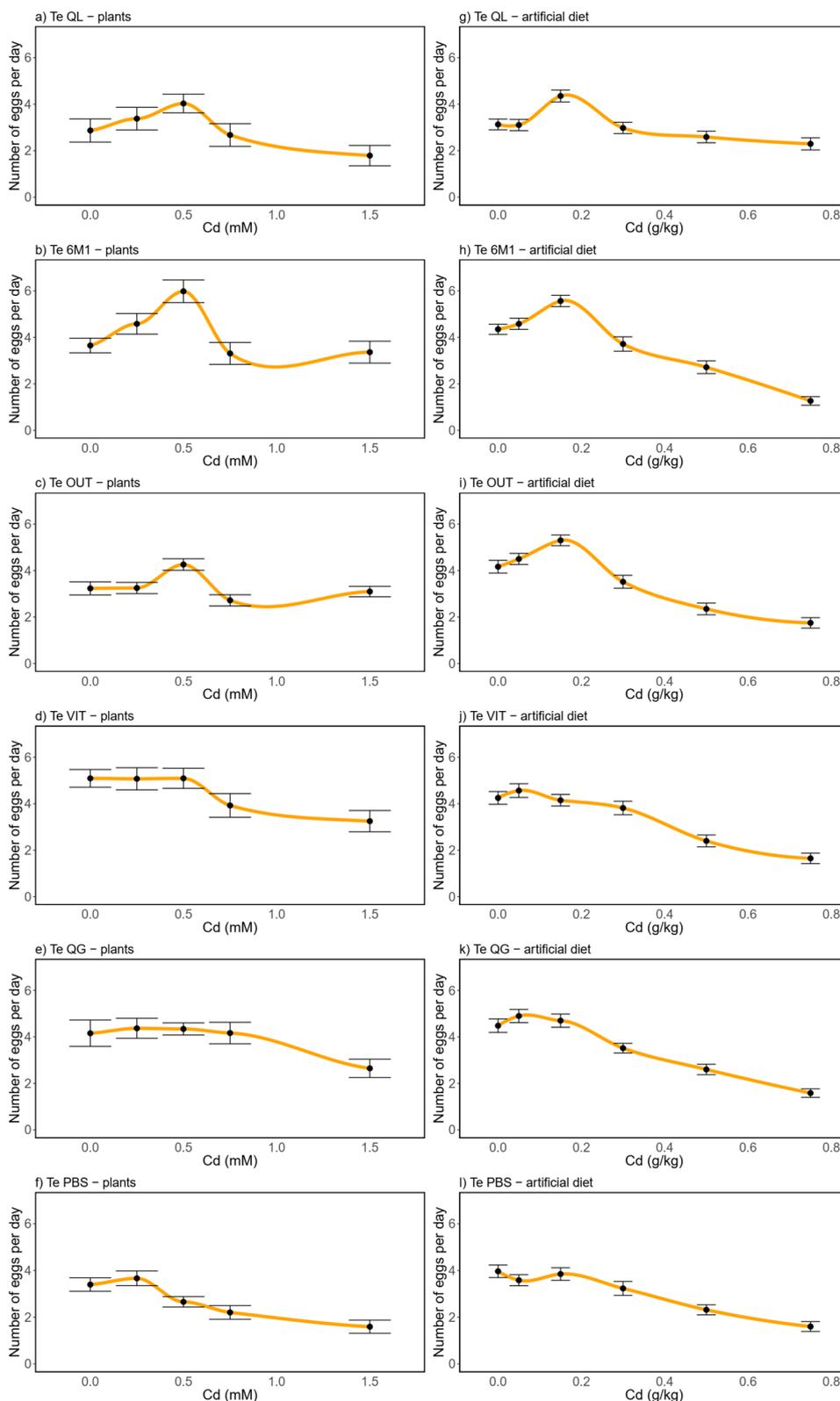
## Discussion

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), may be 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 a 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

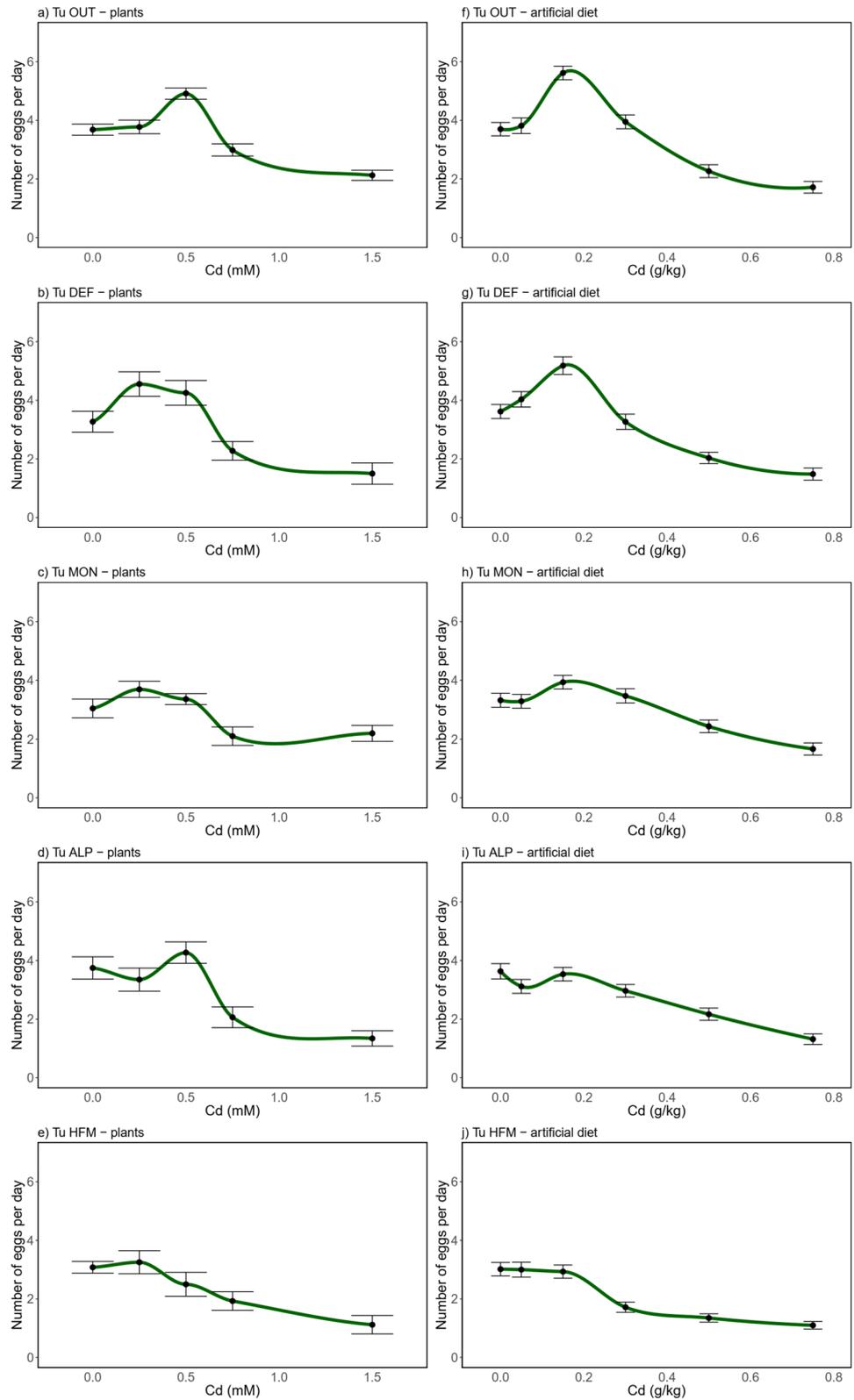
**Fig. 1** Average oviposition rate ( $\pm$  s.e.) of *T. evansi* populations on plants exposed to different concentrations of cadmium (**a** to **f**; N=7-10 plants per concentration, each tested with 3-5 spider mites per population) and on artificial diets containing different concentrations of cadmium (**g** to **l**; N=6 arenas per concentration, each tested with 10 spider mites)



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.

**Fig. 2** Average oviposition rate ( $\pm$  s.e.) of *T. urticae* populations on plants exposed to different concentrations of cadmium (**a** to **e**; N=7-10 plants per concentration, each tested with 3-5 spider mites) and on artificial diets containing different concentrations of cadmium (**f** to **j**; N=6 arenas per concentration, each tested with 10 spider mites)



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 on plants using artificial diets with metals suggests that the direct effect of the metal is stronger than the effects stemming from alterations in plant physiology.

**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

Population	model	AIC	ANOVA (m1,m2)
Te QL	m1	375	Chisq= 10.78; <b>P=0.001</b>
	m2	366	
Te 6M1	m1	468	Chisq= 3.83; <b>P=0.050</b>
	m2	466	
Te OUT	m1	153	Chisq=0.41; <i>P=0.524</i>
	m2	154	
	m3	151	
Te VIT	m1	380	Chisq=0.08; <i>P=0.775</i>
	m2	381	
Te QG	m1	373	Chisq= 2.90; <i>P=0.088</i>
	m2	373	
Te PBS	m1	297	Chisq=0.51; <i>P=0.473</i>
	m2	298	
Te CG	m1	481	Chisq= 3.73; <b>P=0.050</b>
	m2	479	
Te ER	m1	415	Chisq=0.38; <i>P=0.529</i>
	m2	416	
Te VC	m1	470	Chisq=0.78; <i>P=0.375</i>
	m2	471	
Tu OUT	m1	1145	Chisq= 11.97; <b>P&lt;0.001</b>
	m2	1135	
Tu DEF	m1	432	Chisq= 2.76; <b>P=0.046</b>
	m2	431	
Tu MON	m1	435	Chisq=0.01; <i>P=0.953</i>
	m2	437	
Tu ALP	m1	416	Chisq=0.63; <i>P=0.427</i>
	m2	417	
Tu HFM	m1	182	Chisq=0.04; <i>P=0.850</i>
	m2	183	
Tu 6M2	m1	229	Chisq= 6.64; <b>P&lt;0.001</b>
	m2	224	
Tu LIM	m1	360	Chisq=0.47; <i>P=0.492</i>
	m2	361	
Tu LNEC	m1	-90	Chisq=0.05; <i>P=0.819</i>
	m2	-89	

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

If this is the case, the elemental defence is a strong selective pressure on herbivores regardless of within and between plant species variability in physiological responses. On the one hand, metal contamination is expected to lead to similar herbivore responses across plant species, since the effect of a given concentration of metal accumulated may be the same across metal-accumulating plant species. On the other hand,

**Table 2** Comparison of the models explaining the oviposition rate of each spider mite population when feeding on artificial diets with different cadmium concentrations

Population	model	AIC	ANOVA (m1,m2)
Te QL	m1	1094	Chisq= 3.28; <b>P=0.050</b>
	m2	1091	
Te 6M1	m1	1504	Chisq= 7.26; <b>P=0.007</b>
	m2	1499	
Te OUT	m1	793	Chisq= 1.15; <i>P=0.283</i>
	m2	794	
	m3	780	
Te VIT	m1	650	Chisq= 1.65; <i>P=0.198</i>
	m2	650	
Te QG	m1	864	Chisq= 1.33; <i>P=0.251</i>
	m2	864	
Te PBS	m1	625	Chisq=0.87; <i>P=0.352</i>
	m2	626	
Tu OUT	m1	1499	Chisq= 6.53; <b>P=0.009</b>
	m2	1495	
Tu DEF	m1	639	Chisq= 4.16; <b>P=0.041</b>
	m2	637	
Tu MON	m1	1449	Chisq= 8.85; <b>P=0.003</b>
	m2	1442	
Tu ALP	m1	668	Chisq= 3.13; <i>P=0.089</i>
	m2	668	
Tu HFM	m1	266	Chisq= 2.96; <i>P=0.091</i>
	m2	265	

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

plant variability for the amount of metal accumulated may create a heterogeneous landscape for herbivores, which may depend on the limits of their tolerance to metal toxicity and on the presence or absence of hormetic responses. In turn, the intraspecific variability for the response of herbivores to metal accumulation is also possibly a mediator of the relationship between the amount of metal accumulated and how much a plant is eaten.

The fact that the presence or absence of hormesis is explained by a direct effect of the metal on spider mites suggests that populations have been exposed to different amounts of cadmium in their recent evolutionary history. Indeed, hormesis may be a compensatory response to abiotic stress (Calabrese 1999), thus populations previously exposed to cadmium or other contaminants may be adapted to such stress, not presenting the hormetic response. However, the fact that hormetic responses to pesticides have been observed in resistant and susceptible populations of herbivorous arthropods (Guedes and Cutler 2014; Deng et al. 2016; Cutler et al.

2022) runs counter this hypothesis. It is possible, still, that this hormetic response to pesticides correlates with the hormetic response to metals reported here, as many pesticides contain metals, including cadmium (Gimeno-García et al. 1996; Iltis et al. 2022). Additionally, hormesis may be advantageous in populations growing in heterogeneous environments (Forbes 2000), whereas in more homogeneous environments with no cadmium or with high amounts of cadmium, such response would not be selected for. The populations used in this study were collected on tomato plants throughout the same growing season (except for TeQI, collected in 2013 and TeOut and TuOut, created in the laboratory) in different locations, which varied from tomato monoculture, to organic farms and urban communal vegetable gardens and were composed of different plant species. Possibly, these different environments have shaped the evolutionary history of these populations, which may explain the observed differences in their responses. Further studies including more locations and several samplings throughout the growing season, for example before and after pesticide application, are needed to pinpoint the causes of such variation. Alternatively, it is possible that hormesis results from a negative effect on parasites or diseases if those have a lower threshold for metal toxicity than their hosts (Forbes 2000). Thus, differences in the microbiome may explain the presence or absence of hormesis in spider mite populations. This hypothesis may be tested with a detailed characterization of the microbiome of spider mite populations presenting or not an hormetic response. 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 spider mites, 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 nonlinear responses in studies focusing on how metals, and other pollutants, may affect herbivorous arthropods.

## 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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**Data availability** Data are deposited in Figshare (<https://doi.org/10.6084/m9.figshare.17032595>).

## Declarations

**Conflict of interest** The authors declare no conflict of interests.

**Human or animal rights** The animals used in this research are not vertebrates or invertebrates with ethical and care limitations. No human subjects were involved in this study.

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