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The dark side of organic farming: Copper sulphate compromises the life history and behaviour of the walking stick insect, *Bacillus rossius*

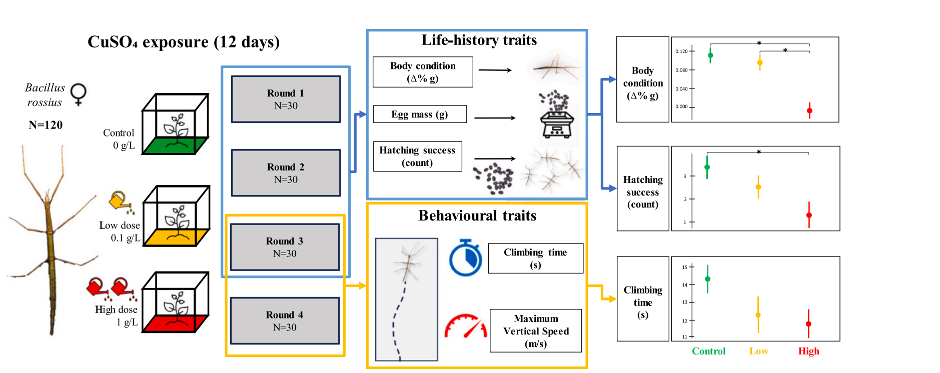
Claudio Pardo [a], Adriana Bellati [a], Giovanni Polverino [a,b,c,1,*], Daniele Canestrelli [a,1]

[a] *Department of Ecological and Biological Sciences, University of Tuscia, Italy*
[b] *School of Biological Sciences, Monash University, Australia*
[c] *School of Biological Sciences, The University of Western Australia, Australia*

HIGHLIGHTS

- Pollution by copper sulphate is global in agricultural ecosystems.
- We test its effects on behaviour and life history of parthenogenetic stick insects.
- We find strong negative effects, at high dosages, on both survival and reproduction.
- Some effects are immediate, but others only appear after 12 days of exposure.
- We reveal key ecological and evolutionary impacts of copper sulphate on wildlife.

GRAPHICAL ABSTRACT



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ABSTRACT

Organic farming is considered the most sustainable form of modern soil cultivation. Yet it often relies on the use of chemical compounds that are not necessarily harmless for the surrounding wildlife. In this study, we tested the effects of realistic concentrations of copper sulphate—largely used in organic farming as a fungicide—on ecologically-relevant traits of the walking stick insect *Bacillus rossius*, a species commonly found in the proximity of cultivated fields across Europe. By using second-generation progeny of wild-caught parthenogenetic females bred in common gardens, we measured the impact of copper sulphate ($CuSO_4$) on both the life-history (body condition, number of eggs, and hatching success) and behavioural traits (activity and maximum vertical speed) of the individuals. We observed strong negative effects of high, realistic concentrations of copper sulphate on most traits within 12 days of exposure, while effects were less evident at lower concentrations of the pollutant. Our results reveal that realistic concentrations of copper sulphate can compromise important traits that regulate both the survival and reproduction of animals in the wild, with such effects that are, however, dose dependent. We suggest that common practices in organic farming require further consideration on their ecological and evolutionary impact on wildlife.

* Corresponding author at: Viterbo, 01100, VT, Italy.
E-mail addresses: giovanni.polverino@unitus.it, gio.polverino@gmail.com (G. Polverino).
[1] These authors contributed equally to this work.

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1. Introduction

Environmental contaminants alter biological processes and entire ecosystems, and are considered as one of the six major contributors to the global biodiversity decline (Jaureguiberry et al., 2022). Mounting evidence indicates that the exposure to dangerous pollutants at environmentally-realistic concentrations can disrupt a wide range of fundamental biological processes in wildlife (e.g. cell transformation and growth; Gore et al., 2015) and ultimately impact both survival and reproduction of living organisms (Aulsebrook et al., 2020). Moreover, behaviour can be remarkably sensitive to perturbation even by low concentrations of the contaminants that are not typically included as conventional endpoints in classic ecotoxicology studies (Melvin and Wilson, 2013). Therefore, understanding the effects of realistic concentrations of widespread contaminants on exposed organisms remains a major goal in ecology and environmental monitoring (Bertram et al., 2022).

Within species, individuals who are unable to escape polluted environments can quickly respond to changing conditions by adjusting their phenotypic traits, such as behaviour and life history (Saaristo et al., 2018; Dominoni et al., 2020). Despite the ecological and evolutionary importance of individual-level variation in life history and behaviour (Réale et al., 2007; Wolf and Weissing, 2012), very few studies have empirically tested the impact of pollutants within animal groups (Royauté et al., 2015; Tan et al., 2020; Polverino et al., 2021; Henry et al., 2022; Polverino et al., 2023).

Among the contaminants of major concern, heavy metals are known to have strong, negative effects on environmental health through their negative impacts on living organisms, including humans (Rehman et al., 2019). While modern farming is inherently associated with the implementation of sustainable cultivation practices, it often relies on the use of metal-based compounds. Among those, copper sulphate ($CuSO_4$) is frequently used in organic farming practices as a fungicide (Katsoulas et al., 2020), especially when validated alternatives are not available or less cost-effective (Tamm et al., 2022). Yet copper sulphate is toxic to living organisms (including humans and animals; La Torre et al., 2018; Rehman et al., 2019; also see Appendix 1, Paragraph 4 of the EU Regulation 1165/2021) and it has the potential to severely contaminate the soil, since it is sprayed as an aqueous solution on plant leaves (Bordeaux mixture; CAS Number: 8011-63-0) and it disperses into adjacent grasslands and river systems (Pavlovic, 2011). The widespread use of copper sulphate, and its dispersion into natural habitats, raises questions on the collateral effects that such pollutant might have on non-target species, especially insects, including pollinators (Di et al., 2016; Bernardes et al., 2022; Oliveira et al., 2022).

The walking stick insect *Bacillus rossius* (Phasmatodea: Bacillidae) is among the non-target species that are more likely to be exposed to copper-sulphate pollution in the wild in the Mediterranean region. This long-lived (6–12 months) flightless insect has, in fact, low dispersal capacity and it feeds almost exclusively on leaves of bramble bushes (*Rubus ulmifolius*), which are typically found at the edges of agricultural fields. Moreover, the species performs facultative parthenogenesis (Scali et al., 2003), so that genetic variation is often low within populations and phenotypic adjustments are particularly important for the individuals to survive under changing conditions. We used the stick insect *B. rossius* as a model to test whether and how the exposure to ecologically-relevant concentrations of copper sulphate had collateral effects on key life-history (body condition, number of eggs, and hatching success) and behavioural traits (activity and maximum vertical speed) of the animals. Our analysis offers a comprehensive, realistic assessment of the effects of a global environmental contaminant on the wildlife, and sheds light onto the sensitivity of stick insects for biomonitoring practices.

2. Methods

2.1. Experimental animals

We used adult females of the European walking stick insect *Bacillus rossius*—second-generation parthenogenetic progeny of wild-caught individuals from regions free from intensive farming. Animals were bred and maintained at controlled temperatures at 25 ± 1 °C under a 12:12 light-dark cycle. These conditions provided a stable environment for the animals over time.

We raised the experimental individuals into social enclosures (60 × 60 × 60 cm) with approximately 50 individuals per enclosure. We provided fresh branches of the bramble bush *Rubus ulmifolius* to the animals twice a week and submerged the lower portion of the stems into 500 ml water bottles before offering them to the insects, to keep them fresh across days. Therefore, animals were raised in standardised conditions and fed ad libitum throughout the experiment.

Experimental animals ($N = 120$) were randomly selected from the social enclosures. Each experimental individual, in post-absorptive state, was carefully weighed to the nearest 0.01 g and measured for its body length with a ruler, to the nearest mm. Then animals were housed into individual enclosures (25 × 25 × 25 cm) and kept in isolation for the duration of the study. Specifically, a subset of 90 animals was used for the life-history assays, while 60 animals participated to the behavioural tests.

2.2. Treatment exposure

The experiment was conducted across four consecutive rounds, with 30 animals tested per round. In each round, we randomly assigned 10 individuals to each of the three experimental treatments, which varied from each other in their concentration of copper sulphate ($CuSO_4$): control (0 g/l; 0.00 mmol/l), low treatment (0.1 g/l; 0.63 mmol/l), and high treatment (1 g/l; 6.27 mmol/l; Fig. 1A). A range-finding test was conducted as a preliminary step to calibrate the ecologically-relevant concentrations of the pollutant onto our model species. To do so, copper sulphate was diluted in distilled water to reach the concentration needed to prepare the Bordeaux mixture (CAS Number: 8011-63-0), a fungicide routinely used to spray crops in the EU (Katsoulas et al., 2020). The Bordeaux mixture contains copper sulphate in a concentration of 10 g/l (62.66 mmol/l), which we have further gradually diluted to obtain lower concentrations and to calculate, through a mortality assay (Oliva et al., 2009), the LC50 for our model species (6.9 g/l; 43.23 mmol/l). We chose the LC10 (i.e. Lethal Concentration 10; the concentration at which 10 % of the sample is expected to die) as our nominal concentration for the high treatment, since we wanted to repeatedly measure our animals over time and, therefore, minimize their deaths. Notably, such concentration is comparable in magnitude with the concentration of copper sulphate used in Bernardes et al., 2022 (5 g/l) for similar analyses, corresponding to concentrations routinely used to spray Brazilian tomato crops. Similarly, the concentration of the pollutant in the low treatment approximated the concentration already validated for such ecotoxicological analyses on the stingless bee *Partamona helleri* (0.145 g/l, LC50, Bernardes et al., 2022), which inhabits similar habitats as our model species.

Solutions of the two experimental concentrations of copper sulphate were applied onto the bramble branches before they were offered to the animals, by spraying the solution upon the leaves. Control bramble branches were, instead, sprayed with clean water. Sprayed branches were allowed to air-dry before being placed in the experimental cages. The stock solutions were freshly prepared for each replicated experiment, and branches were replaced twice a week for each round (see below).

Before life-history and behavioural assays started, animals were kept unfed for 24 h to ensure that they were hungry and to prevent potential rejection of the treated bramble leaves during the early stages of the exposure in response to the presence of copper sulphate (Chapman and Bernays, 1989; Chapman, 2003).

2.3. Life-history traits

Overall, 90 individuals were individually assayed for their life-history traits across three separate rounds (i.e. 30 individuals per round). Individuals were assessed one-by-one four times, that is, once at each time point: T0 (before exposure started), T1 (4 days after exposure started), T2 (8 days after exposure started), and T3 (12 days after exposure started). Therefore, each individual was measured once before the exposure treatments started and once every 4 days after exposure for a total of four data points per animal. Testing the effects of pollutants four days after the initial exposure is common to assess acute toxicity in non-target species (Xiong et al., 2011; Fisher et al., 2014). We also chose to extend the test up to 12 consecutive days of exposure to test whether effects varied over time. All individuals were assayed within 2 h at each time point, to reduce variation arising from measurements performed early and late in the day.

At each time point, we measured the body condition (weight in g; Fig. 1B) of every individual by using a digital scale with two decimals (NiftyPlaza, Superior Mini Digital Scale). The number (count) and mass (g; Fig. 1B) of the eggs laid by each female between subsequent time points were systematically measured respectively with a Nikon D3500 camera, equipped with a 55 mm 3.5 Micro-Nikkor Macro lens, and an analytical scale with four decimals (Acculab ATL-224-I Atilon Analytical Balance 220 g). Eggs were then counted with a dedicated software, DotDotGoose (version 1.6.0; https://biodiversityinformatics.amnh.org/open_source/dotdotgoose). To evaluate hatching success, eggs collected from each female in the first and second round ($N = 60$ females, that is, 20 females per treatment), were stored within 15 ml falcon tubes replacing the tube caps with paper balls to facilitate aeration. The hatching success (count of hatched eggs) was determined per each female after three months, that is, one month since hatching started.

2.4. Behavioural traits

Overall, 60 individuals were assayed for their behavioural traits (i.e. 20 individuals per treatment). We conducted a climbing activity test hypothesising that copper sulphate might have negative effects on the activity pattern and functional performance of the exposed animals, since the pollutant is known for its neurotoxic effect on exposed animals (Habotta et al., 2022; Oliveira et al., 2022). To do so, we assessed the following behaviours: total distance covered (m), time spent climbing (s), and maximum vertical speed (m/s; Fig. 1C). The behavioural tests were conducted on a polystyrene arena 113 cm high and 80 cm long. Each individual was positioned at the bottom of the arena and startled by a single tap on the abdomen to initiate the trial—as per validated protocols for triggering evasive responses in both stick insects (Polverino et al., 2022) and mountain katydids (Umbers and Mappes, 2015). Each trial was started soon after the startle stimulus and was terminated after 20 s. All trials were video recorded using a Nikon D3500 camera equipped with 35 mm 1.8 DX lenses (in FullHD) at 30 frames per s. Behavioural trials were scored with a dedicated software (BORIS, version 7.13.5; Friard and Gamba, 2016) and maximum vertical speed was calculated, at each trial, with the software Tracker (version 6.1.3; https://physlets.org/tracker/).

2.5. Statistical analysis

Overall, 120 individuals were assayed, with a total of 326 life-history measurements and 155 behavioural trials (20 females did not complete the study due to early mortality, with 10 % higher mortality in the high treatment compared to the controls by the end of the experiment).

Egg mass was normalised for the body size of the individual, since egg production typically varies with body size. Under the same perspective, the weight variation of the individuals (i.e. Δ% body condition) was calculated as the percentage of weight variation between consecutive time points. The analysis was performed with *R* (version 4.3.1), using the *lme4* and *emmeans* packages (Bates et al., 2015; Lenth, 2023).

We wanted to test whether copper sulphate had a significant effect on the body condition and the egg mass of the exposed animals. Accordingly, we built two models in which body condition and egg mass were the dependent variables respectively, and included treatment, time point, and round as the fixed effects in each model. We accounted for repeated measures of each individual over time by including individual IDs as the random effect in each model. We also wanted to test the effect of the exposure treatment on the hatching success. To do so, we built a model in which treatment, time point, and round were included as fixed effects, and adjusted hatching success by also including the number of eggs produced by the females as a covariate in the model. Again, we included random intercepts (IDs) as the random effects of the model to account for multiple measurements of the same individuals over time.

A main goal was to test whether copper sulphate had a significant impact on important behaviours of these insects. Accordingly, we built two models in which time spent climbing and maximum vertical speed

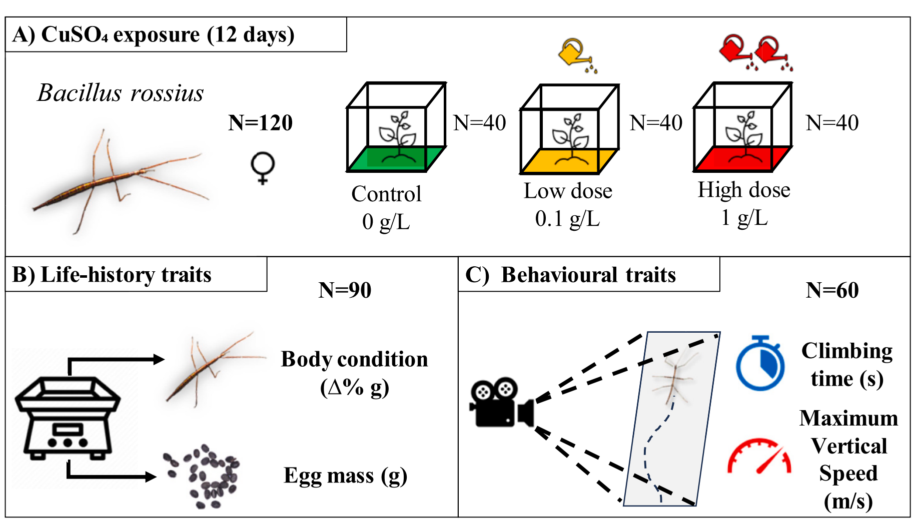

Fig. 1. Experimental design. (A) We used 120 laboratory reared female stick insects and exposed them to realistic concentrations of copper sulphate for 12 consecutive days. (B) Every four days we measured the life-history traits of the individuals and (C) assayed their activity behaviour and climbing performance.

were the dependent variables, respectively. Each model included treatment, total distance covered, and their interactions as the fixed effects. We also included body length as a fixed effect since locomotor performance is likely to vary as a function of body size. Round was not included in the behavioural models since all animals were tested at the same time. Each model also included random intercepts (IDs) to account for repeated measures of each individual over time.

3. Results

3.1. Life-history traits

Copper sulphate significantly altered the body condition of the animals (Table 1 and 2). In particular, post-hoc comparison revealed that body condition was substantially lower in the high treatment than both in the control ($p < 0.001$, SE ± 0.023; Fig. 2A) and low-dose group (p < 0.001, SE ± 0.024; Fig. 2A). Such a dose-dependent effect of the pollutant on body condition did not depend on the length of the exposure, since high-treatment animals consistently had lower body condition than both control (T1: p < 0.001, SE ± 0.025; T2: p < 0.001, SE ± 0.026; T3: p < 0.001, SE ± 0.027) and low groups (T1: p < 0.001, SE ± 0.025; T2: p < 0.001, SE ± 0.026; T3: $p = 0.006$, SE ± 0.027) across the sampling periods.

The number of eggs laid by each female was strongly correlated with its egg mass (Pearson's correlation; $t = 65.807$; df = 234; p < 0.001). Therefore, we only used egg mass in the analysis. We found that, overall, treatment did not affect egg mass, but egg mass decreased as the experiment proceeded (Table 1 and 2; Fig. 3A). Notably, egg mass was significantly lower in the high-treatment than both in control- and in low-treatment females at T3 (Fig. 3A), that is, after 12 consecutive days of exposure.

Hatching success was also negatively affected by the exposure treatment and, as expected, it was related to the total number of eggs laid by the females (Table 1 and 2; Fig. 2B). In particular, post-hoc comparisons revealed that females exposed to the high concentration of copper sulphate had lower hatching success than control females (Fig. 2B).

Table 1
Results from the fixed-factor structure of the generalised linear-mixed models with life-history (body condition, egg mass, and hatching success) and behavioural traits (climbing and maximum vertical speed) as the dependent variables. Significance was α < 0.05 and significant results are in bold.

Model	Mean Square	df	*F*	*p*
Body condition				
Treatment	0.030	2760	13.797	**<0.001**
Time Point	0.021	2138	9.618	**<0.001**
Round	0.003	2760	1.647	0.199
Treatment × Time Point	0.001	4138	0.546	0.701
Egg mass				
Treatment	0.001	2720	1.391	0.255
Time Point	0.009	2142	22.396	**<0.001**
Round	<0.001	2720	0.184	0.832
Treatment × Time Point	0.004	4142	5.071	**<0.001**
Hatching success				
Treatment	13.308	2590	3.349	**0.041**
Time Point	5.718	2109	1.439	0.241
Body Condition	3.824	1123	0.962	0.328
Number of eggs	135.716	1146	34,150	**<0.001**
Round	1.905	1530	0.479	0.491
Treatment × Time Point	2.072	4101	0.522	0.720
Climbing				
Treatment	147.460	2111	4.780	**0.010**
Time Point	15.790	2111	0.511	0.601
Space covered	2667.730	1111	86.540	**<0.001**
Body length	66.780	1111	2.165	0.144
Treatment × Time Point	5.640	4111	0.183	0.947
Treatment × Space Covered	90.450	2111	2.932	0.057
Treatment × Space Covered	29.730	2111	0.960	0.385
Treatment × Time Point × Space Covered	11.890	4111	0.389	0.819
Maximum vertical speed				
Treatment	<0.001	2810	1.221	0.300
Time Point	<0.001	2810	2.639	0.077
Space covered	<0.001	1970	145.598	**<0.001**
Body length	<0.001	1400	0.497	0.485
Treatment × Timepoint	<0.001	4800	2.461	0.052
Treatment × Space Covered	<0.001	2980	2.541	0.084
Time Point × Activity	<0.001	2000	2.662	0.075
Treatment × Timepoint × Activity	<0.001	4000	2.607	**0.042**

Table 2
Results from the random portion of the generalised linear-mixed models with life-history (body condition, egg mass, and hatching success) and behavioural traits (climbing and maximum vertical speed) as the dependent variables.

Dependent variable	Variance	SD
Body condition		
Intercept (ID)	0.007	0.085
Residual	0.002	0.047
Egg mass		
Intercept (ID)	<0.001	0.009
Residual	<0.001	0.015
Hatching success		
Intercept (ID)	2.936	1.714
Residual	3.973	1.993
Climbing		
Intercept (ID)	<0.001	<0.001
Residual	30.850	5.554
Maximum vertical speed		
Intercept (ID)	<0.001	<0.001
Residual	<0.001	<0.001

3.2. Behavioural traits

The total time spent climbing (i.e. activity behaviour) strongly varied across exposure treatments (Table 1 and 2; Fig. 2C). In particular, post-hoc comparisons indicated that activity was lower, albeit not statistically significant, in both the high- and low-exposure treatment than in the control group (Fig. 2C). As expected, the total distance covered by the animals during a trial explained a significant portion of the variance observed in the climbing behaviour (Table 1).

Our analysis also revealed that copper sulphate did not have an overall effect on the maximum vertical speed of the animals (Table 1 and 2). However, animals from different exposure treatments tended to differ in their vertical speed across trials (Fig. 3B), although the interaction between the two factors was marginally non-significant (Table 1).

4. Discussion

Our analysis revealed that realistic concentrations of a widespread pollutant commonly used in agricultural treatments can exert a strong, negative impact on important phenotypic traits of non-target insects, such as the stick insect *B. rossius*. However, we found that effects were dose dependent, whereby high concentrations of copper sulphate had strong negative effects on most of the life-history and behavioural traits measured, while effects were less evident at lower concentrations of the pollutant.

Our analysis revealed that insects exposed to high concentrations of copper sulphate had ~12 % lower body condition than control and low treatment animals. Such variation in weight is noteworthy, particularly because a substantial component of the body of these adult animals is exoskeleton, and it is likely not affected by our treatment (Lease and Wolf, 2010). The exposure to food sources contaminated with realistic concentrations of copper sulphate has been found to be detrimental for the antioxidant activity and even altered the midgut morphology of stingless bees (Bernardes et al., 2022) and fruit flies (Halmenschelager

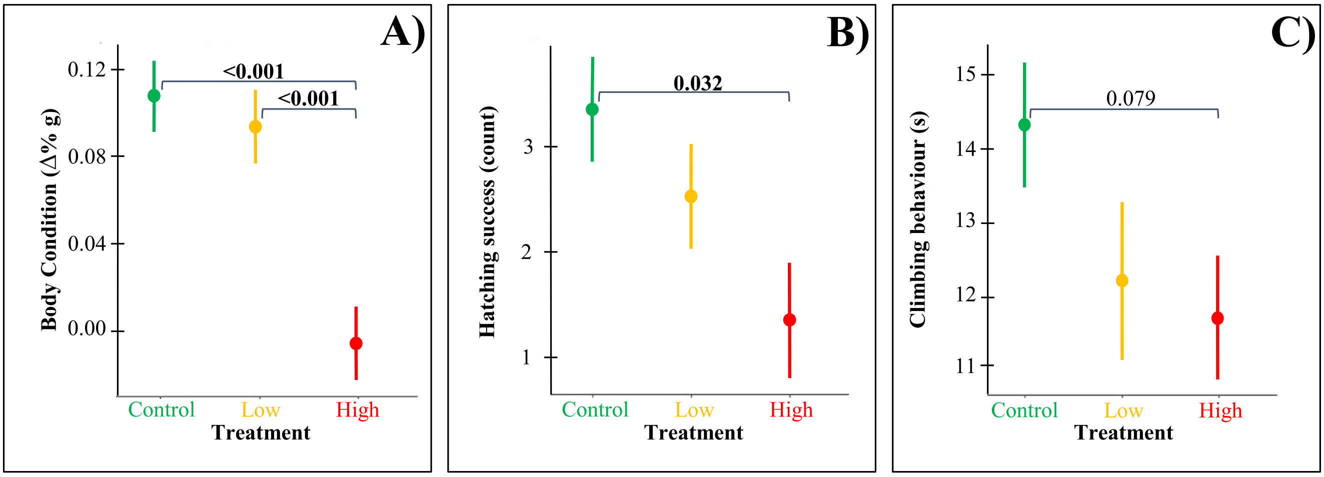

Fig. 2. Estimated marginal means for the three exposure treatments (control, low, and high treatment) with respect to variation in body condition (A), hatching success (B), and climbing behaviour (C). Error bars represent the SE, and significant differences are in bold.

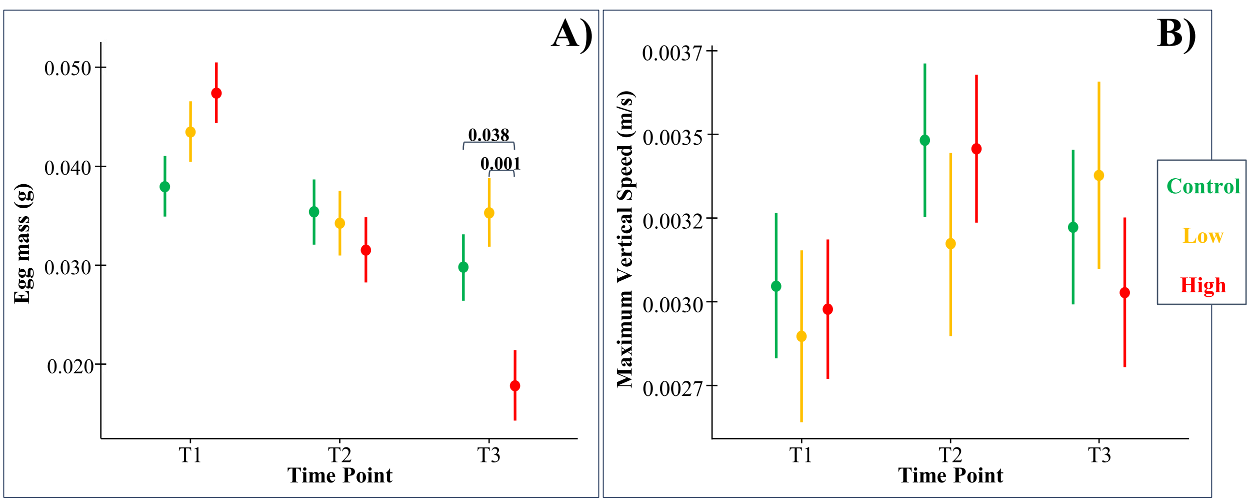

Fig. 3. Estimated marginal means for the three exposure treatments (control, low, and high treatment) with respect to variation in egg mass (A) and maximum vertical speed (B) across time points (Ts). Error bars represent the SE, and significant differences are in bold.

and da Rocha, 2019). Other metal-based compounds used as pesticide agents have been also found to negatively impact the metabolic functions of exposed grasshoppers (Augustyniak et al., 2005). But copper sulphate is also known to have neurotoxic effects on exposed insects, disrupting activity-related behaviours of exposed stingless bees (Bernardes et al., 2022), fruit flies (reviewed by Oliveira et al., 2022), and fish (Habotta et al., 2022). Therefore, it is likely that relatively high, realistic concentration of copper sulphate might have altered the metabolic machinery of the individuals in our high-treatment group—an effect also described in fish exposed to aquatic pollutants (Tan et al., 2020)—which, in turn, could explain their lower body condition and their tendency to be less active compared to control- and low-treatment animals. The tendency of insects to be less active when exposed to high doses of the pollutants is important, especially for animals such as *B. rossius* that necessarily rely on their activity behaviour to evade predators when crypsis fails (Polverino et al., 2022). It is unlikely that the presence of the toxicant reduced the feeding behaviour of our insects, as instead observed in other phytophagous insects (Chapman and Bernays, 1989) and honeybees (Burden et al., 2019). In fact, our animals were deprived of food for 24 h before the experiment started, which is known to prevent insects, such as grasshoppers, from rejecting contaminated food (Bernays and Chapman, 1975). In support of this interpretation, differences in body condition across treatments did not vary over time. Yet whether the uptake of plant material differed among our animals in response to different exposure treatments is unknown.

Interestingly, we found strong, negative effect also on the hatching success of the animals exposed to high concentrations of copper sulphate, suggesting that effects span beyond the life history and behaviour of the exposed individuals. Such a reduction in fitness induced by copper-sulphate pollution was also observed in freshwater snails (Das and Khangarot, 2011) and is concerning, because it suggests that relatively short exposures to this widespread contaminant are sufficient to threaten the long-term survival of entire populations (Chichorro et al., 2022). A similar effect has been also reported in fruit flies, in which the development of larvae declined with increasing concentrations of copper sulphate (Halmenschelager and da Rocha, 2019). However, contrary to Halmenschelager and da Rocha (2019) in which negative effects were evident already at low concentrations of the pollutant (i.e. 0.4 mmol), we found negative effects on hatching success only at relatively higher concentrations (6.27 mmol). Whether the parthenogenetic nature of this stick insect plays a role in explaining its higher tolerance to environmental challenges compared to other organisms is an interesting aspect, but unknown at present (Forbes and Depledge, 1992; Vrijenhoek, 1998).

Collateral effects of copper sulphate on living organisms can also depend upon the exposure length (Wang et al., 2007). Our analysis revealed that, in fact, some effects of the pollutant appeared only after longer exposures. For instance, stick insects in the high-treatment group were less fertile (i.e. produced lower egg mass) than both control and low-treatment individuals after 12 days of exposure, and even variation in their maximum vertical speed depended on the combined effect of exposure treatment and exposure length. Copper sulphate was found to have similar, negative effects on the number of eggs laid also in crustaceans (De Schamphelaere et al., 2007; Wang et al., 2007) and molluscs (Das and Khangarot, 2011) exposed to high concentrations over longer time periods. Such negative effects on fertility rates might be attributed to the cytotoxicity of this pollutant (Schwerdtle et al., 2007), which, in turn, is likely to compromise the reproductive functions of animals, including humans (Roychoudhury et al., 2016).

Overall, our study points out that the exposure to realistic concentrations of the global pollutant copper sulphate induce a substantial, negative response on the life history and behaviour of the stick insect *B. rossius*. Our evidence raises concerns about the collateral effects of copper sulphate on the survival and reproduction of wildlife in agricultural ecosystems that heavily rely on the use of copper-based fungicides (i.e. the organic production; Burandt et al., 2024). We emphasize the need for a more comprehensive evaluation of the ecological consequences of agricultural practices and the potential repercussions on non-target species. As the environmental landscape evolves, it is imperative to re-think current paradigms and reassess the ecological implications of agrochemical use to foster sustainable and biologically harmonious agricultural practices.

Ethical note

All the animals used in the experiments were born in captivity and have been humanely euthanized after the conclusion of the experimental procedure.

CRediT authorship contribution statement

Claudio Pardo: Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Adriana Bellati:** Writing – review & editing, Supervision, Resources, Project administration, Methodology, Conceptualization. **Giovanni Polverino:** Writing – review & editing, Writing – original draft, Validation, Supervision, Software, Project administration, Methodology, Formal analysis, Data curation, Conceptualization. **Daniele Canestrelli:** Writing – review & editing, Resources, Project administration, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

The data and *R* code used to produce the statistics and figures presented in this article are available in Figshare at https://figshare.com/s/f9c176417eceb4b7c49f.

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