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Adverse effects of the Bordeaux mixture copper-based fungicide on the non-target vineyard pest *Lobesia botrana*

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Abstract

BACKGROUND: Bordeaux mixture is a copper-based fungicide commonly used in vineyards to prevent fungal and bacterial infections in grapevines. However, this fungicide may adversely affect the entomological component, including insect pests. Understanding the impacts of Bordeaux mixture on the vineyard pest *Lobesia botrana* is an increasing concern in the viticultural production.

RESULTS: Bordeaux mixture had detrimental effects on the development and reproductive performance of *L. botrana*. Several physiological traits were adversely affected by copper-based fungicide exposure, including a decrease in larval survival and a delayed larval development to moth emergence, as well as a reduced reproductive performance through a decrease in female fecundity and fertility and male sperm quality. However, we did not detect any effect of Bordeaux mixture on the measured reproductive behaviors (mating success, pre-mating latency and mating duration).

CONCLUSION: Ingestion by larvae of food contaminated with Bordeaux mixture had a negative effect on the reproductive performance of the pest *L. botrana*, which could affect its population dynamics in vineyards. Although this study highlighted collateral damage of Bordeaux mixture on *L. botrana*, the potential impact of copper-based fungicides on vineyard diversity, including natural predators is discussed and needs to be taken in consideration in integrated pest management.

Keywords: Bordeaux mixture; copper-based fungicide; heavy metals; *Lobesia botrana*; reproductive performance; viticulture

1 INTRODUCTION

Because of the application of a wide range of pesticides and fertilizers, intensive agriculture has led to a worldwide distribution of heavy metals in ecosystems, mostly copper, zinc, cadmium, and lead.[1] Among heavy-metal based pesticides, the intensive use of copper-based products such as Bordeaux mixture ($CuSO_4$) has a significant role in the dispersion of copper in the environment, leading to substantial environmental contaminations that can potentially alter the functioning of agroecosystems and their biodiversity.[1,2] Bordeaux mixture, developed and used since the middle 19th century in viticulture and fruit production, is widely known for its antifungal and antimicrobial properties, favoring the control of fungal pests in both organic and conventional agriculture.[3] Copper ions (the active substance in Bordeaux mixture) lead to RNA degradation and membrane deterioration, making them highly toxic for bacteria, viruses and fungi.[4,5]

Fungicide exposure may affect untargeted organisms in treated agricultural plots, including beneficial and pest insect species.[6–11] Non-target insects might be negatively affected after consuming contaminated plant material and/or prey during foraging, as well as through direct contact with spray droplets and pesticide residues.[12–15] Excessive accumulation of copper in insects may

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have lethal and sublethal effects,[16] including: (i) the inhibition of enzymes mitigating oxidative damage (e.g., catalase, superoxide dismutase);[6] (ii) behavioral alterations (e.g., feeding and walking behaviors);[6,17] (iii) decreased survival and extended development time;[8,18–20] and (iv) decreased female fecundity and fertility.[18,19] These sublethal effects can even be transgenerational,[21,22] emphasizing the long-lasting impacts associated with the application of copper-based fungicides on non-target insects present in environments contaminated by copper. Hence, the multitude of collateral effects of copper-based fungicides extensively used in agriculture could significantly influence the agroecosystem functioning.[23]

Viticulture is one of the biggest consumers of copper-based fungicides such as Bordeaux mixture, contributing significantly to the accumulation of copper in agricultural soil.[24] Copper-based fungicide is regularly applied to control pathogenic fungi, such as downy mildew *Plasmopara viticola* (Peronosporales, Peronosporaceae), gray mold *Botrytis cinerea* (Helotiales, Sclerotiniaceae) or black mold *Aspergillus* spp. (Eurotiales, Trichomaceae), that decrease yields and wine quality.[25] The use of copper-based fungicides in vineyards is subject to mandatory measures, setting a maximal residue limit copper concentration in grapes of 50 mg.kg^{-1} at harvest.[26] However, owing to many factors that modulate the dispersion of copper in the environment (e.g., geographical context, meteorological conditions),[27] the bioavailable copper concentration in vineyards may exceed 50 mg.kg^{-1}, as observed on grapes with a copper concentration range of 10–100 mg.kg^{-1}.[28-31] Consequently, vineyard entomofauna is likely to be exposed to high copper concentrations. In this context, Vogelweith and Thiéry[32] reported that the use of copper-based fungicides in vineyards negatively impacts leaf arthropod diversity, for both beneficial and pest species. Understanding the copper-related mechanisms that influence a vineyard's entomofauna is essential for formulating sustainable pest management strategies, thereby ensuring the preservation of both biodiversity and agricultural productivity.

Among arthropod grapevine pests, the European grapevine moth *Lobesia botrana* (Denis & Schiffermüller, Lepidoptera: Tortricidae), may be affected by copper-based fungicides. *Lobesia botrana* is one of the most widespread and damaging pests in vineyards worldwide, and is linked to substantial economic losses due to reduced yields.[33,34] However, our understanding of how copper-based fungicides affect the behavior and health of this pest is currently limited. On the one hand, copper-based fungicides may have a positive effect on *L. botrana* populations by altering the population dynamics of their predators.[35] At the individual level, the low copper concentration in Bordeaux mixture exhibits a positive effect on physiological traits such as larval immune defenses.[8] On the other hand, an elevated copper concentration may result in a slowdown of the pest's development time and a decreased pupal mass.[8,20] The complex response of *L. botrana* to Bordeaux mixture is challenging for integrated pest management in vineyards. The impact of copper-based fungicides on adults, particularly in relation to their reproductive capacities, remains unexplored. A thorough assessment of Bordeaux mixture's influence on *L. botrana* reproductive performance is thus essential to anticipate its population dynamics and consequently, crop damage.

The objective of this study was to evaluate the effects of Bordeaux mixture ($CuSO_4$) on the larval developmental and adult reproductive performances of the European grapevine moth, *L. botrana*. During their development, larvae seek refuge inside flower buds and berries, exposing themselves to plant tissues containing or covered by copper-based fungicides through ingestion. To mimic this exposure, we conducted laboratory experiments wherein *L. botrana* larvae developed on diets of either a standard composition or supplemented with Bordeaux mixture. We then assessed the effects of Bordeaux mixture exposure on various physiological traits related to development (development time until emergence, pupal mass, sex ratio, and adult longevity) and reproduction (fertility and fecundity in females and sperm quality in males), as well as its influence on reproductive behavior (mating success, mating latency, and mating duration). *Lobesia botrana* exhibits a capital breeding strategy wherein the quantity and quality of food consumed by larvae are key factors affecting reproductive traits and behavior in adults,[36,37] such as egg-laying capacity,[18,38] egg hatchability,[38,39] sperm quality,[36,40] mating duration, and sexual motivation of partners to mate.[36] We thus hypothesized that copper consumed by larvae negatively impacts *L. botrana* development and therefore, the reproductive performance of adults.

2 MATERIALS AND METHODS

2.1 Insect rearing

Insects used in experiments came from a laboratory-reared population of *L. botrana* (INRAe, Villenave d'Ornon, France). Standard rearing was maintained on a semi-artificial diet [composition for 1000 mL: 1000 mL of water, 15 g of agar, 86.6 g of corn flour, 41.3 g of wheatgerm, 45.5 g of beer yeast, 6 g of ascorbic acid, 3.4 g of mineral salt (Wesson salt mixture), 128 mg of pyrimethanil, 2.7 g of benzoic acid, 2.8 g of methyl 4-hydroxybenzoate, and 5 mL of 95% ethanol; adapted from Thiéry and Moreau].[41] Insect rearing and subsequent experiments were carried out under constant conditions of temperature (22 ± 0.5 °C), relative humidity (60 ± 10%), photoperiod (17 h light, 1 dusk and 6 h dark), and luminosity (650 lx during light periods and 100 lx at dusk). Adults were maintained inside net-cages (35 × 26 × 26 cm) and no diet was required at the adult stage of *L. botrana*. Eggs were collected on waxed paper sheets (8 × 13 cm) suspended inside the cages. They were removed after 24 h and transferred to plastic boxes (18 × 11.5 × 7 cm) that were aerated and humidified to prevent egg desiccation.

2.2 Larval treatments and general procedure

The influence of Bordeaux mixture on *L. botrana* was assessed using newly hatched larvae (<12 h old). To avoid intraspecific competition during experiments, newly hatched larvae were carefully collected with a paintbrush and individually placed in plastic 2-mL microtubes (Eppendorf®) containing 1.5 mL of semi-artificial diet.[42] The semi-artificial diet (see above) was supplemented with either 10 mL of distilled water for the control treatment or 10 mL of different concentrations (expressed in mg of copper per kg of nutrient medium) of Bordeaux mixture solution (RSR Disperss NC, UPL) diluted in distilled water (as described by Iltis *et al.*).[8] Bordeaux mixture consists of 20% copper, in the form of copper sulfate ($CuSO_4$), combined with gypsum ($CaSO_4$) to stabilize copper ions. The chosen treatments covered a broad spectrum of copper concentrations typically found in vineyards, namely 0 mg.kg^{-1} (control group), 25 mg.kg^{-1} (low concentration),[28,30,43] 100 mg.kg^{-1} (high concentration),[31,44] and 225 mg.kg^{-1} (extremely high concentration).[27,45,46] Larvae were reared individually until pupation in their tubes. During their development, several life-history traits (pupal mass, development time and survival rate of larvae until emergence) were measured in all individuals. A subset of individuals reaching the adult stage was randomly selected throughout the emergence period and used for the reproductive experiments (reproductive behavior, female reproductive output, and male reproductive output). To specifically assess Bordeaux mixture's effects on the reproductive performance of both sexes (female and male), we used the procedure developed in previous experiments.[47] Briefly, the mating procedure consists of pairing one individual (male or female) exposed to Bordeaux mixture at the larval stage with one individual of the opposite sex originating from standard rearing. To obtain 'standard individuals', pupae from the standard rearing were individually isolated and weighed. During mating, various reproductive behaviors were measured (mating success, pre-mating latency and mating duration). Following successful mating, reproductive performance was assessed for males and females using various proxies (fertility, fecundity, spermatophore volume, and number of fertilizing spermatozoa).[36]

2.3 Impact of Bordeaux mixture on developmental life-history traits

A total of 1620 newly hatched larvae were randomly allocated to one of the four treatments ($n = 440$ for 0 mg.kg^{-1}, $n = 430$ for 25 mg.kg^{-1}, $n = 388$ for 100 mg.kg^{-1}, and $n = 362$ for 225 mg.kg^{-1}), and larvae were checked daily until pupation. Each pupa was collected and weighed (± 0.1 mg; Pioneer PA214C, OHAUS, Greifensee, Switzerland), isolated in a glass tube (70×9 mm diameter) and inspected daily. Newly emerged males and females were sexed by visual examination of the ventral tip of their abdomen. The following life-history traits were measured in all individuals: (i) survival rate from hatching to adult emergence, expressed as the ratio of viable adults to the number of larvae initially deposited; (ii) development time, expressed as days from egg hatching to adult emergence; (iii) pupal mass; and (iv) sex ratio of emerging adults.

2.4 Impact of Bordeaux mixture on reproductive behavior

To assess the influence of the fungicide on the reproductive performance of *L. botrana*, a total of 406 individuals, exposed to the different Bordeaux mixture treatments at the larval stage, were mated with 409 individuals from the standard rearing as sexual partners. Mating was monitored in 204 females exposed to the fungicide ($n = 53$ for 0 mg.kg^{-1}, $n = 53$ for 25 mg.kg^{-1}, $n = 55$ for 100 mg.kg^{-1}, and $n = 43$ for 225 mg.kg^{-1}) and 202 males exposed to the fungicide ($n = 64$ for 0 mg.kg^{-1}, $n = 46$ for 25 mg.kg^{-1}, $n = 47$ for 100 mgkg^{-1}, $n = 45$ for 225 mg kg^{-1}). At dusk (under red light), one randomly selected virgin individual from each copper treatment was placed in a mating tube (100×15 mm diameter) with a single virgin partner of the opposite sex from the standard rearing. As described by Muller *et al.*,[48] mating procedure involved a 2 ± 1-day-old female mated with 3 ± 1-day-old male. Pairs were observed continuously until mating occurred (formation and separation of the pair), or for up to 4 h if mating did not occur. When individuals mated, we measured various reproductive behaviors: (i) the pre-mating latency period, defined as the time elapsed from the introduction of the male and the female into the tube to mating; (ii) mating duration, defined as the time during which the male is attached to the female's abdomen; and (iii) the mating success, defined for males as the transfer of the spermatophore to his partner (determined by dissecting the female partner), and for females as the successful laying of at least one fertile egg. Following mating, reproductive output was assessed for males (spermatic quality) and females (fecundity, fertility) (see below).

2.5 Impact of Bordeaux mixture on female reproductive output

To assess the reproductive output in females, standardized males were removed from the tube just after mating and were no longer used for experiments. Females were left in the mating tube without food and water and allowed to oviposit on the inner surface of the tubes until they died (see Muller *et al.*[36] for full details). Females were examined daily to determine their longevity. Following their death, all eggs laid were incubated for 7 days under the same conditions as adult maintenance. Eggs were then counted to record: (i) female fecundity (total number of eggs laid during a female's lifespan), (ii) fertility (proportion of hatched eggs) and (iii) longevity.

2.6 Impact of Bordeaux mixture on male reproductive output

To evaluate the reproductive traits in males, standard-rearing females that mated with the tested males were collected and frozen at −25 °C immediately after mating. Males were left in the tubes until they died to determine their longevity. The frozen females were then dissected on a glass slide. The bursa copulatrix, which contained the male spermatophore, was removed and measured. The spermatophore volume was estimated in accordance with previous studies on *L. botrana*.[8,37,49] Considering the ellipsoid shape of the spermatophore, the length (l), width (w) and thickness (t) were measured using a stereomicroscope (Stemi 508, Ziss, Göttingen, Germany) at a magnification of ×50 (accuracy ± 0.1 μm) and a camera (Axiocam 105, Zeiss) to determine the volume V [$V = \pi/6\ (l \times w \times t)$]. Photographs were analyzed using ImageJ (version 1.53, Bethesda, MD, USA). The sperm-containing ampulla inside the spermatophore was then ruptured in a drop of distilled water, allowing the dispersion of fertile eupyrene and non-fertile apyrene spermatozoa.[37] For this study, only eupyrene spermatozoa, encysted in bundles of 256, were counted and the number of bundles was multiplied by 256 to estimate the total number of eupyrene spermatozoa.[50,51] Hence, the reproductive output of males was recorded using: (i) spermatophore volume, (ii) the number of eupyrene sperm and (iii) male longevity.

2.7 Statistical analyses

To assess the effect of copper treatment on *L. botrana* developmental and reproductive metrics (pupal mass, development time, longevity, mating latency, mating duration, female fecundity, female fertility, male spermatophore volume, and number of eupyrene sperm), we applied linear models (LM) for residuals following normal distribution or otherwise generalized linear models (GLM), testing different distribution families to best fit the data (Poisson, quasi-Poisson, and negative binomial). For LM, we also tested whether a log or inverse transformation improved (normalized) the distribution of the response data. When outliers were present in the data, LM were fitted using robust regression.[52] Weighted least squared regression was used when residuals exhibited a normal distribution but were heterogeneous.[53] For proportion data (larvae survival rate until adult emergence, adult sex ratio, and mating success of males and females), we applied a GLM with a binomial error structure.

Given our *a priori* expectation that the pupal mass of tested individual affects all developmental and reproductive metrics, as well as pupal mass of their sexual partners for reproductive metrics,[8,36,37] we included this covariate within an analysis of covariance (ANCOVA), analyzing males and females separately for all adult responses. When significant effect sizes were detected, ANCOVAs were followed by *post-hoc* tests to identify which treatment levels differed from one another. Finally, we performed a variation partitioning to assess the combined *versus* unique contribution of copper concentrations and pupal mass on the suite of continuous response variables. All statistical analyses were performed using R software (version 4.2.0, R Core Team 2022). The *MASS* package[54] and the *car* package[55] were used for the corresponding analyses.

3 RESULTS

3.1 Impact of Bordeaux mixture on developmental life-history traits

Survival of larvae until adult emergence was significantly affected by Bordeaux mixture ($X^2_3 = 281.51$, $p < 0.001$). The initial survival rate of larvae observed in the control treatment, at 84.5% ± 1.73% (mean ± SEM) decreased significantly to 66.8% ± 2.39% when the larvae were reared in medium containing 100 mg.kg^{-1} copper in comparison with copper-free or 25 mg.kg^{-1} copper (Fig. 1(a)). Similarly, the pupal mass of males and females decreased with increasing copper concentrations within the rearing medium, particularly when copper concentrations reached or exceeded 100 mg.kg^{-1} (Table 1; Fig. 1(b)). Development times of females and males were significantly extended with the increase in copper concentration in the rearing medium (Fig. 1(c); Table 1). In addition, development times were globally positively correlated to pupal mass, and the influence of pupal mass interacted with the impact of copper concentrations (Table 1). Depending on the pupal mass, the effect of Bordeaux mixture on development time is modulated either positively or negatively. Finally, Bordeaux mixture exposure did not affect the sex ratio of emerging individuals with an average proportion of females at 0.44 [95% confidence interval (CI) = 0.38–0.50].

3.2 Impact of Bordeaux mixture on female reproductive performance

Mating success, pre-mating latency, and mating duration of females were not influenced by exposure to Bordeaux mixture during the larval stages (Tables 1 and 2). However, mating duration significantly decreased when their male partners were heavier ($\beta = -0.04$, $p = 0.03$; Table 1). Bordeaux mixture exposure significantly decreased the fecundity and fertility of females only at 225 mg.kg^{-1} (Table 1; Fig. 2). Finally, female longevity was significantly impacted both by copper and female pupal mass (Table 1; Fig. 1(d)). Variation partitioning analysis showed that pupal mass was the dominant factor explaining the longevity of females (12.3% unique variation explained), whereas copper uniquely or co-jointly with pupal mass explained 1.2% and 2.9%, respectively, of the variation in female longevity.

3.3 Impact of Bordeaux mixture on male reproductive performance

Mating success, pre-mating latency, and mating duration of males were not impacted by exposure to Bordeaux mixture during the larval stages (Tables 1 and 2). Nevertheless, mating duration increased significantly when female partners were heavier ($\beta = 0.01$, $p < 0.01$). Furthermore, exposure ranging from the lowest tested copper concentration, 25 mg.kg^{-1}, to the highest, 225 mg.kg^{-1}, resulted in a significant decrease in spermatophore volume and the number of eupyrene spermatozoa in males (Table 1; Fig. 3). In addition, heavier males produced larger spermatophore and more eupyrene spermatozoa ($\beta = 0.01$, $p \leq 0.001$ and $\beta = 557.61$, $p \leq 0.001$, respectively) (Table 1). Finally, Bordeaux mixture exposure did not affect the longevity of males (Table 1), but longevity increased when males were heavier ($\beta = 0.07$, $p < 0.01$) (Fig. 1(d)).

4 DISCUSSION

The aim of this study was to evaluate the impact of a copper-based fungicide on a non-target vineyard pest, the European grapevine moth *Lobesia botrana*. According to our predictions, larvae consuming Bordeaux mixture experienced an increased mortality and alterations both in larval development and adult reproduction. Overall, the application of Bordeaux mixture negatively impacted the reproductive performance of the pest, with potential implications for long-term population dynamics.

Our results highlight the importance of copper-based fungicide exposure through ingestion on larval development. As previously documented,[8] we found that consumption of Bordeaux mixture during larval stages led to an extended duration of both larval and pupal development time, associated with a final decrease in pupal mass. Furthermore, Bordeaux mixture induced a decrease in female longevity. These detrimental effects can be attributed to several potential drivers and mechanisms. Copper is a key micronutrient involved in the maintenance of insect vital functions, including oxygen transport and storage in the hemolymph, immunity, homeostasis, and hormone transport.[56] Despite these vital roles of copper, excessive accumulation within the insect body may activate energy-intensive detoxification mechanisms that drastically reduce the allocation of energetic resources available to development, growth, and survival.[7] In addition to this increased physiological demand, insects can modify their feeding behavior to avoid contaminated diets, with ultimate impacts on their weight and development. For instance, Lepidoptera and Coleoptera larvae have been shown to assess the nutritional quality of their food, including the copper content[17,57–59] and either avoid diets contaminated with Bordeaux mixture or reduce feeding frequency.[17,59] Such decreases in food consumption can reduce the energetic resources allocated to development.[60] Taken together, a decrease in food intake is expected to lead to a reduction in survival rate, extended development time, and a reduction in pupal mass and longevity. However, in our study, because of the prevailing influence of pupal mass, we did not identify the specific copper concentrations affecting female longevity. In addition, we did not observe any significant effect of Bordeaux mixture on male longevity, suggesting that the relationship between copper exposure and female longevity may be more complex than initially anticipated. Quantification of the copper accumulation in tissues and characterization of the molecules involved in the detoxification processes of *L. botrana* larvae combined with an observation of feeding behavior would be needed to better understand the implication of these physiological mechanisms during developmental stages. Other components in Bordeaux mixture, such as gypsum, may also help to explaining the detrimental effects on Lepidoptera.[61] Further studies are essential to isolate the specific effects of copper and gypsum on *L. botrana*.

Larval exposure to the copper-based fungicide had further detrimental effects on the reproductive performance of both *L. botrana* females and males. Females laid fewer eggs and the

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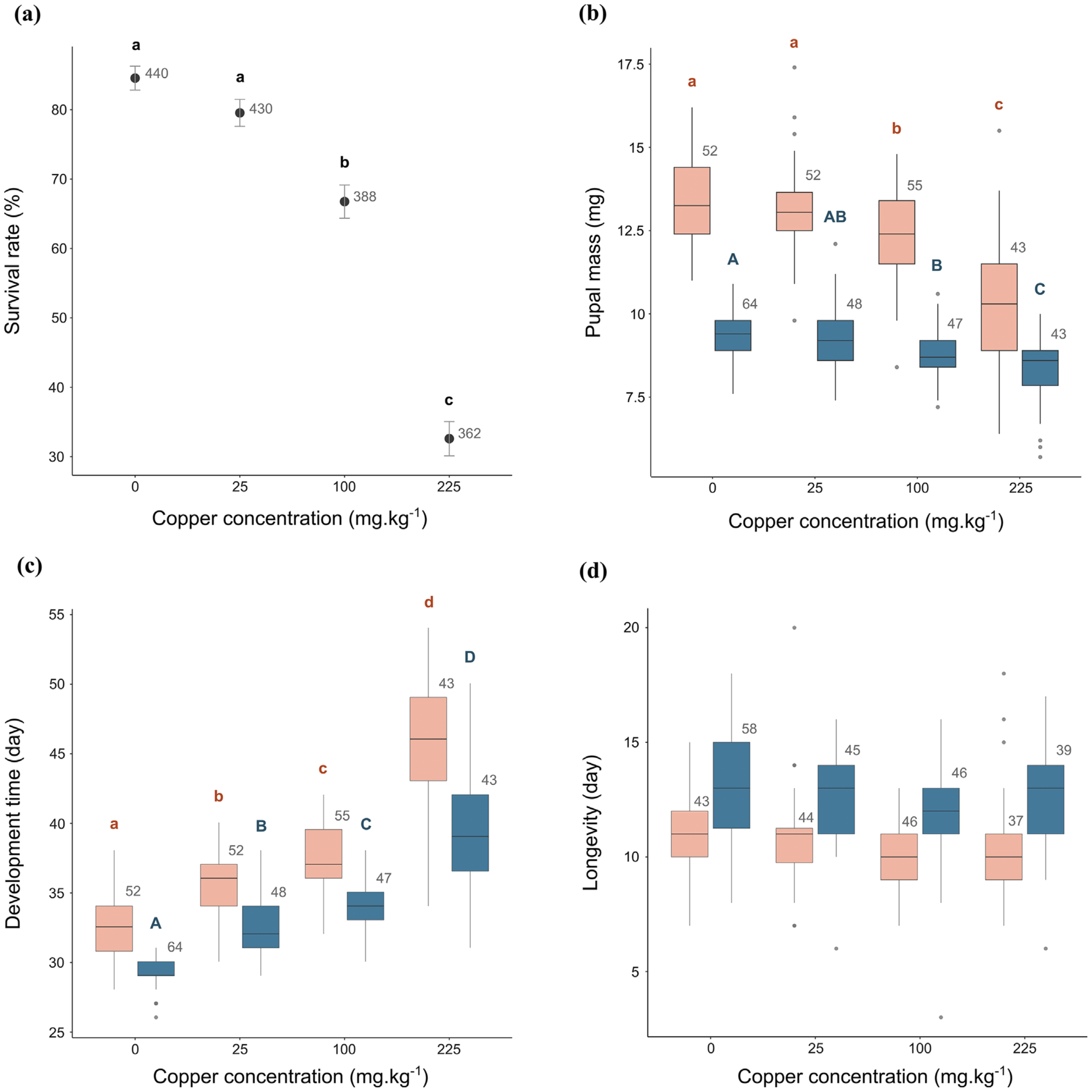

Figure 1. Effect of the exposure to copper contained in the artificial diet (0, 25, 100 and 225 mg.kg^{-1}) on (a) the survival rate of larvae until adult emergence (mean ± SEM) and three developmental traits measured on *Lobesia botrana* females (pink) and males (blue): (b) pupal mass (mg), (c) development time from hatching to emergence (day), and (d) adult longevity from emergence to death (day). For boxplots (b–d), the edges of the rectangles represent the first and third quartiles, the central features are the medians, the lines are the maxima and the minima, and circles represent outliers. Letters (lowercase for females and uppercase for males) indicate significant differences on measured traits between concentrations ($p < 0.05$, *post-hoc* test), and gray numbers associated with points or rectangles correspond to the respective sample sizes.

hatching rate was lower under high copper treatments, whereas males showed a decrease in spermatophore volume and in the number of fertile sperm transmitted to females. This may result from a reduction in the energy allocated to reproduction and/or an alteration of gametogenesis. In capital breeding species, such as *L. botrana*, energy reserves are accumulated during the larval stages.[37] Our results suggest that exposure to Bordeaux mixture could disrupt the nutrient availability (reduction in food intake, energy consumption for detoxification processes) necessary for reproductive performance in adults.[36,47,62] In addition, the decrease in pupal mass, indicative of reduced nutrient availability, has a negative impact on female fecundity and male sperm quality in *L. botrana*.[36] Quantifying energy compounds (e.g., lipids, proteins, glycogen, soluble carbohydrates) at different stages life of *L. botrana* individuals could provide insights into energy allocation for development and reproduction.[63,64] Furthermore, the fungicide has the potential to directly disrupt gametogenesis by affecting a wide range of physiological processes. In lepidopterans, gonad development initiates during embryogenesis[65] and continues through larval growth and metamorphosis.[66,67]

Table 1. Effects of copper concentrations (0, 25, 100 and 225 mg.kg^{-1}) and covariates (mass of tested individual and mass of sexual partner) on the traits and behavior of *Lobesia botrana*

	Copper		Pupal mass of tested individual		Pupal mass of sexual partner		Interaction (copper: pupal mass of tested individual)	
FEMALES	Test value	p	Test value	p	Test value	p	Test value	p
Development time[a]	$F_{3,194} = 8720.52$	**< 0.001**	$F_{1,194} = 1568.50$	**< 0.001**	—	—	$F_{3,194} = 193.38$	**< 0.001**
Adult longevity[b]	$F_3 = 3.51$	**0.017**	$F_1 = 25.48$	**< 0.001**	—	—	—	—
Pupal mass[c]	$F_{3,198} = 44.33$	**< 0.001**	—	—	—	—	—	—
Mating success[d]	$X^2{}_3 = 3$	0.391	$X^2{}_1 = 1.23$	0.267	$X^2{}_1 = 0.92$	0.338	—	—
Mating duration[e]	$F_{3,169} = 2.35$	0.074	$F_{1,168} = 0.10$	0.746	$F_{1,167} = 5.07$	**0.026**	—	—
Pre-mating latency[f]	$\mathrm{LR}_3 = 4.24$	0.237	$\mathrm{LR}_1 = 1.00$	0.317	$\mathrm{LR}_1 = 0.27$	0.603	—	—
Fecundity[e]	$X^2{}_3 = 610.84$	**< 0.001**	$X^2{}_1 = 374.06$	**< 0.001**	$X^2{}_1 = 9.71$	0.268	—	—
Fertility[e]	$F_{3,156} = 13.11$	**< 0.001**	$F_{1,155} = 2.48$	0.117	$F_{1,154} = 0.47$	0.492	—	—
MALES								
Development time[g]	$F_{3,194} = 200.54$	**< 0.001**	$F_{1,194} = 18.51$	**< 0.001**	—	—	$F_{3,194} = 4.27$	**0.006**
Adult longevity[e]	$X^2{}_3 = 1.94$	0.586	$X^2{}_1 = 7.47$	**0.006**	—	—	—	—
Pupal mass[c]	$F_{3,198} = 14.87$	**< 0.001**	—	—	—	—	—	—
Mating success[d]	$X^2{}_3 = 6.78$	0.079	$X^2{}_1 = 1.83$	0.176	$X^2{}_1 = 3.87$	**0.049**	—	—
Mating duration[b]	$F_{3,185} = 0.23$	0.879	$F_{1,185} = 0.23$	0.630	$F_{1,185} = 7.52$	**0.007**	—	—
Pre-mating latency[f]	$\mathrm{LR}_{3,187} = 2.82$	0.420	$\mathrm{LR}_{1,186} = 4.04$	**0.045**	$\mathrm{LR}_{1,185} = 0.87$	0.350	—	—
Spermatophore volume[c]	$F_{3,182} = 17.48$	**< 0.001**	$F_{1,182} = 25.32$	**< 0.001**	—	—	—	—
Eupyrene sperm number[c]	$F_{3,182} = 24.38$	**< 0.001**	$F_{1,182} = 17.49$	**< 0.001**	—	—	—	—

Significant effects ($p < 0.05$) are given in bold. For each of the measured traits, the statistical test and distribution family that best fit the data are shown by superscript letters (a, b, c, d, e, f and g).

[a] Weighted least squared regression.
[b] Linear model, log-transformation.
[c] Linear model.
[d] Generalized linear model, binomial distribution.
[e] Generalized linear model, quasi-Poisson distribution.
[f] Generalized linear model, negative binomial distribution.
[g] Generalized linear model, robust regression.

Table 2. Effects of copper concentrations (0, 25, 100 and 225 mg.kg^{-1}) on reproductive behaviors (mean ± SE) in male and female *Lobesia botrana* exposed to Bordeaux mixture

	Mating success (%)		Pre-mating latency (min)		Mating duration (min)	
Copper concentration (mg.kg^{-1})	Females	Males	Females	Males	Females	Males
0	83.0 ± 5.21 (53)	92.2 ± 3.38 (64)	4.14 ± 0.66 (44)	3.58 ± 1.05 (59)	65.1 ± 2.60 (44)	63.0 ± 2.35 (59)
25	83.0 ± 5.21 (53)	100 ± 0.00 (46)	6.20 ± 1.46 (44)	3.33 ± 0.96 (45)	72.8 ± 2.69 (44)	61.7 ± 2.27 (45)
100	83.6 ± 5.03 (55)	97.9 ± 2.13 (47)	4.54 ± 0.81 (46)	2.59 ± 0.30 (46)	71.8 ± 3.36 (46)	64.7 ± 2.48 (46)
225	93.0 ± 3.93 (43)	93.3 ± 3.76 (45)	4.92 ± 0.91 (39)	3.17 ± 0.43 (41)	63.8 ± 3.15 (39)	64.8 ± 3.62 (41)

Numbers in parentheses are the sample size.

Previous studies reported that larval copper exposure may induce apoptosis damage in testes of *Anopheles arabiensis* (Diptera, Culicidae),[22] a reduction in females' follicular development in *Aedes aegypti* (Diptera, Culicidae),[7] and vitellogenesis inhibition in *Nasonia vitripennis* (Hymenoptera, Pteromalidae).[19] An enhanced understanding of anatomical and physiological damage could yield additional insights into the effects of copper on organisms. Moreover, this study revealed that the copper-based fungicide exposure impacted male reproductive performance at a lower concentration (25 mg.kg^{-1}) compared with females (225 mg.kg^{-1}). This sensitivity difference to the fungicide exposure between males and females suggests potential sex-specific mechanisms.

In contrast to our hypothesis that Bordeaux mixture negatively affects the life-history traits of *L. botrana* and consequently its reproductive behavior, we did not observe any significant effect of Bordeaux mixture on the reproductive behavior. During reproduction, females emit sexual pheromones perceived by sensory receptors of male antennae, enabling males to locate and mate with females.[68] Lepidopteran chemical communication can be influenced by several environmental factors (e.g., chemicals, temperature and pollutants).[69,70] In our experiments, fungicide exposure did not show any

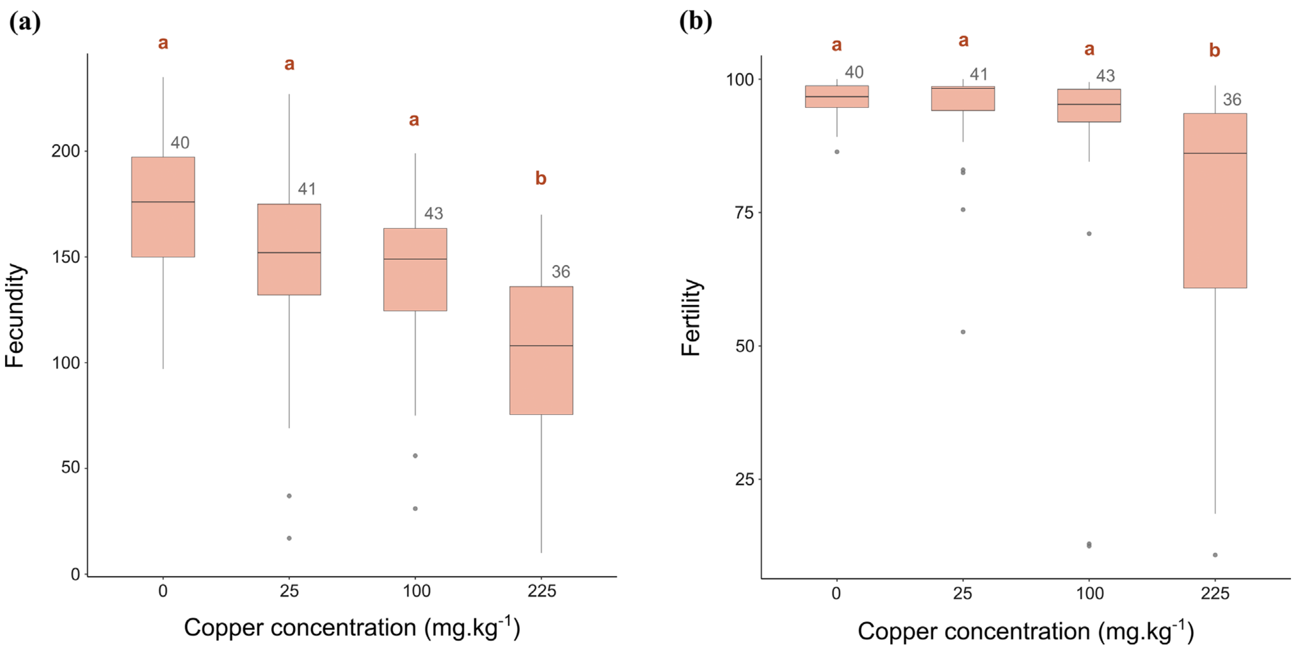

Figure 2. Effect of concentrations of copper contained in the artificial diet (0, 25, 100 and 225 mg.kg^{-1}) on the two *Lobesia botrana* female reproductive traits: (a) fecundity (total number of eggs laid), and (b) fertility (percentage of hatched eggs). The edges of the rectangles represent the first and third quartiles, the central features are the medians, the lines are the maxima and the minima, and circles represent outliers. Lowercase letters indicate significant differences on measured traits between concentrations ($p < 0.05$, *post-hoc* test), and gray numbers associated with rectangles correspond to the respective sample size.

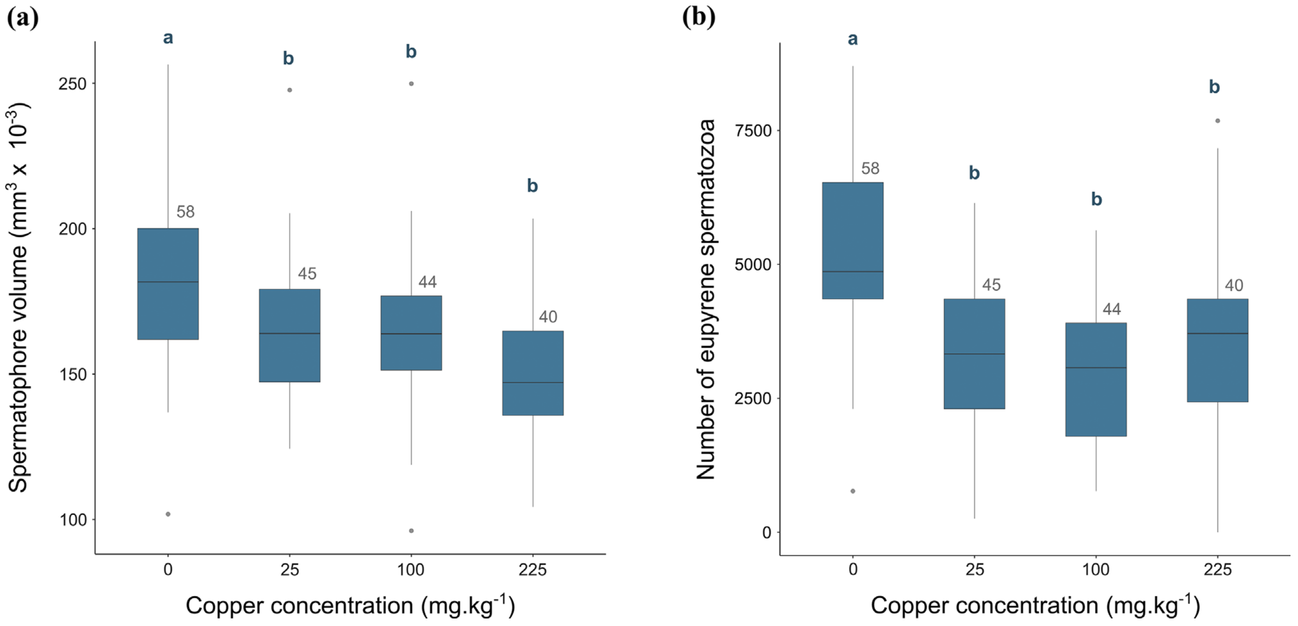

Figure 3. Effect of Bordeaux mixture (0, 25, 100 and 225 mg.kg^{-1}) on the two male reproductive traits measured on *Lobesia botrana*: (a) spermatophore volume (mm^3 x 10^{-3}) and (b) number of eupyrene spermatozoa. The edges of the rectangles represent the first and third quartiles, the central features are the medians, the lines are the maxima and the minima, and circles represent outliers. Lowercase letters indicate significant differences on measured traits between concentrations ($p < 0.05$, *post-hoc* test), and gray numbers associated with rectangles correspond to the respective sample size.

modulating effects on pre-mating latency. This observation could indicate that our experimental setup did not enable the detection of the influence of copper on pheromones (saturation), or that Bordeaux mixture had no detectable impact on the emission and chemical composition of pheromones emitted by *L. botrana* females, as well as on male detection of these pheromones.

In summary, this study highlights a relatively and unexpected strong effect of a copper-based fungicide on the non-target pest, *L. botrana*. A high concentration of Bordeaux mixture in the diet had a lethal effect on larvae. Sublethal effects, including slower growth and impaired reproductive performance, were observed in surviving individuals exposed to copper concentrations currently measured in vineyards.[24] These results suggest a decline in the population dynamic of the European grapevine moth caused by the intensive use of copper-based fungicides in vineyards. Interestingly, Nusillard *et al.* reported a positive effect of a high copper concentration (225 mg.kg^{-1}) accumulated in *L. botrana* on the emergence rate and size of its emerging parasitoid *Trichogramma cordubensis* (Hymenoptera, Trichogrammatidae), a biocontrol agent used against *L. botrana*. Although these fungicides may provide practical benefits by controlling pest populations and enhancing biocontrol, the potential collateral consequences associated with their excessive use in agroecosystems cannot be ignored. As reported by Pennington *et al.*,[35] lower copper-based fungicide applications promote predation of *L. botrana* and therefore minimize the damage on grape berries. Nevertheless, global warming enhances the tolerance of *L. botrana* to Bordeaux mixture exposure by improving larval survival and immunity.[8] This finding underlines the need to use copper-based fungicides with caution to minimize the adverse effects on non-target insects and promote biocontrol programs. Our results call for future studies to better understand the interactions between fungicide application and non-target insects to implement efficient strategies for viticulture pest management in the context of global change.

5 CONCLUSION

This study provides new insights into the impact of the copper-based fungicide Bordeaux mixture on the insect pest *L. botrana*. Application of this fungicide negatively affected the development and reproductive performances of *L. botrana*, suggesting a potential decrease in this pest's population. In addition to its antifungal properties, Bordeaux mixture could help reduce damage associated with the pest. However, the use of copper-based fungicides can alter the structure and functioning of the ecosystem through undesirable effects on the non-target fauna (e.g., pollinators, natural pest enemies).[6,32] Field verification is imperative to confirm the existence of collateral damage related to fungicide used. Moreover, repeated use of copper-based fungicides may select for resistant pests.[71,72] This could decrease the efficiency of these substances against fungi, minimize their impact on insect pests and contribute to environmental pollution.[24] In this regard, copper-based fungicides do not appear to be a sustainable method for viticultural strategies management in the future. Considering future climatic conditions, copper treatments may have a counterproductive effect because of hormetic effects increasing the density of *L. botrana*.[8] Therefore, a better understanding of the response of *L. botrana* to copper treatments under different temperature conditions combined with a physiological approach (e.g., copper accumulation, detoxification processes) is needed to anticipate the effects of fungicides in the context of global warming. These results would provide valuable information for the design of effective pest management strategies in vineyards facing environmental change and the potential impact of pests such as *L. botrana*.

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CONFLICT OF INTEREST

The authors declare they have no conflict of interest.

ETHICS STATEMENT

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

AUTHOR CONTRIBUTIONS

TG and PL conceived and designed the research. TG, WN and PL conducted the experiments. TG, YL, ZET and PL analyzed the data. All authors contributed to the writing process and revised the manuscript.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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