- 1 Warming increases tolerance of an insect pest to fungicide
- 2 exposure through temperature-mediated hormesis
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#### 26 Abstract

27 Pest management strategies relying on agrochemicals could be altered by climate change, 28 because of the temperature-dependent toxicity of the compound involved. Many studies have 29 explored the response of targeted pests to pesticide and temperature. Pesticides are seldom 30 strictly selective and also affect non-target pests. Surprisingly, the way temperature may 31 shape these side effects of pesticides remains overlooked, limiting our understanding of the 32 net impacts of future chemical treatments on the overall damage induced by different pests. 33 We investigated how temperature modulates the response of a major grape insect pest (the 34 tortricid moth Lobesia botrana) to a copper-based fungicide. We examined the lethal (larval 35 survival) and sublethal (larval development, pupal mass, immune parameters) effects of 36 exposure to different concentrations of copper in larval food. We found that copper 37 concentration had negative linear effects on larval development and pupal mass. In addition, 38 copper concentration had biphasic curvilinear effects on total phenoloxidase activity, which is 39 indicative of hormesis (stimulation and inhibition of insect performance at low and high **40** copper concentrations, respectively). Temperature stimulated development, while 41 compromising immunity (total phenoloxidase activity). Significant interaction between 42 copper concentration and temperature was detected for larval survival and phenoloxidase 43 activity: warmer conditions improved pest tolerance to copper through temperature-driven 44 hormesis (larval survival) or by shifting the hormesis-related peak of performance towards 45 higher copper concentrations (phenoloxidase activity). This combination of simple and 46 interactive effects could propagate to populations, communities and agroecosystem, with 47 implications for future management of viticultural pests.

**48** 

## 49 Keywords

50 Climate change, Copper, European grapevine moth, Fungicide, Hormesis, Viticulture.

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- 67 Data involved in this study are available upon request.
- 68 Code availability
- 69 R scripts used to analyze the data are available upon request.

### 70 Authors' contribution

71 All authors conceived and designed the research. PH conducted the experiments. CI, PH and

72 PL analyzed the data. CI, JM and PL led the writing of the manuscript. All authors

radiation significantly contributed to the intellectual content of the manuscript and gave their final

74 approval for submission.

75

# 76 Key message

77 . Temperature affects the toxicity of pesticides for targeted pests and non-target auxiliaries.

78 . Less is known about the thermal modulation of pesticide toxicity for non-target pests.

79 . We studied the biological response of an insect pest to a fungicide (copper) and temperature.

80 . We found additive and interactive effects of the two stressors on insect larval performance.

81 . Temperature improved the insect tolerance to copper in terms of survival and immunity.

82

#### 83 Introduction

84 Pesticides can affect exposed organisms by inducing short-term mortality, due to acute 85 toxicity (i.e. lethal effects), and by altering several life-history traits for individuals that 86 survived the chemical stress (i.e. sublethal effects). In insects, sublethal effects have been 87 documented for a broad range of physiological (e.g., development, reproduction, immunity) 88 and behavioral traits (e.g., learning, orientation, feeding, oviposition) (Desneux et al. 2007). 89 The biological impacts of pesticides can be understood as a dose-dependent balance between 90 lethal and sublethal effects, as illustrated by the concept of hormesis (Cutler and Guedes 91 2017). This term describes a reversed response of a given performance trait to low 92 (stimulation) and high doses (inhibition) of pesticide, and has been observed for several 93 biological endpoints including survival, growth, reproduction and immunity (Calabrese and 94 Blain 2005, 2011; Cutler 2013; Cutler and Guedes 2017).

95 Most pesticides are seldom selective and, apart from targeted pests, may affect several 96 organisms (termed as non-target fauna) in treated agroecosystems, including auxiliary biota 97 (pollinators, soil engineers, natural enemies) and other pests not targeted by the chemical 98 treatment (Hardin et al. 1995; Biondi et al. 2012; Guedes et al. 2016). The collateral effects of 99 broad-spectrum pesticides on non-target organisms may have important undesired 100 consequences at the community and agroecosystem levels. This includes a decline in the 101 services provided by beneficial arthropods that contribute towards maintaining agricultural 102 productivity (e.g., pollination, natural pest control), as well as secondary pest outbreaks if 103 pesticide spreading benefit these noxious species (Brittain et al. 2010; Guedes and Cutler 104 2014; Guedes et al. 2016; Hill et al. 2017). Therefore, the agricultural and ecotoxicological 105 risks of pesticide use have to be evaluated by balancing the benefits and drawbacks associated 106 with these pest management strategies (Zhao et al. 2011; Gill and Garg 2014).

107 Temperature influences the physiological processes involved in the detoxification and 108 excretion of harmful compounds, such that the impacts of chemicals on organisms are largely 109 temperature-dependent (Noyes et al. 2009; Holmstrup et al. 2010; Laskowski et al. 2010; 110 Hooper et al. 2013). Several studies have reported the thermal modulation of pesticide toxicity 111 (lethal and sublethal effects) for both targeted pests (Musser and Shelton 2005; Yu et al. 2012; 112 Li et al. 2014) and non-target auxiliaries, such as predators (Ricupero et al. 2020) and 113 parasitoids (Abbes et al. 2015). From an applied perspective, such findings imply that climate 114 change may have the potential to alter the benefits/costs balance of pesticide use within the 115 agricultural context, through temperature-driven modulation of the susceptibility of the 116 different organisms exposed to the active ingredient (Delcour et al. 2015; Matzrafi 2019). In 117 this regard, there is surprisingly little detailed knowledge about the thermal modulation of 118 pesticide side effects on non-target pests, which may also determine the efficiency and

ecological impacts of future pesticide treatments by affecting the overall herbivory pressureexerted on agricultural commodities under warmed climates.

121 Viticulture relies on intensive fungicide schedule to control downy mildew epidemics 122 that may cause considerable yield losses in susceptible cultivars if left untreated (Gessler et al. 123 2011; Pertot et al. 2017). Hence, fungicides can account from up to 96% of pesticides used in 124 vineyards (Bereswill et al. 2012), and the largest amounts of these fungicides are represented 125 by copper-containing products sprayed for more than 150 years and at rates up to 80 kg.ha<sup>-1</sup> 126 year<sup>-1</sup> (Dagostin et al. 2011; Mackie et al. 2012; Lamichhane et al. 2018). Copper fungicides 127 are disruptive for arthropod communities present in vineyards, and can benefit grape insect 128 pests through relaxation of top-down control exerted by their natural enemies (Pennington et 129 al. 2018; Vogelweith and Thiéry 2018). For instance, an increased number of successive 130 fungicide applications was found to exacerbate the damage caused to berries by caterpillars of 131 the European grapevine moth Lobesia botrana (Lepidoptera: Tortricidae) through adverse 132 effects on its egg predators (e.g. ants) (Pennington et al. 2018). Of note, this tortricid moth is 133 already recognized as a primary grapevine pest worldwide with respect to its densities (up to 134 20-30 larvae per bunch in heavily infested vineyards) and associated levels of economic 135 injury (13.3-27% yield losses over a four-year period in Mediterranean vineyards) (Moschos 136 2006; Delbac and Thiéry 2016; Thiéry et al. 2018). This indicates that copper as a major 137 contaminant in the agroecosystem could make this insect pest even more problematic for 138 grape industry, but additional data on the direct and indirect influences of fungicides on L. 139 *botrana* life history would be required to ascertain this statement. This is especially true **140** within the context of anthropogenic climate change that may modify the deleterious impacts 141 of the moth on grape stock (Reineke and Thiéry 2016; Gutierrez et al. 2017). Filling this 142 knowledge gap should improve our understanding of the potential net benefits associated with 143 future copper spraying within the viticultural context.

144 The European grapevine moth is a multivoltine species with non-obligatory diapause, 145 able to fulfill between two and five generations during growing season depending on latitude. 146 Grape damage are caused by larvae feeding upon vine fructiferous organs (floral buds, 147 ripening and mature berries) and alternative Mediterranean host plants (e.g., Rosmarinus 148 officinalis, Daphne gnidium) (Thiéry and Moreau 2005). Adults are non-feeding and rely on 149 nutritional resources stored as larvae for their reproductive output, which may reach up to 80-150 100 eggs for field-collected females (Savopoulou-Soultani et al. 1990; Muller et al. 2016). 151 The main reported natural enemies of this phytophagous insect are predators (arthropods, 152 birds and bats) and parasitoids, which may specifically parasitize eggs (Trichogrammatidae) 153 and larvae (e.g., Ichneumonidae, Braconidae, Tachinidae) (Thiéry et al. 2018). Parasitism 154 rates as high as 90% were locally reported for L. botrana larvae, suggesting that parasitoids 155 probably act as the main natural agents of biological control of this pest in vineyards (Moreau 156 et al. 2010). In response to this significant parasitism pressure, L. botrana larvae have evolved 157 a defensive system largely relying on an important investment in the immune component 158 (Vogelweith et al. 2014; Iltis et al. 2018). 159 We intended to assess the lethal and sublethal effects of a copper-based fungicide used

160 extensively worldwide on L. botrana larvae under warming conditions associated with 161 climate change scenario. Considering the dual role of copper (as an oligo-element and a heavy 162 metal), we expect a concentration-dependent hormetic response (stimulation at low 163 concentrations, inhibition at high concentrations) of many traits to copper treatment, as 164 demonstrated by previous experimental investigations in insect models (Servia et al. 2006; 165 van Ooik et al. 2008; Baghban et al. 2014). Additionally, provided that larval daily body mass 166 gain (i.e. larval growth rate) increases alongside rearing temperature in L. botrana (Iltis et al. 167 2018), we conjecture that copper intake and accumulation in insect tissues should be higher in 168 warmer conditions. For this reason, the insect should be increasingly sensitive to the amount

169 of copper contained in its food as temperature rises (i.e. the copper concentrations for which

170 maximal insect performance is observed should be lower in warmer conditions).

171

# 172 Materials and methods

# **173** Insect rearing and larvae collection

All insects originated from a laboratory-reared *L. botrana* population maintained diapausefree since 1998 (INRAe, Villenave d'Ornon, France). This insect stock was regularly

176 supplemented with wild individuals collected in nearby vineyards to preserve genetic

177 diversity. Preceding the experiments, the population was grown under standardized conditions

178 of temperature ( $20 \pm 0.5^{\circ}$ C), light (photoperiod of L17:D6 and 1 h of dusk, luminosity: 650 lx

during photophase and 100 lx at dusk), and relative humidity ( $60 \pm 5\%$ ). Eggs were collected

180 via bands of waxed paper hung inside the rearing cages, transferred to plastic boxes and

181 placed under a daily moistened paper towel to limit egg mortality caused by desiccation.

182 Neonate larvae (age < 12 h) were collected in large numbers (n = 1905 individuals) for

183 the purpose of the experiments. They were delicately manipulated with a soft paintbrush to be

isolated into microtubes with pierced cap and filled with 1.5 ml of artificial medium used for

the laboratory rearing of this species (composition for 1000 ml: 1000 ml water, 15 g agar,

186 86.6 g corn flour, 41.3 g wheat germ, 45.5 g beer yeast, 6 g ascorbic acid, 3.4 g mineral salt,

**187** 128 mg pyrimethanil, 2.7 g benzoic acid, 2.8 g methyl 4-hydroxybenzoate and 5 ml 95%

**188** ethanol) (Thiéry and Moreau 2005).

189

**190** *Diet composition* 

**191** Larvae were exposed to one of the four copper concentrations through feeding on artificial

192 media elaborated according to the original recipe previously described and supplemented with

**193** either 10 ml of distilled water (control) or 10 ml of Bordeaux mixture (20% copper content,

194 RSR Disperss NC, UPL) diluted in distilled water to obtain the final desired copper concentrations of 25, 50 and 75 mg.kg<sup>-1</sup>. Bordeaux mixture is a derivative of copper in the 195 196 form of copper sulfates (CuSO<sub>4</sub>) and is widely used in viticultural areas for its antifungal 197 properties (Mackie et al. 2012). Although the control is not completely copper-free (because 198 of the presence of mineral salts and plant material as potential copper sources), its copper 199 concentration can be deemed negligible (closer to 0 mg.kg<sup>-1</sup>) in comparison with the three 200 other copper concentrations included in this study. We chose to contaminate the larval food 201 with Bordeaux mixture instead of pure copper to mimic an ecologically realistic insect 202 exposure to the chemical in terms of formulation, exposure route (ingestion) and 203 concentrations.

204 The different copper treatments were selected to reflect variations in field-relevant 205 copper concentrations found in the skin and pulp of grape berries consumed by L. botrana. In 206 Europe, the Maximal Residue Levels (MRL) of copper compounds in grape berries at harvest is set at 50 mg.kg<sup>-1</sup> (EFSA, 2018). However, during the summer period and grape ripening, 207 208 fluctuations around the permitted MRL threshold can be observed in relation to geographical 209 context, local agricultural policies (amounts and frequencies of fungicide application, 210 cultivated grape varieties), meteorological conditions (causing successive copper leachings), 211 and grape physiology (Mackie et al. 2012). For instance, copper concentrations ranging from less than 10 mg.kg<sup>-1</sup> to more than 100 mg.kg<sup>-1</sup> have been quantified in treated berries 212 213 collected from several vineyards in Europe and elsewhere (Angelova et al. 1999; García-214 Esparza et al. 2006; Lai et al. 2010; Miotto et al. 2014). We did not examine insect response to copper concentrations higher than 75 mg.kg<sup>-1</sup> because these values remain less commonly 215 216 observed in field conditions.

217

218 Thermal regimes

219 Larvae were reared inside cooled incubators (ST 2/2 BASIC, Pol-Eko Aparatura). Abiotic 220 conditions inside these devices were controlled (temperature:  $\pm 0.1$  °C, relative humidity: 50  $\pm$ 221 10%, photoperiod: L16:D8, luminosity: 650 lx) and monitored weekly with an independent 222 data logger (Hobo, Onset Computer Corporation) to ensure they conformed with the regime 223 programmed during the whole course of the experiments. Individuals were exposed to one of 224 two realistic fluctuating thermal regimes (each constituted by six thermal segments of 4 h) 225 designed to mimic the current and expected future thermal conditions (2081–2100 period) 226 observed in Burgundian vineyards (Longvic-Dijon weather station, 47.27°N; 5.09°E; altitude 227 = 219 m) under a local scenario of warming associated with climate change. A 20-year 228 recording (1995–2014) of in situ meteorological data was used to build the 'current regime', 229 simulating the thermal conditions currently observed during one 'mean' summer day in the 230 region of interest (Table 1). The 'future regime' was generated on the basis of fine-grained 231 downscaled predictions provided by six climate simulations involving different climatic 232 models (for more details, see Iltis et al. 2019) (Table 1).

233

234 General measurement procedure

235 Within each of the eight experimental blocks involved in the full factorial design of this study 236 (four copper concentrations and two thermal regimes), newly-hatched larvae were randomly 237 split into two experimental groups because the array of traits included in this study could not 238 have been measured on the same individuals. In one group, larvae were allowed to develop 239 freely until pupation in order to record larval development time, pupal mass and larval 240 survival. In another group, larvae were collected once they reached their fifth instar to 241 quantify immune effectors (hemocyte load, activity of the phenoloxidase/prophenoloxidase 242 system) expressed by this pest as a protective barrier against parasitoid attacks. Immune

bioassays were conducted during the last larval instar as a way to evaluate the biological
impacts of copper for individuals being exposed to this metal during their entire immature
development. Moreover, measurements of immunity are usually performed at the end of
larval development in *L. botrana* (Vogelweith et al. 2014; Iltis et al. 2018, 2019).

247

# 248 Larval development time, pupal mass and larval survival

249 Initially, this group involved a total of 1546 newly-hatched larvae split among the experimental blocks (current regime: n = 154 for 0 mg.kg<sup>-1</sup>, n = 220 for 25 mg.kg<sup>-1</sup>, n = 223250 for 50 mg.kg<sup>-1</sup>, n = 158 for 75 mg.kg<sup>-1</sup>; future regime: n = 151 for 0 mg.kg<sup>-1</sup>, n = 223 for 25 251 mg.kg<sup>-1</sup>, n = 232 for 50 mg.kg<sup>-1</sup>, n = 185 for 75 mg.kg<sup>-1</sup>). For minimizing larval disturbance, 252 253 daily monitoring of development started at a larval age of 14 days (for the future regime) and 254 21 days (for the current regime) because larvae are unlikely to reach pupation earlier given the 255 thermal conditions experienced (Iltis et al. 2019). Larval development time was expressed in 256 days as the time elapsed between egg hatching and pupation. Immediately following pupal 257 transformation, chrysalises were weighed to the nearest 0.1 mg with a balance (Pioneer PA214C, OHAUS) to obtain pupal mass. We also calculated larval survival rates, expressed 258 259 as the ratio between the number of pupae collected and the number of larvae initially 260 deposited for each experimental block.

261 Immune parameters

Immune biochemical tests were conducted on a total of 359 fifth-instar larvae in the dedicated 262 experimental blocks (current regime: n = 42 for 0 mg.kg<sup>-1</sup>, n = 45 for 25 mg.kg<sup>-1</sup>, n = 44 for 263 50 mg.kg<sup>-1</sup>, n = 44 for 75 mg.kg<sup>-1</sup>; future regime: n = 45 for 0 mg.kg<sup>-1</sup>, n = 43 for 25 mg.kg<sup>-1</sup>, 264 n = 49 for 50 mg.kg<sup>-1</sup>, n = 47 for 75 mg.kg<sup>-1</sup>). These tests focused on two immune parameters 265 266 used to infer the susceptibility of this pest to its larval endoparasitoids: hemocyte load and 267 activity of the phenoloxidase/prophenoloxidase system (Vogelweith et al. 2014; Iltis et al. 268 2018). Hemocytes are freely-flowing cells able to form a multilayered cellular structure 269 around a parasitoid egg during the encapsulation process. These immune cells release the 270 phenoloxidase (PO), a copper-containing enzyme produced through cleavage of its inactive 271 precursor (the prophenoloxidase, PPO). The phenoloxidase/prophenoloxidase enzymatic 272 system catalyzes a cascade of reactions leading to the synthesis of melanin (melanization) 273 acting as a cytotoxic compound against the parasitoid egg (González-Santoyo and Córdoba-274 Aguilar 2012).

275 Fifth-instar larvae were chilled on ice for 15 min to facilitate handling, placed on a 276 cold surface and delicately wounded in the dorsal part of the abdomen with a sterile 277 entomological pin to collect a droplet of hemolymph (~ 1 µl) using a cold, sterile glass 278 capillary (Hirschmann Laborgeräte). This hemolymph extract was immediately mixed with 20 279 µl of cold, filtered phosphate-buffered saline (PBS, 10 mM, pH 7.4) to obtain diluted 280 hemolymph samples. Immediately following hemolymph collection, 10 µl of diluted 281 hemolymph samples were pipetted into a Neubauer hemocytometer under a phase contrast 282 microscope (Primo Star, Zeiss) at 400x magnification to conduct a standardized count of 283 hemocyte load. The remaining 11 µl of diluted hemolymph samples were supplemented with 284 10  $\mu$ l of PBS and tubes were homogenized before being fixed in liquid nitrogen and stored at 285 -27°C for subsequent estimation of PO/PPO system activity. Pricked larvae were transferred

286 at -27°C to be euthanized and stored for latter measurements of larval body size, in an attempt 287 to control for allometric effects on larval immunity. Larval body size was extrapolated 288 through head capsule width (i.e. the distance between the most distant margins of the 289 sclerotized capsule) using a stereomicroscope (Stemi 508, Zeiss) at 12.5x magnification. 290 The enzymatic activity of the PO/PPO system was assessed through 291 spectrophotometric method formerly developed. We quantified the activity of naturally 292 activated PO (i.e. PO activity) and the sum of PO activity and activity of proenzyme stock 293 (i.e. total PO activity) (Vogelweith et al. 2013). The distinction between PO and total PO 294 activity enables to quantify the levels of enzymatic activity expressed by larvae at a given 295 time (PO activity) along with the maximal activity that might be potentially mobilized upon 296 an actual infection (total PO activity). In addition, PO and total PO activities can be 297 differentially affected by temperature in *L. botrana*, thereby providing different information 298 about the thermal sensitivity of immune function in this species (Iltis et al. 2018, 2019, 2021). 299 Diluted samples of hemolymph were centrifuged (4000 g, 15 min, 4°C) before two 300 supernatant aliquots (5 µl each) were collected and transferred into microplate wells. One of 301 these two aliquots was mixed with 160 µl of diluted PBS solution (35 ml ultrapure water, 5 ml 302 filtered PBS) and was reserved for PO activity measurement. The other was supplemented 303 with 160 µl of chymotrypsin solution (35 ml ultrapure water, 5 ml filtered PBS, 2.45 mg 304 trypsin) to quantify total PO activity. The reaction started after the addition of 20 µl of L-305 Dopa solution (40 ml ultrapure water, 160 mg L-Dopamine). It was left for 40 min at 30°C in 306 a microplate reader (Versamax, Molecular Devices). Absorbance curves were generated based 307 on regular absorbance readings (every 15 s at 490 nm), before being analyzed using the 308 SoftMax Pro software (version 4.0, Versamax, Molecular Devices). These analyses aimed to 309 extract maximum enzymatic activity  $(V_{max})$ , approximated by the maximum slope of the 310 absorbance curve during its starting linear phase. All immune measurements were reported to

311 1 μl of pure hemolymph for comparison purposes, and corrected for allometric effects through
312 division by body size (head capsule width) (Vogelweith et al. 2013).

313

# **314** *Statistical analyses*

315 All statistical models encompassed the effects of the copper concentration (covariate), 316 temperature regime (factor), and their interaction on the response traits measured. The 317 influence of copper concentration on each response trait was conceptualized in two different 318 ways, generating two candidate models further compared to select the one best suited to fit the 319 data distribution. On the one side, it was modeled through linear terms only (linear function: 320 y = ax + b, thereby yielding a monotone relationship between the trait investigated and 321 copper concentration. On the other side, the copper effect was simulated using both linear and second order polynomial terms (quadratic function:  $y = ax^2 + bx + c$ ). This latter equation 322 323 is more relevant for modeling biphasic functions displaying a transient peak, thereby allowing 324 for the proper identification of hormesis (Cedergreen et al. 2005; Deng et al. 2001). Following 325 an AIC-based model selection procedure, we retained (i) the model with linear function for 326 larval development time and pupal mass; and (ii) the model with quadratic function for larval 327 survival and all three immune traits (hemocyte load, PO activity, total PO activity). 328 Data of larval development time and pupal mass did not require preliminary

**329** transformation to comply with the assumptions of parametric tests (normal distribution and

330 homogeneity of variances) and were studied with Analyses of Covariance (ANCOVAs).

331 Larval survival was compared between copper and temperature treatments with a Generalized

**332** Linear Model (GLM) assuming a binomial error structure and a logit function. The three

- 333 immune traits met the conditions required for parametric tests following different
- 334 transformations (logarithm transformation for hemocyte load, square root transformation for
- 335 PO and total PO activity). They were then processed through ANCOVAs. Parametric

336 assumptions were ascertained through Shapiro-Wilk (normality) and Levene tests

337 (homoscedasticity) conducted on the residuals of the fitted model. All statistical analyses were338 performed with R software (version 4.1.0).

339

#### 340 Results

341 Larval development time, pupal mass and larval survival

342 Larval development time was significantly modulated by copper concentration and thermal 343 regime in an additive way (Table 2). Larval development was significantly slowed with an 344 increasing amount of copper in larval food (positive linear relationship between larval 345 development time and copper concentration in the two thermal regimes) (Fig. 1A). In 346 addition, larvae developed significantly faster within the future regime (warmer conditions) 347 (Fig. 1A). Pupal mass was only significantly affected by copper concentration (Table 2): it 348 declined linearly with an increasing amount of copper in larval food (Fig. 1B). Larval survival 349 was significantly affected by the interaction between copper concentration and thermal regime 350 (Table 2). In the current regime, copper concentration tended to slightly decrease larval 351 survival (Fig. 1C). In the future regime, a hormetic (curvilinear) effect of copper on larval 352 survival was apparent: as copper concentration increased, survival rates rose to a maximal value reached for a copper concentration of 25 mg.kg<sup>-1</sup>, and then progressively decreased 353 354 (Fig. 1C). Accordingly, the highest differences between the two thermal regimes in terms of 355 larval survival were observed at intermediate copper concentrations (25-50 mg.kg<sup>-1</sup>): over that 356 range of values, larvae were more likely to survive until pupation in the future regime (Fig. 357 1C).

358

## 359 *Immune parameters*

360 Hemocyte load was not significantly mediated by each of the variables included in the model 361 (Table 2, Fig. 2A). PO activity was significantly affected by the interaction between copper 362 concentration and thermal regime (Table 2). The hormetic effect of copper on PO activity was 363 apparent in both regimes (Fig. 2B). Nonetheless, the peak of PO activity was reached for higher concentrations of copper in the future regime (c.a. 50 mg.kg<sup>-1</sup>) in contrast with the 364 current regime (c.a. 25 mg.kg<sup>-1</sup>). Hence, when comparing the two thermal regimes, significant 365 366 differences regarding this immune trait were only observed at low copper concentrations (0-25 mg.kg<sup>-1</sup>), with larvae displaying lower PO activity in the future regime (Fig. 2B). Total PO 367 368 activity was significantly impacted by the additive effects of copper concentration and 369 thermal regime (Table 2). Maximal values of total PO activity were observed for intermediate 370 copper concentrations (25-50 mg.kg<sup>-1</sup>) in both of the two regimes, and larvae had reduced levels of total PO activity in the future regime (Fig. 2C). 371

372

## 373 Discussion

374 We performed this study to investigate how temperature (in a climate change context) may 375 affect the balance of lethal and sublethal effects of a systemic fungicide on a major viticultural 376 insect pest (the tortricid moth L. botrana). Exposing larvae to rising concentrations of copper 377 via contaminated food resulted in negative linear effects (larval development, pupal mass) and 378 hormetic curvilinear effects (total PO activity). Our simulation of local warming expedited 379 larval development, but compromised immune function (total PO activity). More important, 380 some traits were affected by the interaction between copper concentration and temperature, 381 suggesting that copper toxicity for the insect pest is to some extent temperature-dependent. 382 For larval survival, a hormetic effect of copper was apparent only in the future regime. For 383 PO activity, hormesis was observed in both thermal regimes, but warmer conditions in the

384 future regime shifted the hormesis-related peak of performance (maximal values of PO385 activity) towards higher copper concentrations.

386 Congruent with our hypothesis, we found evidence for hormetic effects of copper on 387 several larval traits – especially for immune parameters (PO and total PO activity) – but this 388 response pattern was not consistently observed among life-history traits (e.g. a negative linear 389 effect of copper on larval development and pupal mass). The transient peak in immunity 390 observed for some copper concentrations could result from increased PO/PPO synthesis, 391 because copper is a major component of the active site of PO and PPO (van Ooik et al. 2007; 392 González-Santoyo and Córdoba-Aguilar 2012). These findings confirm the dual role of 393 copper as a micronutrient and toxicant, and highlight inter-trait variations in response to metal 394 exposure, probably arising from a strong covariation (trade-offs) between those traits (van 395 Ooik et al. 2007; Pölkki et al. 2014; Debecker and Stoks 2019). They also demonstrate that, 396 over a range of field realistic concentrations of a chemical, the description of dose-response 397 might be best fitted by linear or hormetic model depending on the biological trait under 398 investigation (Calabrese 2004; Cutler 2013; Guedes and Cutler 2014). 399 Further, by contrast with our hypothesis, we found that the presumed elevated food **400** and copper intake observed under warmer conditions was associated with an increase (instead 401 of an expected decrease) in pest's tolerance to copper. It thus could be proposed that 402 temperature increased the efficacy of copper-handling mechanisms (e.g., distribution, 403 sequestration, degradation) contributing towards maintaining copper homeostasis, which limited its toxicity for the organism (Pölkki et al. 2014; Op de Beeck et al. 2017). Another 404 405 plausible explanation is that faster larval development in warmer conditions mechanistically 406 reduced the duration of exposure to copper-contaminated food, which often positively **407** correlates with a chemical treatment's toxicity (Connell et al. 2016). Biochemical 408 quantification of copper accumulation in larval tissues should help assess the actual doses of

409 copper assimilated by larvae and identify the physiological mechanisms underlying increased410 insect tolerance to copper in a warmer environment.

411 The pest physiological response to the stressors should determine its population 412 dynamics and local abundance in agroecosystems affected by both copper pollution and 413 warming. For instance, the accelerated larval development observed in the future regime 414 should imply a shortened biological cycle and, subsequently, a higher number of L. botrana 415 generations during growing season, as predicted by phenological models (Caffarra et al. 2012) 416 and witnessed by field records (Martín-Vertedor et al. 2010). By increasing pest voltinism, 417 climate change has the potential to exacerbate the herbivory pressure exerted by L. botrana on 418 the viticultural output (Gutierrez et al. 2017; Castex et al. 2018). Higher pest virulence under 419 a climate change scenario is further supported by our findings for larval survival, since we 420 noted that warming triggered a hormetic response of this trait (which culminated for a concentration of 25 mg.kg<sup>-1</sup>) to copper concentration. This indicates that *L. botrana* larval 421 422 abundance on infested bunches could benefit from copper treatments in the future, particularly 423 at concentrations below the MRL.

424 However, larval settlement is also determined by adult reproductive success. Though 425 our study did not include measures directly pertaining to the reproductive compartment, we 426 reported that copper linearly decreased pupal mass, which may adversely impact female's 427 fecundity since this reproductive trait is positively correlated with pupal mass in L. botrana 428 (Iltis et al. 2020). Moreover, there is experimental evidence highlighting the negative impacts 429 of warming on male's sperm quality and female's mating success in this species (Iltis et al. 430 2020). Though these potential adverse effects of copper and temperature on L. botrana 431 reproduction might mitigate the above-mentioned conclusions, it is worth noting that stressors 432 applied in combination often yield interactive rather than simply additive biological effects, 433 especially in a climate change context (Hooper et al. 2013; Kaunisto et al. 2016). Specifically,

434 pesticide exposure and temperature can interact in non-easily foreseeable ways to modulate 435 pest reproductive performance (Yu et al. 2012; Li et al. 2014). In the brown planthopper, 436 Nilaparvata lugens (Hemiptera: Delphacidae), for example, males and females benefited the 437 most from a low-concentration pesticide treatment (in terms of reproductive performance) at 438 relatively low and high temperatures (Yu et al. 2012). This example convincingly illustrates a 439 temperature effect on the intensity of pesticide-driven hormesis that could drive *in situ* pest **440** resurgence phenomena (i.e. an increase in pest abundance following pesticide application) 441 (Yu et al. 2012). Considering such ecological and agricultural consequences of pesticide-by-442 temperature interaction, we argue for further investigations examining the combined impacts 443 of these two stressors on L. botrana reproduction.

444 Variations in the abundance of L. botrana populations are also driven by interactions 445 with organisms belonging to adjacent trophic levels within the tritrophic system (natural **446** enemies and host plant), all being exposed to copper pollution as well. We found that the 447 hormesis-related peak of PO activity was shifted towards higher copper concentrations when 448 larvae were exposed to warmer conditions. This indicates that intense copper application 449 could improve the pest's resistance to its parasitic fauna (especially during the larval stage) in 450 a future climate, which may lead to a surge in pest damage to crops through relaxation of top-451 down regulatory control exerted by the upper trophic level. This is especially true considering 452 the possible deleterious effects of copper on performance and populations of natural enemies 453 (Vieira et al. 2001; Butler et al. 2009; Garcia et al. 2009; Bengochea et al. 2014), including 454 predators of L. botrana eggs in the viticultural system (Pennington et al. 2018). Interestingly, 455 among these auxiliaries, oophagous parasitoids appear relatively tolerant to the contamination 456 of host eggs by copper-based products. In Trichogramma cordubensis (Hymenoptera: 457 Trichogrammatidae) for instance, application of copper sulfate during parasitoid immature 458 stages had non-significant to slightly detrimental effect (depending on timing of exposure

459 after egg parasitism) on parasitoid development, emergence rates and adult fecundity, 460 suggesting the safe ecotoxicological profile of this chemical towards the beneficial insect 461 (Vieira et al. 2001; Garcia et al. 2009). It could therefore be postulated that biological control 462 relying on inundative release of egg-attacking parasitoids may prove increasingly important 463 for the future regulation of *L. botrana* populations facing copper pollution and climate 464 change. Additional data about the impacts of different copper concentrations on parasitism of 465 L. botrana eggs would be required to ascertain this statement, especially if copper stimulate 466 egg immunity in a similar temperature-dependent way as we evidenced for larvae (through **467** direct exposure or transgenerational effects).

468

### 469 Conclusions

**470** We demonstrated that thermal conditions can alter the response of a major insect pest to a 471 commonly used fungicide through temperature-dependent hormetic effects. Of note, L. **472** botrana is known to exacerbate plant susceptibility to fungal diseases through larval feeding 473 on plant tissues (Pavan et al. 2014). Hence, copper treatments used for centuries could 474 become counterproductive in future climatic conditions due to hormesis-related increase in 475 the density of *L. botrana* populations that may facilitate epidemics of fungal pests, even at 476 copper concentrations below the MRL. Careful consideration about the combined effects of 477 copper and temperature on L. botrana life history and its associated ecological context should 478 be incorporated in future strategies for the management of different viticultural pests. 479 Pesticide-induced hormesis is increasingly recognized as a crucial concept for the

understanding of the ecotoxicological and agricultural risks associated with chemical control
of arthropod pests (Cutler 2013; Guedes and Cutler 2014; Cutler and Guedes 2017). With our
particular case example, we reported that hormetic responses to realistic concentrations of a
chemical can occur for several key traits related with both population (larval survival) and

**48**4 trophic dynamics (immunity). Layered upon these trait-dependent conclusions, we also 485 showed that hormetic responses can be shaped by environmental conditions, in the present **486** case temperature (Yu et al. 2012). Extrapolating these laboratory-based conclusions to field **487** conditions with practical reference to pest control decision-making is challenging due to the 488 complex interaction between the different stressors. This strengthens the need for multiple 489 stressors experiments to develop our mechanistic and predictive understanding of insect **490** response to climate change in multi-stress environments (Kaunisto et al. 2016). In this regard, 491 one promising approach would be to integrate one supplementary level of trophic complexity 492 by examining the role played by the host plant in the bottom-up regulation of pest response to 493 the combined chemical and temperature stresses (Xie et al. 2011; Kleynhans et al. 2014). This 494 is especially relevant for studying polyphagous pests like L. botrana, which is able to feed 495 upon a large diversity of grape cultivars and alternative host plants, with demonstrated **496** impacts on insect life history.

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Table 1 Temperature conditions (°C) for the two daily fluctuating regimes used in this study:
the current regime and the future regime, each divided into six thermal segments of 4 h
(white: photophase, grey: scotophase). Current regime was based on a large dataset of *in situ*recorded climatic conditions. Future regime was generated on the basis of six fine-grained
climate simulations run with the most pessimistic scenario of greenhouse gas emission. Daily
thermal range is defined as the difference between maximum and minimum daily

740 temperatures.

	Current regime	Future regime
0h – 4h	16.4	21.4
4h - 8h	17.8	22.9
8h – 12h	22.3	27.8
12h – 16h	24.9	30.5
16h – 20h	22.9	28.3
20h – 0h	19.0	24.2
Mean temperature	20.5	25.8
Daily thermal range	8.5	9.1

Table 2 Effects of copper concentration (0, 25, 50 or 75 mg.kg<sup>-1</sup>), thermal regime (current regime or future regime) and their interaction on the
traits measured in the two experimental groups. For larval development time and pupal mass, the effect of copper concentration was expressed as
a linear function. For larval survival, hemocyte load, PO and total PO activities, it was modeled by a quadratic function (indicative of hormesis).
Significant effects are evidenced by bold values.

	Copper concentration		Thermal regime		Interaction	
	Test value	Р	Test value	Р	Test value	Р
Larval development time <sup>a</sup>	<i>F</i> <sub>1,447</sub> = <b>6.64</b>	0.01	$F_{1,447} = 456$	< 0.001	$F_{1,447} = 0.24$	0.63
Pupal mass <sup>a</sup>	$F_{1,447} = 5.22$	0.02	$F_{1,447} = 0.96$	0.33	$F_{1,447} = 0.71$	0.40
Larval survival <sup>b</sup>	$\chi^2_2 = 34.0$	< 0.001	$\chi_1^2 = 80.5$	< 0.001	$\chi^2_2 = 10.4$	0.006
Hemocyte load <sup>a</sup>	$F_{2,353} = 1.25$	0.29	$F_{1,353} = 3.64$	0.06	$F_{2,353} = 2.94$	0.05
PO activity <sup><i>a</i></sup>	$F_{2,353} = 5.80$	0.003	$F_{1,353} = 22.9$	< 0.001	$F_{2,353} = 3.22$	0.04
Total PO activity <sup>a</sup>	$F_{2,353} = 5.50$	0.004	$F_{1,353} = 17.8$	< 0.001	$F_{2,353} = 0.67$	0.51

**746** <sup>*a*</sup> ANCOVA <sup>*b*</sup> GLM with binomial error structure

## 747 Figure captions

748 Fig. 1 Effects of copper concentration and thermal regime on: A larval development time 749 (from egg hatching until pupation), **B** pupal mass and **C** larval survival (between egg hatching 750 and pupation). Equations are given to describe significant copper effect for the two regimes 751 (larval development time and pupal mass: linear regression; larval survival: polynomial 752 regression with a quadratic function). For pupal mass, the parameters of the equation did not 753 differ between the two thermal regimes, hence only one linear regression was plotted. 754 Represented values are means and their 95% confidence intervals. Numbers associated with 755 points refer to sample sizes 756 Fig. 2 Effects of copper concentration and thermal regime on the three immune parameters 757 inspected in fifth-instar L. botrana larvae: A hemocyte load, B PO activity and C total PO 758 activity. All values were reported to one microliter of pure hemolymph and size-corrected 759 through division by larval head capsule width. Equations are given to describe significant 760 copper effect for the two regimes (polynomial regression with a quadratic function). 761 Represented values are means and their 95% confidence intervals. Numbers associated with 762 points refer to sample sizes

▲ Future regime



