



# Adverse effects of the fungal biopesticide *Beauveria bassiana* on a predatory social wasp

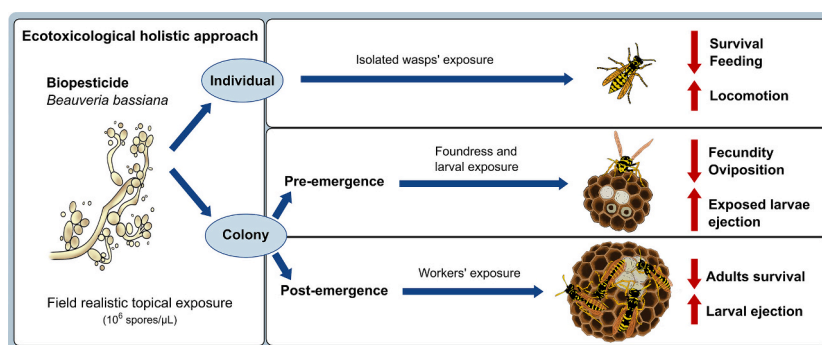
Federico Cappa<sup>\*</sup>, Livia De Fazi, David Baracchi, Rita Cervo

Dipartimento di Biologia, Università degli Studi di Firenze, Via Madonna del Piano, 6, 50019 Sesto Fiorentino, Italy

## HIGHLIGHTS

- Adverse effects of *Beauveria bassiana* on a social wasp are shown for the first time.
- Ecotoxicological holistic approach revealed an impact on individuals and colonies.
- Biopesticide reduced individual survival and altered locomotion and feeding rate.
- Biopesticide impaired wasp reproductive ability and elicited nest larval ejection.
- Natural plant protection products are not always environmentally safe.

## GRAPHICAL ABSTRACT



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## ABSTRACT

Biopesticides are considered eco-friendly alternatives to synthetic agrochemicals. However, their impact on non-target organisms is still poorly understood. Social wasps, in particular, are a largely neglected group when it comes to risk assessment of plant protection products, despite the relevant ecological and economic services provided by these insects. In the present study, we evaluated the impact of a common biopesticide, the entomopathogenic fungus *Beauveria bassiana*, on the paper wasp *Polistes dominula*. We adopted a holistic approach in ecotoxicology by focusing not only on the detrimental effects on isolated individuals, but also on the whole colony. Both adult wasps belonging to different castes and immature larvae were topically exposed to a field-realistic concentration of fungal spores from the commercial strain of *B. bassiana* ATCC 74040 to assess the impact of the biopesticide on their survival, behavior and physiology. Our results showed that the fungus causes a number of adverse effects on *P. dominula*, that include increased mortality, altered locomotion and feeding rate, selective ejection of exposed larvae from nests, reduced oviposition rate and ovary development in foundresses, and colony failure. Our findings provide new insights on the often-neglected sublethal effects of pollutants that can jeopardize not only individual beneficial insects, but also the delicate social balance of their colonies and their valuable ecosystem services, highlighting that the natural origin of plant-protection products does not always guarantee environmental safety.

<sup>\*</sup> Corresponding author.

E-mail address: [federico.cappa@unifi.it](mailto:federico.cappa@unifi.it) (F. Cappa).

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## 1. Introduction

The Anthropocene is often viewed as an epoch of progressive biodiversity loss (Turvey and Crees, 2019). More and more species are disappearing, and there is a broad consensus that anthropogenic global change is the main driver of this dramatic decline (Turvey and Crees, 2019). The alarming biodiversity loss affects both plants and animals and has not spared arthropods (Sánchez-Bayo and Wyckhuys, 2019). Insect populations are declining worldwide in terms of occurrence, taxonomic richness, abundance, biomass, and geographical distribution of different species (Sánchez-Bayo and Wyckhuys, 2019), leading to the definition of the phenomenon as the “insect apocalypse” (Goulson, 2019). Habitat destruction and fragmentation, increasing urbanization, climate change, introduction of invasive alien species, pollution, intensive land use and agriculture appear to be all involved in the process at different scales (Seibold et al., 2019).

The massive use of agrochemicals to control insect pests plays a paramount role in the decline of non-target beneficial species that provide key ecosystem services such as pollination and predation of harmful insects (Ndakidemi et al., 2016). Over the years, various strategies have been developed to mitigate the impact of agrochemicals (Egan et al., 2020). There has been increasing interest in biological pesticides (i.e., biopesticides) of natural origin as a sustainable alternative to reduce the prevalence of synthetic products used for pest control (Gupta and Dikshit, 2010; Glare et al., 2012). The use of biopesticide can be combined with control agents to improve crop protection while minimizing the impact on beneficial organisms in the context of an integrated pest and pollinator management (IPPM) (Egan et al., 2020). Both predators and entomopathogens can act simultaneously as pest control agents, thus increasing the potential for biological control through synergistic action and conservation of natural enemies in the agroecosystem (Dara, 2019).

Despite the reduced impact of biopesticides when compared to synthetic compounds, increasing evidence has been gathered on the actual compatibility and the adverse effects of these natural products on non-target beneficial insects (Desneux et al., 2007; Cappa et al., 2022). Most of the studies have focused on pollinators or natural enemies used as biocontrol agents, such as beetles, hoverflies, or parasitoid wasp and have demonstrated a plethora of detrimental effects. These range from reduced survival, abnormal development and morphology, impaired immune function and reproduction, altered microbiome, behavioral and cognitive deficits at the individual level, up to disruption of social organization, division of labor and colony integrity in species with social habits (Desneux et al., 2007; Cappa et al., 2022).

Social wasps (Hymenoptera: Vespidae) can be effective biological control agents (Prezoto et al., 2019; Southon et al., 2019; Brock et al., 2021). Because of their large colony sizes and the high demand for arthropod protein to feed their brood, the ecological and economic impact of social wasps as generalist predators of agricultural pests appears to be relevant (Donovan, 2003; Brock et al., 2021). The yellow-jacket *V. vulgaris* can capture up to 4.8 million prey loads per hectare per season (Brock et al., 2021). Similarly, a single colony of the paper wasp *Polistes versicolor* is estimated to capture >4000 insect prey in a year (Prezoto et al., 2006).

The effectiveness of social wasps as natural enemies in IPPM systems is also favored by their foraging strategy. *Polistes* paper wasps, for example, are known to nest and forage in different agroecosystems (De Souza et al., 2012), and to return to profitable sites where they have previously found a particular prey type (Armstrong and Stamp, 2003; Brock et al., 2021). Furthermore, foraging wasps intensively patrol infested plant, locating and capturing insect pests even when they are hidden within plants, where pesticides cannot easily reach their targets (Southon et al., 2019). Finally, the efficient action of social wasps as pest control agents is empirically confirmed by the relocation of colonies to artificial shelters within agroecosystems with a subsequent reduction in pest populations (Elisei et al., 2012; Southon et al., 2019; Brock et al., 2021). Thus, it is immediately clear that a negative impact of plant

protection products on these insects would have adverse consequences for the ecosystem and economic services that they provide and for the effectiveness of IPPM strategies (Feldhaar and Otti, 2020; Egan et al., 2020; Brock et al., 2021).

Exposure of social wasps to agrochemicals can occur through direct spraying or contact with contaminated resources (e.g., nest building material, sugar/protein food, water) during foraging, and it can target not only workers, but also foundresses, if the products are applied in the field during the delicate phase of colony founding (Brown et al., 2012). Foragers that survive direct exposure can return to their colonies, and thus transfer contaminated items (e.g. nest material or food) to both adult nestmates and developing brood via trophallaxis exchanges (Feldhaar and Otti, 2020; De Souza et al., 2023). Thus, exposure to agrochemicals is likely to impact not only the individual, but the entire colony through nest provisioning and social interactions.

The entomopathogenic fungus *Beauveria bassiana* is a biopesticide widely used to control many arthropod pests in agriculture, veterinary medicine, and forestry (Mascarin and Jaronski, 2016). The microbial insecticide is considered safe for non-target insects (Zimmermann, 2007), as its impact on natural enemies and beneficial soil insects appear to be negligible (González-Mas et al., 2019; Sayed et al., 2021). Nevertheless, other studies have shown detrimental effects in predators, parasitoids, and pollinators following exposure to the fungus conidia (for a review see Cappa et al., 2022). The fungal biopesticide can affect individual survival, behavior and reproduction in solitary species (Roy et al., 2008), and it also affects the chemical signature as well as recognition and learning abilities of social pollinators (Cappa et al., 2019; Carlesso et al., 2020). At present, however, no study has evaluated the potential impact of this biopesticide on predatory paper wasps.

In the present study, we investigate the effects of *B. bassiana* on the social wasp *Polistes dominula*, the most abundant and widespread species of paper wasp species in temperate Europe, which has also successfully established itself in North America and South Africa (Cervo et al., 2000; Roets et al., 2019). Besides its generalist predatory habit and potential as biocontrol agent (Armstrong and Stamp, 2003; Brock et al., 2021), the species is a model for sociobiological studies (Starks and Turillazzi, 2006), and a promising candidate as a bioindicator of environmental quality and pollution (Urbini et al., 2006).

Here, we adopt a holistic approach to assess not only the effects of field-realistic biopesticide exposure on the survival and behavior of individual *P. dominula*, but also to evaluate the sublethal effects of exposure on the whole colony. We focused our attention on the potential adverse effects of the biopesticide both in the sensitive pre-emergence phase, when colonies consist only of foundresses and immature brood, and in the more advanced post-emergence phase, after worker emergence, when the nest should rapidly increase its size and population. We hypothesize that exposure to the biopesticide could have adverse effects on adult or immature wasps, affecting both their behavior and physiology. In particular, we aim to investigate: (i) the impact of the biopesticide on individual survival, locomotor activity, feeding, and grooming rates of adult wasps; (ii) the potential effects of the fungus on the reproductive abilities of foundresses and their response to exposed larvae; (iii) what happens to a colony when adult workers are exposed to a field-realistic concentration of spores. Our ultimate goal is to provide reliable information on the possible coexistence of the fungal biopesticide and the predatory paper wasps as synergistic biocontrol agents in IPPM systems.

## 2. Materials and methods

### 2.1. Ethical guidelines

*Polistes dominula* is not a protected or endangered species and is common in rural and suburban areas of Italy. According to Italian legislation, research on invertebrates does not require ethical approval. Furthermore, our rearing environment and experimental procedure are

in line with generally accepted animal welfare standards.

## 2.2. *Polistes dominula* life cycle

*P. dominula* is a temperate paper wasp species whose small colony size and phenotypic plasticity have made it a model organism for studies of social evolution and communication (Pardi, 1948; Starks and Turillazzi, 2006; Cappa et al., 2020). In the annual colony cycle, reproductive females (i.e., foundresses) leave their hibernacula in spring to build new nests; workers start to emerge at the end of spring and are active on nests until autumn, taking over the tasks of foraging, nest construction and colony defence; reproductive individuals (i.e., sexuals) emerge in summer (Pardi, 1948). Both foundresses and workers forage intensively for prey and nest building material and during their foraging flight they may visit crop fields in search of insect pests (i.e., caterpillars) and are likely to come into contact with agrochemicals (Elisei et al., 2012). In addition, *Polistes* build their nests without a protective envelope (London and Jeanne, 2000) and pesticides sprayed in the field could reach the brood inside the cells directly or be carried into the nests by returning foragers.

## 2.3. Wasp collection and laboratory rearing

Foundresses of *P. dominula* ( $N = 48$ ) were collected in mid-April 2022 during the founding phase, when wasps that survived the previous winter establish their seasonal nests. Individuals were captured in the field with an entomological net and transferred into 50 mL Falcon tubes and transported to the laboratory. Once there, the wasps were divided into groups of approximately 5 individuals and kept in  $15 \times 15 \times 15$  cm glass boxes provided with ad libitum sugar and water and maintained at  $23 \pm 2$  °C, under an artificial light-dark cycle (L:D = 12:12).

Colonies ( $N = 93$ ) were collected in May 2022 during the pre-emergence phase, i.e., before the emergence of the first cohort of workers, and from five different sites in the surroundings of Florence, that were at least 9 km apart. Among the collected colonies, 58 nests were monogynous (i.e., nests founded by a single wasp), while the remaining 35 were polygynous (i.e., nests founded by two or more cooperating wasps). The nests were at a comparable stage of development and contained eggs and immature brood, from small larvae to pupae. Colonies were transferred to the laboratory and individually housed in  $15 \times 15 \times 15$  cm glass boxes and maintained under the same environmental conditions as previously reported. Each nest box was provided with ad libitum sugar, water, and *Sarcophaga carnaria* larvae, as well as blotting paper for nest construction. Foundresses were marked on the wings with different acrylic colors (Testor Enamel) for individual identification.

## 2.4. Isolation of *Beauveria bassiana* spores and individual exposure

The biopesticide suspension was prepared by isolating infectious conidia from the commercial product *Naturalis*® (Intrachem Bio Italia) containing viable conidiospores of *Beauveria bassiana* strain ATCC 74040, isolated from *Anthonomus grandis* (Boheman), the cotton boll weevil. The formulated product contains a minimum of  $2.3 \times 10^7$  spores/mL. From the product, 100  $\mu$ L were plated on Petri dishes containing Malt Extract Agar (MEA) as culture medium and incubated at room temperature for three days. Fungal conidia emerging from the plates were collected and resuspended in sterile water, and thus diluted to a known concentration of  $10^9$  spores/mL, comparable to those commonly used in the field for pest control (for a review see Cappa et al., 2022). We chose to expose individuals to a concentration of fungal spores that falls within the range of those applied in the field, which can also be higher ( $10^{10}$ – $10^{11}$  conidia/mL) (Alves et al., 1996; Kapongo et al., 2008a, 2008b; Meikle et al., 2012). Thus, while it is possible that an air dilution of spores might happen after application in the field, we

believe that  $10^9$  conidia/mL represents a realistic concentration that foraging wasps could encounter in the field. Indeed, comparable concentrations of conidia have been topically applied to non-target insects ( $10^6$ – $10^8$  conidia/individual) in different studies (Goerzen et al., 1990; Alves et al., 1996; Kapongo et al., 2008a, 2008b).

For each experiment, adult individuals were topically exposed to the fungal biopesticide by applying 1  $\mu$ L of spore suspension (exposed:  $10^6$  spores/ $\mu$ L; hereafter “E”) to the dorsal side of the thorax using a 10  $\mu$ L micropipette, while control wasps (hereafter “C”) received 1  $\mu$ L of sterile water. To simulate the realistic field conditions where wasps might come into contact with the biopesticide during their foraging flights, the suspension was applied topically. Larvae were exposed directly inside the nest cells by topical application of 1  $\mu$ L of spore suspension (E) or sterile water (C) to the back of their head capsule. As with adults, fungal spores were applied to the body of the larvae to simulate the natural exposure route by which immature brood in the nest might be exposed to the biopesticide following direct spraying or spores brought back to the nest by foraging adults (De Souza et al., 2023a). *Polistes* larvae develop inside their cells, but nests have no outer envelope and finely dispersed substances sprayed in the field could easily reach larvae, especially when nests are built in proximity of crop fields. Furthermore, the degradation of *B. bassiana* spores is not particularly fast as the fungus conidia can remain viable for months at a temperature between 8 and 28 °C (Mascarin et al., 2016; Garcia-Riano et al., 2022). Thus, foraging wasps could come into topical contact and gather fungal conidia when they land on plants or other surfaces sprayed with the biopesticide and they could bring spores back to their nests, potentially infecting the developing brood through contact (De Souza et al., 2023a, 2023b).

## 2.5. Biopesticide effects on adult survival and individual behavior

We first evaluated the effects of *B. bassiana* on individual survival, locomotor activity, feeding, and grooming rate on *P. dominula* foundresses and workers. The former ( $N = 48$ ) were collected in the field during their foraging flights while the latter ( $N = 48$ ) were first generation workers that emerged in the laboratory from 15 nests. For both cases, the wasps were divided into two groups in order to obtain 24 fungus-exposed (E) wasps and 24 controls (C). After exposure (see above), each wasp was placed in a plastic observation arena ( $7.5 \times 5.5 \times 4$  cm) covered with a transparent glass and provided with ad libitum sugar and water. A transparent grid sheet was placed on top of the cover glass to divide the arena into 15 equally sized cells. The grid allowed the quantification of individual locomotor activity: the position of each wasp within the grid was recorded at 1 min intervals for 15 min and used as a proxy for activity, calculating the distance between all subsequently visited cells. Observations were made twice daily by an observer blinded to the individual wasp treatment, for five days after exposure. Individual activity was recorded live, once every minute for a period of 15 min between 12.00 a.m. to 1.00 p.m., and for a further 15 min between 3.00 p.m. to 4.00 p.m. For each minute of observation, the observer recorded the location of the wasp and the individual's behavior, which was categorized as follows:

- Move (M): the wasp moves within the arena
- Stop (S): the wasp is motionless
- Grooming (G): the wasp performs hygienic behavior
- Feeding (F): the wasp feeds on the sugar/water

Mortality rate was recorded daily during the activity assessment and for a total of two weeks after exposure.

## 2.6. Biopesticide effects on foundresses' reproductive ability

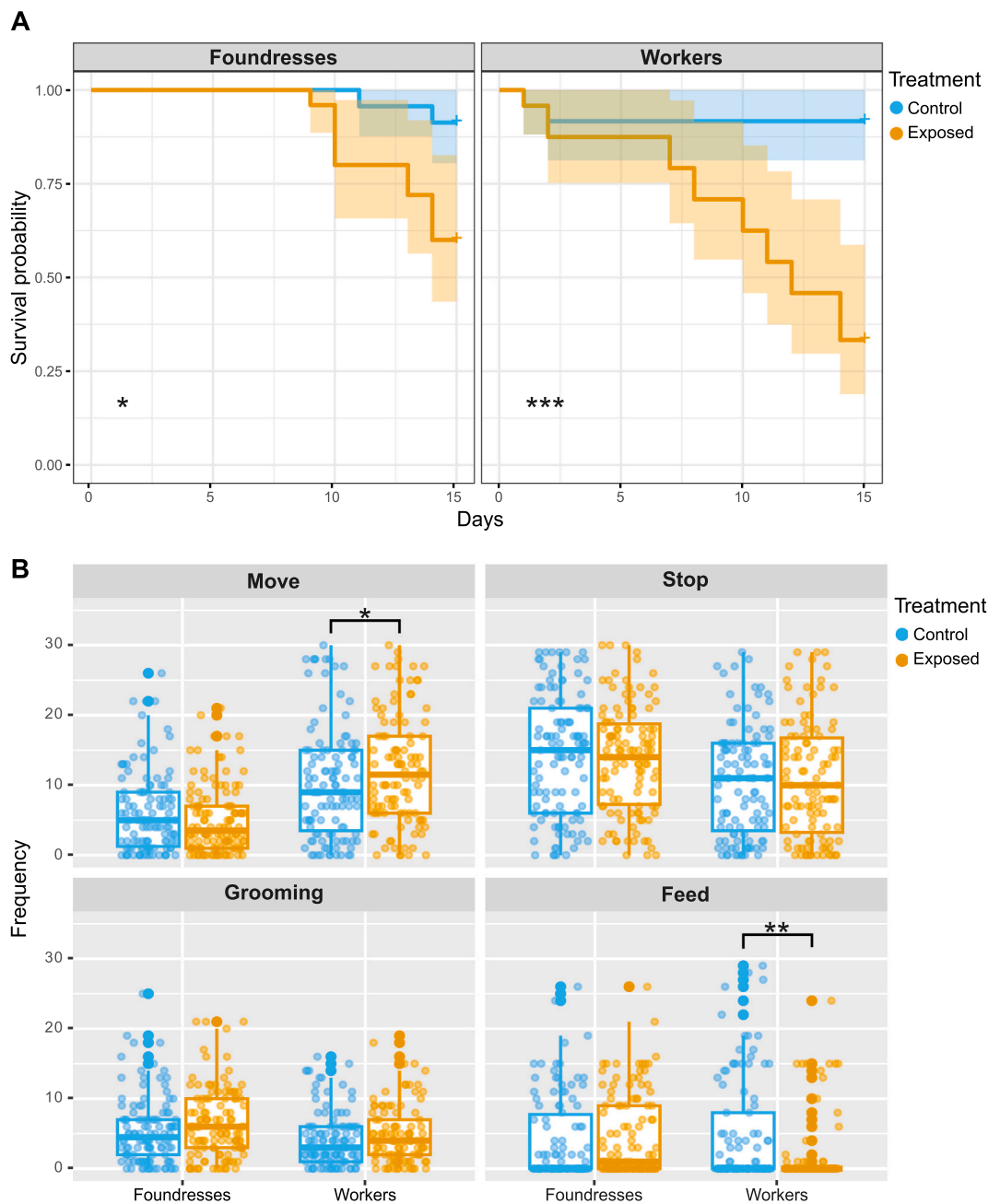
To evaluate the effects of *B. bassiana* on the fitness of *P. dominula* foundresses in terms of reproductive ability, we selected 37 monogynous nests in the pre-emergence phase from two different populations.

Nineteen foundresses were exposed topically to the fungal suspension (E), while the other 18 were treated with the control solution (C). Three days after exposure, all the eggs in each nest were removed from the comb (Dapporto et al., 2007). Twenty-four hours after egg removal, newly laid eggs were counted as a proxy for the reproductive ability of the foundress. After a further interval of 48 h, we repeated the eggs removal procedure, followed by a counting of newly laid eggs after a further 24 h. We chose the initial three-days interval, because a such a period is sufficient for the fungus to (Vilcinskas and Götz, 1999; Zimmermann, 2007) and induce detectable physiological changes that could result in a measurable trade-off between immune activation and

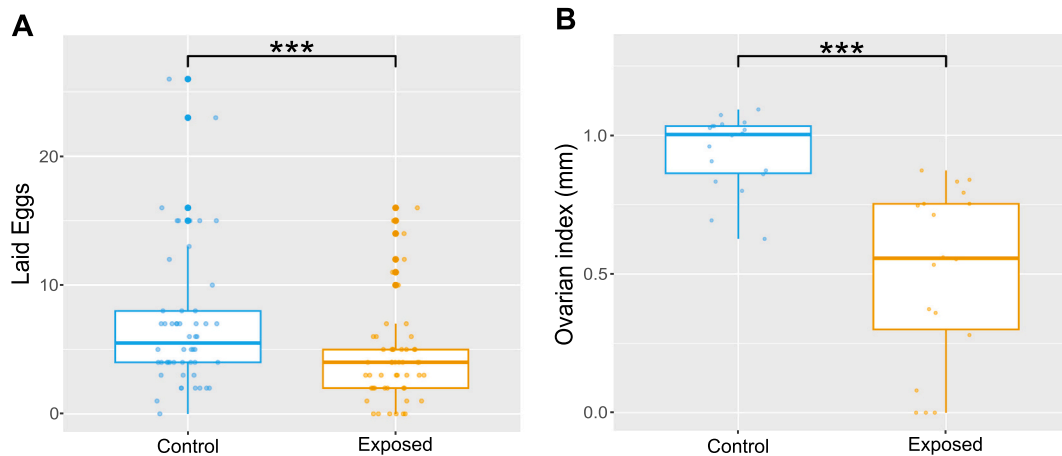
reproductive potential. We repeated the procedure after five days after exposure to evaluate whether the foundresses' egg-laying ability was further impaired by the progression of the fungal infection. Then, the tested foundresses were then killed by freezing (-22 °C) and dissected to assess the degree of ovarian development by calculating the average length of the six largest oocytes in each ovary (ovarian index, Pardi, 1948).

2.7. Biopesticide effects following larval exposure

To evaluate the effects of the biopesticide on the immature brood, a



**Fig. 1.** Biopesticide effects on foundresses (N = 48) and workers (N = 48) survival and individual activity. (A) Survival curves showed a significant increase in mortality rate of both exposed foundresses and workers over time, reported as proportion of live wasps after 15 days from biopesticide exposure. (B) Data obtained from individuals during the 150 min of observations for each caste highlighted differences between the two castes: biopesticide exposure increased workers' locomotor activity but drastically decreased their feeding behavior. Boxplots show the frequency of observed behaviors in each caste. Thick horizontal lines represent medians, boxes are upper and lower quartiles and whiskers indicate the highest and lowest values excluding outliers (large circles), small dots are individual values scattered to prevent overlap. \*:  $p < 0.05$ ; \*\*:  $p < 0.01$ ; \*\*\*:  $p < 0.001$ .



**Fig. 2.** Biopesticide effects on foundresses' reproductive ability ( $N = 37$ ). (A) Individual oviposition was significantly lower in exposed foundresses. Boxplots show the number of laid eggs per nest in each treatment. \*:  $p < 0.05$ ; \*\*:  $p < 0.01$ ; \*\*\*:  $p < 0.001$ . (B) Ovaries of exposed foundresses showed a reduced Ovarian Index (mean length of the six largest oocytes) with respect to controls after five days from exposure. Boxplots show the mean length of the six largest oocytes of each foundress in the two treatments. Thick horizontal lines represent medians, boxes are upper and lower quartiles and whiskers indicate the highest and lowest values excluding outliers (large circles), small dots are individual values scattered to prevent overlap. \*:  $p < 0.05$ ; \*\*:  $p < 0.01$ ; \*\*\*:  $p < 0.001$ .

total of 103 larvae from 19 monogynous nests from two populations were exposed to either the *B. bassiana* spore suspension ( $N = 52$  'E' larvae) or to the control solution ( $N = 51$  'C' larvae). We chose to treat larvae in the nest pre-emergence phase, because this stage represents a delicate phase of the colony cycle in monogynous nests, when only the foundress is present on the nest and any delay in workers' emergence might result in a slow colony development and increase the chances of colony failure. All treated broods consisted of large larvae at the same stage of development exposed on the same date. 'E' and 'C' larvae in each nest were selected for exposure in non-adjacent cells to keep the two treatments separate. According to the treatment ('E' or 'C'), the paper wall of the cells containing the target larvae were marked with a line of acrylic color to allow immediate recognition, alternating the two colors in different nests. Larvae in marked cells was checked daily for one week after exposure to evaluate larval death or removal by the foundress. In addition, 44 larvae from a further 11 nests were similarly exposed to the biopesticide or the control solution and removed from the comb after three days from exposure to examine their cuticular hydrocarbons (CHCs). CHCs extracts were obtained by washing larvae in a vial tube in 1 mL of pentane for 15 min. The extracts were then allowed to evaporate and stored at  $-20$  °C for coupled gas–chromatography mass spectrometry (GS-MS) analysis. We chose to analyze the CHCs of exposed larvae because these compounds are responsible for brood and health status recognition in these wasps (Cotoneschi et al., 2007; Beani et al., 2019), they might be altered by the fungal infection (Cappa et al., 2019) and they could be used by foundresses as cues of larval health condition (Beani et al., 2019). Larvae used for chemical analyses were collected from the nests after three days after exposure, as foundresses started to remove them from the nest on this day (see Results).

## 2.8. Biopesticide effects on post-emergence colonies

To evaluate the effects of biopesticide exposure on the whole colony in the post-emergence phase, we treated 30 nests from five different populations. We selected colonies in the post-emergence phase with a comparable number of workers in each treatment (from 6 to 10 per nest). Workers from 15 nests were exposed topically to the fungal suspension, while workers from the other 15 nests were treated with the control solution ( $N = 106$  workers in "C" nests;  $N = 107$  in "E" nests). Before exposure, tested workers were marked on the wings with different acrylic colors to distinguish them from foundresses and workers that emerged after exposure. Foundresses were not treated to simulate a more realistic situation, as in the post-emergence phase,

workers leave the nest to forage and may come into contact with the biopesticide, while foundresses remain on the nest and have less chance of exposure. Adult mortality rate and larval removal were monitored for two weeks after exposure.

## 2.9. Chemical analysis

Dried extracts of all larvae specimens ( $N = 44$ ) were thawed at room temperature and resuspended in 100  $\mu$ L of pentane and transferred to a conical glass insert inside the original vial used for extraction. The solvent was then dried under a stream of nitrogen and the samples were resuspended in 20  $\mu$ L of heptane to which 40 ng/ $\mu$ L of hexadecanol ( $C_{16}OH$ ) was added as an internal standard. One  $\mu$ L of the extract was injected into a Hewlett Packard (Palo Alto, CA, USA) 5890A gas chromatograph (GC) coupled to an HP 5970 mass selective detector (using a 70 eV electronic ionization source). A fused ZB-WAX-PLUS (Zebtron) silica capillary column (60 m  $\times$  0.25 mm  $\times$  0.25 mm) was installed in the GC. The injector port and transfer line temperatures were set at 200 °C and the carrier gas was helium (at 20 PSI head pressure). The temperature protocol was from 50 °C to 320 °C at a rate of 10 °C/min, and the final temperature was held for 5 min. Injections were performed in splitless mode (1 min purge valve off). Data acquisition and analysis were performed using the Agilent Masshunter Workstation software (version B.07.00) — and the open-source software OpenChrom (Wenig and Odermatt, 2010), respectively. We used the area of the internal standard as a reference for the relative abundance of each peak and selected only peaks with at least 1 % of the peak area of the internal standard. Chromatograms were aligned using the "GCalgnR" R package (Ottensmann et al., 2018), which allowed an initial alignment of the peaks based on their retention time. Peaks, corresponding to different compounds in each chromatogram, were then manually realigned based on their mass spectra. The mass spectra, with their corresponding molecular weights, were compared with electronic mass spectra libraries (NIST MS Search v.2.0). Thus, CHCs were identified based on their mass spectra and equivalent chain length. For the preparation of the dataset used in statistical analysis we only included compounds quantified in at least 25 % of specimens.

## 2.10. Statistical analyses

To test the individual mortality rate, we performed survival analyses. Survival curves were obtained from Kaplan-Meier estimates generated from the proportion of individual wasps (workers and foundresses)

surviving daily from the start until the end of the experiments, using “survival” package (Therneau and Grambsch, 2000). All the other experiments were analyzed using Generalized Linear Mixed Models. To fit each model, we used the “glmmTMB” package (Brooks et al., 2017), including colony membership and individual identity as random effects in each. We used a zero inflated Poisson distribution for the individual activity model, a Gaussian distribution for ovary development model, and a Poisson distribution for the evaluation of foundress oviposition and larval exposure models. As predictor variables for ovary development, we used treatment, while for oviposition assessment, we used treatment, days from last eggs removal and days from exposure. Instead, for the larval exposure experiment we used as predictors treatment, total number of larvae per nest and number of larvae per treatment. We tested model fit using the “DHARMA” package (Hartig, 2022) and then used the “car” package (Fox and Weisberg, 2019) to test which factors of the model had a significant effect on the dependent variable. Then, we carried out a post hoc analysis using estimated marginal means with Bonferroni correction with the “emmeans” package (Lenth, 2022) to examine the statistical differences between the experimental groups. Collected CHCs data were analyzed with a GLMM to evaluate differences in the amount of single compounds between fungus-exposed and control larvae, including colony membership and individual identity as random effects. To verify the possibility of attributing fungus-exposed and unexposed specimens to their colony based on chemical composition, we performed a Partial Least Square Discriminant Analysis (PLSDA), as implemented in the “mixOmics” R package (Rohart et al., 2017) to check whether the first four principal components were able to capture the differences between groups. All statistical analyses were carried out in R 4.2.1 (R Development Core Team, 2008).

### 3. Results

#### 3.1. Biopesticide effects on adult survival and individual behavior

Exposure to the biopesticide caused a significant decrease in individual survival in both castes (Survival analysis post-hoc: foundresses, SE = 0.1429, z.ratio = -2.181,  $p = 0.0292$ ; workers, SE = 0.0615, z.ratio = -3.329,  $p = 0.0009$ ) (Fig. 1A). Data obtained from individual observations showed differences between the two castes with respect to exposure. In general, regardless of treatment, foundresses tended to move less than workers (GLMM post hoc, Bonferroni corrected: SE = 0.0184, z.ratio = -18.692,  $p < 0.0001$ ). Within castes, our analysis

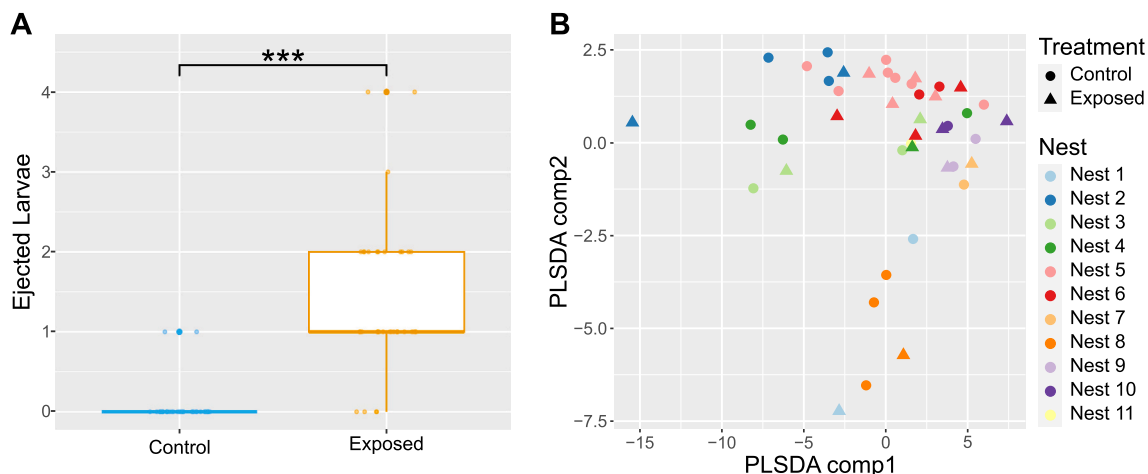
showed that biopesticide exposure increased locomotor activity in workers (GLMM post hoc, Bonferroni corrected: SE = 0.0367, z.ratio = -3.009,  $p = 0.0446$ ), but not in foundresses (GLMM post hoc, Bonferroni corrected: SE = 0.0671, z.ratio = 1.929,  $p = 0.9139$ ) (Fig. 1B). The increase in locomotor activity was further confirmed by the measures of the total distances covered by workers, which increased their activity over the days after exposure (GLMM post hoc, Bonferroni corrected: Total Distance control vs exposed workers' SE = 1.246, t.ratio = -3.344,  $p = 0.0025$ ) (for the full analyses, see supplementary material S1, section 2.1.3). Regarding grooming behavior, there were no particular differences according to exposure in either castes (GLMM post hoc, Bonferroni corrected: 'grooming foundresses control vs foundresses exposed' SE = 0.0479, z.ratio = -2.808,  $p = 0.0797$ ; 'grooming control vs. exposed workers' SE = 0.0581, z.ratio = -1.755,  $p = 1.000$ ) (Fig. 1B). On the other hand, exposure had a marked effect on feeding in the worker caste, with exposed workers showing a lower feeding rate compared to unexposed controls, a sign that *B. bassiana* might have an effect on their health status (GLMM post hoc, Bonferroni corrected: SE = 0.2183, z.ratio = 3.784,  $p = 0.0025$ ) (Fig. 1B).

#### 3.2. Biopesticide effects on foundresses' reproductive ability

*B. bassiana* affected the reproductive ability of monogynous foundresses. Regardless of time from exposure, three or five days, the number of eggs laid by exposed foundresses was significantly lower than controls (model estimate  $\pm$  SE of the number of laid eggs: C =  $6.66 \pm 0.83$ ; E =  $3.39 \pm 0.49$ ) (GLMM post hoc, Bonferroni corrected: SE = 0.372, z.ratio = 3.567,  $p = 0.0004$ ) (Fig. 2A). Moreover, exposed foundresses showed a reduced level of ovary development compared to unexposed controls (Ovarian index: model estimate  $\pm$  SE of the length six largest oocytes, C =  $0.94 \pm 0.05$  mm; E =  $0.50 \pm 0.05$  mm) (GLMM post hoc, Bonferroni corrected: SE = 0.0787, t.ratio = 5.559,  $p < 0.0001$ ) (Fig. 2B).

#### 3.3. Biopesticide effects following larval exposure

The biopesticide elicited the selective removal of exposed larvae by the foundress (GLMM post hoc, Bonferroni corrected: SE = 0.0354, z.ratio = -4.172,  $p < 0.0001$ ) (Fig. 3A). Of all control larvae, only 1.96 % (1 larva out of 51) were removed by foundresses on the tested nests. On the other hand, a much higher removal rate was observed in exposed larvae, as 80.77 % of them (41 larvae out of 52) were removed from the nest cells by their respective foundress (model estimate  $\pm$  SE of the



**Fig. 3.** Biopesticide effects following larval exposure. (A) Exposure induced a selective removal of exposed larvae from each nest. Boxplots show the number of removed larvae per nest with respect to the two treatments. Thick horizontal lines represent medians, boxes are upper and lower quartiles and whiskers indicate the highest and lowest values excluding outliers (large circles), small dots are values from single nests scattered to prevent overlap. \*:  $p < 0.05$ ; \*\*:  $p < 0.01$ ; \*\*\*:  $p < 0.001$ . (B) Scatterplot for the first two components of a Partial Least Squares Discriminant Analysis based on larval chemical profiles shows weak separation between exposed and unexposed larvae (for more details, see S1 - section 2.3.2).

number of ejected larvae per nest:  $C = 0.06 \pm 0.04$ ;  $E = 1.34 \pm 0.19$ ; Fig. 3A). However, no differences were found in the CHCs between fungus-exposed larvae and unexposed controls (for the full analyses see S1, section 2.3.2). Analyses of the chromatograms for all larvae allowed the identification of 53 peaks corresponding to different compounds. We reduced the starting dataset by selecting compounds present in at least 10 % of samples. Statistical analyses were thus performed on 49 compounds. The identified compounds belonged to different classes: alkanes ( $N = 11$ ), alkenes ( $N = 5$ ), methyl- or dimethyl-branched hydrocarbons ( $N = 22$ ), and 11 unidentified compounds. When plotted for the first two principal components, the scores obtained by individuals of different colonies in a Partial Least Squares Discriminant Analysis showed that exposed and unexposed individuals were grouped together according to their colonies showing no distances between groups (Fig. 3B).

### 3.4. Biopesticide effects on post-emergent colonies

Regarding the exposure of workers in post-emergence phase, our analyses showed a significant increase in mortality rate in exposed nests (GLMM post hoc, Bonferroni corrected:  $SE = 0.106$ ,  $z.ratio = -3.322$ ,  $p = 0.0009$ ), as 56.07 % of the exposed workers (60 out of 107) died in the two weeks after exposure. Conversely, of all control workers, only 19.81 % (21 workers out of 106) died during the same period (model estimate  $\pm SE$  of the number of dead workers per nest:  $C = 0.42 \pm 0.16$ ;  $E = 1.61 \pm 0.31$ ) (Fig. 4A). Moreover, we found a difference in larval removal (GLMM post hoc, Bonferroni corrected:  $SE = 0.109$ ,  $z.ratio = -3.139$ ,  $p = 0.0017$ ): nests with exposed workers removed 11.85 % of the total larvae (25 out of 211) while in the control nest only 2.4 % of the larvae were removed (6 out of 250) (model estimate  $\pm SE$  of the number of ejected larvae per nest:  $C = 0.4 \pm 0.16$ ;  $E = 1.67 \pm 0.33$ ) (Fig. 4B).

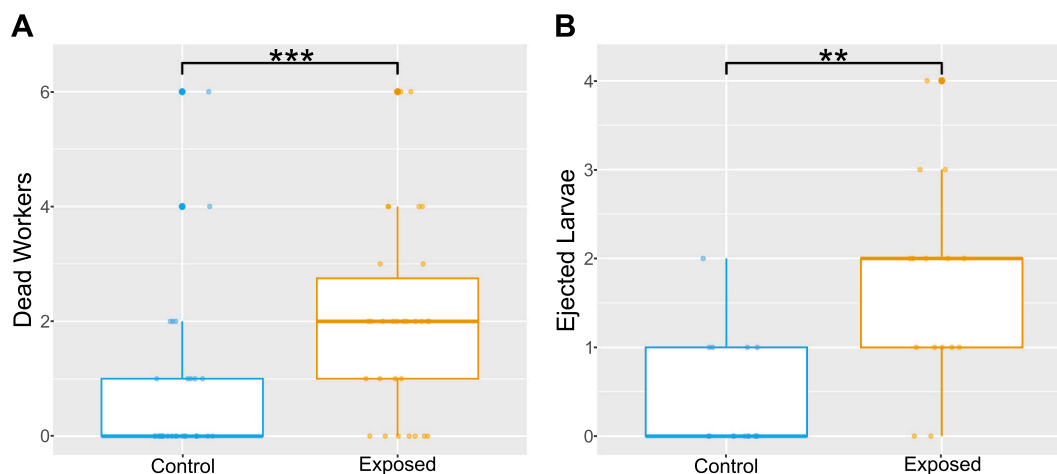
## 4. Discussion

In our study, we adopted a holistic approach (Sgolastra et al., 2020), focusing not only on the potential adverse effects of the biopesticide on the individual but also on the social insect colony as a whole. The adoption of a holistic approach is a crucial step to conduct more realistic ecotoxicological studies on non-target beneficial social insects (Chapman, 2002; Sgolastra et al., 2020). Thus, we used the small colonies of the *Polistes dominula*, a predatory paper wasp that serves as a natural enemy of agricultural pests (Southon et al., 2019; Brock et al., 2021) easy to maintain and manipulate under laboratory conditions (Starks and Turillazzi, 2006). Our study clearly showed that field-

realistic exposure to *Beauveria bassiana*, generally considered safe for non-target organisms (Zimmermann, 2007), can alter individual activity, reduce the survival of exposed workers either isolated or maintained on their natal colonies, impair the reproductive ability of foundresses, and induce the removal of exposed brood, ultimately leading to premature colony failure.

In our first experiment, exposure of wasps isolated from their nests reduced individual survival in foundresses and workers. Moreover, exposed workers showed higher levels of locomotor activity and covered greater distances in the confined arena with respect to unexposed controls, while their feeding rates decreased significantly. Similar effects of increased individual activity induced by synthetic pesticides used in seed dressing was documented on collembola by Zaller et al. (2016), while exposure to *B. bassiana* as well as other entomopathogenic fungi used as biopesticides, such as *Metarhizium anisopliae*, *Cordyceps fumosorosea*, *Cladosporium tenuissimum*, *Penicillium citrinum* reduced food consumption in caterpillars of *Spodoptera frugiperda* and *Ocinara varians* (Hussain et al., 2009; Idrees et al., 2021). Similarly, *Anopheles gambiae* mosquitoes, inoculated with a moderately high dose of *M. anisopliae* fungal conidia, exhibited reduced feeding rate compared to controls (Scholte et al., 2006). An altered foraging activity and feeding behavior in *P. dominula* has been observed also in workers infected with the strepsipteran parasite *Xenos vesparum* (Beani et al., 2018, 2020), but in this case, parasitized wasps showed a preference for specific food sources, rather than a variation in feeding rate. The increased activity, coupled with reduced feeding, observed in our fungus-exposed workers could disrupt their energy balance, leading to poor colony task performance and high mortality when engaged in costly nest maintenance tasks (Markiewicz and O'Donnell, 2001). Indeed, about half the workers exposed and reintroduced into their colonies in post-emergent phase died within two weeks, underlining the importance of evaluating the impact of agrochemicals and other pollutants on individuals maintained in their social environment. It is likely that colony tasks required an energetic cost that exposed wasps were unable to sustain due to fungal infection, resulting in their premature death and ultimately leading to colony failure due to an insufficient workforce.

Regarding the effects of *B. bassiana* on reproductive foundresses, we observed a reduction in oviposition and ovary development following exposure. Foundress egg-laying was already reduced compared to controls as early as three days after exposure. Furthermore, after another two days, their ovaries were significantly less developed compared to unexposed controls. Previous research on other species has shown that such a limited time interval is enough for the fungus to infect the host



**Fig. 4.** Biopesticide effects on post-emergent colonies. (A) Nests with exposed workers ( $N = 19$ ) showed a higher worker mortality and (B) larval ejection. Boxplots show the number of dead workers (A) and ejected larvae (B) per nest. Thick horizontal lines represent medians, boxes are upper and lower quartiles and whiskers indicate the highest and lowest values excluding outliers (large circles), small dots are values from single nests scattered to prevent overlap. \*:  $p < 0.05$ ; \*\*:  $p < 0.01$ ; \*\*\*:  $p < 0.001$ .

and induce physiological and behavioral alterations (Mascarin and Jaronski, 2016). A reduction in fecundity following exposure to *B. bassiana* conidia was observed also in target insect pests such as *Aphis gossypii* and *Nezara viridula* (Gurulingappa et al., 2011; Portilla et al., 2022). Conversely, the effects of *B. bassiana* on the reproduction of non-target insects have been hardly investigated (Cappa et al., 2022). Chronic oral administration of another biopesticide, the Neem tree-derived azadirachtin, caused a reduction in oviposition also in the tropical paper wasp *Polistes versicolor* (Teixeira et al., 2022), but apart from the study of Teixeira et al. (2022) and the present one, no research has investigated the effects of biopesticides on predatory paper wasps.

The reduced egg-laying ability and ovary development observed in exposed foundresses may be the results of an energetic trade-off between immunity and reproduction (Schwenke et al., 2016). The fungal infection might activate the wasp's immune system or deplete nutrients from the host, thereby reducing its reproductive output. It is worth noting that a high egg-laying rate and well-developed ovaries are a trademark of dominant individuals in *Polistes* colonies (Pardi, 1948; Röseler et al., 1985; Sledge et al., 2001; Dapporto et al., 2007). These traits, along with dominance interactions and the cuticular chemical profile of the foundress, are crucial for maintaining reproductive hegemony over the colony (Pardi, 1948; Dapporto et al., 2007). Thus, a reduction in ovary development and a decreased rate of egg-laying, could have a considerable impact on the social environment of the nest, particularly when the first workers start to emerge, as they may not recognize the exposed foundress as rightful dominant individual in the colony.

In addition, our larval exposure experiment demonstrated that exposed larvae were systematically removed from the nest by foundresses within a few days from topical application of spores. The detection and removal of diseased brood or adult nestmates has been largely observed in social insects and interpreted as a hygienic strategy performed at colony level (Qiu et al., 2015; Cappa et al., 2016; Liu et al., 2019; Spivak and Danka, 2021;). In our experiment, the selective removal of exposed larvae does not seem to be elicited by chemical alterations in the larval cuticular profile, as our GC-MS analyses did not find any qualitative or quantitative difference in CHCs between biopesticide-exposed and unexposed nestmate larvae. We observed that these larvae were not dead at the time of ejection, therefore, we hypothesize that other chemical or behavioral cues may be responsible for triggering the selective removal by foundresses, as reported for ergosterol, a key fungal membrane component that induces sanitary grooming in workers of the ant *Linepithema humile* (Stock et al., 2023). Infected larvae exposed to *B. bassiana* could produce volatile compounds signaling their compromised health status, or their behavioral repertoire might be affected by infection, leading to reduced activity in terms of motility inside their nest cells, reduced food requests, or decreased trophallaxis exchanges.

The highly efficient nest ejection of fungus-exposed larvae, combined with the reduced reproductive ability of exposed foundresses, clearly poses a significant threat to colony survival and fitness, especially if exposure to the biopesticide occurs during the sensitive pre-emergence phase, when the single foundress carries out all the tasks of nest building and provisioning (Turillazzi, 1980). In fact, if exposed larvae are promptly removed from the colony, and if the foundresses themselves become exposed to the biopesticide and their reproduction is impaired, they may not be able to replace the ejected brood with new eggs. This would result in a significant delay or a complete halt in colony development (Martin, 2006; Manfredini et al., 2013). It may be superfluous to point out that in temperate conditions, where the entire colony cycle of *P. dominula* lasts only a few months, an abrupt interruption of colony growth would almost certainly result in colony failure. This concern is not limited to the pre-emergence phase alone, as our experiment involving worker exposure in the post-emergence phase also resulted in increased mortality rate of workers and larval ejection from the nests. In this case, however, it is not clear whether the larvae were removed from nests because exposed workers spread the infection to the

brood via spore transfer or if the increased number of ejected larvae was due to the inefficient workforce in the nest unable to cope with the colony needs (Martin, 2006). Indeed, around half of the workers in exposed colonies died over a two-week period following exposure, and it is possible that the remaining workers were unable to provide sufficient care to the brood in advanced post-emergent colonies, leading to deterioration of larval health and subsequent ejection of dying larvae (Martin, 2006).

Overall, our experiments demonstrated that *B. bassiana* has a plethora of detrimental effects on the non-target predatory paper wasp *P. dominula*. Therefore, our findings highlight that the natural origin of plant-protection products does not guarantee environmental safety (Haddi et al., 2020; Cappa et al., 2022). Our study is one of the pioneering investigations that focused on the impact of allegedly safe biopesticides on non-target natural agents of control represented by social wasps. Previous research on the adverse effects of biopesticides on non-target organisms has primarily focused on other natural enemies, soil invertebrates, and pollinating insects (Cappa et al., 2022; Giunti et al., 2022). Social wasps, on the other hand, have been largely overlooked. Exceptions include a couple of studies investigating the impact of baculoviruses on *Polistes metricus* (McNitt et al., 1995) and azadirachtin on *P. versicolor* (Teixeira et al., 2022).

The lack of scientific attention given to the impact of plant-protection products on social wasps may appear surprising given the ecosystem services they provide in both natural and managed ecosystems. These services extend beyond pest control and include pollination, decomposition of organic matter, seed dispersal, as well as source of nutrition and medicinal compounds for human consumption and use (Brock et al., 2021). The paucity of ecotoxicological studies on non-target social wasps becomes even more evident when comparing the scant literature on the topic with the considerable number of studies investigating the impact of pesticides on bees (Arena and Sgolastra, 2014; Cappa et al., 2022). This manifest bias finds a likely explanation in the fact that bees are commonly perceived by the human eye as beneficial insects, while wasps are universally disliked by the public, and they represent unpopular research taxa among researchers (Sumner et al., 2018). Thus, in addition to its other objectives, our study aims to not only assess the effects of a biopesticide on a non-target natural enemy but also to promote ecotoxicological research on social wasps. By highlighting their valuable ecological and economic services, we seek to challenge the stereotypical image of wasps as despised and dangerous animals.

## 5. Conclusions

In conclusion, our work has shown the importance of adopting a holistic approach when assessing the impact of agrochemicals on non-target organisms. It is crucial to investigate not merely the direct toxicity of a bioactive substance but also the subtle sublethal effects that can pose a threat to individual survival and compromise colony integrity in social species. We underline that allegedly safe products can still affect non-target species in different ways, and that a careful evaluation of their risk is necessary to select the best candidates with the lowest impact in IPPM practices (Egan et al., 2020). Finally, we emphasize that predatory social wasps, apart from their beneficial role as selective and sustainable agents of pest control in agroecosystems, can also serve as suitable models for ecotoxicological research. Their ease of rearing and maintenance under laboratory-controlled condition allows for comprehensive insights into the diverse effects of plant-protection products on non-target organisms.

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## CRedit authorship contribution statement

**Federico Cappa:** Conceptualization, Writing – original draft, Writing – review & editing, Methodology, Investigation, Data curation,



Supervision, Project administration, Funding acquisition. **Livia De Fazi:** Methodology, Investigation, Visualization, Data curation, Formal analysis, Writing – review & editing. **David Baracchi:** Methodology, Investigation, Writing – review & editing. **Rita Cervo:** Conceptualization, Supervision, Investigation, Writing – review & editing, Project administration, Funding acquisition.

### 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

Full statistical analyses and raw data will made available as supplementary materials.

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