



Behavioral Responses of the Social Wasp *Polistes myersi* to Prey Infected with Fungi Used in Biological Control

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Abstract *Metarhizium anisopliae* and *Beauveria bassiana* are commonly used entomopathogenic fungi, but their non-target effects over generalist predatory insects, which can contribute to pest control, are not well known. We studied the capacity of the social wasp *Polistes myersi* to detect the pathogens in either a powdered form or in *Galleria mellonella* larvae infected with either of the two pathogens offered as prey. The effects of these treatments were compared considering wasp behaviors such as prey preference, frequency, duration and transitions of both hunting and grooming behaviors. Additionally, the effects of each entomopathogenic fungus on the wasp's mortality were measured. Wasps seem not to detect the pathogens in powdered form but preferred healthy over infected larvae. Seventeen behavioral units for hunting and 34 for grooming were recognized. There were no differences in grooming frequency but there

were significant differences on grooming duration, hunting behaviors and the patterns of transitions. Exposure of wasp colonies to either *B. bassiana* or *M. anisopliae* had no detectable impact on the mortality of adults, but mortality of larvae increased. For the first time, this study documented behavioral changes that indicated the capacity of social wasps to detect pathogens before physical contact and the display of hygienic strategies once contact occurs. The study also suggested a potential non-target effect of these entomopathogenic fungi on a generalist predator.

Keywords *Metarhizium anisopliae* · *Beauveria bassiana* · Non-target effects · *Polistes myersi* · Olfactory capacity · Hunting behavior · Grooming behavior

Introduction

Modern agriculture has faced pest problems with different strategies. The application of pesticides has been the most common strategy. However, despite initial successes, there are multiple examples of pests that have shown resistance (Van Leeuwen et al. 2010; Casida and Durkin 2013). Thus, alternative approaches have been developed seeking greater efficacy, specificity, and lower environmental impacts. Entomopathogenic organisms have been extensively used seeking these goals; however, effects on non-target organisms have also been detected. For example,

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Otterstatter and Thomson (2017), reported spillover infections and population reductions in native bumble bees from infected commercial bee populations with *Crithidia bombi* (Euglenozoa). Prior (1997), Espinosa-Ortiz et al. (2011), and de Conceição et al. (2014) recorded increased mortality of pollinators from areas where pathogens were used. Similarly, Danfa and Van Der Valk (1999) and Hokkanen et al. (2003) detected increased mortality on herbivores and parasitoids.

Metarhizium anisopliae and *Beauveria bassiana* have been extensively used as control agents (Cruz et al. 2017; Meikle and Nansen 2007) and are important fungi used in biological control (Lacey et al. 2015; Joop and Vilcinskis 2016). These have been targeted towards a wide array of species, including orthopterans, coleopterans, lepidopterans, and mites (Mesquita and Lacey 2001). However, the results of these applications are variable and depend on the strain, the species, the dose, and the timing of application (Prior 1997). For example, Harris et al. (2000) have observed strong differences in the efficacy of pathogenic species and strains for the control of *Vespa vulgaris*. Thus, studies to analyze the effect of entomopathogens on generalist predators, such as social wasps, need to have increased knowledge on the consequences of entomopathogens use on these predators.

There are many publications on the effective responses of insects towards pathogens, such as the hygienic behaviors of honey bees or the use of metapleural gland products in ant grooming (Roy et al. 2006). In social wasps a large array of defensive strategies against pathogens have been detected ranging from behavioral responses (Sumana and Starks 2004), beneficial microbial associations (Madden et al. 2013), secretion of antimicrobial substances (Baracchi et al. 2012), phagolysosomes, and humoral responses (Manfredini et al. 2008; Sadd and Barribeau 2013). However, the study of olfactory capacities has focused on beneficial microorganisms, food baits, and sexual selection of the wasps (Mackenzie et al. 2008; Davis et al. 2013; Landolt et al. 2014; Elmquist and Landolt 2018; Babcock et al. 2019), while behavioral responses against entomopathogens have been characterized by classic selection tests with very broad descriptions of the animal's responses (Meyling and Pell 2006; Mburu

et al. 2009). Detailed analyses of the behavioral response of an insect towards challenges of infection are relatively scarce.

Many species of the eusocial wasp genus *Polistes* have been studied as generalist predators in agroecosystems identifying them as important factors in pest control (Andrade and Prezoto 2001; Elisei et al. 2011). In Colombia *Polistes myersi* (8.5–15 mm long) is a common species in crops, farmlands, and in generally perturbed habitats. The nests of this species can have up to 100 cells. This species is distributed from Panama to south Venezuela. It is a widespread species in Colombia, found up to 2000 m above sea level (Sarmiento 1997). Thus, it is a good model to understand the effects of entomopathogens on a generalist predator.

The goal of this research was to assess the effect of prey larvae infected with either *B. bassiana* or *M. anisopliae* on the generalist predator wasp *P. myersi* and to compare their behavioral defensive response to these pathogens. The study could shed light on the non-target effects of these fungi on beneficial elements of the agroecosystem.

Materials and Methods

Wasp Colony Maintenance

A total of 20 colonies of *Polistes myersi* were collected from the field (Colombia: Cundinamarca, Fusagasugá, 1309 m, 4°18'996"N, 74°26'475"W) looking for nests that were highly distanced from each other along an area of 3×1 km; colonies were collected at three field trips along two years. Each nest was established in transparent PVC cages (25×16×16 cm) and stored in a rearing chamber under controlled conditions that resemble the climate of their environment (28 °C, 44% RH, 12/12 h photoperiod). Collected colonies ranged in size from two adults and 10 cells to 15 adults and 100 cells. All colonies had either late larvae or pupae. Strips of different paper qualities were provided for nest construction. Colonies were provided with water and a mixture of water, honey and pollen *ad libitum*. Later instar larvae of the greater wax moth *Galleria mellonella* (Pyralidae) were offered every other day. *Galleria* larvae are commonly reared moths that naturally attack honeycombs, and the species is also

a common insect model for diverse studies (Mylonakis et al. 2005; Mukherjee et al. 2010). A single larva was provided to most of the colonies, but large colonies received two larvae. No additional larvae were given if these were not hunted by the wasps. PVC cages were thoroughly cleaned every other day. Colonies were used for the studies if they regularly hunted and fed on the food items provided for approximately a month after being established in the rearing chamber. All the colonies increased their nest size and their larvae reached adulthood. In three cases new colonies were begun within the same cage. These observations suggest that they were maintained under appropriate captivity conditions.

Larvae Infection Procedure

Galleria mellonella larvae were infected through an immersion in a water-based solution with either of the two pathogens. The immersion lasted two seconds. In order to replicate field conditions, the pathogen solution was prepared at the concentration recommended by the producer (Biológicos y Ecológicos de Colombia Company, Colombian Institute of Agriculture certificate number 2593 of 1999) as follows: for *B. bassiana* at 1×10^8 spores/g (Strain BEC01) added to a liter of water, and for *M. anisopliae* at 1×10^9 spores/g (Strain BEC12) added to a liter of water. This means that we add a gram of product to a liter of water. According to the producer, germination rate is 85% for both *B. bassiana* and *M. anisopliae*.

The infection rate for each pathogen was characterized by exposing a separate batch of larvae ($n = 10$ each) to the pathogens following the above described protocol. The infection rate was of 80% for *B. bassiana*, and 100% for *M. anisopliae*. Infected larvae always showed the same symptoms: slower movement, clear delay in their response to tactile stimulus, and their body became less turgent and irregularly darker. In both cases, after 36 h of infection larvae showed the first symptoms but were still active, a key characteristic to induce hunting behavior by the wasps, thus, this lapse of 36 h was established as the appropriate timing to offer the larvae to the wasps. Late instar larvae of *Galleria mellonella* were used as prep in all tests.

Olfaction Tests

Given the preliminary results, tests to proof whether foragers showed any behavioral differences towards the odor of larvae infected with the pathogen were performed. Protocols by Mackenzie et al. (2008), Landolt et al. (2014), and Elmquist and Landolt (2018) were considered. Colonies used for these tests were different from those used for the hunting tests. Foragers were starved 24 h before olfactory tests and were extracted from the colony and placed individually in small containers for 30 min within the white styrofoam cage used for the video recording described above. In this way stress due to manipulation was reduced. The glass Y-tube (4 cm diameter, 19 cm stem length, 16 cm branch length) used for the olfaction tests was placed horizontally inside the styrofoam cage described before for the behavioral repertoire analyses.

Constant airflow was provided into each branch of the Y-tube with an aquarium pump. Air passed through each treatment container to provide the corresponding odor. To avoid infection of wasp foragers by the pathogens, standard filter paper (Whatman®) was placed between the exit of the treatment container and the Y-tube branch. No nest or wasp showed signs of infection after the olfaction tests. A total of six treatments were applied in order to compare between pathogen and control as follows. Three treatments used the pathogens in the powder presentation accompanying the commercial pesticide, and a control of rice flour, since this is used as an excipient for the pesticide commercial presentation. Three treatments used infected larvae as described previously (see 2.2.) and healthy larvae were used as controls. The pairs compared by treatment were as follows: 1. *M. anisopliae* vs. rice flour; 2. *B. bassiana* vs. rice flour; 3. *M. anisopliae* vs. *B. bassiana*; 4. Larvae infected with *M. anisopliae* vs. healthy larvae (control); 5. Larvae infected with *B. bassiana* vs. healthy larvae; 6. Larvae infected with *M. anisopliae* vs. larvae infected with *B. bassiana*.

Each treatment was tested with 30 forager wasps taken randomly from any of the 16 colonies available. Tested wasps were individually marked with color paints (either Revell® color 312 or red acrylic polish) and then were returned to their nests. Thirty-three percent of wasps were used a second time but tests

were separated by at least three days. The effect of these individuals tested twice was explicitly analyzed as explained below. Given preliminary results, treatments four and five were tested 30 times more.

Following Mackenzie et al. (2008), a series of five wasps were tested successively with a single treatment and then the location of both odor sources was switched to eliminate left or right potential bias. After performing ten tests all the equipment was cleaned with Micro 90 Solution 2% (International Products Corp., Burlington, NJ) and then rinsed with deionized water, then again with acetone and then finally with hexane.

Individual foragers were placed into the stem of a Y-tube and observed for 2 min. If the wasp moved upwind until its entire body was inside any of the branches it was scored as a positive result. Data were analyzed with standard chi-squared tests available in R. The effect of using foragers more than once on the selection outcome was assessed using mixed model analysis where a forager was a random effect and the treatment was a fixed effect. This analysis was performed using the packages *car*, *MASS*, and *lme4* of R (Fox and Weisberg 2019; Venables and Ripley 2002; Bates et al. 2015; respectively).

Hunting Tests

A total of 15 hunting tests were conducted for each entomopathogen as follows: an infected larva and a healthy larva were placed simultaneously on the floor of the PVC cages separated from each other by a 15 mm tall × 25 cm long transparent acetate wall to avoid cross contamination. Tests were videotaped after placing the PVC box with the colony into a white styrofoam cage (36 cm height, 77 cm width, 70 cm depth) at 26 °C and 3276 lm. This cage facilitated a similar temperature to that of the rearing chamber and a neutral visual background during the tests. Colonies were randomly selected for each treatment. An equal number of colonies was used for every test (healthy- *B. bassiana* and healthy - *M. anisopliae*) of the same size as given by the number of adults. Hunting tests were used to resemble natural conditions as wasps in the field may face an array of prey varying in health. In this sense, a wasp may be in an area where entomopathogens have been applied but never select infected prey, avoiding pathogen contact. Single treatment tests, with either infected or

healthy larva, would show the wasp's immune resistance but will not reveal whether they have an array of behaviors to prevent disease.

Infected larvae were handled with separate tweezers. Tests were video recorded until the wasp chewed the selected prey turning it into a ball and then left the hunting place. Half of the 20 colonies studied were tested up to two times. Colonies were included in a second test if the wasps did not attack a larva or if only healthy larvae were attacked the first time. Following the studies of Landolt et al. (2014) and Elmquist and Landolt (2018) on *Polistes* behavior, tests on the same colony were separated by three days to reduce the effect of previous experiences. The effect of these repetitions on the selection outcome was assessed using mixed model analysis where the colony was a random effect and the treatment was a fixed effect. This analysis was performed using the statistics packages described previously for the olfaction tests.

Characterization of Wasp Behavioral Repertoire

Hunting and grooming behaviors were identified and typified by looking for clear changes in the activity and the movement of specific body parts. Repertoire completeness for grooming and hunting behaviors were analyzed using the Chao1 estimator run under the package EstimateS version 9.1.0. (Colwell 2013). Duration and frequency of each behavior, and the sequences of behaviors were recorded and compared, discriminating whether it occurred with healthy or infected larvae. Comparisons were also performed with the baseline of either the hunting or grooming behaviors.

Behavioral sequences per treatment were analyzed as follow: the frequency of a transition from one behavior to another was recorded and that value was divided by the total of observed transitions. In this way, the frequency of a specific transition regarding all records was assessed. The inclusion of behaviors and sequences in the graphs followed two criteria: behaviors with higher frequencies and most common transitions between behaviors. Thus, a graph included those behaviors that summed about 80% of the total frequency observed and other behaviors that were less frequent but part of often repeated transitions.

As normality and homogeneity of variances requirements were not met from our four data sets for

hunting frequency, hunting duration, grooming frequency, and grooming duration, the Wilks' Lambda type nonparametric multivariate test available in the nrmv package of R (Liu et al. 2011; Ellis et al. 2017) was performed. The method also allowed us to identify changes in probability of occurrence between treatments for every single behavior (Ellis et al. 2017).

The baseline of the hunting behaviors was established after 13 hunting events as follows: two healthy larvae of *Galleria mellonella*, were placed on the floor of the PVC cages separated by 3 cm from each other and videos of the adult wasp behavior were recorded under the conditions described previously. Videos lasted until the wasp chewed the prey, turning it into a ball and left the hunting area. When larvae were offered, usually a single wasp from the colony attacked one larva; only in three cases a second wasp simultaneously flew to the hunting area and in all three cases each wasp attacked a different prey.

A baseline of wasp grooming behavior was established using 68 units of video recording as follows: five-minute video recordings were focused on individuals displaying grooming. Following classical ethological techniques (Lehner 1979; Wagner-Ziemka et al. 2008) grooming behaviors were classified into different types by observing within a sequence what body regions were cleaned and what body parts were used to clean those regions.

Adult and Larvae Wasp Mortality

Adult and larvae wasp mortality was recorded up to 24 days after exposure to the infected prey larvae discriminating by treatment (healthy larvae, larvae infected with *M. anisopliae*, or larvae infected with *B. bassiana*). Colonies were labeled for each treatment depending on the prey selected by the wasps the first time: If a single wasp of a colony attacked an infected larva the first time, the colony was used in a second test offering the same pathogen and a healthy larva; thus the mortality results were attributable to a single pathogen in this case. Data were then compared with standard chi-squared tests available in R. As colonies were producing new adults constantly, there was difficult to establish the size of a starting cohort and it requires to mark every single individual increasing the level of stress in the colonies potentially affecting more the behaviors recorded.

Results

Olfactory Selection Tests

The mixed model analysis showed that the use of a colony more than once has no effect on the results (variance=0.0214, SD=0.14); and that, overall, there were no differences between treatments (ANOVA, chi-squared=3.6608, df=1, $P=0.05$). Consequently, the chi-squared analysis of the selection tests with the pathogens presented as powder showed no differences, while tests with larvae suggested a trend towards healthy prey. This trend was significant only in those cases where larvae were infected with *M. anisopliae* (Table 1) and no difference was observed when both pathogens were present in the infected larvae.

Hunting Selection Tests

Of the 20 colonies tested, six did not attack larvae. Of the three that attacked a healthy larva the first time, one attacked an infected larva the second time, one attacked a healthy larva the second time, and one did not attack a larva the second time. One colony that did not attack larvae the first time, attacked a healthy larva the second time. Consequently, the mixed model analysis indicated that repetitions have no effect on the outcome of the tests (variance=0.0416, SD=0.2149). Of the 15 hunting tests with *B. bassiana* two infected larvae and five healthy larvae were selected. In the tests with *M. anisopliae* six infected larvae and one healthy larva were selected. However, overall there were no differences between treatments (ANOVA, chi-squared=0.6656, df=1, $P=0.41$).

Table 1 Olfactory test results with *P. myersi* foragers. Bb: *B. bassiana*, Ma: *M. anisopliae* C: Control (either excipient powder only or healthy larva). Most tests were repeated 30 times, * $n=60$. Significant differences are marked in bold

Treatment	Selection	Chi ² test
Powder C vs Bb	13, 17	$X^2=0.53$, df=1, $P=0.4$
Powder C vs Ma	15, 15	$X^2=0$, df=1, $P=1$
Powder Bb vs Ma	14, 16	$X^2=0.13$, df=1, $P=0.7$
Larvae C vs Bb*	32, 28	$X^2=0.26$, df=1, $P=0.60$
Larvae C vs Ma*	36, 19	$X^2=5.2545$, df=1, $P=0.02$
Larvae Bb vs Ma	15, 15	$X^2=0$, df=1, $P=1$

Behavioral Responses to Pathogens

Hunting Behaviors A total of seventeen hunting behaviors were recorded (Table 2), the chao1 index estimated 20 behaviors implying that our sampling covered 85% of the repertoire. A total of 34 grooming behaviors were recorded (Table 3); the chao1 index estimated 34.5 behaviors implying that our sampling covered 98.6% of the repertoire.

The frequency of hunting behaviors for healthy prey (control) showed the following patterns: prey dragging (HE, 19.6%), food ball making (HF, 16.9%), and kneading (HD, 14.2%) accounted for the 50.7% of the total recorded (Fig. 2a). Only antennal waving (HB, 24%), observing the prey (HA, 22.7%) and attacking (HC) were required to reach 61.3% of the total behaviors in wasps exposed to larvae infected with *B. bassiana* (Fig. 2b). For *M. anisopliae*, antennal waving (HB, 19%), kneading (HD, 16.5%), observing the prey (HA, 10.1%), and antennal cleaning (HI, 8.9%) summed up 54.4% of the total frequency (Fig. 2c, Supplementary Fig. S1). These differences were statistically significant (Wilks' lambda=2.711, df=20,40, $P=0.004$). The relative effects of the analysis indicate that HD, HDG,

HE, and HF exhibited higher frequencies in wasps exposed to healthy larvae while other behaviors did not. Kneading (HD) and prey dragging (HE) decreased with larvae infected with *B. bassiana* (Supplementary Fig. S1). These data described changes related to direct interaction with the prey and not to previous or posterior behaviors.

The duration of separate hunting behaviors showed the following patterns: food ball making (HF, 41.3%) and prey handling II (HDG, 26.8%) made up to 68.1% in wasps exposed to healthy larvae (Fig. 2a). In wasps exposed to *B. bassiana* 64.8% was reached with food ball making (HF, 29.1%), communal food ball making (HFH, 18.8%) and kneading (HD, 20.1%) (Fig. 2b). Finally, for wasps exposed to *M. anisopliae* kneading (HD, 43.7%) and food ball making (HF, 13.8%) comprised 57.5% of the time spent (Fig. 2c, Supplementary Fig. S2), which was a similar pattern to that of *B. bassiana* exposed wasps. Differences between treatments were statistically significant (Wilks' lambda=3.003, fd=20, 40, $P=0.001$). The relative effects showed that prey handling II (HDG) and prey dragging (HE) decreased with larvae infected with *B. bassiana* (Supplementary Fig. S2).

Table 2 Repertoire of hunting behaviors observed in *Polistes myersi* exposed to healthy (control) and infected larvae of *Galleria mellonella*

Code	Behavior name	Description
HA	Observing the prey	The wasp moves the body towards the larva, raises antennae and wings are held just above the body
HB	Antennal waving	The wasp slowly moves the antennae while remaining still towards the larva
HC	Attack	The wasp attacks biting, opening the larvae exoskeleton, and finally kills the larvae
HD	Kneading	With its mandibles the wasp kneads the larva along its length often breaking the skin
HE	Prey dragging	The prey is dragged a short distance over the floor of the cage
HF	Food ball making	The prey is carefully chewed until it is transformed into a ball
HG	Metasoma movements	Obvious contraction and distension of the metasoma segments beyond those related to breathing
HI	Antennal cleaning	Cleaning of antennae and front legs with mouth
HJ	Hind leg cleaning	Cleaning of hind legs with metatibial spurs
HX	Abandonment	The worker leaves the prey remains without carrying obvious pieces in its mouth
HK	Head removal	The worker carefully cuts away the cephalic capsule of the larvae
HBDF	Food antenation	Antenation over the prey remains while chewing these remains
HDEG	Prey handling, I	Simultaneous display of food ball making, dragging and metasoma pumping movements
HDG	Prey handling II	Simultaneous display of food ball making and metasoma movements
HFH	Communal food ball making	More than one worker participates in the food ball making of a single prey
HED	Dragging and food ball making	Simultaneous display of food ball making and dragging
HBC	Antenation and attack	The wasp performs antenation over the prey and attacks it by biting, and kills the larvae

Table 3 Repertoire of grooming behaviors observed in *Polistes myersi* exposed to healthy (control) and infected larvae of *Galleria mellonella*

Code	Cleaned body part	Cleaning body part
A	Antennae	Front legs
AA	Metasoma	Mid and hindlegs
AN	Antennae and front legs	Front legs and mouth
B	Eyes	Front legs
BB	Upper area of genae and base of maxillae	Front legs
BN	Eyes and front legs	Front legs and mouth
C	Posterior and dorsal sides of head	Front legs
CC	Clypeous	Front legs
CN	Posterior and dorsal sides of head and front legs	Front legs and mouth
D	Pro and mesonotum	Front legs
DA	Pro, mesonotum and antennae	Front legs
DN	Pro, mesonotum and front legs	Front legs and mouth
F	Metanotum	Front legs
FJ	Metanotum and anterior part of metasoma	Front legs
FN	Metanotum and front legs	Front legs and mouth
H	Base of the front wings	Front legs
I	Hind legs	Mid legs
J	Metasoma	Hind legs
K	Metasoma	Hind legs and one of the mid legs
L	Tip of the front wings	Hind legs
M	Hind legs	Metatibial spurs
N	Front legs	Mouth
Ñ	Mid legs	Metatibial spurs
Q	Hind legs and one of the mid legs	Metatibial spurs
QQ	Hind legs and mid legs	Metatibial spurs
S	Ventral area of mesosoma	Front legs
SA	Ventral area of mesosoma and antennae	Front legs
SN	Ventral area of mesosoma and front legs	Front legs
T	Dorsal area of mesosoma	Front legs
U	Mid legs	Front legs
V	Front legs	Front legs
VV	Front legs and one of the mid legs	Front legs
WN	Gena and front legs	Front legs and mouth
WOW	Allogrooming	Mouth

Transitions of hunting behaviors also showed strong differences between the three treatments (Fig. 2). Wasps exposed to healthy larvae displayed 17 behavioral types and a total of 46 transitions representing 16% of the potential events. Wasps exposed to larvae infected with *B. bassiana* displayed 11 behavioral types and a total of 25 transitions representing 20.7% of the potential events to occur. Wasps exposed to larvae infected with *M. anisopliae* displayed 12 behavioral types and a total of 37

transitions representing 26% of the potential events to occur. Overall, wasps exposed to healthy larvae displayed the following sequence: observing the prey (HA) followed by antennal waving (HB) (7.0%), then two behaviors showing the same transition probability, attacking (HC) (4.3%) or direct kneading (HD) (4.3%). The latter was followed by food ball making (HF) (8.7%) and this was followed by simultaneous food ball making while performing strong metasoma pumping (HDG) (7.0%) or prey dragging (HE) (5.2%)

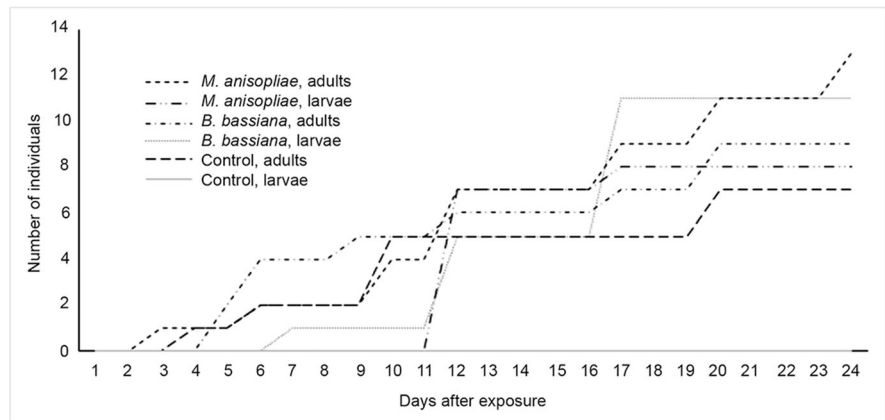
(Fig. 2a). Wasps exposed to larvae infected with *B. bassiana* started from observing the prey (HA) to either antennal waving (HB) (13.3%) or antennal cleaning (HI) (1.7%). Antennal waving (HB) was followed by attacking (HC) (8.3%) which could be followed by kneading (HD) (8.3%), prey dragging (HE) (3.3%) or food ball making (HF) (3.3%) (Fig. 2b). Three behavioral loops were recorded: observing the prey (HA) (11.7%); antennal waving (HB) (5.0%); and attacking (HC) (1.7%). The last one was due to a series of pauses that the wasps performed while attacking. Wasps exposed to larvae infected with *M. anisopliae*, started from observing the prey (HA) followed by either antenna waving (HB) (7.58%) or attacking (HC) (3.03%). Those that proceeded through antennal waving continued with attacking (HC) (3.03%) followed by kneading (HD) (6.06%), then prey dragging (HE) (4.55%), Prey handling II (HDG) (3.03) or food ball making (HF) (3.03) and finally, metasoma movements (HG) (3.03%), antennal cleaning (HI) (1.52%), and abandonment (HX) (7.58%) (Fig. 2c).

Grooming Behaviors Five grooming behaviors evenly contributed to 48.6% of the frequency of all patterns observed in colonies of wasps that attacked healthy larvae (Fig. 3a). These behaviors were grooming of front legs with the mouth (N, 12.3%), hind legs with the metatibial spurs (M, 9.4%), antennae with front legs (A, 9.4%), genae and front legs with the front legs and mouth (WN, 9.4%), and front legs and one of the midleg with the front legs (VV, 8.1%). In contrast, only three behaviors were required to reach over 50% of the total frequency of activities in colonies of wasps which attacked larvae infected with either *B. bassiana* (54.3%, Fig. 3b, Supplementary Fig. S3) or *M. anisopliae* (55.2%, Fig. 3c, Supplementary Fig. S3). These overall differences between treatments were not statistically significant (Wilks' lambda = 1.786, df = 18, 30, $P = 0.078$). However, the most frequent behaviors differed between treatments. In colonies exposed to *B. bassiana* grooming of hind legs with the metatibial spurs (M, 18.8%), anterior wings with the anterior legs (L, 18.5%), and grooming of front legs with front legs and mouth (AN, 17%) were the most frequent, while in colonies exposed to *M. anisopliae* grooming of hind legs with the metatibial spurs (M, 25.1%), grooming of anterior wings with the anterior legs (L, 17%), and grooming

of metasoma with hind legs (J, 13.1%) were the most frequent (Fig. 3, Supplementary Fig. S3). The relative effects of the nonparametric Wilks' lambda analysis concurred with most of this description indicating that A, N, VV, and WN exhibited higher frequencies when exposed to healthy larvae while other behaviors do not. However, grooming of the hind legs with the metatibial spurs (M) was not suggested by this test as a high frequency behavior. Interestingly these behaviors involved cleaning the anterior parts of the body (Fig. 3, Supplementary Fig. S3).

Differences between treatments were detected for the duration of grooming behaviors (Wilks' lambda = 2.308, fd = 18, 30, $P = 0.02$). Wasps that selected healthy larvae spent up to 54.4% of the time with the following five behaviors (Fig. 3a, Supplementary Fig. S4): anterior wings with the anterior legs (L, 13.2%); hind legs with the metatibial spurs (M, 11.8%); gena and front legs with the front legs and mouth (WN, 9.9%); grooming of front legs with front legs and mouth (AN, 9.8%); and hind legs and one of the mid legs with metatibial spurs (Q, 9.8%). In wasps exposed to larvae infected with *B. bassiana* spent 66.3% of the time in the following four behaviors (Fig. 3b, Supplementary Fig. S4): grooming of anterior wings with the anterior legs (L, 23.2%); grooming of front legs with front legs and mouth (AN, 18.3%); hind legs with the metatibial spurs (M, 13.1%); and grooming of hind legs and one of the mid legs with metatibial spurs (Q, 11.7%). In wasps exposed to larvae infected with *M. anisopliae* 74.5% of grooming time was invested in the following five behaviors (Fig. 3c, Fig. S4): cleaning of anterior wings with the anterior legs (L, 21.4%); hind legs with the metatibial spurs (M, 19.5%); grooming of front legs with front legs and mouth (AN, 15.1%); hind legs and one of the mid legs with metatibial spurs (Q, 11.2%); and grooming of antennae with front legs (A, 7.4%) (Supplementary Fig. S4). These changes concurred with the variation in the relative effects of three behaviors as given by the Wilks' lambda analysis, although these behaviors were not among those of higher duration for healthy larvae. They showed higher duration for A, N, and VV in wasps exposed to healthy larvae (Supplementary Table S5). Only cleaning the metasoma with hind legs (J) increased in *B. bassiana* treatment, and cleaning front legs with the mouth (N) was strongly reduced in *M. anisopliae* treatment (Supplementary Table S5).

Fig. 1 Accumulated number of dead *Polistes myersi* larvae and adults after different days of treatment with either *M. anisopliae*, *B. bassiana* or water as control



The transition for grooming behaviors of wasps exposed to healthy larvae showed very important

differences compared to those of wasps exposed to either *M. anisopliae* or *B. bassiana* (Fig. 3). Wasps

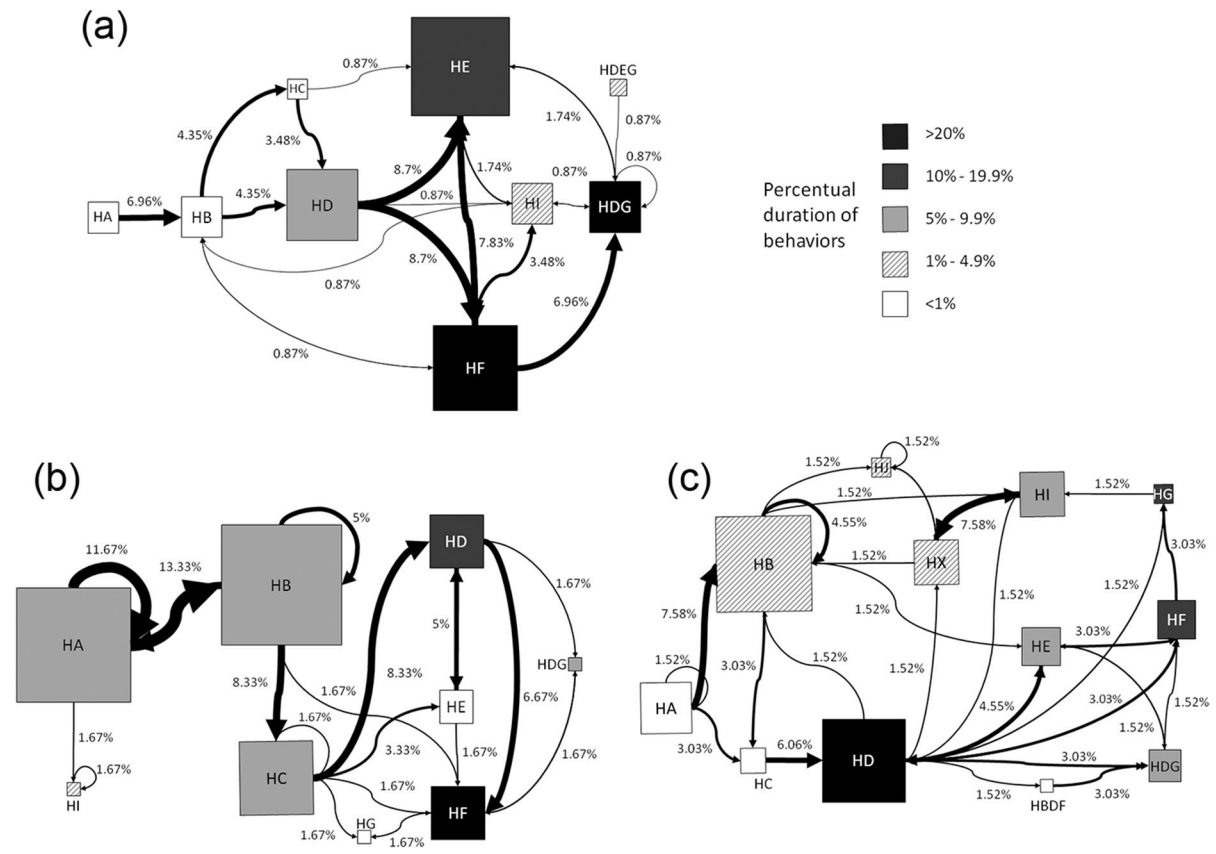


Fig. 2 Flowcharts of the hunting behaviors observed in *Polistes myersi* colonies exposed to healthy (control) (a), and infected larvae of *Galleria mellonella* with *B. bassiana* (b) and *M. anisopliae* (c). Behaviors are indicated by the codes provided in Table 2. The size of the boxes indicates the approximate frequency of each behavior expressed as a percentage. The duration of each behavior is expressed by the filling of

the boxes. The thickness of the arrows shows frequency of transitions. The exact percentile distribution of the transitions is indicated by the lines, those above and to the right go with the arrowhead, those below and to the left show the transition value against the arrowhead. Transitions below 0.5% are not shown

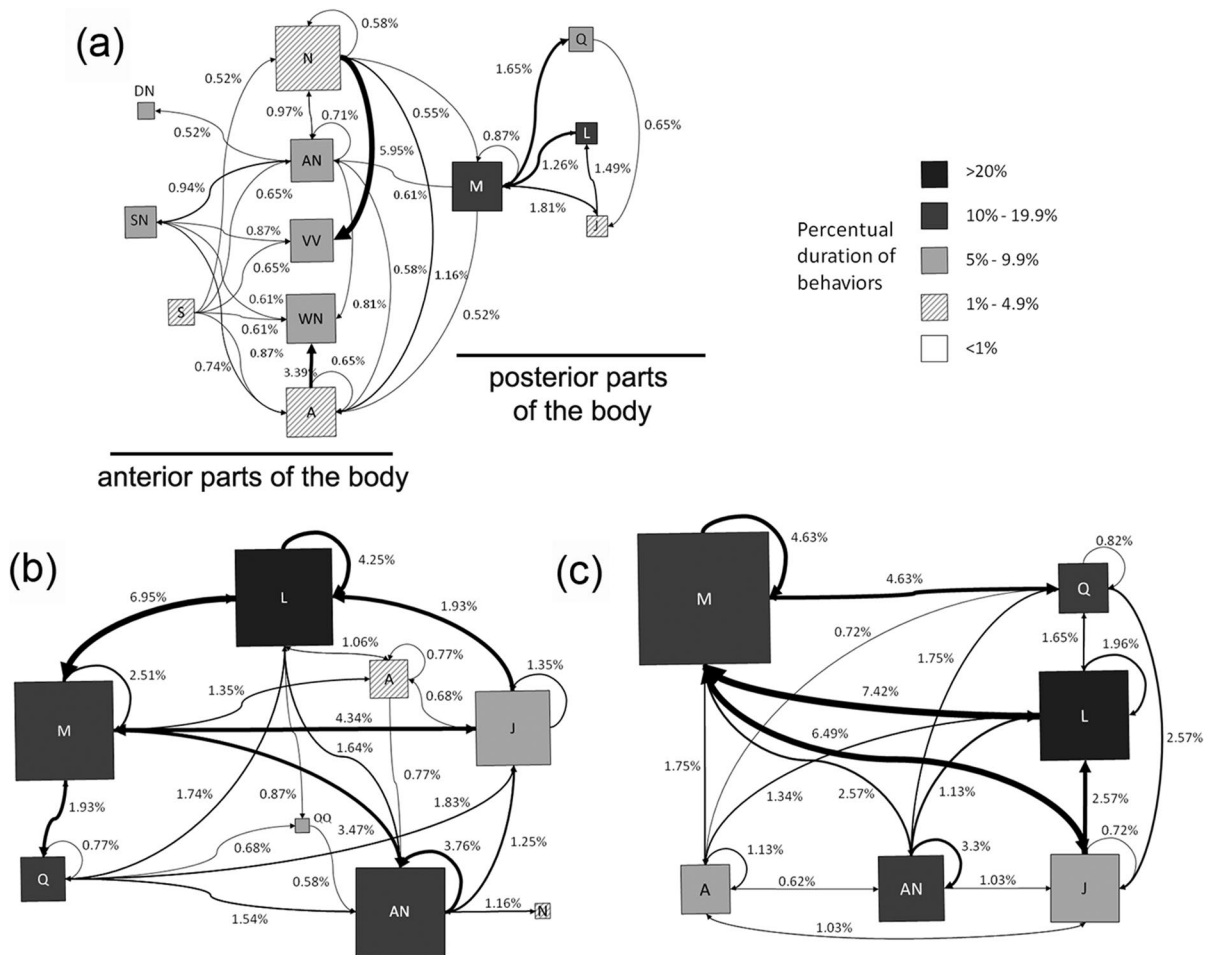


Fig. 3 Flowcharts of the grooming behaviors observed in *Polistes myersi* colonies exposed to healthy (a) and infected larvae of *Galleria mellonella* with *B. bassiana* (b) and *M. anisopliae* (c). Behaviors are indicated by the codes provided in Table 3. The size of the boxes indicates the approximate frequency of each behavior expressed as a percentage. The duration of each behavior is expressed by the filling of the boxes.

exposed to healthy larvae displayed 31 behavioral types and a total of 374 transitions representing 39% of the 961 potential events. However, only 47 of the observed frequencies showed values above 0.5% representing 13% of observed events (Fig. 3a). Wasps exposed to larvae infected with *B. bassiana* displayed 29 behavioral types and a total of 191 transitions representing 23% of the 841 potential events to occur. Of these observed 39 showed frequencies above 0.5% and represented 20% of observed events (Fig. 3b). Cleaning of the tip of the front wings with the hind legs (L) transitioned to grooming of the hind legs with the metatibial spurs

The thickness of the arrows shows the higher frequency of the transitions. The exact percentile distribution of a transition is indicated by the lines, those above or to the right go with the arrowhead, those below or to the left show the transition value against the arrowhead. Transitions below 0.5% are not shown. Dashed squares in (a) enclose behaviors classified by the region of the body cleaned

(M) or the other way around, and these behaviors reached 10.42% of the events (Fig. 3b). Repeated sequences of behaviors, named behavioral loops, were recorded in four of the five more frequent behaviors. Wasps exposed to larvae infected with *M. anisopliae* displayed 25 behavioral types and a total of 172 transitions representing 28% of the 625 potential events to occur. However, only 39 of these observed showed frequencies above 0.5% and they represented 23% of observed events. Loops were also observed with grooming of the hind legs with the metatibial spurs (M) as one of the most common behaviors (Fig. 3c).

Survival of Individuals

After 24 days of observation of colonies exposed to larvae either healthy or infected with pathogens, the number of dead adults did not differ between treatments (chi-squared=1.931, $df=2$, $P=0.381$), while, there were differences in the number of dead wasp larvae (chi-squared=10.211, $df=2$, $P=0.006$) (Fig. 1).

Discussion

Olfactory Selection Test

Overall, foragers of *Polistes myersi* seemed not to detect the pathogen in its powdered form; however, larvae infected with the pathogens, especially with *M. anisopliae*, were not preferred over healthy larvae. These differences suggested that, even before physical contact, the forager might detect the pathogen's metabolic activity or the physiological changes in the infected larvae. Davis et al. (2013) report several types of interactions between microorganisms and insects where compounds produced by the microorganism's act as semiochemicals for the insects. *Vespula pensylvanica* and *Vespula germanica* are attracted to the fungus *Aureobasidium pullulans* as some of the chemicals it produces may simulate a food source for wasps. They also report that parasitoid wasps preying on bark beetles (*Ips pini*) use the odor of their symbiotic fungi to locate the prey. *Sirex noctilio* (Hymenoptera: Siricidae) avoid localities with the odor of the symbiotic fungi of *I. pini* as they compete for the same resources. Babcock et al. (2019) also report the capacity of *V. pensylvanica* to detect yeast-produced semiochemicals to locate food sources. Mburu et al. (2009) report that the termite *Macrotermes michaelseni* (Termitidae) discriminates and avoids the most virulent strains of both *B. bassiana* and *M. anisopliae* found in the soil where they forage.

Hunting Selection Tests and Behavioral Changes

The presence of the entomopathogen in prey larvae had significant effects on hunting behavior for *Polistes myersi*. The number of attacked larvae was reduced by half when they were infected with any

of the entomopathogens. The time spent in hunting behaviors also showed important differences; wasps spent 1% of the time displaying surveying behaviors such as observing the prey (HA) and antennal waving (HB). Wasps exposed to larvae infected with either *B. bassiana* or *M. anisopliae* increased those exploratory behaviors up to 13.4% and 2.2% respectively. In addition, kneading (HD) reached 7.5% in control tests, while it reached 16.9% when the wasps were attacking *B. bassiana* infected larvae and 43.7% when exposed to *M. anisopliae* infected larvae. These changes revealed a reluctance of the wasps towards the use of infected prey and the results agree with studies on other predators such as *Anthocoris nemorum* (Heteroptera, Anthocoridae) which reject prey infected with pathogens (Meyling and Pell 2006). Likewise, Rännbäck et al. (2015) found that the parasitoid *Trybliographa rapae* lays more eggs in healthy larvae of *Delia radicum* than in infected larvae.

Despite the lack of statistical differences for grooming frequencies between the three treatments, data suggest that wasps altered their grooming behavior. When they are in contact with healthy larvae, a richer and more evenly distributed array of behaviors and their transitions are observed. In contrast, wasps exposed to infected larvae concentrated their grooming activities on a few behaviors, especially cleaning the hind legs with metatibial spurs (M), cleaning the apex of wings with the hind legs (L) and cleaning of the antennae and front legs with front legs and mouth (AN).

These differences might imply a response of the wasps for protecting themselves from the pathogen through exocrine gland products or behavioral alterations because of the effects of the pathogen on the nervous system. There is a large body of evidence showing that hymenopterans reduce pathogenic effects through grooming, such as the hygienic behaviors in *Apis mellifera* that deter the spreading of disease (Arathi et al. 2000; Masterman et al. 2001; Cheruiyot et al. 2018). In ants, antimicrobial compounds are produced with their metapleural glands. For example, in *Solenopsis invicta* spray alkaloids protect the brood and nestmates against fungi and other pathogens (Oi and Pereira 1993). Poulsen et al. (2003) describe how leaf cutting ants deter microorganisms through focused grooming, spreading compounds present in their metapleural gland. To our knowledge this is the first report where detailed

records of behavioral changes have been analyzed when a predator faces an immunological challenge.

Wasp Mortality

Exposure of wasp colonies to *B. bassiana* and *M. anisopliae*, prepared at the commercially recommended concentrations, did not impact the mortality of adults but did affect larvae. Even though the cause of death of adults and larvae was not identified, the trends observed concur with the expectations. In addition, these results agree with Espinosa-Ortiz et al. (2011) who report that larvae of *Apis mellifera* are more susceptible than adult bees. Similarly, Lord (2001) finds that adults of *Cephalonomia tarsalis* (Hymenoptera: Bethyridae) experience lower mortality (68.6%) when exposed to beetle hosts infected with *B. bassiana*, while none of the wasp larvae complete development in such hosts. However, other experiments report different results. For example, Harris et al. (2000), studying *V. vulgaris*, show no differences in mortality between larvae and adults.

The generality of these mortality results must be handled with caution as both the pathogen strain and the application method have profound effects on the results. Espinosa-Ortiz et al. (2011), for example, report mortalities between 12 and 100% for adult honey bees depending on the strain of *B. bassiana* applied. Likewise, Harris et al. (2000) report changes in larvae survival and adult longevity of *V. vulgaris* depending on the strain used of *B. bassiana* or *Aspergillus flavus*. Poidatz et al. (2018) compare different infection methods on adults of *Vespa velutina* and report that direct immersion is more efficient than other approaches, including consumption of cooked tuna infected with spores of either *B. bassiana* or *Metarhizium robertsii*. Harris et al. (2000) observe that adult longevity of *V. vulgaris* is reduced if infection occurs through dipping, compared to other infection strategies. These studies on vespids were designed to test methods of controlling predator species that have become a nuisance to human populations or ecosystem invaders, while our goal, like Espinosa-Ortiz et al. (2011), was to understand the effects of accidental infection by entomopathogens of a species that naturally exhibits positive benefits to crops. Thus, our protocol was closer to the field conditions experienced by these predators and may have provided a better picture of

field conditions. Considering both the wasp's attack preferences and larvae mortality, *M. anisopliae* appeared as a less damaging entomopathogen for the generalist predator than *B. bassiana*.

The consequences of our study towards the use of entomopathogens for biocontrol heavily rely on their field persistence (Thompson et al. 2006). Castrillo et al. (2010) report a persistence of up to 28 days for *B. bassiana* conidia, while Vänninen et al. (2000) report a three-year persistence for *M. anisopliae* and at least a year for *B. bassiana*. In our experiments, every colony was exposed to a single infection event and even though larvae mortality increased immediately after the infection, no colony died; and they remained actively producing new individuals for six weeks up to when they were sacrificed. These data suggested that the two entomopathogens may have had a temporary effect on the populations and that these populations might be at risk if a pathogen is used on a regular basis. Field testing is required to assess these predictions.

Our study is in line with previous analyses that document the capacity of social insects to detect and deter pathogens, and in our case these pathogens also change the behavior of *Polistes myersi*. Our results pointed towards a non-target effect case for a generalist predator. However, the detection and survival depend on the pathogen; foragers rejected larvae infected with *M. anisopliae* and their colonies recorded lower larvae mortality compared with *B. bassiana*. Our results showed no capacity to detect larvae infected with *B. bassiana*, and consequently higher larvae mortality was recorded in their colonies. Even though time invested in grooming is similar for both pathogens, strong differences were recorded for the frequency and duration of each behavioral repertoire. These results pointed towards a pathogen, *B. bassiana*, that passes undetected, but the latter negatively affects the colony while the other pathogen, *M. anisopliae*, is readily detected and consequently has weaker effects on a colony's survival. This study provided useful information for developing sustainable pest control, as native wasp species can be fundamental for controlling herbivore populations.

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Authors' Contributions **DM-Ch.:** conceptualization, data curation, formal analysis, research, methodology, validation, visualization, writing - original draft, writing - review and editing. **NCCC:** Colony maintenance and data curation. **CR:** conceptualization, data curation, formal analysis, funding acquisition, research, methodology. **CES:** conceptualization, formal analysis, Funding acquisition, methodology, project administration, resources, supervision, visualization, writing - original draft, writing - review and editing.

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Data Availability Authors are willing to provide data if required.

Code Availability Not applicable.

Declarations

Ethics Approval All applicable national, and/or institutional guidelines for the care and use of animals were followed. This article does not contain studies with human participants.

Consent to Participate Not applicable.

Consent for Publication All authors agree.

Conflicts of Interest/Competing Interests The authors declare that they have no conflict of interest.

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