Infestation by phorid flies disrupts behavior and immune function in honey bees monitored by radio-frequency identification

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Research Article

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Abstract

Honey bees (*Apis mellifera* Linnaeus) and global food security face increasing threats from diverse biotic and abiotic stressors. *A. mellifera*, known for its remarkable adaptability, thrives across varied environments while encountering novel challenges. In this study, we examined the effect of phorid fly infestation on *A. mellifera* under field conditions. We recorded the daily activity of honey bees, both incoming and outgoing records, in healthy and infested hives. This monitoring involved the use of radio-frequency identification (RFID) tags on individual worker bees to assess the effects of fly infestations on bee performance weekly. We also analyzed worker bee survival, additional coinfections, and immune responses including humoral and cellular parameters. Our findings revealed: (1) reduced frequency and duration of foraging trips, (2) increased nocturnal activity, and (3) elevated immune activity due to fly invasion. Here, we highlight the detrimental consequences that a phorid fly invasion can have on the health and colony performance. Although specific outcomes may vary depending on hive management practices, it is evident that colonies in which bees must allocate significant resources to safeguard their nests from intruders exhibit reduced vigor and performance. Furthermore, knowledge of phorid flies and their invasion impact on honey bees could help prevent their spread to beehives that may be easily susceptible to attack.

Keywords

Colony losses / RFID monitoring / *Apis mellifera* / *Megaselia* / Behavior / Immune system

Introduction

Over recent decades, bees and other pollinators have faced significant global declines, threatening pollinator-dependent ecosystems and food crops (Ashworth et al. 2009; Novais et al. 2016; Simmons et al. 2019; Hristov et al. 2020; Balvino-Olvera et al. 2024). Currently, managed bees continue to experience colony losses, which remain poorly understood in various parts of the world, including the tropics (vanEngelsdorp et al. 2009; Currie et al. 2010; Brodschneider et al. 2018; Gray et al. 2023; Medina-Flores et al. 2023; Lamas et al. 2024; Requier et al. 2024). These losses have been attributed to multiple factors including habitat degradation, land use change, pesticide exposure, extreme weather events, and parasitism, which are believed to synergistically alter bee performance (vanEngelsdorp et al. 2009; Perry et al. 2015; Potts et al. 2016; Roy et al. 2018).

Honey bees exhibit behaviors that significantly increase their vulnerability to parasites. Specifically, worker bees, the most numerous and active members of a colony, perform a wide range of age-related tasks essential for colony survival, including brood care, nest maintenance, defense, and foraging. Foraging bees, in particular, frequently interact with other colonies (Oliveira et al. 2021) and drone congregation areas (Ayup et al. 2021), thus increasing their exposure to parasites. This not only makes them susceptible to infection but also the likelihood that foragers act as vectors within the colony (Graystock et al. 2015; Nunes-Silva et al. 2019). Consequently, parasitic pressure can alter essential worker functions such as foraging activity, nest-related tasks, and defense-related behavior, and may compromise overall colony performance, particularly under conditions of limited floral resources (Polatto et al. 2014; Koch et al. 2017).

To counter parasites and pathogens, honey bees rely on a suite of behavioral and immune defenses. Behavioral defenses operate at the colony level, including social immunity mechanisms that mitigate pathogen spread and damage (Cremer 2019). In parallel, the immune response involves both cellular (e.g., hemocytes mediating phagocytosis and encapsulation) and humoral components (e.g., antimicrobial peptides, lysozymes, and the prophenoloxidase system) that target a wide range of invaders including viruses, bacteria, fungi, and parasitic arthropods (Gillespie et al. 1997; Kanost et al. 2004; Tang et al. 2006). Among these parasites, phorid flies have been recognized as a significant mortality factor in stingless bee colonies in Central America (Robroek and Jong 2003) and are increasingly reported as emerging threats to honey bee health across several regions,

including the United States, Italy, Saudi Arabia, Egypt, Algeria, and Central America (Core et al. 2012; Dutto and Ferrazzi 2014; Khattab and El-Hosseny 2014; Menail et al. 2016; Cham et al. 2018; Mohammed 2018; Dias de Freitas et al. 2023). Although most studies focus on the presence of these flies within honey bee colonies (see Table 1), little is known about their actual impact on colony health and defense mechanisms.

In Mexico, beekeepers have observed phorid flies during episodes of high colony mortality. In 2016, for example, the Comarca Lagunera region in northern Mexico reported the loss of approximately 3066 of 6256 hives. Similar events occurred in central (San Luis Potosí, Querétaro), southern (Oaxaca, Chiapas) and southeastern (Campeche) regions, based on personal reports from beekeepers. While the parasitic behavior of *Apocephalus borealis* is well documented (Core et al. 2012), other phorid species have also been observed infesting weak or decaying *A. mellifera* colonies (Disney 2008; Dutto and Ferrazzi 2014), and occasionally even in apparently healthy hives (Ricchiuti et al. 2016). However, no field studies have evaluated how these infestations affect bee behavior and immune function under natural conditions.

In this study, we evaluated the effect of phorid fly infestation on honey bee behavior and immune responses under field conditions. While conducting routine monitoring in ten apiaries in Mexico as part of the Global Initiative for Honey Bee Health (GIHH), led by the Australian Commonwealth Scientific and Industrial Research Organisation, we identified a case of phorid infestation in one hive (H2-infested) adjacent to a healthy reference hive (H1-healthy). We intensified monitoring of these two colonies by equipping individual worker bees with RFID tags to record their activity patterns (Susanto et al. 2018), capturing detailed records of incoming and outgoing movements. We evaluated the impact of phorid infestation by comparing survival, behavior, and immune response between hives. Immune parameters included humoral (phenoloxidase, prophenoloxidase, and lytic activity) and cellular (hemocyte counts) components, which reflect physiological condition of bees and their investment in defense (Hill 2011). We predicted that the H2-infested hive would show elevated immune activity and reduced survival, despite being managed under the same conditions as the H1-healthy hive. To control for confounding factors, we also screened for prevalent pathogens such as *Ascosphaera apis*, *Melissococcus plutonius*, and *Vairimorpha* (*Nosema*) *ceranae* in both colonies. To our knowledge, this is the first field-based study to explore the phorid fly infestation on behavior and defense in *A. mellifera* under a natural coinfection scenario.

Table 1 Reviewed works (from 2010 to 2024) about the parasitic behavior of phorid flies observed in honey bees. The slash indicates missing information in each case

Region	Phorid fly species	Bee species	Parasitic behavior	Effect of infection	Fly origin	Detection method	Parasitism percentage	Co-infections	Bee defense trait	Reference
USA (California, South Dakota)	*Apocephalus borealis*	*Apis mellifera*	Parasitoid	Behavioral manipulation, disorientation, nocturnal abandonment, death	Field / Lab	Morphological, DNA barcode (COI), rRNA	Up to 77% of sites	*Nosema ceranae,* DWV, BQCV	/	Core et al. 2012
Italy (Piedmont)	*Megaselia rufipes*	*Apis mellifera*	Parasitoid	Limited movement	Field	Morphological	23% of adults	DWV, *Varroa destructor*	/	Dutto & Ferrazzi 2014
Egypt	*Apocephalus borealis*	*Apis mellifera*	Parasitoid	Abnormal behavior, abandonment	Field	Morphological	Up to 37% of colonies	/	/	Khattab & El-Hosseny 2014
Algeria (Annaba)	*Megaselia scalaris*	*Apis mellifera intermissa*	Parasitoid	/	Field	RT-qPCR	/	DWV in bees and flies	/	Menail et al. 2016
Italy (Central-South)	*Megaselia scalaris*	*Apis mellifera*	Parasitoid	/	Field	Morphological	Up to 80% of apiaries	/	/	Ricchiuti et al. 2016
Cameroon	*Megaselia scalaris*	*Apis mellifera*	Parasitoid	/	Field	Morphological, DNA barcode, qPCR	9.2% of colonies	/	/	Cham et al 2018
Slovakia	*Megaselia spp.*	*Apis mellifera*	Parasitoid, beehive parasite (on eggs and larvae in capped cells)	/	Field	Morphological	/	Fungi	/	Sabo et al. 2020
South Korea	*Apocephalus borealis*	*Apis mellifera*	/	/	Field	RT-qPCR	1.71% of adults	*Aspergillus flavus,* ABPV, KBV	/	Truong & Yoo et al. 2023

Fig 1 Left: RFID reader installed at the entrance of each hive for data collection. Right: A worker honey bee with an RFID tag attached on the top of the thorax. As the bee passes through the only hive entrance, the tag is detected and the bee's ID, date, and time are recorded in daily CSV files

Material and methods

Study site, observations, and sampling

This study was conducted in July and August 2016 at an apiary located in Los Tigrillos, Michoacán (19° 41' 58.68" N, 101° 0' 2.46" W) within a pine-oak forest at an elevation of 2023 m.a.s.l. Two hives were intensively monitored: H1 represented the healthy hive not infested by flies (and no other parasites), and H2, the hive colonized by phorid flies. Both hives were located nearby in the same apiary. The H1-healthy hive was monitored for eight consecutive weeks, while the H2-infested hive was monitored for five consecutive weeks (until it collapsed). In the H2-infested hive, phorid eggs, larvae, and pupae were directly collected from naturally parasitized bees at different developmental stages. Adult worker bees and pupae (aged 12 - 14 days) were sampled from both hives. The brood frames and the collected samples were transported to the laboratory and stored at -70ºC until use.

Taxonomic and molecular classification of Phorid flies

Phorid flies were classified based on morphological traits described by Disney (1994, 2008) and by molecular barcoding using the cytochrome oxidase I (COI) DNA sequence, that distinguishes between Phoridae species (Boehme et al. 2010). The genomic DNA of 21 individual adult flies was extracted using Doyle's (1991) protocol, modified by adding 1% PVP in the extraction buffer. Samples were incubated with 2.5 µL Proteinase K (20 mg/mL per 500 µL extraction buffer), for 3 h at 65ºC with constant agitation. The COI gene was amplified in

a 12.5 µL reaction using the QIAGEN Multiplex PCR Kit with primers LCO1490 and HCO2198 (Folmer et al. 1994), and the PCR conditions adapted from Boehme et al. (2010), including an initial activation step of 15 min at 95ºC for the HotStarTaq DNA polymerase. All PCR reactions were performed on a Mastercycler® nexus gradient (Eppendorf). PCR products were analyzed using the QIAxcel Advanced system-(QIAGEN), where a ~740 bp fragment was determined using the Screening Kit (QIAxcel-QIAGEN) and method OM400 described in the QIAxcel DNA Handbook (sample uptake 10 s at 5 kV, separation 400 s at 6 kV). Amplicons were sequenced in both directions by Macrogen (Seoul, Korea), and assembled using Sequencher.

DNA extraction and detection of parasites

The thorax of adult bees and entire pupae were homogenized in liquid nitrogen in pools of 10 individuals, (adults and pupae processed separately). We obtained seven pools for the H1-healthy hive and twelve for the H2-infested hive. Each pool's DNA was extracted using the CTAB method (cetyltrimethylammonium bromide) with 1% PVP and 0.01% β-mercaptoethanol. Samples were incubated with 2.5 µL Proteinase K for 1 h at 65ºC (600 rpm). The extracted DNA was quantified using an Eppendorf Biospectrometer® basic. The prevalence of parasites such as microsporidian *Vairimorpha* (*Nosema*) *ceranae* in adults, *Melissococcus plutonius*, and *Ascosphaera apis* in pupae was assessed to determine the proportion of positive pools. Each pathogen was targeted with a single diagnostic primer (see Table 2 for references and PCR conditions).

Table 2 Prevalence of the parasites *Ascosphaera apis*, *Melissococcus plutonius*, and *Vairimorpha* (*Nosema*) *ceranae* expressed as the percentage of positive pools in adult worker bees and pupae of the H1-healthy hive and the H2-infested hive. The dashes mean that those parasites were not tested at that developmental stage. Primers references: [a]Garrido-Bailón et al., 2013; [b]Martín-Hernández et al., 2007

			Parasites prevalence (% of positive pools)		
Hive	**Age**	**No. Pools (No. bees)**	***Ascosphaera apis*[a]**	***Melissococcus plutonius*[a]**	***Vairimorpha* (*Nosema*) *ceranae*[b]**
H1-healthy	**Pupae**	7 (35)	0	0	-
	Adults	6 (30)	-	-	100
H2-infested	**Pupae**	12 (60)	0	0	-
	Adults	13 (65)	-	-	0

Bee activity

As part of the Global Initiative for Bee Health (GIHH) implemented by the Australian Commonwealth Scientific and Industrial Research Organisation (CSIRO; https://www.csiro.au/en/news/All/News/2015/August/Honey-Bee-Health), the activity of the bees (incoming and outgoing records) was monitored using radio-frequency identification (RFID) tags that were glued to the thorax of adult bees with superglue (see: shellac has produced better results (Toppa et al. 2020). The tag's size was 2.5 x 2.5 x 0.4 mm; each tag weighed 2.4 mg (Hitachi Chemical, Japan) and allowed the recording of a unique bee identification number in hexadecimal format (de Souza et al. 2018). One hundred tags per hive (H1-healthy and H2-infested) were tagged weekly over eight consecutive weeks. An RFID reader (USB Desktop RU-824) installed at each hive's entrance recorded the daily tags' signals, capturing data on each individual bee's movements (Fig 1).

The RFID system was autonomously controlled by an Edison Intel Atom Processor microcomputer (1 GB memory, 5 GB storage, Wi-Fi, and Bluetooth enabled), powered by solar cells. Data were wireless downloaded weekly. The system recorded each tagged bee's unique ID upon passing through the reader. Activity was analyzed according to four behavior categories suggested by Susanto et al. (2018): 1) By the entry (BTE) or successive readings of the same bee within less than three minutes indicates that this particular bee is near the RFID reader, 2) Short mission (SM) or the individual bee engaged in an activity of short duration (between three and six minutes), for example, making orientation or defecation flights and inspecting the hive surroundings, 3) Foraging (FG) or the individual bee engaged in activities to search for or exploit food/water sources (6 min to more than 6 h), example bee roles undertaking such activities are scout, exploiter, recruit, water carrier, and 4) Departed bee (DB), the last detection of a bee during its lifetime. Daily activity was categorized according to the day and nighttime it was recorded: 1) Sunrise, from 7 a.m. to 12 a.m., 2) Sunset, from 12 a.m. to 7 p.m. (Susanto et al. 2018), 3) Night, from 7 p.m. to 12 p.m., and 4) Midnight, from 12 p.m. to 7 a.m. The activity duration was considered as the difference in time between one reading and the subsequent reading.

Immune response and health parameters

A sample of the homogenized tissue of adults and pupae was used to assess immune system parameters including phenoloxidase activity (PO), prophenoloxidase activity (proPO), and lytic activity. Each sample (30 mg) was homogenized in 400 µL of PBS (Phosphate Buffered Saline, pH 7.4, Sigma), vortexed, and centrifuged

(17,000 xg for 15 min). The supernatant was used for subsequent protein and immune analyses (Nicoletti et al. 2020). Total protein content was also measured as a proxy of nutritional condition (Contreras-Garduño et al. 2007; Lee et al. 2008; Nicoletti et al. 2020; Burciaga et al. 2023).

Prophenoloxidase (proPO) and phenoloxidase (PO) activity

The PO activity in adults and pupae from each hive was quantified by the oxidation of *L*-dihydroxyphenylalanine (L-DOPA; Sigma-Aldrich) to dopachrome (Contreras-Garduño et al. 2007). The proPO is the inactive zymogen precursor to PO, an important enzyme to innate immune function that results in the formation of melanin (Wilson-Rich et al. 2009), which leads to the cuticle sclerotization, wound healing, and cellular defense responses against infectious agents (Ratcliffe et al. 1984; Söderhäll and Cerenius 1998; Chan et al. 2009; Laughton et al. 2011). For each reaction, 50 µL of sample, 40 µL PBS, and 10 µL of L-DOPA solution (4.0 mg/mL) were added to a 96-well plate. Absorbance was read every 3 min for 60 min at 490 nm in a Multiskan GO Microplate reader (Thermo Scientific). In each well of a 96-well microplate, 50 µL of the sample, 40 µL of PBS, and 10 µL of L-DOPA solution were added. Fifty µL of PBS was added to the control wells instead of the sample. Each plate was read at 490 nm every 3 min for 60 min in a Multiskan GO Microplate Spectrophotometer (Thermo Scientific) after 20 min of incubation at room temperature. Due to the cytotoxic nature of the products in the melanization cascade, PO is commonly stored in the inactive precursor proPO and activated naturally following the recognition of foreign compounds or artificially by using chymotrypsin that activates PO production (Ratcliffe et al. 1984; Söderhäll and Cerenius 1998; Laughton et al. 2011). Thus, proPO activity was activated using 2 µL of α-chymotrypsin (0.25 mg/mL) as an artificial activator (Ratcliffe et al. 1984; Söderhäll and Cerenius 1998; Eleftherianos et al. 2006; Laughton et al. 2011), and 38 µL of PBS, instead of 40 µL. The PO and proPO activity were expressed as the slope of the absorbance curve during its linear phase (Nicoletti et al. 2020).

Lytic activity

To quantify lytic activity for each sample, 30 µL of each sample was added to 200 µL of a bacterial suspension (72 mg of lyophilized *Micrococcus lysodeikticus* in 20 mL PBS). Absorbance at 490 nm was recorded every 3

min for 60 min following a 20-min incubation at room temperature. Activity was calculated as the rate of OD reduction over time (Kortet et al. 2007).

Total hemocyte counts

Hemolymph was extracted from anesthetized worker bees by puncturing between abdominal segments III and IV (Chan et al. 2006). Approximately 5 µL per bee was collected in a glass capillary and transferred to a PCR tube containing 5 µL of trypan blue 0.4% in a 1:1 ratio. For total hemocyte counts, samples were transferred to dual chambers of a counting slide. Cell counts and viability were determined using the TC10 automated cell counter (Bio-Rad) following manufacturer protocols.

Protein content

The total protein content in adults and pupae bees was determined using the Pierce BCA Protein Assay Kit (Thermo Scientific). For each sample, 5 uL was mixed with 45 uL of PBS and 150 uL of BCA reagent (A:B were placed in a radius of 50:1). Absorbance at 562 nm was measured after 20 min incubation at room temperature using a Multiskan GO Microplate reader (Thermo Scientific). The total protein content (µg/5 µL) was estimated using a standard BSA calibration curve.

Statistical analyses

Statistical analyses were performed using RStudio (version 2023.12.1+402). Chi-square tests were used to compare incoming and outgoing records, behavior category, and daytime-scale frequencies. T-tests were used to compare activity duration between hives. Generalized Linear Models (GLMs) were applied to assess immune parameters by hive and age. Data transformations were applied where necessary according to model assumptions, with proPO, PO, and lytic activity data adjusted for Gamma distribution by adding ‘1’ to each value to manage zeros in the data set. Prevalence of parasites was analyzed by comparing the proportion of positive samples between the hives, and Levene's test was used to assess variance homogeneity.

Results

Taxonomic and molecular classification of phorid flies

Adult phorid flies were classified based on morphological traits as outlined by Disney (1994, 2008). The specimens exhibited characteristic morphology of small flies (~2.5 mm), including a humpbacked torax, laterally flattened hind femora, and distinctive wing venation indicative of the genus *Megaselia* (Fig 2). Confirmation of their taxonomic classification was achieved through cytochrome oxidase I (COI) gene sequencing, which matched *Megaselia* sequences in the NCBI BLAST database (Online Resource 1).

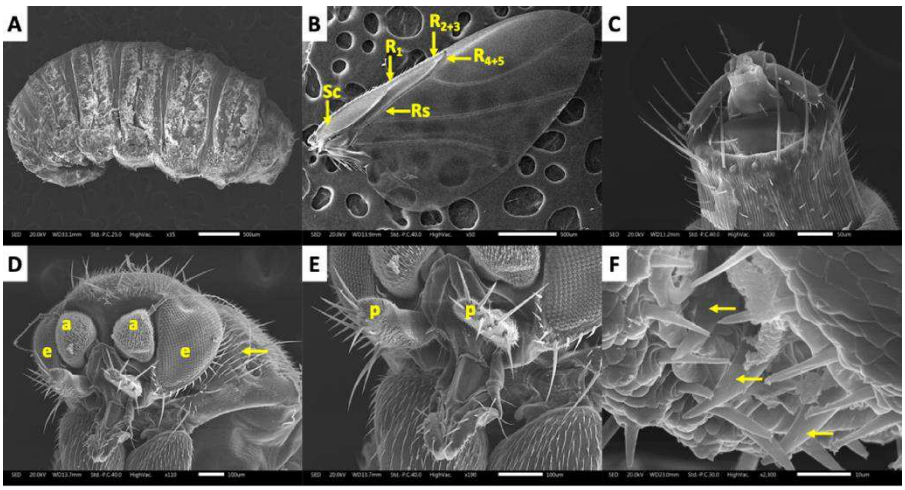

Fig 2 Morphological characteristics of *Megaselia sp.* by SEM micrographs. A: Entire body view of a third-instar larva. B: Wing venation highlighting radial veins (R), radial sector (Rs), and subcostal (Sc). C: Apex of the female terminalia. D: Propleura with numerous scattered hairs (yellow arrow), compound eyes (e), and antennae (a). E: Maxillary palpi (p). F: Mouthparts of *Megaselia sp.*, ventrolateral view of labellum showing large, sharply pointed teeth (yellow arrows)

Observation of parasitic behavior in honey bee hives

In the field, *Megaselia* larvae were observed on both adult bees and brood. Eggs, larvae, pupae, and adult phorids were observed in the brood frames of the H2-infested hive, whereas only larvae were observed on the hive's stored food (Fig 3). The density of *Megaselia* was estimated at 318 larvae/10 cm^2 and 291 pupae/10 cm^2, averaging four pupae per cell. The eggs were rare on the adult bees and were laid mainly on capped and

uncapped brood cells. The developmental stages observed suggest that the ovipostion occurred approximately three weeks prior, while the bees were still alive. No phorid flies were detected in the H1-healthy hive.

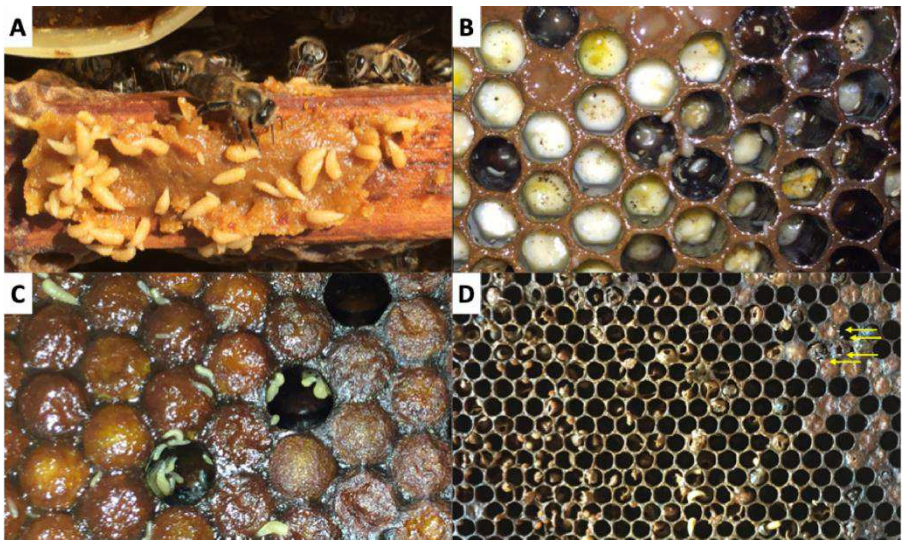

Fig 3 The H2-infested hive with *Megaselia sp.* eggs, larvae, and pupae. A: Phorid fly larvae feeding on *Apis mellifera* food. B: Phorid fly larvae of different ages feeding on *Apis mellifera* larvae. C: Phorid fly larvae on capped brood cells. D: Frame of the H2-infested hive showing the remains of *A. mellifera* and *Megaselia sp.* after infestation (yellow arrows indicate some hatched eggs of phorid fly)

Bee activity

The bees of the H2-infested hive recorded a significantly higher number of incoming and outgoing RFID detections (n = 77380; 93.1 %) compared to the H1-healthy hive (n = 5762; 6.9 %) (χ^2 = 53344, df = 7, p < 0.0001; Fig 4, Table 3). Weekly comparisons showed similar numbers of detected bees in W1 (χ^2 = 2.79, df = 1, p = 0.09), W3 (χ^2 = 3.31, df = 1, p = 0.07), W4 (χ^2 = 0.29, df = 1, p = 0.59), and W5 (χ^2 = 0.33, df = 1, p = 0.56). However, during W2, the H1-healthy hive displayed a significantly higher activity level than the H2-infested hive (χ^2 = 10.13, df = 1, p = 0.001). By W5, the H2-infested hive had collapsed following the onset of *Megaselia sp.* oviposition in W2, leading to a subsequent surge in larvae activity and pupation in W3. Post-collapse, the H1-healthy hive showed increasing bee activity, whereas the H2-infested hive recorded no further activity.

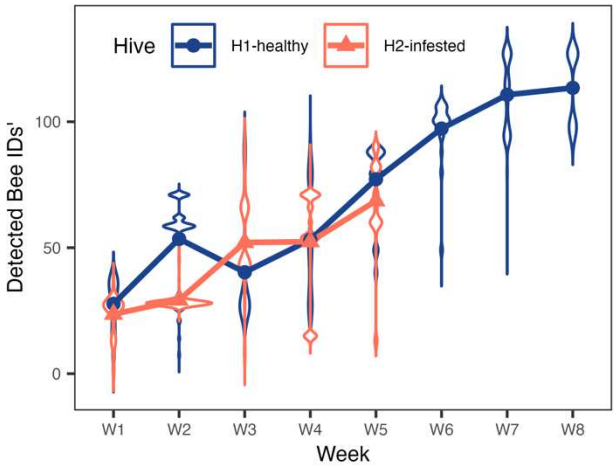

Fig 4 Violin plot with error bar shows the honey bee IDs detected weekly during monitoring. Both H1-healthy and H2-infested hives had a similar number of detected honey bees, except for week W2 when the H1-healthy hive (blue circles) had a higher number of active honey bees than the H2-infested hive (orange triangles)

Table 3 Total filtered readings, weekly counts of RFID-detected honey bees, and behavioral category frequencies observed in H1-healthy and H2-infested hives. Statistical results include Chi-square values, degrees of freedom, and *p* values

	H1-healthy	**H2-infested**	**χ^2**	**df**	***p* value**
Total filtered readings	5762	77380	53344	7	< 0.0001
Number of bees detected by week					
W1	10	19	2.79	1	0.09
W2	25	7	10.13	1	0.001
W3	19	32	3.31	1	0.07
W4	14	17	0.29	1	0.59
W5	5	7	0.33	1	0.56
W6	20	0	20.00	1	< 0.0001
W7	26	0	26.00	1	< 0.0001
W8	9	0	9.00	1	< 0.0001
Bees' activity (behavior scale)					
BTE	4071	76657	65265	1	< 0.0001
SM	173	297	24.9	1	< 0.0001
FG	1433	373	622.2	1	< 0.0001
DB	85	53	7.4	1	0.006

Bee behavior categories also differed between hives ($\chi^2 = 16454$, df = 3, $p < 0.0001$). The H2-infested hive had more short activities "by the entry" (BTE; $\chi^2 = 65265$, $p < 0.0001$) and "short mission" (SM; $\chi^2 = 24.9$,

p < 0.0001) behaviors (76657 and 297 detections for H2 *versus* 4071 and 173 detections for H1), while the H1-healthy hive showed more “foraging” (FG; $\chi^2 = 622.2$, p < 0.0001) and “departed bee” (DB; $\chi^2 = 7.4$, p = 0.006) behaviors (53 and 373 detections for H2 -infested hive *versus* 85 and 1433 detections for H1-healthy hive, respectively) (Table 3).

There was also a significant difference in behavior duration between each category (F = 7.04, p < 0.0001). BTE and FG behaviors were significantly shorter in the H2-infested hive than in the H1-healthy hive (p < 0.0001, Fig 5). Regarding the daily activity schedule, it was observed that the H1-healthy hive was active during the "sunrise" and "sunset" daytime categories, having a longer activity duration during the "sunset" (t = -13.11, p < 0.0001). For hive H2, two additional daytime categories were necessary since it was also recorded during the "night" and "midnight". At "midnight", the activity duration was short and was only recorded in weeks W4, and W5, while the "night" included longer trips in week W3 (t = -35.54, p < 0.0001) and short trips in weeks W4 (t = 3.73, p < 0.0001), and W5 (Fig 6).

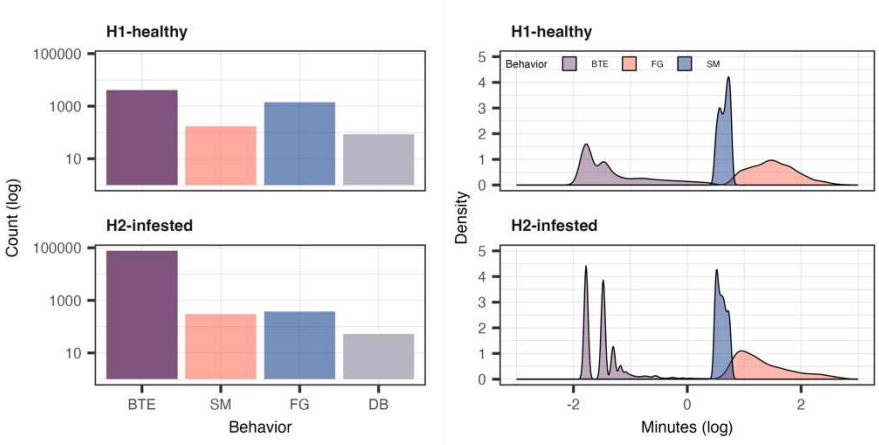

Fig 5 Behavioral activity over eight weeks in the H1-healthy and H2-infested hives. The figure shows a histogram with the number of behaviors (log of count) and a density plot with each behavior's duration (log of minutes): BTE; By the entry, DB; Departed bee, FG; Foraging, and SM: Short mission. The H2-infested hive had more BTE and SM behaviors and fewer DB and FG behaviors than the H1-healthy hive. The BTE and FG behaviors had a shorter duration in the H2-infested hive than in the H1-healthy hive. DB behavior is excluded from the density plot as it represents unique detections without a duration. The log scale was required to display numerical data over a very wide range of values compactly

Fig 6 Frequency of bee activity at different daytime for both hives. The H1-healthy hive was active at sunrise and sunset, while the H2-infested hive was active at sunrise, sunset, night, and midnight

Immune response and health

Molecular analysis by end-point PCR using specific diagnostic primer showed that adult worker bees and pupae pools from the H2-infested hive had a 0 % prevalence of the *Ascosphaera apis*, *Melissococcus plutonius*, and *Vairimorpha (Nosema) ceranae*. In contrast, the H1-healthy showed no detection of *A. apis* and *M. plutonius* in pupae, but it presented a 100 % prevalence of *V. ceranae* in adult worker bee pools, indicating that at least one individual per pool was infected (Table 2). Bees from H1-healthy and H2-infested hives had a similar protein content (GLM, $\chi^2 = 1.0$, $p = 3.17$), suggesting that the physiological condition of bees at the time of sampling was comparable, regardless of whether the colony was infested with phorid flies.

Immune assays showed significantly higher proPO (GLM, $\chi^2 = 13.98$, $p < 0.0001$) and PO activity (GLM, $\chi^2 = 22.05$, $p < 0.0001$) in h2-infested hive compared to H1-healthy hive. However, lytic activity was higher in the H1-healthy hive (GLM, $\chi^2 = 11.84$, $p < 0.001$; Fig 7). In all cases, the age of the bees did not significantly affect PO ($\chi^2 = 0.008$, $p = 0.927$), proPO ($\chi^2 = 0.0001$, $p = 0.999$), or lytic activity ($\chi^2 = 1.71$, $p = 0.19$). Total hemocyte counts (cells/mL), were significantly higher in the H1-healthy hive ($\chi^2 = 145.22$, $p < 0.0001$), whereas the number of viable hemocytes did not differ between hives ($\chi^2 = 0.95$, $p = 0.33$; Fig 7).

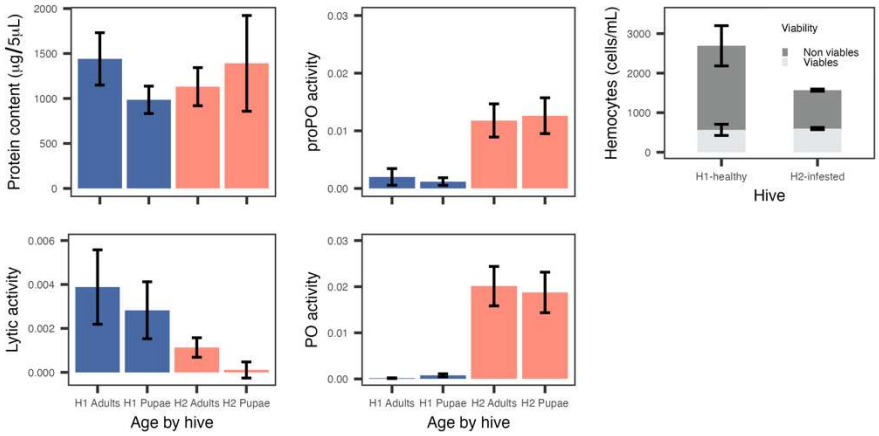

Fig 7 Humoral and cellular immunity of honey bees from H1-healthy and H2-infested hives. Protein content was similar between adult honey bees and pupae from both hives. However, the phenoloxidase (PO) and prophenoloxidase (proPO) activity were significantly higher in pupae and adult honey bees from the H2-infested hive. In contrast, the lytic activity was significantly higher in pupae and adult honey bees from the H1-healthy hive. Honey bees in the H1-healthy hive had the highest total hemocyte count, with no significant difference in the number of viable hemocytes between both hives

Discussion

Bees are generally more affected by macro than microparasites. Boomsma et al. (2005) analyzed the relative interactions between social hosts (bees, wasps, ants, and termites) and ten different parasites and diseases, showing that, after mites, dipterans are the most common. Phorid flies, in particular, are a highly diverse dipteran group, often associated with social insects. Although their initial interactions are primarily detritivores or commensals (Hölldobler and Wilson 1990), increased population density or reduced food availability can trigger parasitic behavior (Wojcik 1994, Robroek and Jong 2003). Several recent reports have documented the presence of phorid flies in *A. mellifera* colonies (see Table 1). However, the physiological and behavioral impacts of these parasites on honey bees remain largely unexplored and are often underestimate. Although Cham et al. (2018) and Ricchiuti et al. (2016) provided preliminary evidence of *Megaselia* infestations in honey bees, no detailed studies have evaluated how phorid flies affect the honey bee's behavior and defense under field conditions. Like other Phoridae species, *Megaselia* larvae can feed on bee tissues and hemolymph, potentially reducing the longevity of bees and impairing overall colony performance (Abou-Shaara and Darwish 2021). Our findings are the first to describe this interaction in detail, evaluating the effect of *Megaselia* fly invasion markedly alters bee activity patterns and honey bee colony condition.

The H2-infested hive showed increased short-duration activities (BTE and SM), shorter foraging trips (FG), and fewer departed bee events (DB) compared to the H1-healthy hive, possibly due to increased hive cleaning activity and short trips for essential functions such as excretion, resource exploration, and feeding in nearby points (Susanto et al. 2018). In addition, a decrease in honey bees possibly altered the caste structure within the H2-infested hive. In this case, foraging bees, which normally make long trips in search of food, may be forced to stay and defend the colony. A similar phenomenon was found by Mehdiabadi and Gilbert (2002) in the phorid fly *Pseudacteon tricuspis* that decreased in half the worker population and reduced foraging rates for the social ant *Solenopsis invicta* (fire ants). Feener and Brown (1992) reported a similar result where the *Pseudacteon spp.* phorid flies can even reduce the foraging rate (by up to 50%) in the ant *Solenopsis geminata*. Similarly, Bragança et al. (1998) showed that relatively few leafcutters' ants *Atta sexdens* were attacked by the phorid fly *Neodohrniphora sp.*, but the presence of flies caused the outgoing ants to return to the nest to defend the colony, causing a significant reduction in the number of foragers.

The *Megaselia sp.* invasion also affected the daily activity. H1-healthy hives had activity during sunrise and sunset, while the H2-infested hive was active during sunrise, sunset, night, and midnight. Similar behavior has been observed in the ant *Pheidole titanis*; the foraging workers stop foraging during the day because they defend themselves from the oviposition attacks of parasitic phorid flies (Fenner 1988). In this case, ants forage at night when flies do not interfere and take turns in their activity times to avoid parasitoids (Fenner 1988). Core et al. (2012) associated the nocturnal activity of bees infested by phorids with hive abandonment, and they observed that bees that left the hive at night had higher parasitism than those that left during the day. They also propose that the phorid *A. borealis* could have manipulated the bees to the point of changing their circadian rhythm.

The activity duration in the H2-infested hive was short at "midnight" and was only recorded in the weeks W2, W4, and W5, while the "night" time included longer trips in W3 and short trips in W4 and W5. Accordingly, it has been reported that *Megaselia* has nocturnal oviposition (Zulaikha and Zuha 2016), which may have influenced the activity pattern of the H2-infested hive. Susanto et al. (2018) suggest that occurrences after sunset and before sunrise are likely associated with bees fitted with RFID tags that died inside the hive and were transported out by worker bees. The high hygiene habits of Africanized bees (Aumeier et al. 2000) and the

nocturnal oviposition of flies in bees probably caused healthy worker bees to prioritize defense over foraging. In this way, they spent the night removing infested bees and phorid eggs or pupae, cleaning and defending the hive, leading to more reads by repeatedly passing near the RFID reader. In addition, the bees could sometimes alter their activity at high temperatures (Rowel 1986; Ayup et al. 2021). According to the climatic data from the nearest meteorological station reported by CONAGUA-DGE (08/14/2019), this could not have influenced our results since the maximum temperature did not exceed 26ºC and the minimum temperature did not drop below 9ºC during the monitoring time of H1-healthy and H2-infested hives.

Regarding the number of bees registered daily, the H1-healthy hive and the H2-infested hive recorded similar bee IDs per week, except in W2, when fewer detected IDs were found in the H2-infested hive. This period coincided with the oviposition time estimated for phorid flies in this hive. This lower number of detected IDs in W2 could be explained by the fact that most bees were kept captive inside the hive because they spent their time cleaning cells, removing dead bodies and *Megaselia sp.* eggs, instead of going out to forage. It is reasonable to believe that the infested hive had weaker bees or a lower population density than the healthy hive. This could have favored the invasion and subsequent collapse of the H2-infested hive.

Nevertheless, the total protein concentration was similar in the bees from both hives, and the proPO and PO activity was much higher in the bees from the H2-infested hive. Throughout the monitoring, the bees from both hives were managed under the same conditions and had access to the same food sources. As mentioned, the number of detected IDs was similar between both hives in the first weeks. For this reason, it is possible that neither the bees' condition nor the population density of the hives could predispose to the collapse of the H2-infested hive, nor did it influence the bees' ability to respond to the invasion or mount a defense against parasitoids. Although in some cases, phorid flies can be found on honey bees that are dead, dying, or affected by other diseases (Dutto and Ferrazzi 2014), in our study, we suppose that the coinfections did not predispose the H2-infested hive to collapse, with a zero prevalence of three of the most common parasites, *M. plutonius, A. apis,* and *V. ceranae*, which causes the European foulbrood, chalkbrood, and nosemosis diseases, respectively.

It is known that the investment in immune defense should be higher in hosts exposed to higher parasite pressure, and how the host resists parasitoids is by activating the serine protease cascade for the melanization of foreign bodies (Pennacchio and Strand 2006). Many hatched *Megaselia* eggs and larvae in the hive posed a

threat to larvae, pupae, and adult bees, and the absence of coinfections with *A. apis*, *M. plutonius*, and *V. ceranae* allows us to suppose that the higher proPO and PO activity in the H2-infested hive was a response to the parasitoid infestation. Meanwhile, the higher lytic activity and total hemocyte count in the H1-healthy hive may be part of the response against the 100% prevalence of the *V. ceranae* in the H1-healthy hive that was not present in the H2-infested hive. It is possible that bees of the H1-healthy hive activated the lytic activity to confront the microsporidium *V. ceranae* since it is known that microbial components of bacteria, fungi, or microsporidia can activate the expression of lysozyme and other antimicrobial peptides (Cotter et al. 2004). The bees of the H1-healthy hive probably activated a greater production of total hemocytes to counteract the presence of *V. ceranae* but allocated more resources towards humoral defense (lytic activity) than to cellular defense (in terms of the production of viable hemocytes).

The coinfections in social insects are not yet well understood, but it is known that larvae and adult phorid flies can be infected within the hive with some parasites that initially attack bees. Core et al. (2012) found that the parasite *V. ceranae* had a prevalence of 50% in adult phorids and 87.7% in larvae, while the deformed wing virus (DWV) had a prevalence of 25% in adult phorids and 75% in larvae. This suggests that phorids may selectively avoid hosts already harboring coinfections, because they can detect healthy or infected bees in a hive (i.e., via specific volatiles), selecting hives free of other parasites before invading them. Richard et al. (2008) showed that experimentally infected bees with lipopolysaccharides significantly expressed different cuticular hydrocarbon profiles compared to healthy bees, emitting specific volatiles that other parasites might detect. In our case, a previously infected hive with a parasite may not represent a potential quality source for the phorids. Therefore, although our two studied hives were near each other, it is possible that the H1-healthy hive was already infected by the parasite *V. ceranae,* and the phorids selectively avoided this hive and instead infected the hive free of other parasites, selecting a better-quality food source to develop their offspring. In this sense, crops such as corn, beans, squash, and avocado were found near the apiary so that possible pesticide exposure could influence the collapse of the H2-infested hive. This was not recorded, and we could not make a direct association. However, we can intuit that in this case, both hives could show an alteration in their behavior associated with pesticides or some other factor, not only with exposure to the phorid. Hence, our data suggest for the first time that phorids could induce changes in the daily activity pattern of bees.

Finally, several phorid species can be considered as an emerging threat that may also affect native bees (Core et al. 2012). More attention should be given to a potential increment of infestations by phorid flies in honey bees. For beekeepers, early detection of parasites is crucial for maintaining a healthy and productive apiary, and the infected hives are constantly monitored and treated until the hive either collapses or is free of parasites; however, the healthy hives are monitored quickly or less frequently. Our study suggests that these healthy hives may be vulnerable to the attack of phorid flies that might be capable of specifically searching for hives free of other parasites because they represent a better food source. Therefore, we emphasize the importance of giving special attention to the management of healthy hives and not taking for granted their good condition over time. Understanding the interactions between honeybees and their parasites is crucial for developing effective strategies to mitigate the threats faced by these vital pollinators. The information presented in this study sheds light on the consequences of phorid fly invasion and expands our knowledge of honeybee health and hive sustainability. New research and intensive monitoring that utilizes emerging technologies are needed to identify potential treats to pollinators.

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Author contribution

Conceptualization: GRG and MQ; methodology (field and laboratory work): GRG, VPC, ODC, MJAA, and FJBO; formal analysis and investigation: GRG, VPC, ODC, MJAA, FJBO, MQ and PdS; writing original draft preparation: MQ and GRG; writing review and editing: GRG, VPC, ODC, MJAA, and FJBO, MQ and PdS;

funding acquisition: MQ. All authors contributed to the manuscript writing, review and approved the final version.

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Data availability

Data will be available from the corresponding author on reasonable request.

Declarations

Conflict of Interest Statement

The authors declare no competing interests.

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