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IMMUNOLOGY AND HOST-PARASITE INTERACTIONS - ORIGINAL PAPER

The nature of the arena surface affects the outcome of host-finding behavior bioassays in *Varroa destructor* (Anderson & Trueman)

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Abstract

Varroa destructor, an acarian parasite of the Western honey bee *Apis mellifera* L., is a serious threat to colonies and beekeeping worldwide. The parasite lifecycle occurs in close synchrony with its host development. The females have to discriminate between different developmental stages of the host and trigger an appropriate behavioral response. Many studies have focused on these behavioral aspects, whether it is the choice of a precise host stage or the reproduction of female mites. Behavioral tests often require laboratory settings that are very different from the mite's environment. Our first experiment was designed to study the impact of the surface of test arena on the mite behavior. We found that plastic from Petri dishes commonly used as test arenas disturbs the female mites and can cause death. We searched for a substrate that does not harm mites and found that gelatin-coated plastic Petri dishes responded to these expectations. We then investigated the host choice behavior of phoretic mites confronted with larval stages of the bee on gelatin-coated arenas to watch if the well-documented orientation towards 5th instar larva was observable in our conditions. Pupal stages were included in the host choice experiments, initially to act as neutral stimuli. As white-eyed pupae were revealed attractive to the mite, several pupal stages were then included in a series of host choice bioassays. These additional experiments tend to show that the positive response to the white-eyed pupa stage depends on cues only delivered by living pupae. Further investigation on the nature and impact of these cues are needed as they could shed light on key signals involved in the parasite lifecycle.

Keywords *Varroa destructor* · Host choice behavior · Honey bees · White-eyed pupae · Gelatin arenas

Introduction

Varroa destructor Anderson & Trueman, 2000 is an acarian parasite of the Western honey bee *Apis mellifera* Linnaeus, 1758. It is one of the major modern pests in beekeeping and is the cause of important ecological and economic losses each year, by its direct impact on honey bees and consequently by its impact on pollination and agriculture (Beetsma 1994; Losey and Vaughan 2006; Gallai et al. 2009; Smith et al. 2013). In order to better understand the impact of the mite, its lifecycle has been studied and described in detail by many authors (Ifantidis 1983; Donzé and Guerin 1994; Donzé et al. 1996; Rosenkranz et al. 2010; Nazzi and Le Conte 2015) and is known to be closely synchronized with the development of the host (Frey et al. 2013).

For the parasite to achieve synchronization with its host, precise behavioral responses to several host cues are required. In the case of *V. destructor*, temperature (Le Conte and Arnold 1987), cell size (Maggi et al. 2010), or the kairomones released by the bees at different stages of their development (Le Conte et al. 1989) are involved. Many studies have more specifically focused on cues likely used in the host stage selection and recognition by the parasite. Indeed, *V. destructor* females must discriminate between honey bee larval and adult stages from different casts to complete their lifecycle (Nazzi and Le Conte 2015). Larval food, cocoons, worker or drone

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brood and adult nurses are all well-known to attract the parasite (Le Conte et al. 1989; Donzé et al. 1998; Nazzi et al. 2001, 2004; Pernal et al. 2005) whereas queen pheromones, adult foragers, and royal jelly—found in important quantities in queen cells—seem to have the opposite effect (Kraus 1990; Pernal et al. 2005). Further studies on adult bee stages showed that the mite is also able to distinguish between nurse bees and other adult stages such as foragers or newborn bees (Del Piccolo et al. 2010).

Regarding the larval stages, a specific attention has been brought to the larvae from the 5th instar because mites in natural conditions were to our knowledge never found on the first to fourth larval stages, whether in male or female brood (Rembold et al. 1980; Ifantidis 1988; Boot et al. 1992). The positive response towards 5th instar larvae is thought to be based on temperature and a cocktail of cuticular compounds emitted by the larvae, although no consensus has been reached on the nature of these compounds (Le Conte and Arnold 1988; Le Conte et al. 1989; Rickli et al. 1992; Trouiller et al. 1992; Boot et al. 1994; Trouiller and Milani 1999; Calderone and Lin 2001; Pernal et al. 2005). Fatty acid esters have been pinpointed but those eliciting attraction differ between studies (Le Conte et al. 1989; Rickli et al. 1992) and are sometimes not sufficient to trigger clear orientation behavior from the parasite (Rosenkranz 1993; Pernal et al. 2005).

It should be noted that, to this date, most of the host-finding bioassays have been performed in propylene or Plexiglas arenas over limited periods (Le Conte et al. 1989; Rosenkranz 1993; Aumeier et al. 2002) that may not be suitable for the parasite to express its orientation behavior. Indeed, mites do not experience plastic surface in the colony and this could lead to biases, especially when the tests are run over longer periods. Being able to conduct experiments in more adapted arenas could bring complementary information about the host selection decision. The first objective of this study consisted in searching for an artificial experimental arena with the least detrimental effect on *V. destructor*. Bee wax is the most natural surface for such tests but the impossibility to control hydrocarbons naturally present in the wax and the complexity to wash and clean waxed arenas makes it difficult to use it in laboratory bioassays. Gelatin is a surface known to simulate wax in 12-day long rearing experiments without any noticeable side effect on mites or bees (Nazzi and Milani 1994; Piou et al. 2016, 2018; Egekwu et al. 2018). Gelatin-coated arenas were successfully tested in our study.

Using these new gelatin-coated arenas, the second objective was to test if the parasite orientation towards the 5th instar larval stage highlighted in the literature was also observable in our conditions. In these bioassays, pupal stages were included. As they are putatively less attractive to the mite, they were used as neutral control and we expected mites to choose 5th instar over pupal stages. Unexpected results—namely the higher attraction of white-eyed pupae compared with late 5th instar larvae—led us to extensively test the host choice behavior of *V. destructor* with several pupal stages. To assess the sensitivity of the host choice, we differentiated early 5th instar larvae from open brood cell and late 5th instar larvae from sealed cells and tested the parasites when confronted with series of combinations of three successive bee developmental stages from the early 5th instar larva to the emerging bee.

To further understand the response to the white-eyed pupal stage, we then investigated if physical and chemical cues played a role, by using dead pupae. Physical cues associated with the vitality of the bees, like breathing, vibrations, and emission of heat or volatiles, are lost once the pupa is dead. However, freeze killing does not alter the attractive cuticular hydrocarbons (Rosenkranz 1993; Pernal et al. 2005) and is often a preliminary step of cuticular extraction protocols (Boulay et al. 2000; Ayasse et al. 2003). When the choice of *V. destructor* is mainly based on cuticular hydrocarbons, as it is the case for adult nurses (Pernal et al. 2005; Del Piccolo et al. 2010), it remains observable for hours after the death of the bee. In our study, a loss of response in dead pupae would mean that cuticular hydrocarbons would not elicit the parasite choice for the pupal stage on their own.

Material and methods

All of our observational studies and in vitro bioassays were conducted according to the current European laws for scientific research. The experiments started in 2013 and were conducted during spring, summer, and autumn using bees and mites from eight Buckfast colonies bought from local beekeepers. The colonies were maintained on the university campus (INU Champollion, Albi, South of France). They were occasionally fed sugar syrup and pollen and were untreated so *V. destructor* infestation remained high throughout the time. The experiments were repeated over the following 2 years using new sets of eight Buckfast colonies.

Varroa sampling

Prior to each behavioral bioassay, *V. destructor* foundresses were removed during their reproductive phase from randomly selected brood cells of the colony frames. As the physiological status of the parasite can influence its behavioral response (Kraus 1994; Zetlmeisl and Rosenkranz 1994), we standardized the status of the mites before the experiments. The females were thus transferred onto adult bees collected on brood frames and kept within experimental cages. The cages were maintained in a Memmert™ climatic chamber at 35 °C and 60% RH for 3 days to mimic the parasitic phase on adults (called phoretic phase). Through this procedure, all the randomly sampled females used in the behavioral bioassays were in the same stage of their cycle and in the same physiological state, i.e., post-phoresis.

Optimization of the experimental arenas

In spring 2013, we observed that electrostatic discharges affected mites walking on plastic arenas. They displayed jolting movements, staying immobile for a while before being projected some distance away as soon as they started moving again. These mites were often found on their back at the end of the experiment, and the mortality rate was high (Fig. 1, personal observations, Supplementary material: Online Resource ESM1).

To overcome this problem, we developed arenas consisting of 5-cm diameter polypropylene Petri dishes (Nunc™) coated with a 0.5% gelatin solution. Gelatin was preferred over bee wax because its composition is standardized, it has been used successfully in *V. destructor* rearing bioassays, and it is washable. First, the bottom of the Petri dishes was pierced with 4 holes of 1 cm in diameter, 1 cm away from the center of the arena (Fig. 1). The arenas were then dipped into the 0.5% gelatin solution and dried at ambient temperature. Once the arenas were dried, the bottom half of a gelatin capsule (1-cm diameter, 1.5-cm depth, LGA, La Seyne sur Mer, France) was fitted to each hole to simulate brood cells, following the design of Le Conte and Arnold (1988).

We then set up a control experiment to compare mite mortality in classic polypropylene Petri dishes ($n = 47$) and in gelatin-coated Petri dishes ($n = 47$). In this bioassay, a mite was taken from an adult bee in the experimental cages and placed at the center of a coated or a non-coated arena. To make sure the mites would search long enough in order to assess mortality properly, it was offered a worker bee at the white-eyed pupal stage in one of the four artificial cells as we initially considered this stage being a neutral stimulus with a relatively low attractiveness to the mite. A cotton plug was placed at the bottom of every gelatin capsule, including the empty ones to not bias the experiment. This permitted the adjustment of the head of the bee at the level of the surface of the Petri dish. The arena was then kept in an incubator at 35 °C and 70% RH. The initial position of the mite was observed after 1 min and then every 10 min for 3 h. The experiment was repeated 47 times both in non-coated and in gelatin-coated arenas. The mites that died in the arena were counted and the results converted to percentages. After each test, the arenas were washed with water and then hexane to eliminate potential pheromone residues left by previous mites. The gelatin coating, the pupae, the capsules, and the cottons were also renewed between the experiments.

Once gelatin was validated as an appropriate surface, a host choice bioassay was performed in gelatin-coated Petri dishes to check if the parasite was impacted in more subtle aspects of its behavior. As 5th instar larvae are known to attract the females, we tested the mite response to spinning larvae (LS), which corresponds to a late 5th instar stage. The spinning larvae were expected to trigger a positive response close to

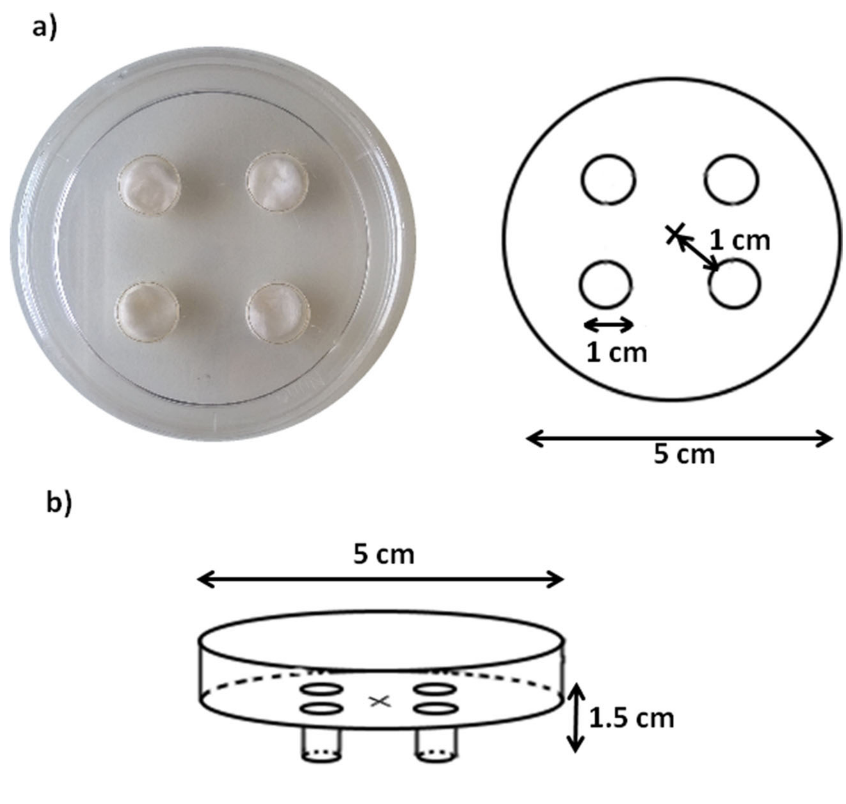

Fig. 1 Test arenas with four artificial brood cells used in the control and host-finding bioassays, adapted from Le Conte and Arnold (1988), viewed from the top (**a**) and from the side (**b**). The surface of the Petri dish was coated or non-coated with gelatin depending on the group. The brood cells consisted in bottom halves of gelatin capsules. The mite was placed at the center (represented by a cross) at the beginning of the experiment

the one observed for early 5th instar larvae (L5) but at this stage, contrarily to the L5, larval food is already consumed and cannot impact the response of the mite (Nazzi et al. 2001, 2004). In this bioassay, two chronologically close stages, a prepupa and a white-eyed pupa (Rembold et al. 1980), were additionally offered to the mite as we assumed these stages being less attractive compared with a 5th instar larva. All the honey bee stages were kept in an incubator (35 °C 80% RH) until the beginning of the test. The three stages were then randomly placed in three of the artificial brood cells 5 min before the start of the experiment. The fourth pit remained empty to act as a negative control. At the beginning of the experiment, a phoretic mite was collected from an experimental cage and placed at the center of the arena using a thin paint brush. The arena was then placed in an incubator at 35 °C and 70% RH. The initial position of the mite was observed after 1 min and then every 10 min for a maximum of 3 h. A mite was considered as having made a choice when it has stayed in the same cell for 60 min straight in the course of the 3 h, following the design of Pernal et al. (2005).

Host choice bioassays

The important positive response for the white-eyed pupal stage initially considered as a neutral stimulus in the first behavioral bioassay (Fig. 3, Table 1, test 2) led us to extend the study to test whether additional stages were attractive to mites. A series of 8 other tests (Table 1) was thus conducted in the same gelatin-coated arenas and experimental procedures.

The different honey bee developmental stages used, according to Rembold et al. (1980), were as follows: 5th instar from unsealed cell (L5), 5th instar larva from freshly sealed cells referred as spinning larvae (LS), prepupa, white-eyed pupa, pink-eyed pupa, brown-eyed pupa, medium-pigmented-body pupa, and emerging bee (see also Table 1). All the larval and pupal instars used in the bioassay were visually identified and removed from a hive brood frame 1 h before the beginning of the experiment. Only healthy individuals free from parasites were used in the experiments. Younger stages were not tested in this bioassay because of the difficulty to ascertain the identification of L1 to L4 stages with simple visual cues (Rembold et al. 1980) and because it is difficult to handle these stages without wounding them. When necessary and more specifically at the L5 stage, larval food was carefully removed from the larvae before the test because of its potential attractive effects on mites (Nazzi et al. 2001, 2004). To reduce the number of assays, tests 1 to 6 focused on combinations of three successive developmental stages to investigate the sensitivity of mite choice when confronted with close host stages (Table 1). Test number 7 gathered the three most frequently chosen stages in the 6 preceding tests.

To further elucidate the mechanisms of the parasite orientation observed towards white-eyed pupae, freeze killed bee larval stages were used in tests 8 and 9 (see Table 1). In this study, white-eyed pupae alone (test 9) or with prepupae and spinning larvae (Test 8) were freeze killed by a 15 min exposure at − 40 °C. In addition, emerging bees used in tests 6 and 7 were also freeze killed because of their propensity to leave the artificial cells and wander into the test arena. Each time a frozen bee developmental stage was used in a test, it was warmed to the incubator temperature (35 °C 80% RH) for an hour before the beginning of the test.

Statistical analyses

The statistical analyses and the graphs were performed using R-3.1.2 (2014).

In the test comparing the behavior of female mites in plastic arenas and gelatin-coated arenas, the mortality rate was analyzed using a GLM with the surface as the explanatory fixed factor.

In the three choices bioassays, the empty control cells were never chosen by the mites. Therefore, only the three wells containing an immature bee are considered in the analysis. The results of the three stages bioassays were analyzed using conformity χ^2 tests comparing the observed distribution with a uniform distribution (with a probability of 1/3 for each cell). In case of significant differences, a contrast analysis allowed us to obtain the 98.3% confidence intervals to determine the stages that significantly differ from the uniform distribution (Neu et al. 1974).

Finally, to compare the three different conditions testing white-eyed pupae, pepupae, and LS larvae, in which either none of the stages, only the pupa, or all three stages are dead (namely tests 2, 8, and 9), a GLM model was used to analyze if the white-eyed pupa choice was dependent of the vitality of the pupa.

Results

Optimization of the experimental arenas

The comparison between the parasite mortality in plastic arenas or gelatin-coated plastic arenas with one host available showed that being kept in plastic arenas for 5 min to 3 h could be harmful or even lethal. During the 3-h test, the mortality rate was reduced from 38.3% [CI 95 24.5–53.6] in plastic arenas to 2.1% [CI 95 0.1–11.2] in gelatin-coated arenas (Fig. 2; GLM; likelihood ratio test, $\chi^2 = 22.39$, $p < 0.001$; $N_{plastic} = N_{gelatin} = 47$).

Surprisingly, over the 117 repetitions of the host choice test offering a late 5th instar larva, a prepupa, and a white-eyed pupa, the parasites chose the white-eyed pupa significantly more often than the two others (49.6%, Fig. 3, Table 1—test 2). This result led to the extension of the stages tested.

Table 1 Table representing the outcome of the 9 behavioral tests performed in gelatin-coated arenas. The results are expressed as percentages of the mite choices for each bee developmental stage († = freeze killed developmental stage). The contingency tables were analyzed using conformity χ^2 tests and the results of the analyses are shown under the test number (< 0.05, < 0.01, < 0.001, *NS* not significant). The brackets indicate the 98.3% confidence intervals obtained from the contrast analysis (Neu et al. 1974); the stages responsible for the significance are presented in italics

Stages used	Test 1 ($\chi^2 = 12.5$, $p < 0.01$)	Test 2 (Fig. 3), ($\chi^2 = 13.9$, $p < 0.001$)	Test 3, ($\chi^2 = 9.8$, $p < 0.01$)	Test 4, ($\chi^2 = 3.8$, NS)	Test 5 ($\chi^2 = 2.7$ NS)	Test 6, ($\chi^2 = 11.8$, $p < 0.01$)	Test 7, ($\chi^2 = 7.7$, $p < 0.05$)	Test 8 ($\chi^2 = 3.8$ NS)	Test 9, ($\chi^2 = 1$, NS)
	Number of mites tested								
	89	117	98	83	75	82	145	35	42
Early 5th instar (L5)	*50.6% [37.3–63.9]*						29.0% [19.6–38.3]		
Late 5th instar (spinning-LS)	28.1% [16.1–40.1]	24.8% [14.8–34.8]						†48.6% [26.7–70.5]	38.1% [18.8–57.4]
Prepupa	*21.3% [10.4–32.4]*	25.6% [15.5–35.8]	*20.2% [10.3–30.1]*					†22.8% [4.2–41.5]	35.7% [16.6–54.8]
White-eyed pupa		*49.6% [38.1–61.1]*	*45.2% [33.0–57.4]*	43.4% [29.7–57.1]			26.9% [17.7–36.1]	†28.6% [8.6–48.5]	†26.2% [8.6–43.8]
Pink-eyed pupa			34.6% [22.9–46.3]	27.7% [15.3–40.1]	40.8% [27.0–54.4]				
Brown-eyed pupa				28.9% [16.4–41.5]	33.3% [20.1–46.6]	24.4% [12.4–36.4]			
Medium-pigmented-body pupa					25.9% [13.6–38.3]	24.4% [12.4–36.4]			
Emerging						*51.2% [37.3–65.1]*	*44.1% [33.9–54.4]*		

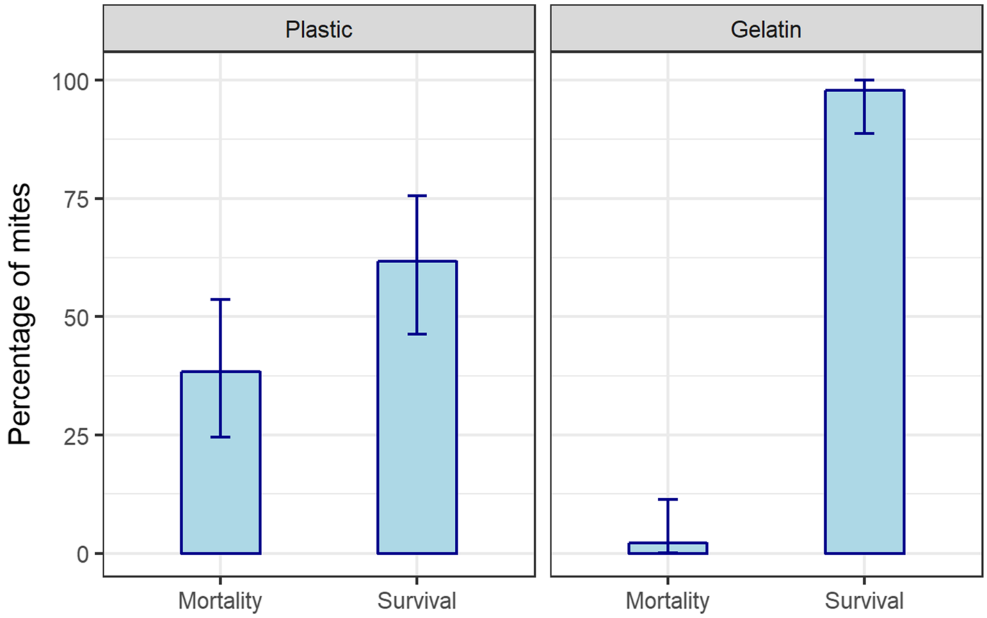

Fig. 2 Effect of the arena surface on the outcome of a control bioassay. The proportions of dead or living female mites at the end of the experiment in plastic arenas (left, $N = 47$) or in gelatin-coated arenas (right, $N = 47$) are expressed in percentage. The error bars represent the 95% confidence intervals. The mortality rates are significantly impacted by the arena surface

Bioassays in gelatin-coated arenas

When confronted with a L5 larva (early 5th instar), a spinning larva (late 5th instar), and a prepupa, the mites chose the L5 larvae (50.6%) and orientated less frequently towards the prepupae (21.3%). As in test 2 (Fig. 3), the white-eyed pupa was also chosen in test 3 gathering prepupae, white-eyed pupae, and pink-eyed pupae (45.2%) even though the significance seems to also be attributable to the less frequent orientation of mites towards prepupae (20.2%). In test 4 comparing the white-, pink-, and brown-eyed pupa stages, the choice for white-eyed pupae is less pronounced and not significant. No significant difference was found in test 5 either when pink-eyed, brown-eyed, and medium-pigmented-body pupae were compared. Finally, in test 6, the emerging bees were preferentially selected by the mites over brown-eyed pupae or pigmented body pupae. Almost 52% of mites' choices were oriented towards the emerging bee when the expected value is 33.3%.

In test 7 gathering early 5th instar larvae, white-eyed pupae, and emerging bees, the emerging bee is again more frequently selected (44.1% of the choices towards emerging bees vs 26.9% towards white-eyed pupae and 29.0% for 5th instar larvae (L5)).

In test 8, dead white-eyed pupae, spinning larvae, and prepupae were offered to the mite. The dead spinning larva

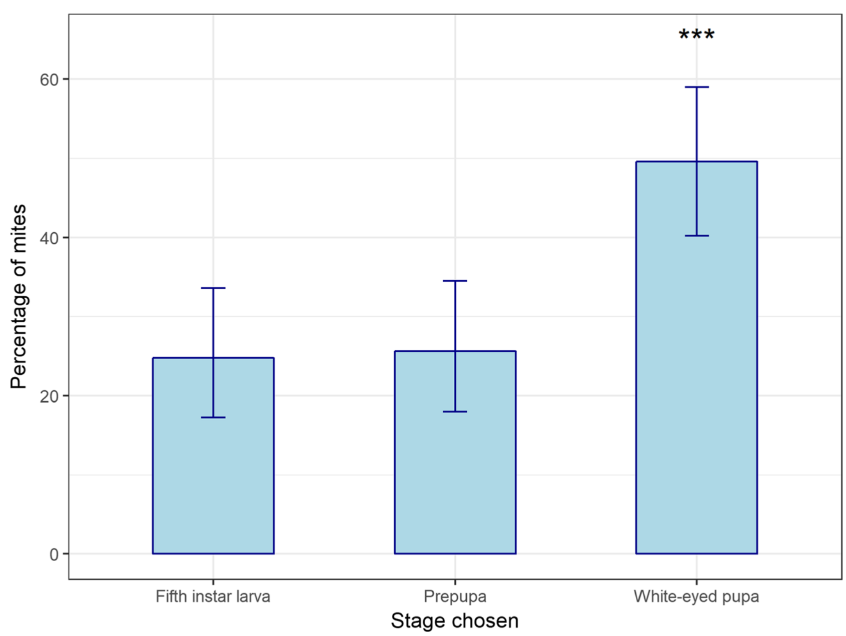

Fig. 3 Results of test 2 gathering a late 5th instar larva (LS), a prepupa, and a white-eyed pupa. The graph shows the repartition of the parasite's choices in percent. The stars show the stage that significantly differs from the uniform distribution

seems to be the stage on which the parasite stayed more often but the differences were not significant (48.6% for LS larvae, 22.9% for prepupae, 28.6% for white-eyed pupae). The choice is no longer oriented towards white-eyed pupae. When only the white-eyed pupal stage is killed by deep freezing, none of the three host instars is significantly chosen over any other even if the spinning larva is again the most frequent choice (Table 1).

Only when presented with a living white-eyed pupa did the females express a significant positive response. The statistical analysis confirmed this idea as the frequency of white-eyed pupae choice was significantly impacted by the vitality of the host (GLM; likelihood ratio test, $\chi^2 = 9.70$, $p < 0.01$).

Discussion

The surfaces classically used in behavioral bioassays on *V. destructor* are very different from the natural substrates experienced by mites, which usually consists of *Apis mellifera* or *Apis cerana* wax or cuticle (Donzé and Guerin 1994; Donzé et al. 1996). We observed electrostatic discharges between the hairy body of female mites and the polypropylene surface of standard Petri dishes, sometimes killing the mites (Supplementary material: Online Resource 1). In a search of a less harmful experimental setting, we found that gelatin-coated plastic Petri dishes did not affect mites so dramatically as uncoated plastic Petri dishes. The gelatin coating indeed diminished the proportion of dead mites to negligible proportions in our control experiment.

The first behavioral bioassay conducted in these new conditions confirmed the absence of mortality but resulted in an unexpected white-eyed pupa preference over a late 5th instar larva. To study this unexplored regained attractiveness for pupae compared with earlier stages, we conducted a series of host choice bioassays to investigate the ability of the mite to discriminate between early and late 5th instar larvae and between pupal stages. We found that three stages, namely the early 5th instar larvae (L5), the white-eyed pupae, and the emerging bees, elicited positive responses from the parasite.

Despite variations in experimental designs, similar positive responses to L5 larvae have been pointed out in several previous studies (Le Conte et al. 1989; Rosenkranz 1993; Pernal et al. 2005). Emerging bees have also been found to elicit orientation behaviors from the mites (Rosenkranz 1993; Ledoux et al. 2000; Pernal et al. 2005). The fatty acid esters emitted by the larvae (Le Conte et al. 1989; Trouiller et al. 1991, 1992; Rickli et al. 1992, 1994; Calderone and Lin 2001;) or more broadly cuticular hydrocarbons (Del Piccolo et al. 2010) are thought to play a crucial role in orientation towards these stages. In our study, the response to emerging bees is still observable after 1 to 3 h, which would be in agreement with the fact that freezing does not impair cuticular hydrocarbons (Rosenkranz 1993; Boulay et al. 2000; Ayasse et al. 2003). Emerging bees are even chosen over early 5th instar larvae or white-eyed pupae in our conditions. The mite may in fact detect the spinning larva or pupal cells as not appropriate for the completion of the cycle so the emerging adult would represent the best alternative as it is both a food source and a possible transport to a better site. We also highlighted that the female mites could distinguish between an early 5th instar larva from an unsealed cell (L5) and a late 5th instar larva from a freshly sealed cell (LS), even though these two stages are separated only by a few hours and are qualitatively close in terms of hydrocarbon profiles (Rembold et al. 1980; Trouiller et al. 1992). This could be explained by quantitative variability in cuticular fatty acids between the two stages. Indeed, most cuticular hydrocarbon amounts seem to increase in the first 12 h post operculation before a decrease in the following 24 to 72 h after the sealing of the cell (Trouiller et al. 1991, 1992; Frey et al. 2013). Furthermore, although the remains of food were removed from the larvae, food-based odorant cues could still be present on the L5 stages, which would make them recognizable and more attractive to the parasite than the spinning stages (Nazzi et al. 2001, 2004). In any case, the positive response to L5 larvae and emerging bees observable in our conditions seems concordant with the natural ability of the mite to discriminate and chose the stages beneficial to its lifecycle.

The attractiveness of white-eyed pupae, especially when compared with stages close to the L5 larvae, is puzzling since this stage occurs usually 3 days after the cell is capped. The white-eyed pupa is thus inaccessible to phoretic female mites. Even though this stage has already been tested for shorter periods (Rosenkranz 1993), it is the first time that the positive response elicited is comparable with the one observed in the presence of early 5th instar larvae. This response can seem illogical and shows the limited comprehension we have on the mechanisms that lead to the parasite's choice. The orientation of the parasite towards a larval stage requires a series of cues, whether they are of chemical or physical nature. In natural conditions, the mite's environment is saturated with many potential cues. Among the vibrations, breathing, and chemicals emitted by the adult bees, the brood, and the different hive matrices, the parasite has to identify a site beneficial for the completion of its cycle. We suggest that white-eyed pupae display several cues the parasite might pick up when searching for a cell to infest. The identification of those cues is of prime importance as it could shed light on the mechanisms driving the behavioral responses of the parasite. Regarding the chemical cues, part of the positive response is lost once the pupa is dead, which could mean that the involvement of the persistent cuticular hydrocarbons is moderate (Trouiller et al. 1991; Frey et al. 2013). The hypothetical generation of a repulsive odor after death was possible but is not consistent with the non-negligible percentage choosing dead pupae in tests 8 and 9. This does not rule out the chemical basis of the response, however, which could still be due to the active emission of kairomones or carbon dioxide (Le Conte and Arnold 1988; Kirchner 1993). Other physical cues specific to living pupa could also be at the origin of the parasite's choice. Slight changes in vibrations (Kirchner 1993), heat (Rosenkranz 1993), hygrometry (Le Conte and Arnold 1987), or even electric charges (Colin et al. 1992) have been shown to be detected by the females and can help the parasite in the host choice procedure. This represents an interesting path to investigate, especially since in closely related acarian parasites, hygrometry, photoperiod, or carbon dioxide emissions were also found to act as useful cues for the completion of the parasite lifecycles (Wilson et al. 1972; Perret et al. 2003).

In conclusion, the host choices of *V. destructor* females have been investigated frequently and yielded different results (Le Conte et al. 1989; Rickli et al. 1992; Rosenkranz 1993; Nazzi et al. 2001, 2004; Pernal et al. 2005). The variability of those results, especially regarding the chemical basis of the choice, is probably due to the lack of common experimental protocol (issue raised by Dietemann et al. (2013)). Even parameters that were not considered in previous *V. destructor* behavioral bioassays such as the viral load of mites should be taken into account in future studies as it has been recently shown to impact their locomotive behavior (Giuffre et al. 2019). Our study and the new design used in our tests also emphasized the importance of the experimental protocol in laboratory behavioral experiments. Our method remains to be perfected and future alternative protocols closer to the natural environment of the mite could represent an interesting source of information regarding the mite's behavior. Being a stage naturally inaccessible in sealed cells, the regained attractiveness of the white-eyed pupae found in our study pinpoints the limits of our current knowledge about this host-parasite relationship. Kairomones are well-known cues able to influence the parasite cycle but our results could imply that physical cues also play an important role in the host-parasite interactions. The nature and impact of those cues, for instance vibrations, heat production, or carbon dioxide emission, remains to be elucidated in further studies.

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Data availability The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Compliance with ethical standards

All applicable international, national, and institutional guidelines currently in force for the care and use of animals were followed.

Conflict of interest The authors declare that they have no competing interests.

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