ARTICLE

Monitoring of parasites in bumblebee colonies developed from controlled nesting of wild queens (Hymenoptera: Apidae: *Bombus*)

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Abstract

Bumblebees (Apidae: *Bombus* spp.) are a major group of wild and domesticated bees that provide crucial ecosystem services through wildflower and crop pollination. However, most of bee populations, including bumblebees, are declining worldwide, partly because of parasite spill-over and spill-back between bumblebee commercial colonies and wild populations. Breeders have to cope with invasions by a vast array of bumblebees' parasites, and techniques need to be developed to prevent such invasions to support breeders and wild bee populations. Our 10-year study is based on 327 nests of seven bumblebee species (*B. humilis*, *B. hypnorum*, *B. lapidarius*, *B. lucorum*, *B. pascuorum*, *B. sylvarum*, *B. terrestris*) reared in outdoor boxes. Some boxes were equipped with parasite-preventing techniques, namely (i) an airlock (n = 2) or (ii) an additional chamber with natural fragrances (n = 74). We recorded the invasion of the nests by the wax moth *Aphomia sociella*, the eulophid *Melittobia acasta* and the cuckoo bumblebees *Bombus* subgenus *Psithyrus* spp. Overall, 8.26 %, 1.53 % and 3.67 % of the colonies were invaded by *A. sociella*, *M. acasta* and *Psithyrus* spp., respectively, without coinfection. Neither the airlock nor the additional chamber with natural fragrances prevented *A. sociella* infestation. Despite that no nest equipped with an airlock or an additional chamber with natural fragrances was invaded by *M. acasta* or *Psithyrus* spp., we lacked replicates to properly demonstrate the efficiency of these techniques. Nest inspection remains a time-consuming but powerful technique to reduce artificial nest spoilage by parasites, yet it is inefficient against tiny invaders (< 1 mm) that are left unnoticed. We therefore encourage further studies to actively seek for parasite-preventing techniques to reduce artificial nest spoilage and to mitigate spill-over towards wild populations.

Keywords | *Aphomia sociella* • *Melittobia acasta* • *Psithyrus* spp. • *Bombus* spp. • parasite-preventing techniques

Surveillance des parasites dans les colonies de bourdons issues de la nidification contrôlée de reines sauvages (Hymenoptera : Apidae : *Bombus*)

Résumé

Les bourdons (Apidae : *Bombus* spp.) représentent un groupe important d'abeilles sauvages et domestiquées qui fournissent des services écosystémiques cruciaux à travers la pollinisation de fleurs sauvages et de cultures. Toutefois, la plupart des populations d'abeilles, bourdons inclus, sont en déclin à l'échelle mondiale, en partie à cause du transfert de parasites entre les colonies domestiquées de bourdons et les populations sauvages. Les éleveurs doivent faire face à l'invasion de nombreux parasites de bourdons, et des techniques doivent être développées pour empêcher de telles invasions afin de soutenir les éleveurs et protéger les populations d'abeilles sauvages. Notre étude s'étend sur 10 ans et se base sur 327 colonies de sept espèces de bourdons (*B. humilis*, *B. hypnorum*, *B. lapidarius*, *B. lucorum*, *B. pascuorum*, *B. sylvarum* et *B. terrestris*) élevées dans des boîtes placées à l'extérieur. Certaines boites étaient équipées de mécanismes permettant d'empêcher l'invasion de parasites, à savoir (i) un sas (n = 2) ou (ii) une chambre additionnelle avec des odeurs naturelles (n = 74). Nous avons enregistré l'invasion de ces colonies par la pyrale du bourdon *Aphomia sociella*, l'eulophide *Melittobia acasta* et les bourdons cleptoparasites *Bombus* sous-genre *Psithyrus* spp. Au total, 8,26 %, 1,53 % et 3,67 % des colonies furent envahies par *A. sociella*, *M. acasta* et *Psithyrus* spp., respectivement, sans trace de co-infection. Ni l'utilisation du sas ni l'utilisation d'une chambre additionnelle avec des odeurs naturelles n'ont empêché l'invasion par *A. sociella*. Bien qu'aucune des colonies équipées d'un sas ou d'une chambre additionnelle avec des odeurs naturelles n'ait été envahie par *M. acasta* ou *Psithyrus* spp., nous manquons de réplicas pour démontrer l'efficacité significative de ces techniques. L'inspection des colonies reste une technique chronophage mais efficace pour réduire la perte de colonies domestiques par des parasites, quoique cette méthode reste inefficace face aux petits envahisseurs (< 1 mm) qui ne peuvent être

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remarqués. Nous encourageons ainsi de futures études à chercher activement des techniques pour faire face aux parasites afin de réduire la perte de colonies domestiques et atténuer le transfert de parasites vers les populations sauvages.

INTRODUCTION

Bees are key animals to preserve in the context of global changes (Potts *et al.*, 2016). They are direct and indirect drivers of human well-being through the cultural value and the ecosystem services they provide by pollinating ~ 75% of globally important crop types and ~ 87% of wildflower species (Klein *et al.*, 2007; Ollerton *et al.*, 2011; Hristov *et al.*, 2020). Among more than 20,000 bee species (Michez *et al.*, 2019), the bumblebees (Hymenoptera: Apidae: *Bombus* spp. Latreille, 1802) represent a moderately large genus comprising around 265 species showing social behaviours (Goulson, 2003; Williams *et al.*, 2008; Cameron & Sadd, 2020). Bumblebees have been increasingly studied in the last few years due to their easy rearing and efficiency in the pollination of greenhouses and outdoor crops (Velthuis & van Doorn, 2006; Kleijn *et al.*, 2015). Annually, individual species of wild bumblebees are estimated to contribute to over $ 5000 per hectare of crop flowers (Kleijn *et al.*, 2015). Along with the domestication of roughly 50 other bee species that are used for crop pollination, including solitary bees (*e.g., Megachile rotundata* Fabricius, 1787), honeybees (*Apis mellifera* Linnaeus, 1758) and stingless bees (Meliponini Lepeletier, 1836) (Klein *et al.*, 2007; Osterman *et al.*, 2021), bumblebee domestication has expanded into a worldwide mass rearing industry with millions of colonies shipped annually around the world (Velthuis & van Doorn, 2006). Six species have been commercially reared but *B. terrestris* Linnaeus, 1758 and *B. impatiens* Cresson, 1863 remain the most common ones in Europe and North America, respectively (Velthuis & van Doorn, 2006; Owen, 2016; Osterman *et al.*, 2021).

Although some bumblebee species are expanding (e.g., *B. terrestris*; Ghisbain *et al.*, 2021), about one third of the species appears to be declining (Goulson *et al.*, 2008, Arbetman *et al.*, 2017). Several anthropogenic drivers of decline have been highlighted including for instance habitat degradation, pesticide use and, to a lesser extent, parasite spread (Goulson *et al.*, 2015; Dicks *et al.*, 2021). Bumblebees suffer from a broad range of parasites such as viruses, bacteria, mites, protozoans, nematodes, and even other insects, with a high degree of diversity in their virulence and transmission mode (Pouvreau, 1993; Macfarlane *et al.*, 1995; Meeus *et al.*, 2011; Goulson *et al.*, 2018; Plischuk *et al.*, 2017). These parasites have coexisted with bumblebees for millions of years and have therefore represented a 'natural' pressure in bumblebee populations. However, an anthropogenic pressure occurs when infected commercially reared bumblebee colonies are carried throughout the globe, and it leads to bee-damaging parasite 'spill-over' and 'spill-back' between commercial colonies and wild populations (Colla *et al.*, 2006; Otterstatter & Thomson, 2008; Cameron *et al.*, 2016; Martin *et al.*, 2021). For commercial colony owners (such as farmers), bumblebee breeders and wild bee populations, it is thus crucial to implement mitigating strategies to detect and cure infected managed bumblebee colonies (Goulson & Hughes, 2015).

We here expose qualitative field observations recorded in bumblebee colonies visited by the larvae of the kleptoparasite and predatory wax moth *Aphomia sociella* Linnaeus, 1758 (Lepidoptera: Pyralidae), the ectoparasite and parasitoid eulophid *Melittobia acasta* Walker, 1839 (Hymenoptera: Eulophidae) and the inquiline cuckoo bumblebees *Bombus* subgenus *Psithyrus* spp. Lepeletier, 1832 (Hymenoptera: Apidae: Bombini; hereinafter '*Psithyrus* spp.'). These are parasites very likely to invade bumblebee breeders' colonies placed in the field. Additionally, we present quantitative observations around several techniques that could be used to prevent bumblebee colonies to be visited by the hereinabove parasites.

MATERIALS AND METHODS

Colony implementation

Seven bumblebee species were observed in this study, consisting of four subgenera, namely the subgenus *Bombus* (*B. terrestris*, *B. lucorum*), the subgenus *Pyrobombus* (*B. hypnorum*), the subgenus *Melanobombus* (*B. lapidarius*) and the subgenus *Thoracobombus* (*B. pascuorum*, *B. sylvarum*, *B. humilis*) (Michez *et al.*, 2019). Gravid wild bumblebee queens were captured (private garden; Sterpenich, Belgium) and colonies were initiated following a method such as described in Przybyla *et al.* (under review). The queen and its brood were then gently placed in small (25 cm × 25 cm × 20 cm; subgenus *Thoracobombus*) or in large (40 cm × 30 cm × 30 cm without additional chamber; 50 cm × 30 cm × 30 cm with additional chamber; subgenera *Bombus*, *Pyrobombus*, *Melanobombus*) wooden boxes with sawdust and wadding as litter (see below for details about the additional chamber). Colonies were placed annually in private gardens (figure 1a) in Sterpenich (Belgium) and Eischen (Grand Duchy of Luxembourg) between 2011 and 2021. Species of the subgenera *Bombus*, *Pyrobombus* and *Melanobombus* were left from March to July while species of the subgenus *Thoracobombus* were left from May to October. Wooden boxes were frequently opened (*i.e.*, weekly the first month and then bimonthly) and colonies were visually checked to examine the symptoms of any parasite invasion. Any colony infested upon inspection was removed off the site. Overall, 327 colonies were examined in this study.
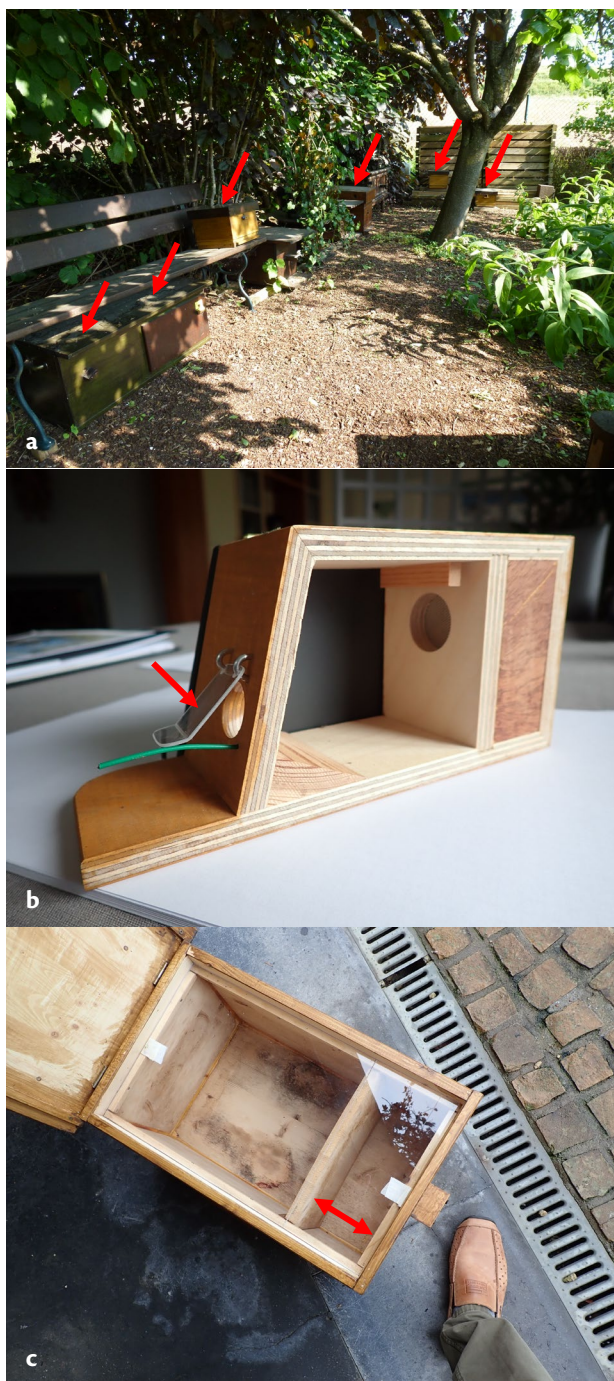
Figure 1. Photos of experimental setups. **a.** Wooden boxes with bumblebee nests (arrows) in a garden. **b.** Airlock apparatus with its plastic lid (arrow) covering the 'exit'. **c.** Empty wooden box with an additional chamber (arrow). Photos J. Habay.

Parasite-preventing techniques

First technique: airlock

This technique was only tested in 2019 in two large colonies of *B. terrestris*. The apparatuses were purchased from Hummeltischler® that year (Gubisch, 2021). The airlock consisted of two openings facing each other at the ends of a cube-shaped compartment: one opening was adjacent to the original flight opening of the colony while the other opening (hereafter referred as to 'exit') was closed by a mobile plastic lid (figure 1b). When the lid was raised, bumblebees could freely go inside and outside the nest while when the lid covered the exit, bumblebees could still freely go outside but had to lift the lid to go inside. For bumblebees to learn this maneuver, we left the lid raised for a few days and then progressively lowered it every day until it completely covered the exit. Bumblebees learnt they had to slip under the lid to reach their nest. Within days, bumblebees were eventually able to lift the lid and enter the wooden box. This technique is expected to prevent parasites from entering the nest by flying or walking through the lid-covered exit.

Second technique: additional chamber and natural fragrances

Assuming that animal parasites find a bumblebee colony to infest relying on olfactory cues emitted by the nest (Ayasse & Jarau, 2014), some colonies were concealed with olfactory cue-masking fragrances. From 2014 to 2021, 32 large wooden boxes were equipped with an additional chamber (10 cm × 30 cm × 30 cm) (figure 1c) containing wadding soaked with three drops of lavandin oil (*Lavandula × intermedia*, Lamiaceae; Pranarôm International SA, 2021) as well as some fresh leaves of *Pelargonium citronellum* van der Walt, 1983 (Geraniaceae). The opening of the additional chamber and the opening leading to the nest were shifted from each other. From 2019 to 2021, 42 small wooden boxes were provided with lavandin oil and *P. citronellum* leaves but these fragrances were directly placed on the litter around the nest. Because these small wooden boxes housed bumblebee species with longer life cycles (*i.e.*, subgenus *Thoracobombus*), the fragrances were replaced in July. Placing these two natural fragrances in the colonies did not lead to any unusual behaviour displayed by the bumblebee individuals (J. Habay, *pers. obs.*).

Statistical analyses

For *A. sociella* data, we recorded the year, the box size and the presence of natural fragrances of every infested colony (we did not record the bumblebee species). *A. sociella* infestation was assessed using a generalised linear model with a binomial distribution and logit link function, with wooden box size, the presence of natural fragrances and their interaction as explanatory variables. Box size is confounded with bumblebee species but it is not an issue for the model since bumblebee species is not considered as an explanatory variable. For *Psithyrus* spp. and *M. acasta* data, we recorded the year and the presence of natural fragrances of every infested colony (we did not record the bumblebee species and the box size). *Psithyrus* spp. and *M. acasta* infestations were assessed using a generalised linear model with a binomial distribution and logit link function with the presence of natural fragrances as explanatory variable. In all models, significance of individual terms was tested with Wald chi-square tests, after checking for overdispersion. We did not include 'year' in the models since the first years of experiment lacked replicates. Because airlock apparatuses were only tested for two colonies, we could not run any statistical analysis to assess their efficacy. All data visualisations and analyses were conducted in R 4.0.3 (R Development Core Team).

RESULTS

Infestation prevalence and symptoms

When considering the full set of observations (i.e., all years, all bumblebee species, all box sizes and all parasite-preventing techniques), 8.26 %, 1.53 % and 3.67 % of the colonies were visited by *Aphomia sociella*, *Melittobia acasta* and *Psithyrus* spp., respectively.

Figure 2. Photos of *A. sociella* (**a–c**), *M. acasta* (**d–e**) and *Psithyrus* spp. (**f**). **a.** Combs destroyed by *A. sociella* larvae (green arrow) and dead non-emerged bumblebees (red arrows). **b.** Web of silk and frass produced by *A. sociella* larvae. **c.** Wooden box gnawed by *A. sociella* larvae. **d.** *M. acasta* adults on bumblebee combs (red arrow) and decaying bumblebee pupae (green arrow) extracted from its *M. acasta* larvae-infested comb. **e.** *M. acasta* female on a bumblebee comb. **f.** *Bombus* spp. queens and their associate *Psithyrus* spp. female inquilines. Photos J. Habay.

A. sociella infestation was easily determined by the presence of dozens of *A. sociella* larvae and their associated hard-wearing web of silk and their frass (figures 2a–b). These larvae were observed to feed from nectar, pollen and wax as well as bumblebee larvae and pupae, thereby destroying the whole nest architecture and the brood (figure 2a). *A. sociella* larvae were also found to feed from adult bumblebees that presented perforated abdomens, but our observations did not enable to determine if these adults were already dead or if *A. sociella* larvae actually killed them. We also noted that

these larvae gnawed the wooden boxes (figure 2c). Most of *A. sociella* infestations were observed in May–June and concerned well-developed colonies.

M. acasta infestation were easily determined by the presence of hundreds of *M. acasta* adults inside the nest, the presence of damaged combs and the presence of hundreds of *M. acasta* larvae inside these combs, specifically on bumblebee pupae and prepupae. The latter were generally decaying or totally decayed (figures 2d–e). *M. acasta* was quicker to contaminate adjacent colonies than A. sociella (J. HABAY, *pers. obs.*). Colonies infected by *A. sociella* or *M. acasta* were completely wiped out within 4–6 weeks.

Psithyrus spp. infestation (figure 2f) was suspected when the host queen or an increasing number of dead workers were found on the litter around the nest. During our regular nest inspection, we captured *Psithyrus* females before they usurped the host queen. A *Psithyrus* female laid eggs the next day in the plastic cup it was caged in, but this behaviour was only observed once. *Psithyrus* females could survive a week in the plastic cup without any food supply. When the *Psithyrus* females were left inside the nests, they started laying eggs within 2–3 days. Most of time, the host queen was found dead, but it happened that the host and the *Psithyrus* females coexisted in the colony. In the latter case, the host queen was clearly subdued (no aggressive behaviour, no egg laying). All *Psithyrus* infestations occurred before June in middle-sized colonies. We also noted that the nest could be subdued by a queen of the same species and that the symptoms were the same as the ones displayed in *Psithyrus*-infested nests. Usurpation by a queen of the same species was yet much rarer than *Psithyrus* infestation.

Parasite-preventing technique efficacy

Statistical tests showed that neither box size, the presence of natural fragrance nor the interaction of these two variables had significant effect on *A. sociella* infestation (figure 3a) (GLM binomial; box size: $\chi^2 = 1.93$, $df = 1$, $p = 0.16$; fragrance: $\chi^2 = 1.17$, $df = 1$, $p = 0.28$; box size × fragrance: $\chi^2 = 0.58$, $df = 1$, $p = 0.45$). Likewise, the presence of natural fragrances did not

have any significant effect on *M. acasta* (figure 3b) (GLM binomial; fragrance: $\chi^2 < 0.001$, $df = 1$, $p = 0.99$) or *Psithyrus* spp. (figure 3c) (GLM binomial; fragrance: $\chi^2 < 0.001$, $df = 1$, $p = 0.99$) infestations. Moreover, one of the two boxes equipped with an airlock got infested by *A. sociella* but none of them got infested by *M. acasta* or *Psithyrus* spp.
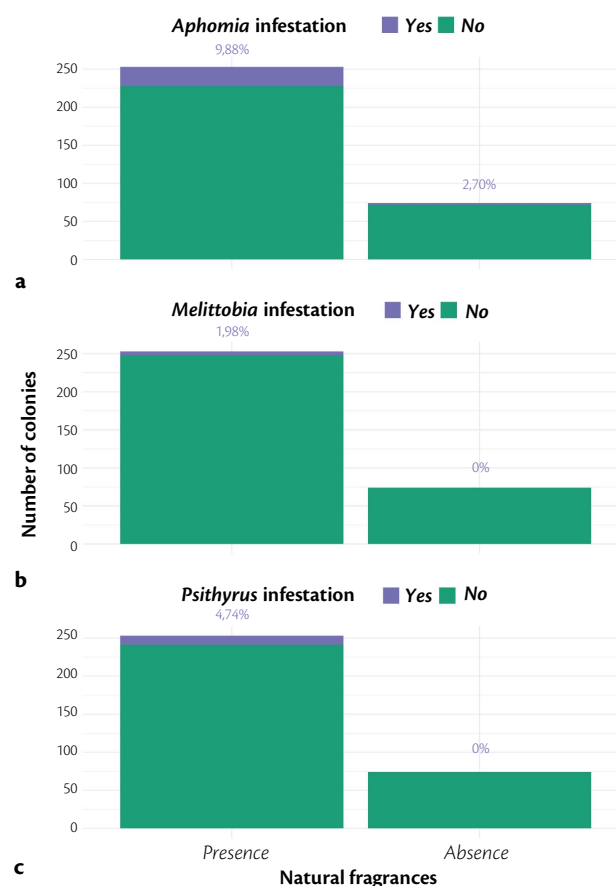

Figure 3. Number of bumblebee colonies infested by parasites. The parasite prevalence (in percent) in the presence (left) or absence (right) of natural fragrances (i.e., lavandin oil and *P. citronellum* leaves) is shown above each barplot. **a.** Infestation by the wax moth *A. sociella*. **b.** Infestation by the euphold *M. acasta*. **c.** Infestation by the cuckoo bumblebees *Psithyrus* spp.

DISCUSSION

The wax moth *A. sociella* is a very specialist parasite of bumblebees and has been suggested as one of the most important natural enemies of bumblebee colonies (ALFORD, 1975). In addition to limiting colony growth by consuming all the provisions, broods and combs (SCHWEIGER *et al.*, 2022), it seems that wax moth larvae induce a non-feeding behaviour in bumblebee queens and workers (KWON *et al.*, 2003). *A. sociella* is univoltine, i.e. the cocoon spinning occurs within the colony and adults emerge the next year after hibernation (POUVREAU, 1988). Here, *A. sociella* infestation was observed in roughly 8 % of the colonies. In previous studies, *A. sociella* was found in eight out of 30 (26 %; GOULSON *et al.*, 2018) and 73 out of 133 wild nests (55 %; GOULSON *et al.*, 2018). In commercial nests placed in the field, GERVAIS *et al.* (2020) found a prevalence of 36 %. Recently, SHARMA *et al.* (2021) showed that *A. sociella* was responsible for 10 % of *B. haemorrhoidalis* queen death out of the 139 dead queens they examined. *A. sociella* prevalence is likely

explained by several environmental factors as higher prevalence has been found in suburban areas and gardens than farmlands (GOULSON *et al.*, 2002; GERVAIS *et al.*, 2020). Although, it is worth noting that SHARMA *et al.* (2021) observed no correlation between *A. sociella* prevalence and temperature as well as relative humidity. Besides, no correlation was observed between *A. sociella* prevalence and landscape composition (GERVAIS *et al.*, 2020; SCHWEIGER *et al.*, 2022). Recently, SCHWEIGER *et al.* (2022) showed that colonies collecting higher pollen diversity had a reduced *A. sociella* depredation. *A. sociella* prevalence also seems bumblebee species-specific. GOULSON *et al.* (2018) found a significant difference in the proportion of infested nests between *B. pascuorum*, *B. pratorum*, *B. terrestris*, *B. lapidarius* and *B. hypnorum*, the two latter being the most frequently infested. The low prevalence observed here remains intriguing as our artificial nests were placed in gardens, and as we further assume that artificial nest boxes should be

more easily detected by *A. sociella* than natural bumblebee nests, which are usually concealed underground (GOULSON *et al.,* 2002). Rather, as proposed by GOULSON *et al.* (2002), we suggest that *A. sociella* prevalence is also dependant of the density of bumblebee nests surrounding the experimental sites, which was an uncontrolled factor in our experiment. Besides, as we removed infested nests upon inspection, we prevented *A. sociella* offspring to hibernate and infest new colonies the next year, and therefore we likely influenced *A. sociella* invasion prevalence over the years.

The parasitic wasp *M. acasta* is a serious generalist parasite of bumblebee colonies as well as other bees and insects (SCHMID-HEMPEL, 1998). While the larvae develop on the hosts' prepupae and pupae, *M. acasta* female adults puncture the hosts' exocuticle with their ovipositor and feed from the oozing haemolymph (KWON *et al.,* 2012). *M. acasta* is multivoltine: dozens of generations are produced each year (MATTHEWS *et al.,* 2009), which enables this parasite to largely spread among adjacent colonies. Here, *M. acasta* infestation was observed in roughly 1.5 % of the colonies. In *B. hortorum,* MACFARLANE & DONOVAN (1989) showed that *M. acasta* ranged from 0 to 13 % in the field but could reached 31 % under laboratory conditions. As outlined by KUMAR *et al.* (2015) who studied four species of megachilids, *Melittobia* spp. can be highly prevalent in nests that are placed in clusters since it is easier for the parasitic wasp to detect and contaminate adjacent nests. The prevalence would have definitely been higher in our study if we had not removed infested nest upon inspection.

After hibernation, the cuckoo bumblebee females (*Psithyrus* spp.) typically invade early nests prior to the emergence of the second brood of workers. While some *Psithyrus* species are specialised on a single bumblebee host, some others can invade bumblebee species from different subgenera (WILLIAMS, 2008). They kill or subdue the host queen, lay eggs and exploit the host workers that will rear a new generation of *Psithyrus* gynes and males. *Psythirus* spp. are univoltine, since mated queens of the new generation will hibernate and invade a new host the next year (BENTON, 2006; LHOMME & HINES, 2019). Worldwide, 27 cuckoo bumblebee species have been described (WILLIAM, 1998) and given our host species, six *Psithyrus* species are concerned here (RASMONT *et al.,* 2021). It is worth noting that many *Psithyrus* species are declining (ARBETMAN *et al.,* 2017) because of the decline of their hosts (SUHONEN *et al.,* 2015). We found that around 3.5 % of our colonies were usurped by *Psithyrus* females. Based on wild colonies, GOULSON *et al.* (2017) observed that one out of 47 nests (2.13 %) had been invaded by a cuckoo bumblebee female over the two years they studied while, over 100 failed nests, GOULSON *et al.* (2018) observed that three of them (3 %) had been usurped by a *Psithyrus* female. By contrast, studies using reared commercial colonies placed in the field found higher prevalence of invasion by cuckoo bumblebees, ranging from 25 to 80 % (MÜLLER & SCHMID-HEMPEL, 1992; CARVELL *et al.,* 2008), probably because the nests were not concealed underground. In a molecular study, ERLER & LATTORFF (2010) found a prevalence of 42 % in wild populations. Given these numbers and our method relying on aboveground artificial nests, the low prevalence of *Psithyrus* invasion we recorded is intriguing. Besides, by capturing dozens of *Psithyrus* females near the entrances of the nests every year, we likely

influenced *Psithyrus* invasion prevalence over the years.

Originally, we postulated that bumblebee nest-seeking parasites should rely on olfactory cues to locate and infest a nest. First, it has been shown that cuckoo bumblebees recognise and locate the nests of their hosts through species-specific scents; the invading female may even be able to acquire the chemical profile of its host after intrusion, thereby performing chemical camouflage (reviewed in AYASSE & JARAU, 2014). The new offspring then produces specific semiochemicals to evade host worker attack and rejection (LHOMME *et al.,* 2015). Second, the parasitic wasps *Melittobia* spp. are polyphagous (MATTHEWS *et al.,* 2009). Every *Melittobia* species displays variation in terms of behavioural responses towards the semiochemicals emitted by their associated hosts, stressing that *Melittobia* spp. exploit and differentiate the semiochemicals when seeking for a specific host (GONZÁLEZ *et al.,* 2018). Yet, to our knowledge, no study has ever tackled the behavioural response of *M. acasta* towards *Bombus*-associated olfactory cues. Third, *A. sociella* is also a polyphagous parasite, invading several bumblebee species nests (GOULSON *et al.,* 2018). It has been shown that *A. sociella* rely on chemical cues for its mating behaviours (KINDL *et al.,* 2012), but its nest-invading mechanism remains hitherto unstudied. It is however likely to assume that nest invasion by *A. sociella* is based on host-emitted olfactory cues. Our study showed that neither lavandin oil nor *P. citronellum* leaves significantly prevented the bumblebee nests from being invaded by *A. sociella*. We propose three rationales: (i) the natural fragrances we used did not conceal the odours emitted by the nests (possibly because they were applied in too small concentrations); (ii) the natural fragrances we used masked the odours emitted by the nests but these natural fragrances were also appealing for the wax moths, even though the natural fragrances did not significantly increase the number of invasions; and (iii) we lacked replicates – and thus increased our β error – to demonstrate the potential effects of these natural fragrances. We also note that the additional chamber was inefficient to prevent *A. sociella* invasion. Even though no nest equipped with natural fragrances was invaded by *M. acasta* or *Psithyrus* spp., we lacked replicates to determine if this method is efficient against these parasites. Using lavandin oil, *P. citronellum* leaves or other natural fragrances to prevent *A. sociella, M. acasta* or *Psithyrus* spp. invasion warrants further investigations. Despite that we only equipped two nests with an airlock, we observed an *A. sociella* invasion in one of them, suggesting that the airlock may not be efficient against wax moth infestation. We postulate that *A. sociella* was able to enter the nest during the learning period (the exit was not completely covered for a few days), and we suggest that other parasites may be able to enter during this period as well. If that is the case, the airlock would render the nest vulnerable during the learning phase, thereby casting doubt over its efficiency. Even though airlocks were only provided to *B. terrestris* nest, this issue is unlikely species-specific.

Overall, the nests we placed in gardens got infected by *A. sociella, M. acasta* and *Psithyrus* spp. The natural fragrances did not prevent *A. sociella* invasion but we encourage further large-scale experiments to test the effects of natural fragrances on *A. sociella, M. acasta* and *Psithyrus* spp. invasion. Besides, while our studies did not discriminate

among bumblebee species, we recommend that further studies consider interspecific variations. By contrast to natural fragrances, we suggest that an airlock at the entrance of the nests may not be efficient against parasite infestation since the nests remain vulnerable as long as the bumblebees do not manage to lift the lid and that the entrance is kept open. Nests that were infected upon inspection were immediately destroyed to avoid contamination of adjacent colonies, which could explain the seeming lower infestation prevalence in our study in comparison with the literature. Frequent and systemic nest inspection remains a very powerful but laborious technique to reduce artificial nest spoilage by parasites. For example, a daily checking enables to prevent *Psithyrus* spp. usurpation since the invading cuckoo bumblebee female can be removed before it kills the host queen. However, visual inspection reaches its limits when looking for small parasites, typically parasitoid wasps usually smaller than 1 mm. Developing parasite-preventing techniques is an outstanding area of research that is crucial to support bumblebee colony owners and reduce parasite spill-over towards wild bee populations.

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