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Hyperparasitoids exploit plant-volatiles to locate their parasitoid host despite non-host herbivore interference

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Abstract:	Hyperparasitoids are ubiquitous components of terrestrial food webs. They must find their inconspicuous parasitoid host in a complex environment to generate offspring, but their foraging behaviour is poorly understood. Herbivore-induced plant volatiles (HIPVs) are the main source of information for their long-range foraging, but the naturally occurring presence of non-host organisms can interfere in HIPV production. It is unknown how hyperparasitoids respond to this source of variation in their foraging behaviour. We studied how the presence of non-host herbivores affected foraging decisions of the hyperparasitoid Lysibia nana. In Y-tube olfactometer bioassays, we compared the preference of hyperparasitoids to volatiles from cabbage plants induced by single herbivory (host- or non-host), dual herbivory (host + non-host) or uninfested cabbage plants. Following this, we conducted a common-garden experiment to study whether preferences observed in the laboratory are also apparent in the field. We found that the hyperparasitoid L. nana preferred plants that were induced by its host, Cotesia glomerata-parasitised Pieris brassicae caterpillars. However, the volatiles of non-host-induced plants hampered the hyperparasitoids' ability to locate host-induced plants in the Y-tube olfactometer. In particular, HIPVs by the non-host caterpillar Mamestra brassicae in both single- and dual herbivory. The presence of other non-host herbivores feeding simultaneously on the same plants as host herbivores did not interfere in L. nana's preference. In the field, hyperparasitoids could locate their host despite non-host presence. Overall, these results indicate that host location errors by hyperparasitoids may occur during HIPV-guided foraging steps, which can be corrected by using more reliable cues after landing on the plant. This demonstrates the ability of higher trophic level organisms to step-wise exploit the scarce amount of available information to locate their host.
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Dear Editor,

Please find enclosed our manuscript entitled **"Hyperparasitoids exploit plant volatiles to locate their parasitoid host despite non-host herbivore interference",** which we submit to be considered for publication as a research article in *Animal behaviour*.

We consider our manuscript to be suitable for this journal because *Animal behaviour* has a long standing track record of publishing studies on parasitoid behaviour in plant-based food webs. This includes studies on parasitoid foraging behaviour when they are faced with the complexity of locating their herbivorous host in presence of non-host herbivores (Bukovinszky *et al.,* 2012; de Rijk *et al.,* 2013, 2016; Croijmans *et al.,* 2022). Our study aims to extend this knowledge to the natural enemies of parasitoids, so called hyperparasitoids. To date, it is unknown how these hyperparasitoids adapt their foraging strategy to this source of variation.

In this study, we tested how the presence of non-host herbivores affected foraging decisions of the hyperparasitoid *Lysibia nana*. We compared the preference of the hyperparasitoids for herbivore-induced plant volatiles (HIPVs) of single herbivore-infested (either host or non-host), dual herbivore-infested (host and non-host), or uninfested cabbage plants. We then conducted a common-garden experiment to investigate whether the preferences observed in the laboratory were also evident in the field.

Our results show that the hyperparasitoid had a preference for plants induced by its host, even in presence of non-host herbivores. Although, non-hot induced plants were preferred over uninfested plants. This suggests that that hyperparasitoids use a multi-level foraging strategy, adjusting their decisions based on the most reliable cues available. This work confirms the significance of HIPVs in information transfer to higher trophic levels, where it acts as an important cue for hyperparasitoid to guide their behaviour. Furthermore, we demonstrate the ability of higher trophic level organisms to exploit scarce information to locate their host. Accordingly, we think that this study will be of broad interest to the readership of *Animal behaviour*.

Thank you for considering our submission. We look forward to the possibility of working with you.

Sincerely,

Mitchel E. Bourne

Hyperparasitoids exploit plant-volatiles to locate their parasitoid host despite non-host herbivore interference

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Results: 1578 words

Discussion: 1411 words

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Declaration of Interest

None

Abstract

Hyperparasitoids are ubiquitous components of terrestrial food webs. They must find their inconspicuous parasitoid host in a complex environment to generate offspring, but their foraging behaviour is poorly understood. Herbivore-induced plant volatiles (HIPVs) are the main source of information for their long-range foraging, but the naturally occurring presence of non-host organisms can interfere in HIPV production. It is unknown how hyperparasitoids respond to this source of variation in their foraging behaviour.

We studied how the presence of non-host herbivores affected foraging decisions of the hyperparasitoid *Lysibia nana*. In Y-tube olfactometer bioassays, we compared the preference of hyperparasitoids to volatiles from cabbage plants induced by single herbivory (host- or non-host), dual herbivory (host + non-host) or uninfested cabbage plants. Following this, we conducted a common-garden experiment to study whether preferences observed in the laboratory are also apparent in the field.

We found that the hyperparasitoid *L. nana* preferred plants that were induced by its host, *Cotesia glomerata*-parasitised *Pieris brassicae* caterpillars. However, the volatiles of non-host-induced plants hampered the hyperparasitoids' ability to locate host-induced plants in the Y-tube olfactometer. In particular, HIPVs by the non-host caterpillar *Mamestra brassicae* in both single- and dual herbivory. The presence of other non-host herbivores feeding simultaneously on the same plants as host herbivores did not interfere in *L. nana's* preference. In the field, hyperparasitoids could locate their host despite non-host presence. Overall, these results indicate that host location errors by hyperparasitoids may occur during HIPV-guided foraging steps, which can be corrected by using more reliable cues after landing on the plant. This demonstrates the ability of higher trophic level organisms to step-wise exploit the scarce amount of available information to locate their host.

Key words:

Dual herbivory; Herbivore-induced plant volatiles (HIPVs); Hyperparasitoid foraging behaviour; *Lysibia nana;* Multitrophic interactions; Non-host; Optimal foraging theory.

INTRODUCTION

Animals have evolved sophisticated sensory systems and foraging strategies to evaluate and navigate their complex environment in search of prey, hosts or food plants (Hassell & Southwood, 1978). According to the optimal foraging theory, foraging decisions are shaped by the available information and the organisms' ability to store and process this information (Pyke, 1984; van Alphen, Bernstein, & Driessen, 2003). However, the available information is constrained because prey are under selection to remain inconspicuous (Vet & Dicke, 1992). Prey-emitted cues are often released in low amounts and have a short detectability range (Webster & Cardé, 2017). Furthermore, (a)biotic variation and the presence of organisms that cannot be used as prey obscure the presence of prey organisms and add to the complexity of the environment (Aartsma, Bianchi, van der Werf, Poelman, & Dicke, 2017; De Rijk, Dicke, & Poelman, 2013; Desurmont et al., 2014).

To navigate within this complex environment and ultimately locate their prey, foraging insects are hypothesised to have multi-level foraging strategies in which they subsequently navigate the i) habitat, ii) patch, iii) microhabitat (such as a single plant) and iv) evaluate the potential prey or hosts for acceptance (Aartsma et al., 2019; Beyaert & Hilker, 2014; Hassell & Southwood, 1978; Lewis & Martin, 1990; Price et al., 1980; Webster & Cardé, 2017). This strategy enables carnivorous insects to use the most reliable cues within detection range to optimise their foraging strategy and allows them to leave the patch when prey availability falls below a profitability threshold (Beyaert & Hilker, 2014; Charnov, 1976; Pyke, 1984; Thiel & Hoffmeister, 2004; van Alphen et al., 2003).

Tritrophic systems with plants, herbivores and their specialised parasitic wasps (parasitoids) are often used as model system to study foraging behaviour. Parasitoids lay their eggs in or on their host and successful development of their offspring ultimately results in host death and a new generation of parasitoids (Godfray, 1994). The link between foraging success

and fitness is particularly clear for parasitoids. For every located host, the parasitoid can deposit eggs, meaning that there is a direct link between host encounter rate and fitness in parasitoids (van Alphen et al., 2003; van Baalen & Hemerik, 2008). Therefore, parasitoid foraging strategies are subjected to a strong selection pressure (Montovan, Couchoux, Jones, Kern Reeve, & van Nouhuys, 2015; Wajnberg, 2006). Their host is often patchily distributed, and they must tailor their foraging strategy to their organismal limits such as longevity and egg load (Rosenheim, 1999; van Baalen & Hemerik, 2008). The foraging success of parasitoids in plant-based food webs is mostly attributed to their ability to navigate, evaluate and learn from their environment through herbivore-induced plant volatiles (HIPVs) (Aartsma et al., 2017; Mumm & Dicke, 2010; Turlings & Erb, 2018). These long-ranged, plant-derived cues are known to be exploited by parasitoids to navigate through the complexity of the environment and help to avoid uninfested plants, non-hosts and (intra- and interspecific) competition (De Rijk et al., 2013; Kafle, Morawo, & Fadamiro, 2020; Tamò, Roelfstra, Guillaume, & Turlings, 2006).

Food webs extend beyond the third trophic level, and at the fourth trophic level so-called hyperparasitoids parasitise the immature stages of parasitoids to complete their lifecycle (Poelman, Cusumano, & de Boer, 2022; Sullivan & Völkl, 1999). Hyperparasitoids can be divided into two categories: 1) true hyperparasitoids that attack larvae of their parasitoid host when they are developing inside a host, and 2) pseudo-hyperparasitoids that attack cocoons of parasitoids emerged from their host (Poelman et al., 2022). Unlike parasitoids, we have little knowledge on how the presence of non-hosts, such as unparasitized herbivores or non-host herbivore species, affects hyperparasitoid foraging behaviour. Virtually all our understanding on hyperparasitoids is based on studies in which plants were infested with a single herbivore species. This poorly represents the natural situation where plants are simultaneously or subsequently infested by herbivores of different species (Mertens et al., 2021; Takabayashi, 2022).

Because hyperparasitoids must find their herbivore-associated parasitoid host, they have a wide suite of information available, originating from plant, herbivore (their secondary host), and their parasitoid host (their primary host) (Aartsma et al., 2019). However, hyperparasitoids face much stronger constraints than parasitoids to locate their host. Their parasitoid host has a low detectability, low availability and may occupy different herbivore species that are feeding on a range of food plants (Aartsma et al., 2019; Ohsaki & Sato, 1994; Poelman et al., 2022; Sullivan & Völkl, 1999). Still, just as for parasitoids, hyperparasitoid fitness is directly tied to their foraging success (Harvey, Wagenaar, & Gols, 2011). We may thus predict that hyperparasitoids evolved multi-level foraging strategies to navigate within the complexity of the environment in which they need to locate their parasitoid host.

Studies on the foraging behaviour of caterpillar-associated hyperparasitoids indicate that they share characteristics with their parasitoid host, especially in the usage of HIPVs for long-distance foraging (Cusumano, Harvey, Dicke, & Poelman, 2019; Poelman et al., 2012; Poelman, Harvey, van Loon, Vet, & Dicke, 2013; Zhu et al., 2018). Plant volatiles induced by parasitised caterpillars allow *Lysibia nana* (Hymenoptera: Ichneumonidae), a specialist pseudo-hyperparasitoid of *Cotesia* spp. (Hymenoptera: Braconidae), to locate their host. They favour a gregarious host species (*Cotesia glomerata*) yielding the highest fitness benefit over a solitary parasitoid (*Cotesia rubecula*) (Cusumano et al., 2019; Harvey et al., 2011; Poelman et al., 2012).

Here, we used the pseudo-hyperparasitoid *L. nana* to study how the presence of non-host herbivores on neighbouring plants or on the same plant as their hosts affects the foraging behaviour of hyperparasitoids. In laboratory Y-tube olfactometer bioassays we compared the attraction of hyperparasitoids to HIPVs from differently infested plant pairs (non-host, host or host + non-host infested) or undamaged plants. We then performed a common garden experiment to investigate how the results obtained in our controlled laboratory experiments

compare to foraging decisions of hyperparasitoids in the field. We expected *L. nana* to be guided by HIPVs to navigate through their environment in which they subsequently: i) locate suitable patches with herbivore-infested plants, ii) avoid non-host-infested plants and iii) locate plants that are infested with hosts, even when these plants are also infested with non-hosts. Hence, we hypothesised the hyperparasitoid to prefer odours of herbivore-induced plants over undamaged plants (even when the inducers are non-host herbivores). We expected hyperparasitoids to favour plants (co-) induced by their parasitised caterpillar host over non-host-induced plants. Finally, we expected hyperparasitoids to be attracted to the plant that represented the highest host availability when given the choice between plants with only hosts against plants with hosts plus non-hosts.

MATERIALS AND METHODS

Plant material and insects

We used *Brassica oleracea var gemmifera cv. Cyrus* (Brussels sprouts) for lab experiments in the Y-tube olfactometer and the wild *B. oleracea* population 'Kimmeridge' for the common garden experiment in the open field. Both plants are known to differentially respond to parasitized and unparasitized *P. brassicae* caterpillars and allow hyperparasitoids to identify the presence of parasitized caterpillars based on HIPVs with similar effect size (Cusumano et al., 2019; Poelman et al., 2012). Seeds from the 'Kimmeridge' population were originally obtained from Dorset, UK (50.36'N, 2.07'W) (Gols et al., 2008) and multiplied by open pollination in a field with multiple wild *B. oleracea* genotypes. All plants were grown in 2L pots containing standard potting soil (Lentse potgrond) under greenhouse conditions (22 ± 2 C, 50-70% RH, 16:8 h LD) and natural daylight supplemented with SON-T light if the level dropped below 500 µmol photons m^{-2} (16:8 L:D).

We studied a cabbage-based food web with four trophic levels (Fig 1), in which plant volatiles are known to be an important source of information to mediate trophic interactions

(Poelman et al., 2012). We used caterpillars of the large cabbage white *Pieris brassicae* (Lepidoptera: Pieridae) (Pb) as the focal herbivore species in the trophic food web. To study effects of non-host herbivores from different feeding guilds, we included caterpillars of the cabbage moth *Mamestra brassicae* (Lepidoptera: Noctuidae) (Mb), and the cabbage aphid *Brevicoryne brassicae* (Hempitera: Aphididae) (Bb). All three species commonly occur on *B. oleracea* and are often found sharing the same plant individual (Stam, Dicke, & Poelman, 2018). Furthermore, we included the parasitoid *C. glomerata* (Hymenoptera: Braconidae) which is a gregarious koinobiont endoparasitoid that uses *P. brassicae* as its main host, while both *M. brassicae* and *B. brassicae* are non-hosts to *C. glomerata* (Geervliet, Vet, & Dicke, 1996). The pseudo-hyperparasitoid *Lysibia nana* (Hymenoptera: Ichneumonidae) was selected as focal study organism. To complete its lifecycle *L. nana* must locate and parasitise the cocoons of braconid wasps like *C. glomerata* (Poelman et al., 2022). It is known to be strongly attracted by HIPVs of plants infested with *C. glomerata*-parasitised *Pieris* spp. (Cusumano et al., 2019; Poelman et al., 2012).

All insects were originally collected from field sites near XXXX. Herbivores were reared on Brussels sprouts plants under greenhouse conditions (22 ± 2 ºC, 50-70% RH, 16:8 h LD). *Cotesia glomerata* was reared on *P. brassicae* under the same conditions. *Lysibia nana* was reared on *C. glomerata* cocoons in the absence of plant-related cues. Hyperparasitised *C. glomerata* cocoons were placed in a climate chamber (22 ± 0.5 °C, 50–70 % RH, 16:8 h L:D) until new adults emerged. Adult *L. nana* were kept in a rearing cage at room temperature, under natural light conditions (22 ± 2 °C, 35-45% RH) and were supplied with 5% honey water.

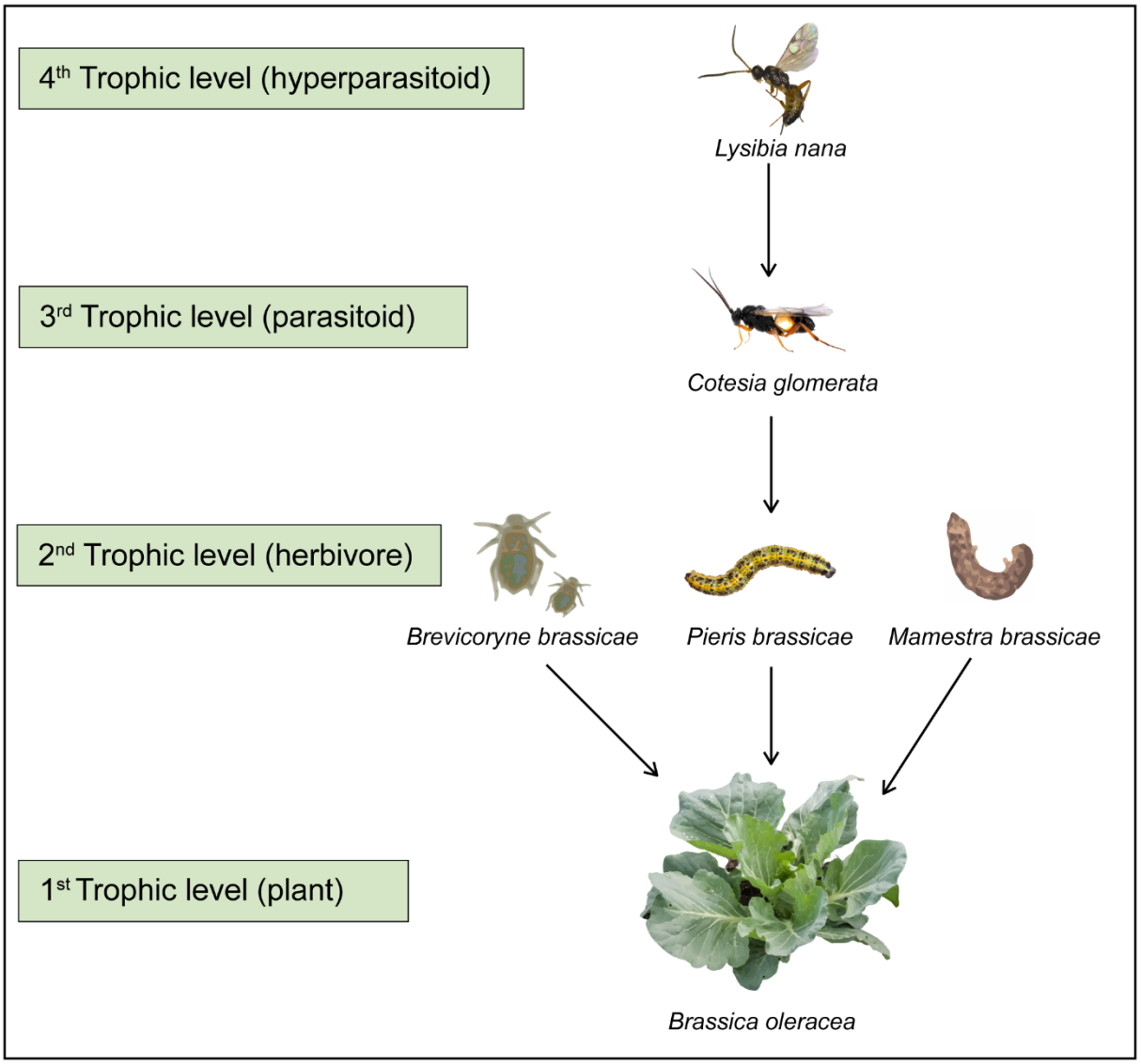


Figure 1: Trophic system used in this study. The food plant *Brassica oleracea* is under attack of multiple herbivores including *Brevicoryne brassicae*, *Pieris brassicae* and *Mamestra brassicae*. *Pieris brassicae* is the preferred host of the parasitoid *Cotesia glomerata*, while *Brevicoryne brassicae* and *Mamestra brassicae* are non-hosts. The hyperparasitoid *Lysibia nana* hyperparasitises cocoons of braconid wasps of which *Cotesia glomerata* is its preferred host. *L. nana* picture credit to Davide Bottacini, *C. glomerata* picture credit to Hans Smid.

Ethical note

Our study with insects (Hymenoptera, Lepidoptera and Hemiptera) did not require a licence. In total, we used about 1700 female *L. nana* hyperparasitoids, 1500 *P. brassicae* larvae of which about half were parasitised by *C. glomerata*, 2200 *B. brassicae* aphids, 600 *M. brassicae* larvae and 200 female *C. glomerata* parasitoids. Larvae and aphids were handled gently with a fine brush or soft tweezers for parasitism and induction of plants. After parasitism,

caterpillars were given a day to recover in a petri-dish with leaf material prior to induction in the field. Herbivores reared by the stock rearing were provided with an excess of plant material at any time. Parasitoids and hyperparasitoids were reared in clean cages and provided with honey-water and water, which were weekly refreshed to avoid the growth of mold. When large numbers were needed, the density of (hyper) parasitoids in cages was limited by dividing them over multiple cages. Furthermore, (hyper) parasitoids were gently moved from their cage by putting a glass vial over them and waiting until they climbed inside the vial. After being used in choice-assays hyperparasitoids were placed back in a separate rearing cage (not to be used again for the experiment) and provided with honey, water and hosts to oviposit. All herbivores were recaptured after induction and perished by placing them in a -20ºC freezer.

Y-tube olfactometer

To characterise the response of *L. nana* to the odours of Brussels sprouts plants in a controlled environment, 5-week-old plants were induced for 24 hours with their corresponding herbivore treatment (see description below). After induction, the herbivores and their excrements were removed, pots were wrapped in aluminium foil to exclude soil odours, and two differently treated plants were placed in separate 30L glass jars. A charcoal-filtered and humidified airflow (4 L min^{-1}) was led through the jars into the Y-tube olfactometer (Ø=3.5 cm, 15 cm arm length, 22 cm basis length). One mated *L. nana* female was released at the basis of the Y-tube and it was considered to have made a choice when it had passed a line set 12 cm into an arm for at least 15 seconds. If a hyperparasitoid failed to make a choice within 10 minutes, it was regarded as no-choice and excluded from the analysis. Each plant pair was subjected to choices of 10 hyperparasitoids and after five tests the airflow through the arms was swapped to correct for an unforeseen bias of the setup.

Experiment 1: Single herbivory

We first studied whether HIPVs of non-host-induced plants are used by *L. nana* to navigate its environment to distinguish between damaged and undamaged plants. In addition, we studied

whether of *L. nana* uses the most reliable cues within detection range to optimise its foraging strategy, by testing its ability to differentiate between the HIPVs of host-infested plants versus non-host-infested plants. Hence, we offered *L. nana* females a choice between odours of a) undamaged plants versus plants induced by a single host or non-host herbivore (Table 1abc) or b) plants induced by a single non-host herbivore versus plants induced by *C. glomerata*-parasitised *P. brassicae* caterpillars (PbCg, referred to as parasitised caterpillars) (Table 1bc). Herbivore-induced plants were infested with 20 adult aphids (Bb) or two caterpillars (Pb, PbCg or Mb) in the late fourth or early fifth instar obtained from XXXX. Parasitised *P. brassicae* caterpillars were obtained from the stock rearing as well; these were visually inspected to contain parasitoid larvae before induction and dissected after induction to confirm their parasitism status.

Experiment 2: Dual herbivory

In experiment 2 we studied how HIPVs of dual-infested plants, with the presence of both host and non-host herbivores on the same plant, affects the preference of *L. nana*. We studied if *L. nana* can locate parasitised caterpillars through HIPVs in the presence of non-hosts on the same plant, when the alternative plant in the Y-tube is infested with non-host herbivores. Additionally, we tested whether the presence of non-host herbivores on the same plant as hosts interferes with the preference of *L. nana*, by testing its ability to differentiate between the HIPVs of host-infested plants versus host- plus non-host-infested plants. We offered *L. nana* females choices between odours of a) plants induced by a single non-host herbivore (Bb, Mb or Pb) versus dual-herbivore-infested plants consisting of the non-host herbivore and parasitised caterpillars (Table 1bd) or b) plants induced by host-containing caterpillars versus dual-herbivore-infested plants consisting of a non-host and host-caterpillars (Table 1cd). Single herbivore-induced plants were infested with 40 adult aphids or four caterpillars (Pb, Pb-Cg or Mb) in the late fourth or early fifth instar. Dual herbivore-induced plants were infested with two parasitised caterpillars, and 20 aphids or 2 unparasitised caterpillars (Pb or Mb).

Common garden experiment:

To investigate whether the results obtained from our controlled laboratory experiments compare to hyperparasitoid foraging in the field, we performed a common-garden field experiment using plants induced with various host and/or non-host herbivores. Four-week-old *B. oleracea* 'Kimmeridge' plants, grown in 2 L pots under semi-field conditions, were transplanted to the field with a 1 x 1 m spacing. The field was located at XXXX. After transplanting in the field, plants were directly individually covered with fine mesh tents (100 x 66 cm, Bugdorm Ltd., Taiwan; 100 x 80 mesh/square inch). The plants were left in the field for seven days to acclimatise.

After acclimation, plants were induced with herbivores. They were randomly assigned to one of ten induction treatments, including the undamaged control (Ud; Table 1a), non-host single herbivory (Pb, Mb, Bb; Table 1b), host single herbivory (PbCg; Table 1c) and host + non-host dual herbivory (Bb + PbCg, Mb + PbCg, Pb + PbCg; Table 1d) as applied in previous choice experiments (eight treatments) and two additional treatments with non-host dual herbivory (Pb + Mb, Pb + Bb; Table 1e) to contrast the host + non-host dual herbivory treatments). Plants were induced with a total of four L2 caterpillars or 20 adult aphids in the case of single herbivory. For dual herbivory, two caterpillars were used per species or parasitism status or 10 aphids. The herbivores were allowed to feed on the plants for a total of 10 days after induction, which corresponds to a large proportion of the development time of the caterpillars and parasitoid larvae prior to pupation (Cuny, Bourne, & Poelman, 2022). During the induction period, plants remained under the mesh tents to avoid predation and infestation by other herbivores (Fig 2).

After the induction period, tents and herbivores were removed and four *C. glomerata* cocoon clutches were attached per plant to measure hyperparasitism on a plant and clutch level (Fig 2) (Poelman et al., 2012). Cocoon clutches consisting of 15-20 cocoons were first attached to a piece of paper (3 x 3 cm) with a droplet of non-toxic glue (HEMA, the Netherlands), then

attached to the abaxial side of the four youngest fully grown leaves of each plant with two metal pins. Typically, 'Kimmeridge' plants had four fully grown leaves after the induction period. On the same day, 100 lab-reared *L. nana* females were released from the four cardinal points (25 wasps per cardinal point) of the field at a distance of 3 meters from the field to assure local presence of a hyperparasitoid community.

The cocoon clutches were exposed for five days to the hyperparasitoid community in the field, after which the clutches were recollected and kept in 2 mL Eppendorf vials capped with cotton wool (Fig 2). Vials were daily checked for emergence of *C. glomerata* or hyperparasitoids. A clutch was considered to be hyperparasitised when at least one hyperparasitoid emerged from it. This common-garden experiment was repeated six times from May 2020 until October 2020 in a complete randomized block design, where every treatment was replicated ten times per block. After each block, all plants were removed, the field was ploughed and left fallow for several days after which the next block was planted.

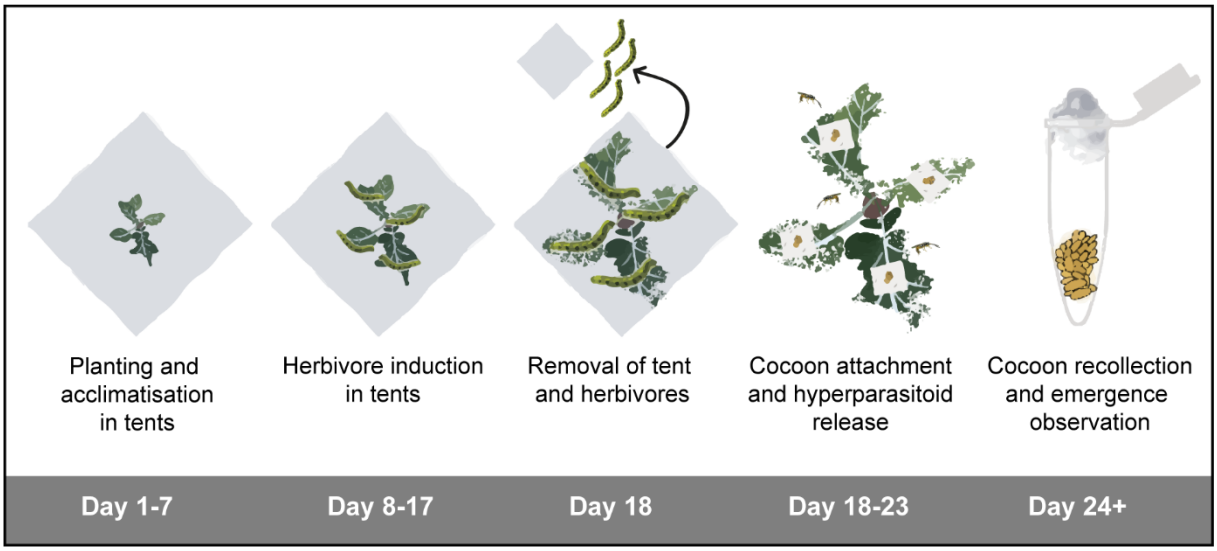


Figure 2: Workflow of the common garden experiment. This represents one block, where a total of six subsequent blocks were performed in the months May-October.

Table 1: Overview of the herbivory types and treatments, as well as the experiment in which they have been tested. Abbreviations used: Ud = undamaged plants, Bb = *Brevicoryne brassicae* aphids, Mb = *Mamestra brassicae* caterpillars, Pb = *Pieris brassicae* caterpillars, PbCg = parasitised *P. brassicae* caterpillars.

Herbivory type	Treatment	Experiment(s) used	Pictogram
(A) Negative control, undamaged plants	Ud	Y-tube exp. 1a Common garden	Ud
(B) Non-host single herbivory	Bb	Y-tube exp. 1ab + 2a Common garden	
	Mb		
	Pb		
(C) Host single herbivory	PbCg	Y-tube exp. 1ab + 2b Common garden	
(E) Host + non-host dual herbivory	Bb + PbCg	Y-tube exp. 2ab Common garden	
	Mb + PbCg		
	Pb + PbCg		
(E) Non-host dual herbivory	Bb + Pb	Common garden	
	Mb + Pb		

Statistical analysis

Y-tube olfactometer

The hyperparasitoids' preference for plant odours, as tested in the Y-tube under lab-conditions, was analysed with a generalized linear mixed model (GLMM). This included the combination of treatments as fixed factor and the tested plant pair on a day as random factor. Because of the singularity effect of the random factor (intercept close to zero), we removed this from the model and conducted statistical analysis with a GLM with a binominal distribution and zero intercept logit link (H_0 = 50:50 distribution) function with Tukey's HSD for *post-hoc* comparisons. In addition, we used a *Type III Wald Chi-square test* on the GLM to determine if there was an overall difference between the tested herbivory types. Models were performed in R version 4.2.0 (R Core Team, 2022) with "glm" function of the "lme4" package (Bates, Mächler, Bolker,

& Walker, 2015). *Post-hoc* tests were performed at the log odds ratio scale with the "emmeans" package (Lenth, 2021). For all tests $\alpha = 0.05$ was used as level of significance.

Common garden experiment

Hyperparasitism was considered as a binary response variable per plant in which: 1 = at least one hyperparasitoid emerged from one of its attached cocoon clutches and 0 = no hyperparasitoids emerged from any of the attached cocoon clutches. In addition to this, we used the hyperparasitism rate of the clutches attached to the plant as a second response variable in a separate model. Here, we used a two-vector response variable, binding the number of clutches hyperparasitised (success) and the number of clutches not hyperparasitised (failure) per plant to the model in separate columns using the "cbind" function in R (Crawley, 2007; R Core Team, 2022). Data at the plant level provided information on the preference of hyperparasitoids depending on the induction of the plant and its associated volatile blend. Data at the clutch level provided information on how frequent the plant was visited by hyperparasitoids and/or if hyperparasitoids were arrested to these plants to parasitize multiple cocoon clutches.

To test the preference of hyperparasitoids for plant volatiles under field conditions we analysed the response variables with a GLMM with 'Block' as random factor with six levels, representing the six temporal blocks of the experiment. In a first model, we tested the effect of the different induction treatments. This was included in the model as a factor with 10 levels (each treatment as a level). We performed a second model to test the effect of the different types of herbivory. Herbivory type was included in the model as a factor with five levels (Undamaged, non-host single herbivory, non-host dual herbivory, host + non-host dual herbivory, host single herbivory) (Table 1). For the second model "Treatment" was initially nested in "Herbivory type", but was removed due to a singularity effect. Both models were performed at plant and clutch level with Tukey's HSD for *post-hoc* comparisons when a model factor was significant.

To study the interaction between non-host identity and the presence of (un-) parasitised *P. brassicae* we used a third model in which we omitted PbCg and Ud from the dataset because these treatments have no interaction with the non-host treatments. The model included three fixed factors which were i) Non-host identity, as factor with three levels (Mb, Bb and Pb), ii) the presence of unparasitised secondary host caterpillars (Pb), as factor with two levels (0 and 1) and iii) The presence of parasitised secondary host caterpillars (PbCg), as factor with two levels (0 and 1). Because the factors Pb and PbCg were expected to interact with non-host identity we initially included the interactions Pb:non-host and PbCg:non-host, but these were found to be non-significant and were removed from the model. Models were again performed on the plant and clutch level.

Data was analysed in RStudio (with R version 4.2.0) (R Core Team, 2022) using the packages "lme4" (Bates et al., 2015) and "car" (Fox & Weisberg, 2019) to build and test the mixed models ("glmer" function of "lme4"). Model assumptions and fit were tested with the "performance" and "see" packages (Lüdecke, Ben-Shachar, Patil, Waggoner, & Makowski, 2021). Estimated marginal means and standard errors were back-transformed from the logit scale, extracted with the "emmeans" package (Lenth, 2021) and plotted using "ggplot2" and "ggpubr" (Kassambara, 2020; Wickham, 2016). *Post-hoc* tests were performed at the log odds ratio scale with the "emmeans" package (Lenth, 2021). For all tests $\alpha = 0.05$ was used as level of significance.

RESULTS

Y-tube olfactometer response of *L. nana* in single and dual-herbivory context

Most of the *L. nana* females tested in the Y-tube olfactometer responded to HIPVs and made a choice. The overall response rate was 75.0%, varying from 57.5% to 81.3% between different test combinations. *Lysibia nana* females preferred volatiles from plants induced by their host-containing herbivore, parasitised *P. brassicae* (PbCg), over volatiles from undamaged plants (Ud) (GLM, $Z = 2.757$, $p = 0.006$; Fig 3a). In addition, the hyperparasitoid preferred volatiles from plants induced by non-host single herbivory over volatiles from undamaged plants (Ud) (GLM, $\chi^2 = 12.216$, df = 3, $p = 0.006$). They preferred the volatiles of plants induced by non-host *M. brassicae* (Mb) caterpillars (GLM, $Z = 2.032$ $p = 0.042$), their secondary host *P. brassicae* (Pb) (GLM, $Z = 2.640$, $p = 0.008$), but did not differentiate between volatile blends of plants induced by the non-host phloem-feeding herbivore *B. brassicae* (Bb) and undamaged plants (GLM, $Z = -0.589$, $p = 0.55$) (Fig 3b). When the complexity of the foraging scenario was increased by testing volatiles of non-host-induced plants against host-induced plants, differences were less pronounced (GLM, $\chi^2 = 6.380$, df = 3, $p = 0.09$). *Lysibia nana* preferred HIPVs of plants induced by parasitised *P. brassicae* over those induced by unparasitised *P. brassicae* (GLM, $Z = 2.149$, $p = 0.032$). However, the hyperparasitoids did not differentiate between volatile blends from plants induced by parasitised *P. brassicae* and plants induced by the non-hosts *M. brassicae* (GLM, $Z = 0.508$, $p = 0.612$) or *B. brassicae* (GLM, $Z = 1.113$, $p = 0.266$) (Fig 3c).

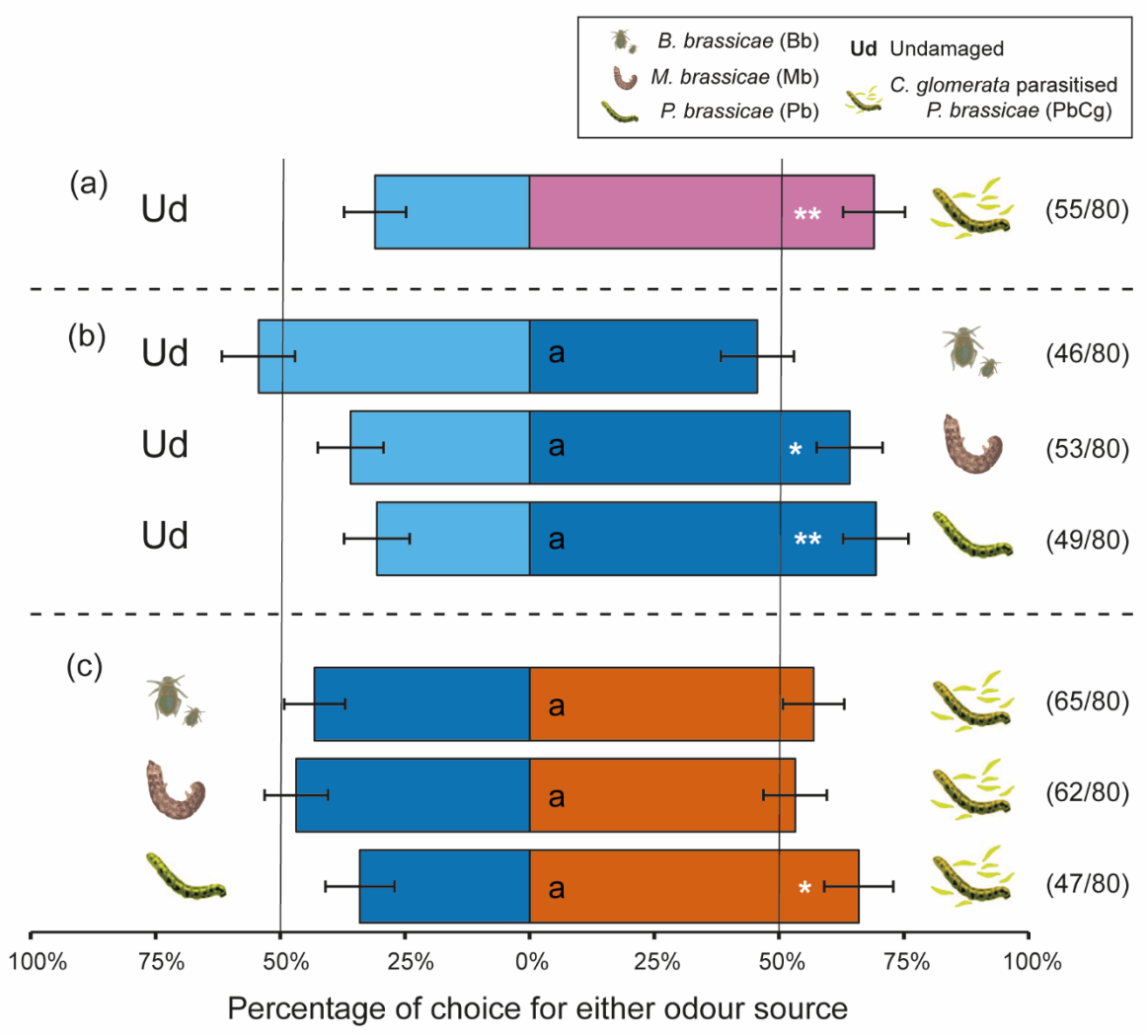


Figure 3: The preference of naive *Lysibia nana* females to plant odours in single herbivory context. This was tested in a Y-tube olfactometer, including pair-wise comparisons of plants induced by: (a) single host herbivory (pink) vs undamaged plants (light blue). (b) single non-host species (dark blue) vs undamaged plants (light blue). (c) single non-host species (dark blue) vs single host herbivory (orange). Bars indicate the percentage of individuals choosing either volatile source. Asterisks indicate significant differences. *$p < 0.05$, **$p < 0.01$. The number between brackets indicates how many of the 80 wasps in each treatment made a choice within 10 minutes. Non-responders were excluded from statistical analyses. Error bars represent the standard error of the estimated marginal mean. Different letters indicate significant differences between treatment groups (GLM with Tukey HSD, $p < 0.05$), different groups subjected to post-hoc tests are separated by dashed lines.

In choice experiments between plants with more complex mixtures of herbivores, the hyperparasitoid preferred the HIPVs of plants induced by host + non-host dual herbivory over

HIPVs induced by single herbivory of the corresponding non-host (GLM, χ^2= 19.76, df = 3, p < 0.01). However, the identity of the non-host affected the hyperparasitoids' choice. The HIPVs of plants induced with unparasitised + parasitised *P. brassicae* (Pb + PbCg) were preferred over *P. brassicae* (Pb) induced plants (GLM, Z = 2.032, p = 0.042) and similarly HIPVs of dual-infested *B. brassicae* + parasitised *P. brassicae* (Bb + PbCg) were preferred over solely *B. brassicae* (Bb) induced plants (GLM, Z = 3.560, p < 0.001). However, *L. nana* did not differentiate between the volatiles of plants induced by *M. brassicae* + parasitised *P. brassicae* (Mb + PbCg) against *M. brassicae* (Mb) induced plants (GLM, Z = -0.894, p = 0.371). Furthermore, contrasts of Bb vs Bb + PbCg and Mb vs Mb + PbCg differed (Tukey HSD, p = 0.002), indicating that the presence of *M. brassicae* caterpillars on the same plant as parasitised *P. brassicae* interferes with this preference, but the phloem-feeding aphid *B. brassicae* does not (Figure 4a). Finally, *L. nana* did not have a preference when offered the choice between the HIPVs of host-induced plants against host + non-host induced plants (GLM, χ^2 = 3.267, df = 3, p = 0.352). The choice of *L. nana* towards plants induced by their host was not affected by the co-presence of any of the three non-hosts tested (Bb: Z = 1.148, p = 0.251; Mb: Z = 1.208, p = 0.227; Pb: Z = -0.673, p = 0.501) (Fig 4b), indicating that *L. nana* does not discriminate between the HIPVs of plants with a similar fitness benefit in a dual-herbivory scenario.

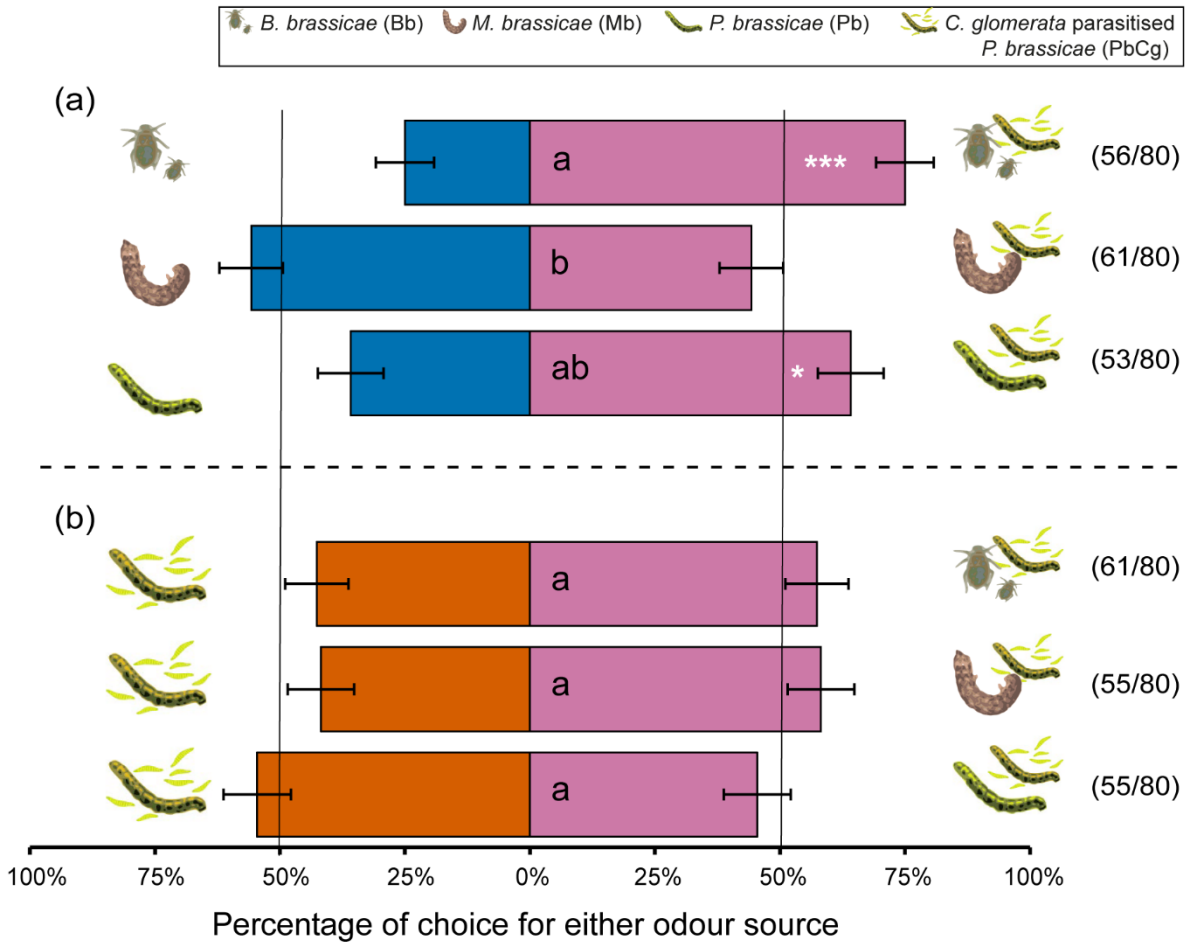


Figure 4: The preference of naive *Lysibia nana* females to plant odours in dual-herbivory context. This was tested in a Y-tube olfactometer, including odours of plants induced by a) host + non-host infested plants (pink) against odours of plants infested with solely the corresponding non-host herbivore (dark blue). b) Host + non-host-infested plants (pink) against odours of plants infested with solely its host (orange). Bars indicate the percentage of individuals choosing either volatile source. Asterisks indicate significant differences calculated with a GLM: *p<0.05, **p<0.01 ***<0.001. The number between brackets indicates how many of the 80 wasps in each treatment made a choice within 10 minutes. Non-responders were excluded from statistical analyses. Error bars represent the standard error of the estimated marginal mean and different letters indicate significant differences between treatment groups (GLM with Tukey HSD, $p < 0.05$), different groups subjected to post-hoc tests are separated by dashed lines.

Common garden experiment

Hyperparasitoids were recovered from parasitoid cocoons in all six blocks of the common garden experiment. A total number of 1181 hyperparasitoids emerged from the recollected

cocoons. Of the hyperparasitoids recovered, 1143 individuals were identified as the released species *L. nana*, 21 individuals as *Pteromalus semotus* (Hymenoptera: Pteromalidae) and 17 belonged to the genus *Gelis* (Hymenoptera: Ichneumonidae) (S1 Figure). We did not find a significant effect of the plant-induction treatment (represented by the 10 different treatments) on total hyperparasitism rate at the plant level (GLMM, $\chi^2 = 12.47$, df = 9, $p = 0.188$). The type of herbivory (five groups) did have a significant effect on the rate of hyperparasitism (GLMM, $\chi^2 = 10.68$, df = 4, $p = 0.030$) (Figure 5a, Table 2). Cocoon clutches that were attached to plants induced by host + non-host dual herbivory were more frequently parasitized by hyperparasitoids than those attached to plants that were only induced by non-host single herbivory (Tukey HSD, $p = 0.014$). All other herbivory types were found to not significantly differ in terms of hyperparasitism rate (Figure 5a). Over time, the hyperparasitism rate was highest in the four middle blocks (2-5). In block one and six, hyperparasitism rates were low where only seven and eight clutches were hyperparasitised respectively (Fig 5b).

The hyperparasitism rate of cocoons at the plant level was significantly different when host-containing herbivores (PbCg) were present during induction (GLMM, $\chi^2 = 9.70$, df = 1, $p = 0.002$) (Figure 6a). Non-host identity (GLMM, $\chi^2 = 1.694$, df = 2, $p = 0.423$) and the presence of unparasitised *P. brassicae* caterpillars during induction (GLMM, $\chi^2 = 1.983$, df = 1, $p = 0.159$) did not have a significant effect and were not found to interact with the factor for host presence (PbCg) (Figure 6a). This indicates that neither non-host presence nor species identity of the non-host interfered in the host-location of hyperparasitoids, but rather that the presence of host-containing herbivores during induction overrules the effect of non-hosts. Over the season as a whole, treatments that had a host included (PbCg) consistently had a higher hyperparasitism rate at the plant level (Figure 6b). Highly similar results were found when the models were performed at the clutch level (Table 2) or when hyperparasitism data was restricted to the species *L. nana* that was released in the field (S1 Table).

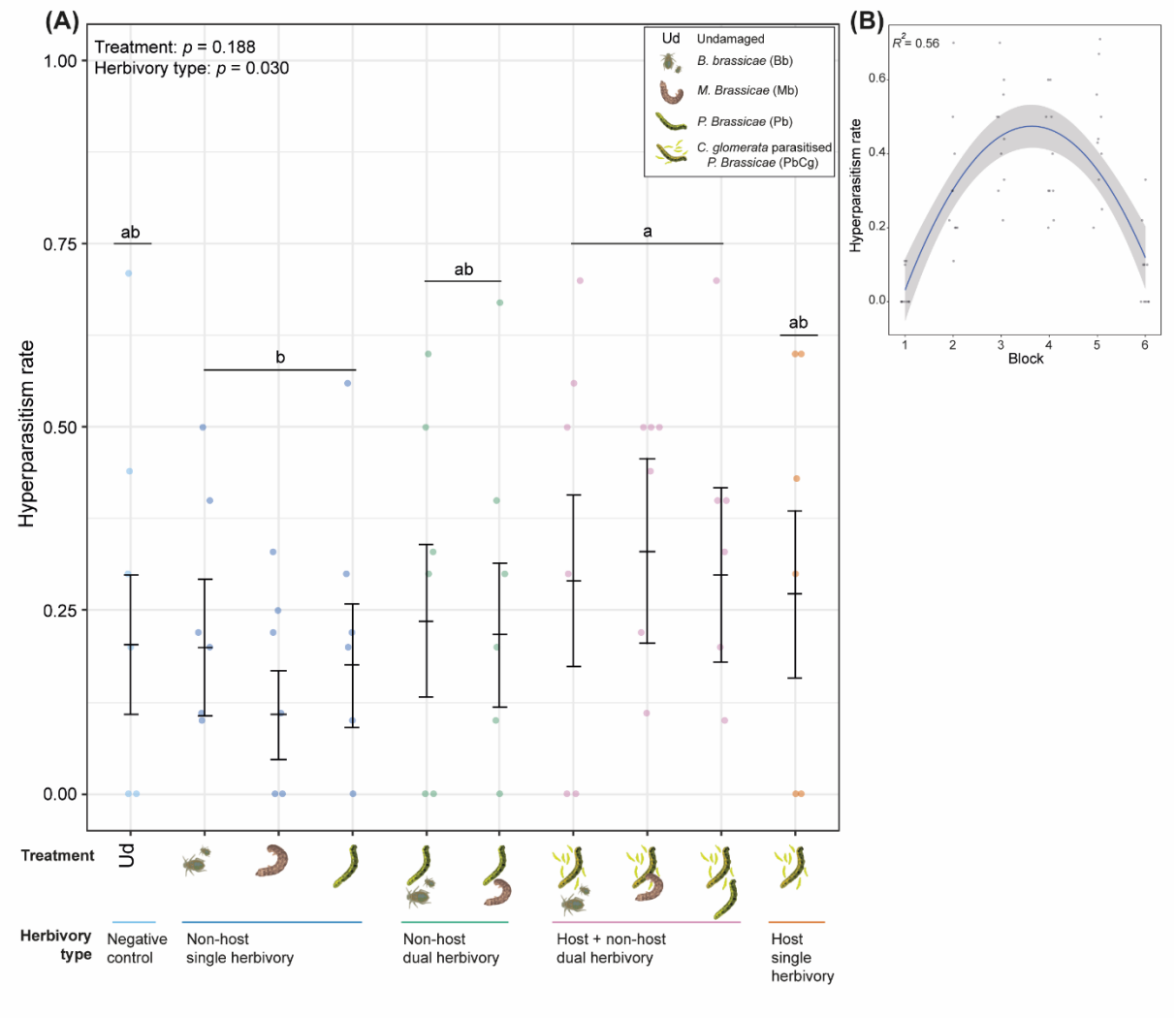
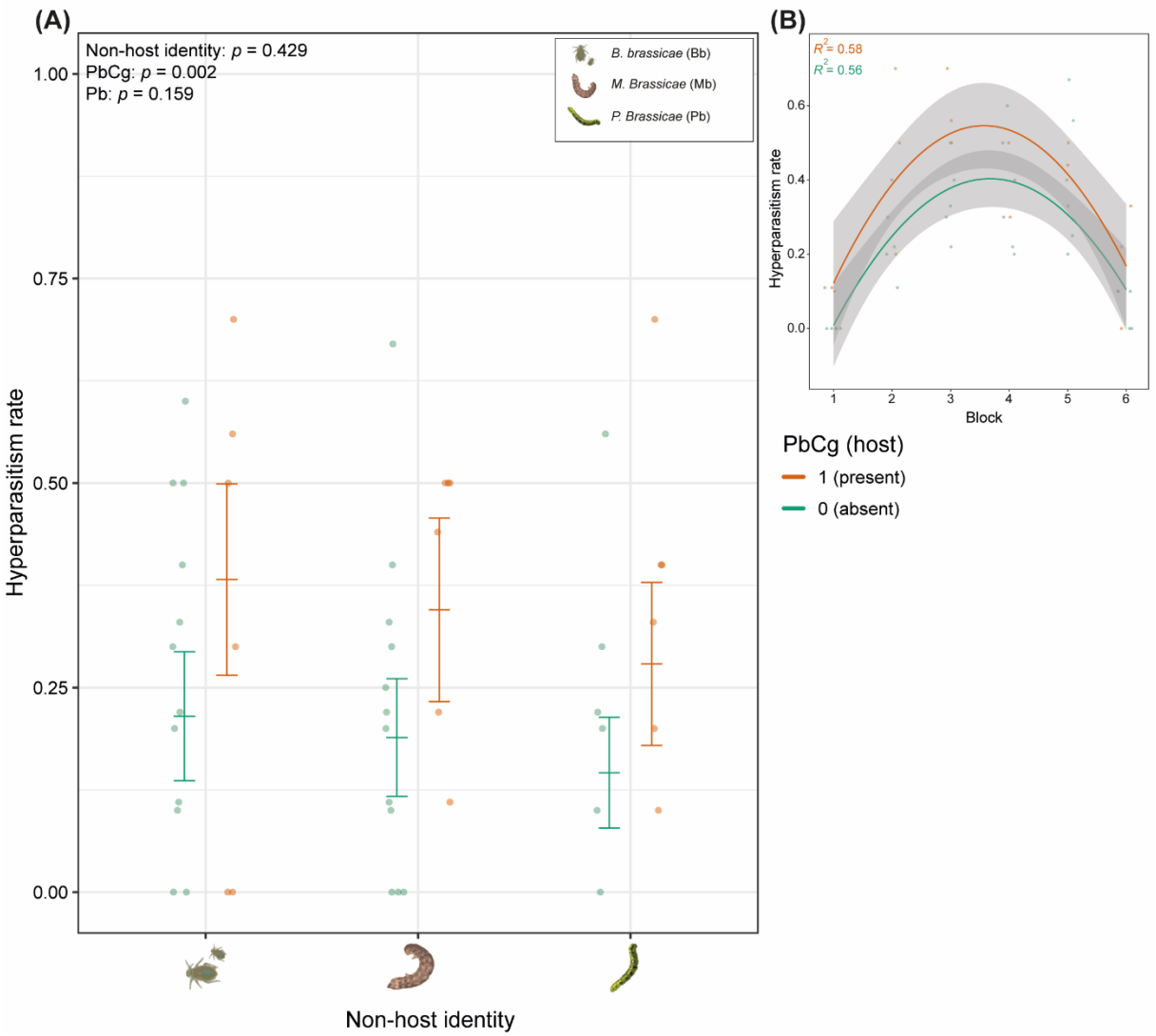


Figure 5: The hyperparasitism rate of *Cotesia glomerata* cocoon clutches on the plant level in the field: Effect of herbivory type and treatment. Clutches were attached and recollected after five days from plants with different herbivore induction treatments (see table 1). (a) The hyperparasitism rate per treatment. Dots indicate the observed values of hyperparasitism for each of the six blocks, the colour of the dots indicates the herbivory type. Light blue = control, dark blue = non-host single herbivory, green = non-host dual herbivory, pink = host + non-host dual herbivory and orange = host single herbivory. (b) The hyperparasitism rate over the course of the six temporal blocks of the experiment. Dots indicate the observed rates of hyperparasitism of each of ten treatments per block. A polynomial line ± its 95% confidence interval was drawn through these points with a R^2 value of 0.56. Crossbars in both figures indicate the estimated marginal mean ± its standard error, letters indicate significant differences between treatment groups (GLMM with Tukey HSD, $p < 0.05$).



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415 **Figure 6: The hyperparasitism rate of *Cotesia glomerata* cocoon clutches at the plant level in the field: Effect**
416 **of non-host identity.** Treatments were grouped by non-host identity (Bb, Pb or Mb) and the presence or absence
417 of (PbCg) host caterpillars during induction. (a) The hyperparasitism rate per treatment grouped by non-host
418 identity. Green = treatments with an absence of PbCg (non-host dual or non-host single herbivory), orange =
419 treatments with a presence of PbCg (host + non-host dual herbivory or host single herbivory). (b) The
420 hyperparasitism rate over the course of the six temporal blocks of the experiment. Dots indicate the observed rate
421 of hyperparasitism of each of eight included treatments per block. Polynomial lines ± their 95% confidence interval
422 were drawn through these points with a R^2 value of 0.56 (PbCg = 0) and 0.58 (PbCg = 1). Crossbars in both figures
423 indicate the estimated marginal mean ± its standard error.

424

Table 2: Overview of the models on both plant- and clutch level.

Dataset	Model (GLMM)	Model factor	Conditional R^2	Marginal R^2	χ^2	Df	*P* value
Plant	~Treatment + (1|Block)	Overall	0.316	0.031			
		Treatment			12.47	9	0.188
Plant	~Herbivory type + (1|Block)	Overall	0.310	0.023			
		Herbivory_type			10.68	4	0.030*
Plant	~ Non-host_identity + PbCg + Pb + (1|Block)	Overall	0.262	0.029			
		Non-host_identity			1.694	2	0.423
		PbCg			9.780	1	0.002**
		Pb			1.983	1	0.159
Clutch	~Treatment + (1|Block)	Overall	0.328	0.023			
		Treatment			13.12	9	0.157
Clutch	~Herbivory type + (1|Block)	Overall	0.320	0.011			
		Herbivory_type			9.608	4	0.048*
Clutch	~ Non-host_identity + PbCg + Pb + (1|Block)	Overall	0.266	0.007			
		Non-host_identity			1.081	2	0.583
		PbCg			4.3964	1	0.036*
		Pb			0.983	1	0.322

DISCUSSION

The foraging behaviour of consumers in terrestrial food webs has been intensively studied, but how top carnivores beyond the third trophic level find their resources in complex environments is still poorly understood. In this study we show that the hyperparasitoid *L. nana* has an innate preference for plants induced by parasitized caterpillars that contain its host *C. glomerata*, in

both laboratory and field conditions. However, especially the presence of *M. brassicae* interfered with this preference in the Y-tube olfactometer. Furthermore, our finding that the hyperparasitoids preferred the volatiles of plants induced by chewing herbivores over undamaged plants in the Y-tube olfactometer demonstrates that non-host-infested plants can also provide important information during early foraging steps to locate a suitable microhabitat (Beyaert & Hilker, 2014; Webster & Cardé, 2017). Foraging decisions of hyperparasitoids in the field were comparable to our controlled laboratory experiments. In the common garden experiment, hyperparasitoids favoured the cocoon clutches attached to host-induced plants. This occurred despite the presence of non-hosts in the environment or on the same plant during induction. However, the volatiles of non-host-infested plants hampered the hyperparasitoid's ability to locate host-induced plants in the Y-tube olfactometer setup. This indicates that foraging errors by hyperparasitoids may occur during HIPV-guided foraging steps, which can be corrected by using more reliable cues after landing on the plant.

In the common garden experiment, hyperparasitoids were attracted to plants induced by their parasitised caterpillar host, whereas induction by non-host herbivores in single- or dual-herbivory did not affect hyperparasitism rates. These findings are in line with previous studies, demonstrating that hyperparasitoids have a strong affinity to HIPVs induced by parasitised *P. brassicae* and can use these HIPVs to fine-tune their foraging behaviour in lab and field settings (Cusumano et al., 2019; Poelman et al., 2012; Zhu et al., 2015). It has been suggested that parasitism overrides herbivore identity in the induction of plant responses, including transcriptomic and metabolomic changes which allow caterpillar hyperparasitoids to locate their host (Zhu et al., 2015). Our results demonstrate that this overriding effect of parasitism can extend to scenarios where also non-host herbivores are present on the same plant. However, the attractiveness of HIPVs in dual infestation is known to depend on the relative densities of

the herbivores and more research is required to study the strength of this overriding effect (Desurmont, Guiguet, & Turlings, 2018).

In line with previous studies, the outcome of the common garden experiment is similar at plant and cocoon level. This finding also indicates that plant odours mostly attract hyperparasitoids but do not cause them to be arrested on the same plant, as this would result in increased hyperparasitism rates at the cocoon level (Cusumano et al., 2019). Similar results were found for both the full hyperparasitoid community and hyperparasitism by *L. nana*. Because we released *L. nana* in the common garden experiment it had an increased local abundance and a competitive advantage over other hyperparasitoids. The emerged hyperparasitoids are thus by no means representative for the natural hyperparasitoid community in the environment, which is known to include a larger diversity of pseudo-hyperparasitoids (Harvey, Snaas, Malcicka, Visser, & Bezemer, 2014; Heinen & Harvey, 2019). *Pteromalus semotus* and *Gelis spp.* were found in similar numbers, of which *P. semotus* is known to use HIPVs to locate their host (Poelman et al., 2013) while *Gelis spp.* are generalistic hyperparasitoids which are not expected to exploit HIPVs (Poelman et al., 2022). In our experiment, their abundance was too low to draw conclusions about their information usage, but our understanding of hyperparasitoid foraging will benefit from future studies including a wider range of hyperparasitoid species.

Non-host herbivores caused no interference in the common garden experiment, regardless of their identity. However, we observed a more subtle pattern in hyperparasitoid responses to plants that were induced with non-host herbivores in controlled Y-tube olfactometer bioassays. *Lysibia nana* females did not differentiate between the volatiles of plants induced by *M. brassicae* + parasitised *P. brassicae* (Mb + PbCg) against solely *M. brassicae* (Mb) induced plants. In addition, they did not prefer the odours of host-infested (PbCg) plants when given the choice against *M. brassicae* (Mb) infested plants. Interestingly,

480 the same phenomenon has been observed in foraging behaviour of the parasitoid *C. glomerata*.
481 The presence of *M. brassicae*-infested plants disrupted the foraging for its host *P. rapae* in a
482 two-choice setup, but this did not result in differences in parasitism rates in mesocosms
483 (Bukovinszky et al., 2012). In addition, we found that no HIPV-mediated differentiation
484 occurred between undamaged plants and plants induced by the phloem feeding insect *B.*
485 *brassicae*. This can be due to its feeding guild (Desurmont et al., 2014) and because *B. brassicae*
486 is a highly abundant non-host in brassicaceous food-webs (Bukovinszky, Van Veen, Jongema,
487 & Dicke, 2008; Stam et al., 2018). Thus, it can be expected that *L. nana* never developed any
488 responsiveness to its HIPV blends, but was rather selected to filter this signal in its foraging
489 strategy. Furthermore, *L. nana* females did not prefer plants induced by their parasitised
490 caterpillar host when *B. brassicae* induced plants were given as alternative option. Yet, *B.*
491 *brassicae* + parasitised *P. brassicae* (Bb + PbCg) infested plants were highly attractive to the
492 hyperparasitoid. While more studies should be carried out to disentangle the complexity of these
493 results, it is also important to note that dual induction by aphids and caterpillars has more often
494 resulted in a stronger attraction to parasitoids compared to plants induced by either herbivore
495 separately (Li, Weldegergis, Chamontri, Dicke, & Gols, 2017; Ponzio et al., 2016).

496 Comparing our lab and field results for plants infested with the non-host herbivore *M.*
497 *brassicae* indicates that *L. nana* can detect differences between host- and non-host-induced
498 plants more accurately in field conditions. First, foraging hyperparasitoids must locate host-
499 infested plants against a background of cues from plants infested by non-hosts. Feeding by hosts
500 and non-hosts on the same plant can lead to the production of HIPVs that differ in their
501 attractiveness to foraging insects compared to HIPVs produced by plants that harbour only hosts
502 (De Rijk et al., 2013; Li et al., 2017; Rowen & Kaplan, 2016; Shiojiri, Takabayashi, Yano, &
503 Takafuji, 2002). This is likely to reduce foraging efficiency (Desurmont et al., 2014;
504 Takabayashi, 2022). However, when a host-plant is selected, microhabitats are explored as well

(Aartsma et al., 2019). At this stage, repelling effects of non-hosts are increased because their close ranged chemical cues (as herbivore damage, frass, silk and cuticular hydrocarbons), with a higher reliability than HIPVs can be encountered (Fatouros, Dicke, Mumm, Meiners, & Hilker, 2008; Vet & Dicke, 1992). These cues can then be integrated by hyperparasitoids to make decisions on the invested time per plant. Similarly, aphid hyperparasitoids are known to use close range cues to identify their host and adapt their residence time on a plant (Grasswitz, 1998).

In conclusion, our findings indicate that *L. nana* employs a multi-level foraging strategy to locate their inconspicuous host. The presence of parasitised caterpillars overrules the presence of non-hosts in field conditions, although non-hosts still can hamper HIPV-guided foraging behaviours. The different nuances, but also large similarities between lab and field approaches highlight the importance of a complementary approach in studying foraging behaviour. Foraging decisions are context dependent and continuously adjusted when more reliable information becomes available (Beyaert & Hilker, 2014; Fatouros et al., 2008; Ishii & Shimada, 2010; Vet & Dicke, 1992). Our results contribute to an improved understanding of different levels of hyperparasitoid foraging in multi-herbivore communities and their infochemical usage while doing this. Future studies can use this knowledge to develop hyperparasitoid management strategies to enhance biological pest control, which is limited by hyperparasitoids (Tougeron & Tena, 2019). For example, bio-inspired bait-and-trap strategies, in which HIPVs can be used as long-range cue to attract hyperparasitoids and close range host-cues to arrest and trap them (Cusumano, Harvey, Bourne, Poelman, & de Boer, 2020). An additional advantage of such a strategy is that cues related to parasitised herbivores, including HIPVs, are known to repel the pest-supressing adult parasitoids, limiting non-target effects (Kafle et al., 2020; Tamò et al., 2006; van Neerbos et al., 2023). Still, hyperparasitoids are currently understudied, while they represent a wealth of lifestyles that have been under a strong

selection to develop foraging- and information integration strategies to deal with scarcity, inconspicuousness and time-limitation of their host. A better understanding of hyperparasitoid ecology will improve our understanding on how organisms at the top of the food web deal with complexity on different scales when foraging.

Data availability

Data will be made available in a public repository upon publication of this manuscript.

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729 Table S1: Common garden experiment: Model outcomes when the results are limited to of *L.*

730 *nana*.

Dataset	**Model (GLMM)**	**Model factor**	**Conditional R^2**	**Marginal R^2**	χ^2	**df**	***P* value**
Plant	~Treatment + (1|Block)	Overall	0.305	0.036			
		Treatment			12.805	9	0.1716
Plant	~Herbivory type + (1|Block)	Overall	0.297	0.025			
		Herbivory_type			10.461	4	0.0333*
Plant	~ Non-host_identity + PbCg + Pb + (1|Block)	Overall	0.247	0.033			
		Non-host_identity			3.7723	2	0.1516
		PbCg			9.4010	1	0.0021**
		Pb			2.2462	1	0.1339
Clutch	~Treatment + (1|Block)	Overall	0.321	0.026			
		Treatment			13.36	9	0.147
Clutch	~Herbivory type + (1|Block)	Overall	0.311	0.012			
		Herbivory_type			9.8185	4	0.0436 *
Clutch	~ Non-host_identity + PbCg + Pb + (1|Block)	Overall	0.252	0.007			
		Non-host_identity			0.6945	2	0.7066
		PbCg			3.5702	1	0.0588 .
		Pb			1.2408	1	0.26531

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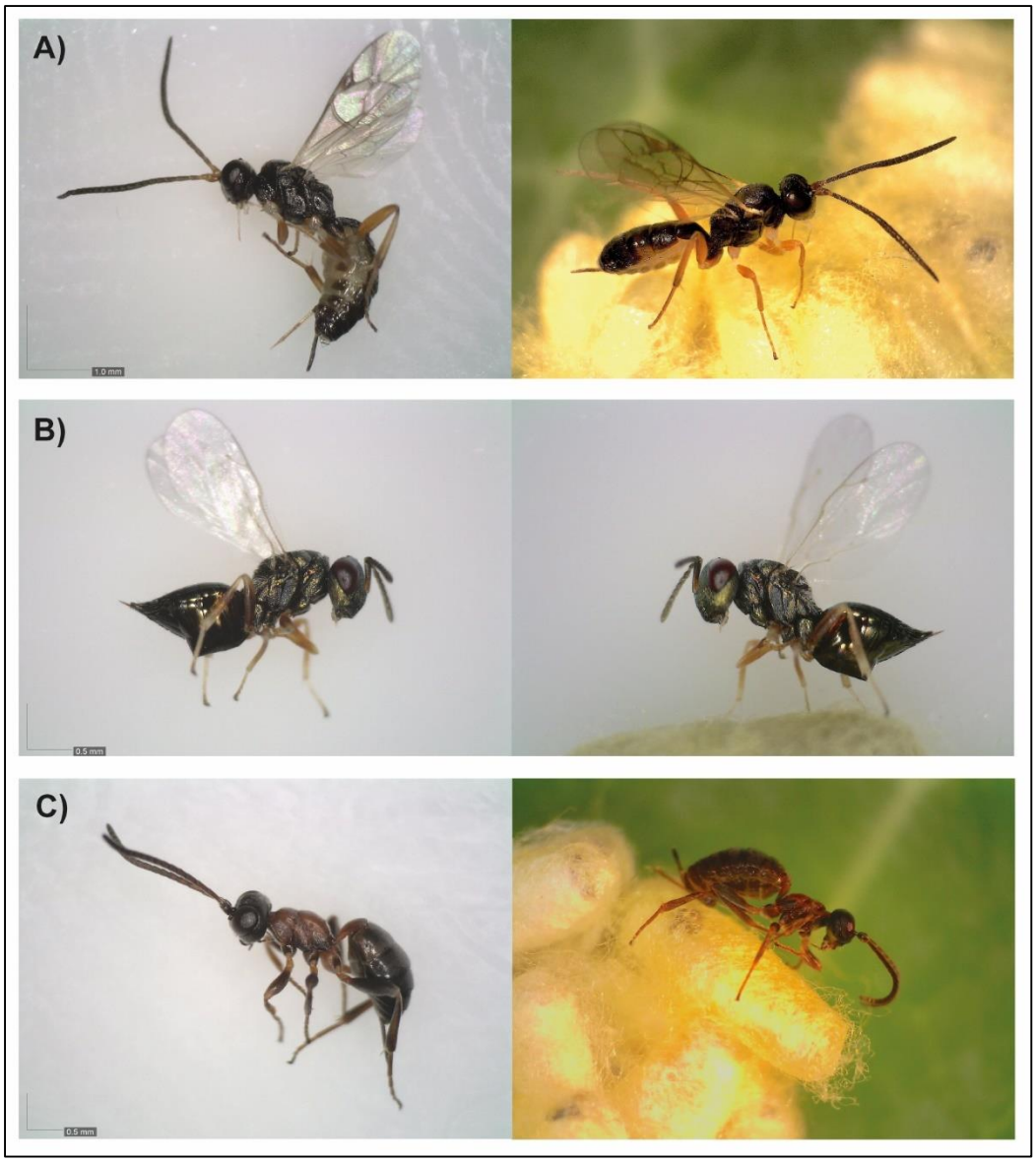

Figure S1: The hyperparasitoids found in the field. A) *Lysibia nana* (96.8% of all samples) B) *Pteromalus semotus* (1.8% of all samples) C) *Gelis sp.* (1.4% of all samples). Scales in the left corner indicate the hyperparasitoid size. Picture credit to Davide Bottacini.

Highlights:

- The presence of host- and/or non-host herbivores on a plant shapes the attractiveness of herbivore induced plant volatiles (HIPVs) to the hyperparasitoid Lysibia nana.
- Non-host species with traits similar to the host most profoundly reduced host location based on HIPVs.
- In the field, hyperparasitoids could locate their host despite non-host presence.
- Hyperparasitoids use a multi-level foraging strategy, adjusting their decisions based on the most reliable cues available.

Y-tube olfactometer data

Common garden experiment data