Investigating headspace extract from aphid infested plants to analyze the role of semiochemicals in the attraction of the natural enemy *Eupeodes corollae* (Diptera: Syrphidae)

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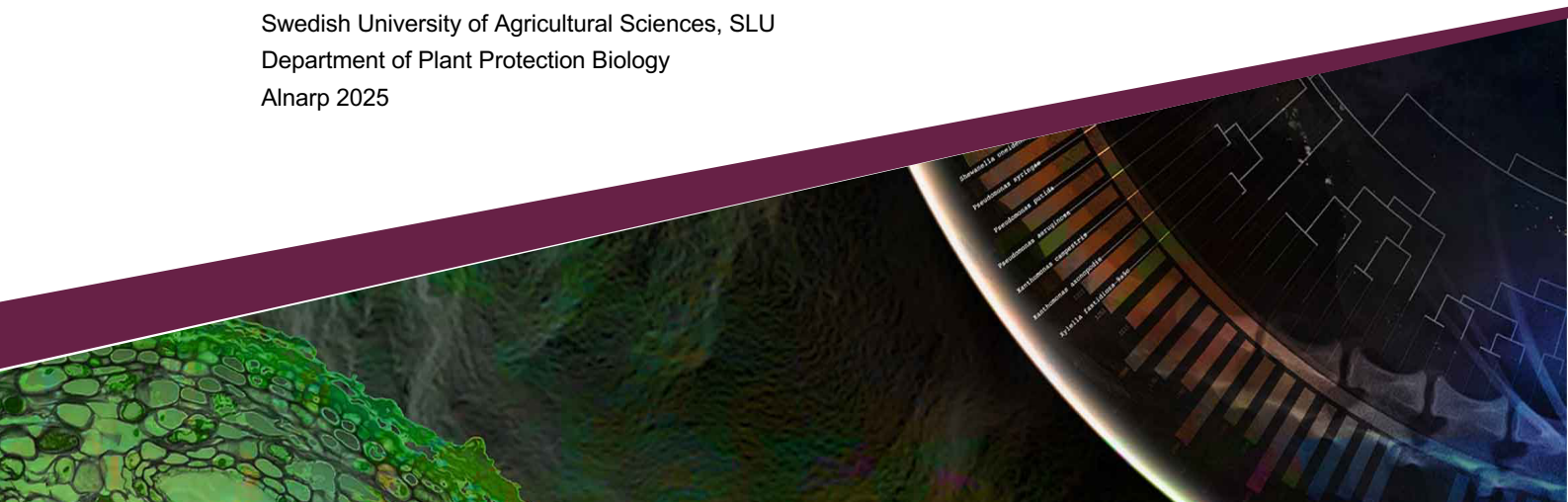
Bachelor thesis 15 hp
Swedish University of Agricultural Sciences, SLU
Department of Plant Protection Biology
Alnarp 2025



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Investigating headspace extract from aphid infested plants to analyze the role of semiochemicals in the attraction of the natural enemy *Eupeodes corollae* (Diptera: Syrphidae)

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Examinar:	Postdoctor Anna Erdai
Extent:	15 hp G2E
Year of publication:	2025
Place of publication:	Alnarp
Keywords:	*Eupeodes corollae,* kairomone, pest management, *Myzus periscae,* natural enemy, *Raphanus sativus,* wind tunnel, oviposition, Syrphidae

Abstract

Plants constitutively emit volatile organic compounds (VOCs) affecting their surroundings. Under attack by herbivores, plants emit additional distinct volatile compounds, so called herbivore induced plant volatiles(HIPVs), that may act as indirect defense by attracting natural enemies of the herbivore. Hoverflies (Diptera: Syrphidae) play a crucial role in biological pest control by preying on aphids. Gravid females using visual and chemical cues—including aphid stress secretions and HIPVs—to locate suitable oviposition sites. We investigate the attraction of *Eupeodes corollae* to VOCs from aphid-infested radish plants to better understand the semiochemicals involved in natural enemy attraction. *E.corollae* (Syrphidae) showed flight attraction towards a stream of headspace volatiles directly injected into a wind tunnel, but not towards headspace samples that were dissolved and vaporized in organic solvents. Headspace volatiles in organic solvent did not induce oviposition, suggesting that the lack of behavioral response towards headspace samples was due to the absence or wrong ratio of behavioral active chemicals, or a negative effect of the solvent.

Keywords: Eupeodes corollae, kairomone, pest management, *Myzus periscae,* natural enemy, *Raphanus sativus,* wind tunnel, oviposition, Syrphidae

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1. Introduction

To defend themselves against predation and parasitism plants have evolved different defenses to evade attack from pests (Agrawal, 1999). The defense strategies generally rely on a cost for the plant which affects future growth and development. Plant defense is divided into direct and indirect defense. Direct defense is classified as an immediate negative impact on herbivores i.e. through leaf structure, trichome density or secondary plant metabolites that impose a physical barrier or chemical mechanism which prevents or inhibits the ability of herbivores to feed on the plant (Arimura, Kost and Boland, 2005; Agrawal, 1999). An induced defense typically leads to a change in the chemical profile in the plant affecting the volatile compounds emitted from the attacked plant (Agrawal, 1999).
Herbivore induced plant volatiles (HIPVs) act as attractants for natural enemies towards the herbivore. Locating herbivorous prey by natural enemies is well known to be mediated by semiochemicals emitted by host plant and many studies have been done on HIPVs (Verheggen et al., 2008). Mono- and sesquiterpenes together with green leaf volatiles (alcohols, aldehydes or esters) are usually what these HIPVs consist of (Verheggen et al., 2008).

Mixtures of odours emitted from herbivore-infested plants have a distinct and complex chemical profile which differs depending on a number of factors. Different plant species often share compounds in their headspace composition, for example, Lima beans and cucumbers which belong to two distinct different families both emit (*E*)-β-ocimene, (*3Z*)-hex-3-enyl-acetate under distress from herbivory (Arimura, Kost and Boland, 2005). Even though compounds emitted from different species are partly overlapping the headspace composition can vary con – and heterospecifically both quantitatively and qualitatively (Arimura, Kost and Boland, 2005). Biotic factors such as genotype, cultivar, damaged plant tissue affect plant chemical headspace profiles, and abiotic conditions might play a role in the production and emission of different compounds from the plant. (Arimura, Kost and Boland, 2005). Time of year, light intensity and water stress poses a regulatory effect on the induced defense which affects the plants' ability to attract natural enemies (Arimura, Kost and Boland, 2005). Moreover, for all the variability occurring on an individual plant level, studies have shown that emissions from herbivorous animals may also vary within a species depending on

ontogenetic stage of the herbivore which influences the overall headspace composition of the plant (Arimura, Kost and Boland, 2005).

Herbivorous animals typically also emit distinct chemical profiles such as body odours, pheromones and also volatiles from associated microorganisms (Vosteen, Weisser and Kunert, 2015). Natural enemies have through evolution learned how to interpret and exploit these cues that prey cannot suppress without a cost in their fitness (Vosteen, Weisser and Kunert, 2015). To alert conspecifics some herbivores emit alarm signals under distress. Alarm signals do not necessarily benefit the signaling individual but reduces the probability of a successful attack by alarming surrounding conspecific individuals and giving them a chance for evasion (Vosteen, Weisser and Kunert, 2015). Aphid species showcase this alarm signaling behavior when under attack (Vosteen, Weisser and Kunert, 2015). A known response to attack from natural enemies is for aphids to secrete droplets from their cornicles which mainly consists of triglycerides but also alarm pheromones to signal information to conspecific individuals (Vosteen, Weisser and Kunert, 2015). The main alarm pheromone compound described in aphid species is *(E)-β*-farnesene (EBF) either alone or in mixture with other compounds (Vosteen, Weisser and Kunert, 2015). EBF has been identified as an alarm pheromone in 16 aphids species including the green peach aphid (*Myzus persicae*) (Almohamad et al., 2008; Vandermoten et al., 2012).

The quest of finding aphid infested plants for natural enemies is divided into three steps; 1) locating plants with aphids, 2) locating aphids on the plant and 3) accepting the aphid as a suitable prey (Vosteen, Weisser and Kunert, 2015). When searching for aphids a s prey the natural enemy faces a problem which is to distinguish between unspecifically detected compounds and chemical cues that reliably inform about the presence of prey. The low body mass of aphids in comparison to the host plant possibly makes the volatile emitted from aphids hard to detect. However, cues emitted by the herbivore could be more reliable compared to constitutive plant-derived volatiles (Vosteen, Weisser and Kunert, 2015). To face this detectability/reliability problem natural enemies are known to respond to HIPVs which are distinct when compared to the chemical headspace profile of a non-infested plant (Vosteen, Weisser and Kunert, 2015). The cues derived from a herbivore attacked plant often include information about said herbivore such as species, instar, duration of attack and previous infestation events which makes the reliability of the olfactory cue much more valuable for the natural enemy (Vosteen, Weisser and Kunert, 2015).

Some species of hoverflies (Syrphidae) are at their larval stage natural enemies to aphids (Amorós-Jiménez et al., 2015). Studies have shown hoverflies' great ability to control and decrease aphid populations in agriculture ecosystems. (Amorós-Jiménez et al., 2015; Verheggen et al., 2008). The syrphid larva has limited dispersal ability and is greatly

affected by the mother's choice of an oviposition site. (Verheggen et al., 2008). Selecting an oviposition site is dependent on the maturity of the female hoverfly and cues emitted from the plant and prey (Verheggen et al., 2008). The selection process includes locating the host plant, confirming said site through various olfactory, visual, and gustatory tests until the final decision, laying eggs or not (A. Raki, FJ. Verheggen and Haubruge, 2009). In agreement, studies confirm that foraging behavior of hoverflies is not a random search for prey (A. Raki, FJ. Verheggen and Haubruge, 2009). Hoverflies exposed to infested and no-infested plants assess both before choosing an oviposition site, in laboratory conditions (A. Raki, FJ. Verheggen and Haubruge, 2009).

Eupeodes corollae (Syrphidae) has since 2020 commercially been used as bio-control agent in agricultural systems. *E.corollae* is the first hoverfly species to be reared in a laboratory environment and is known to feed upon 64 aphid species. (Lillo, Perez-Bañón and Rojo, 2021). The aim of this study was to investigate the role of semiochemicals in attracting the natural aphid predator *E.corollae.* Through trapping the headspace volatiles present during an aphid infestation and analyzing the chemical profile our aim was to understand what compounds an aphid infested radish plant emits and how these compounds attract *E.corollae* and affect oviposition behavior.

The questions which lays ground for the study is:

- Are female *E.corollae* attracted to aphid infested radish plants and are the volatiles emitted by aphid infested radish plants sufficient to attract *E.corollae* females?
- Is it possible to extract the attractive compounds from aphid infested radish plants?
- Can we through bio-assay guided isolation identify the bioactive semiochemical(s)?

2. Method and materials

2.1 *Eupeodes corollae*

Eupeodes corollae, was provided by "Borregard Bioplant" in the stage of pupae. Rearing occurred in a climate-controlled room (*T*=20 C°±3 C°, photoperiod=16L:8D, *RH*=30±20 %). Hoverflies were according to emergence day kept in separate rearing cages (L×W×H=30-33×30-33×30-33 cm) and were provided pollen, water and sugar solution (5%).

2.2 *Myzus persicae*

A colony of *Myzus persicae* was reared in mesh box (L×W×H=92×48×48 cm) together with 10-12 pots of radish plants containing 2 two plants per pot *(T*=23 C°±1 C°, photoperiod=16L:8D, *RH*=30±20 %). Radish plants were replaced by uninfested plant material regularly (2-3 days) to provide the colony with fresh plant material. The room where aphid rearing occurred was semi climate controlled meaning the temperature and light is controlled but the RH is not *T*=23 C°±1 C°, Photoperiod=16L:8D, *RH*=30±20 %).

2.3 *Raphanus sativa*

Raphanus sativus was planted from seed and cultivated in a greenhouse (*T*=23°C±2°C, photoperiod=16L:8D, *RH*=50%). Two seeds were placed in nursery pots (L×W×H=8×8×9.5 cm). Plants were transferred to bigger pots (13-15 cm ∅) when first character leaves were presented. To prevent uncontrolled infestation with potential greenhouse pests, plants were through all stages of development kept in aphid-tight mesh cages (L×W×H=92-60×48-38×48-40 cm) until transfer to the aphid colony or used for experiments.

2.4 Headspace collection, solvent extraction and chemical analysis

To investigate volatile compounds emitted from aphid infested radish plants a dynamic headspace sampling was used where a continuous air stream flows through the sample container. To establish a sampling container an infested radish plant is placed in an oven cooking bag (55×55 cm). Charcoal-filtered air could enter the bag at the inlet through a connected tube. The airflow (150 ml/min) was established by a pump connected at the outlet of the bag and drawing air through the volume of the headspace. The volatile trap connected at the outlet of the bag was a filter made by a plastic tube containing Super Q adsorbent (75 mg) which was held by glass wool plugs in both ends of the plastic tube. Prior-use the filter was conditioned with 1 ml pentane and 1 ml hexane.

The sampling duration was 20-24h after which the filter was removed for elution and preparation for chemical analysis. To extract compounds adsorbed to the filter 500 µl of hexane was used to elute the samples, which were stored in glass vials. A single plant was sampled for 2 rounds (2×24h) at a temperature of 23℃±2℃, a photoperiod of16L:8D and at a relative humidity of *RH*=50%. Age and infestation rate differed slightly between plants. All infested plants were infested 3-5 days prior to the start of headspace sampling. Sampling requires a headspace extraction of a non-infested radish plant which together with a headspace sampling of an empty container served as controls to analyze if the cooking bag or other parts of the system emit volatile contaminants.

Extractions of leaves infested with *M.persicae* were performed with three solvents; methanol, dichloromethane and hexane. A single leaf with aphids was weighed and solvent was added (1 ml/1 g fresh biomass) and placed in 4-ml vials. The extraction time was 72h before removing the biomaterial. An aliquot of the extract was saved for analysis via GC-MS and the remaining extract was used for behavioral assays.

Extractions of only aphids were also conducted. Each extract was done by placing 150 aphid individuals in 1.5-ml vials and adding 500 µl of ethanol. The 500 µl was divided into extracts differing in extraction duration, 250 µl for 6 h and 250 µl for 20 h. For each extract an aliquot was saved for GC-MS analysis. The extracts were stored at -20℃ until usage.

The volatile compounds eluted from the headspace collection and solutions were analyzed on a coupled gas chromatography-mass spectrometer (GC-MS, 6890 GC and 5975 MS, Agilent Technologies Inc., Santa Clara, CA, USA). Samples (2 µL) were injected and analyzed using a non-polar capillary column (60 m x 0.25 mm i.d, 0.25 µm film thickness; HP-5MS UI, Agilent Technologies Inc.). The carrier gas was Helium (35 cm/s), and the oven temperature increased from 40 °C (held for 3 min) to 300 °C (held for 2 min) at 8 °C/min. Injector and MS transfer line temperatures were set at 225 °C. Compounds were tentatively identified based on their mass spectra and retention time using the NIST17 database (NIST MS search v2.3).

2.5 Behavioral assays

2.5.1 Oviposition behavior

Single mated female hoverflies (>5 day old) were placed in open glass containers (11.5 cmØ) covered with mesh to prevent hoverflies from escaping together with pollen and water. Placed in the container along with hoverfly and resources for the hoverfly was a dispenser containing test solutions. A non-infested radish leaf was placed vertically to mimic the natural position of the leaf acting as oviposition site for the hoverfly. The behavioral system was kept for 24 h in a climate controlled room (T=23°C±2°C, Photoperiod=18L:8D, RH=50%) and eventual eggs were counted upon the end of the experiment.

2.5.2 Flight response

The flight assay was conducted in a wind-tunnel (WT) made of Plexiglas (L×W×H=1.7×0.86×0.56 m). The airstream in the tunnel blown by a fan through charcoal filters and flowing through a compartment with a piezoelectric sprayer at an airflow of 0.3m/s was based on an earlier flight experiment conducted with the same species. Following the compartment with the sprayer and divided by a metal grid was the flight section where the hoverfly's response to odor was observed. Artificial light was lit above the WT through a diffusing filter and the light intensity was set at 1200 lux. Temperature in the tunnel ranged from 23±1°C and relative humidity 30-40°C.

Headspace solution was sprayed from the piezoelectric sprayer which is connected to a microinjection pump delivering the solutions from a 1 ml syringe at a rate of 10µl/min. At the end of the sprayer the solution was pumped through a glass capillary which vibrates creating an aerosol from the tip of the capillary. The capillary was placed at the upwind end of the WT and 20 cm above the WT floor at the same height as the release tube containing the hoverfly.
Hoverflies were also tested towards life volatiles. An aphid infested radish plant was enclosed in a glass dome and sealed with a glass floor. By connecting a pump forcing air into the dome, pushing headspace volatile compounds out through a plastic tube leading to the WT. The volatiles were released at the same location as when the sprayer was used. A non-infested radish plant was placed upwind close to the metal grid of the tunnel. All hoverflies were 5 days old when tested in the WT and as a control treatment hoverflies were tested towards non-infested plants (without headspace stimuli from infestation).

Hoverflies response to odor was scored as flies 1) Hoverflies taking off, 2) Hoverflies landing on upwind wall or plant, 3) Hoverflies taking off and escaping the WT.

Individual hoverflies were placed in glass tubes (L×Ø=7×3 cm), sealed with cotton balls at each end. Single glass tubes were placed in the center of the WT, 23 cm from downwind end and 20 cm raised in the air on a release position. The floor of the WT was marked with colorful marks to help the hoverfly to navigate within the WT. Each hoverfly was given 10 minutes to respond to the odor.

2.6 Stastical analysis

The up-wind flight towards odors sources in the wind tunnel was modeled with a GLM fitted with a binomial error distribution. Model fit was assessed using residental diagnostics, including checking for overdispurtion and examining residual plots for patterns or deviations from expected behavior. A Tukey's contrast pairwise comparison between the different treatments.

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3. Results

3.1 Chemical analysis

Analyses through injection of solvent extraction showed tentatively identified compounds (figure 1).

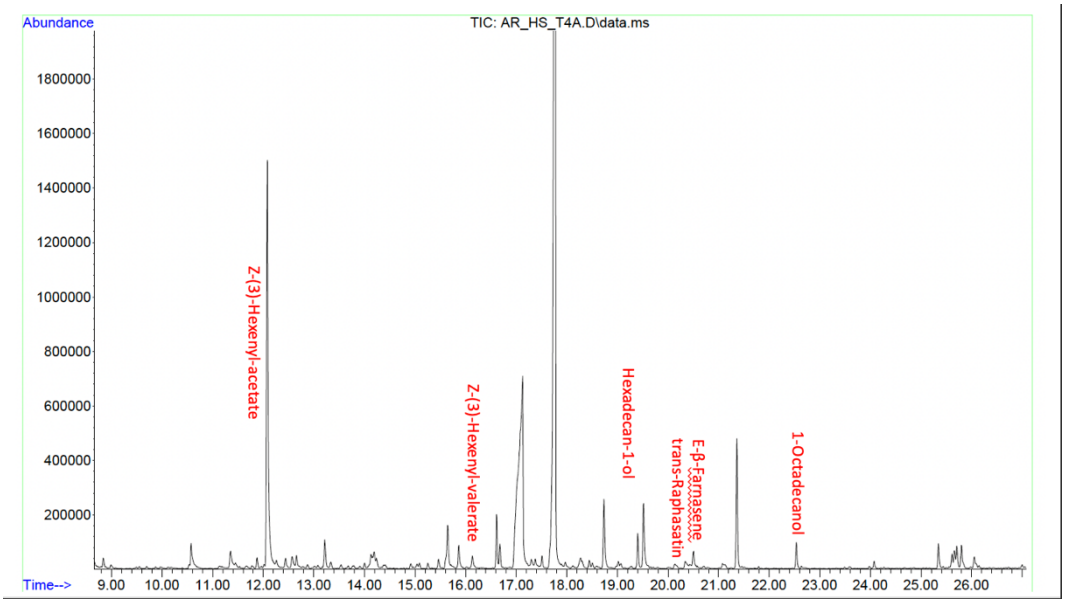

Figure 1: Chromatogram showing identified compounds from headspace extraction from *R.sativus* infested by *M.persicae* eluted in hexane. Infestation duration was 98 h when sampling was initiated.

3.2 Behavioral assays

3.2.1 Flight response

Hoverfly gravid females showed no attraction towards headspace samples applied in organic solvent and hoverflies which responded to the odor stimuli escaped the wind tunnel. When tested against headspace volatiles 36.9 % showed attraction

and flew and landed on the radish plant similar to the attraction gravid female hoverflies had against infested plant material (37.2%). Figure 2 shows the flight response of the hoverfly towards treatments. A significant difference in landing between mated female hoverflies tested towards present headspace and control treatment ($p<0.05$). No significant difference in landing between treatments was recorded where virgin flies flow towards infested radish plant and control, also no significant difference between mated flies towards infested radish plant or present headspace volatiles ($p>0.05$). There were no significant differences across all treatment in take off rate.

Table 1: Take off, upwind flight attraction and landing behaviour of E.corollae towards different sources of odor tested in a wind tunnel assay. The treatments were mated female towards present headspace volatiles, mated female towards infested radish plant, mated female towards non infested radish plant (control) and virgin female towards infested radish plant. The flight section was 170 cm long i.e 75cm=reaching half the distance to odor source, 135cm=reaching 90% of the distance to the odor source and landing=landing on the radish plant or within 20cm of it.

Treatment	Take off (%)	75cm (%)	135cm (%)	Landing (%)
Mated->Headspace	63.0	54.3	43.5	36.9 (b)
Mated->Infested	70.6	62.7	47.1	37.2 (b)
Control	60.6	36.4	12.1	9.1 (a)
Virgin->Infested	64.7	37.3	15.7	3.9 (a)

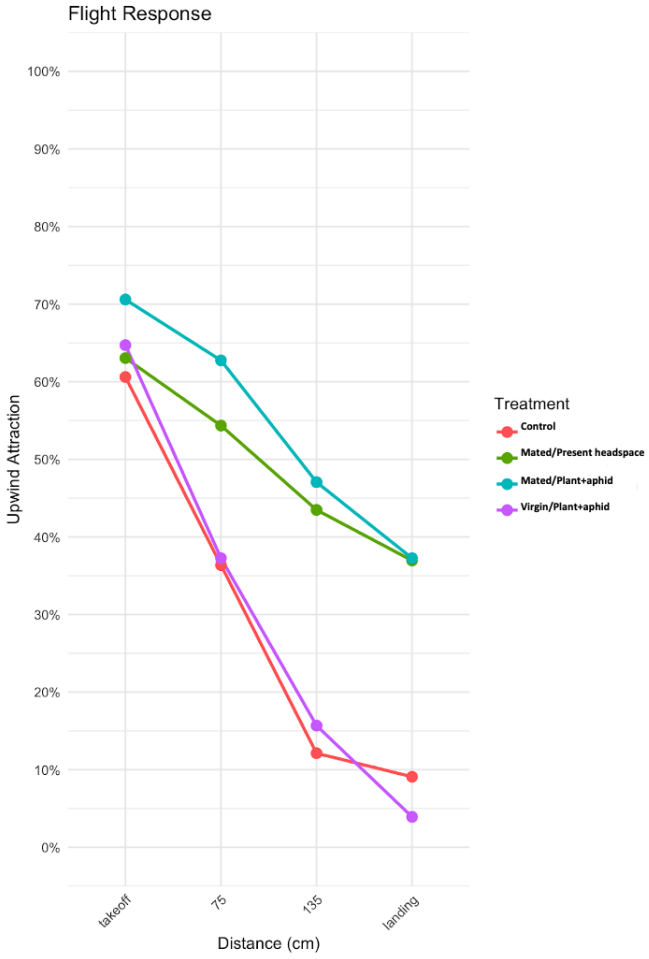

Figure 2. Flight pattern of E.corollae towards different sources of odor in wind tunnel.

3.2.2 Time before take off

Female hoverflies time before taking off; Mated towards headspace volatiles 282 s (sd=156, n=46), Mated towards solvent extract 69 s (sd=59, n=8), Control 219 s (sd=117, n=42), Mated towards infested radish plant 249s (sd=193, n=51), and virgin towards infested radish plant 205 s (sd=180, n=50) (figure 3).

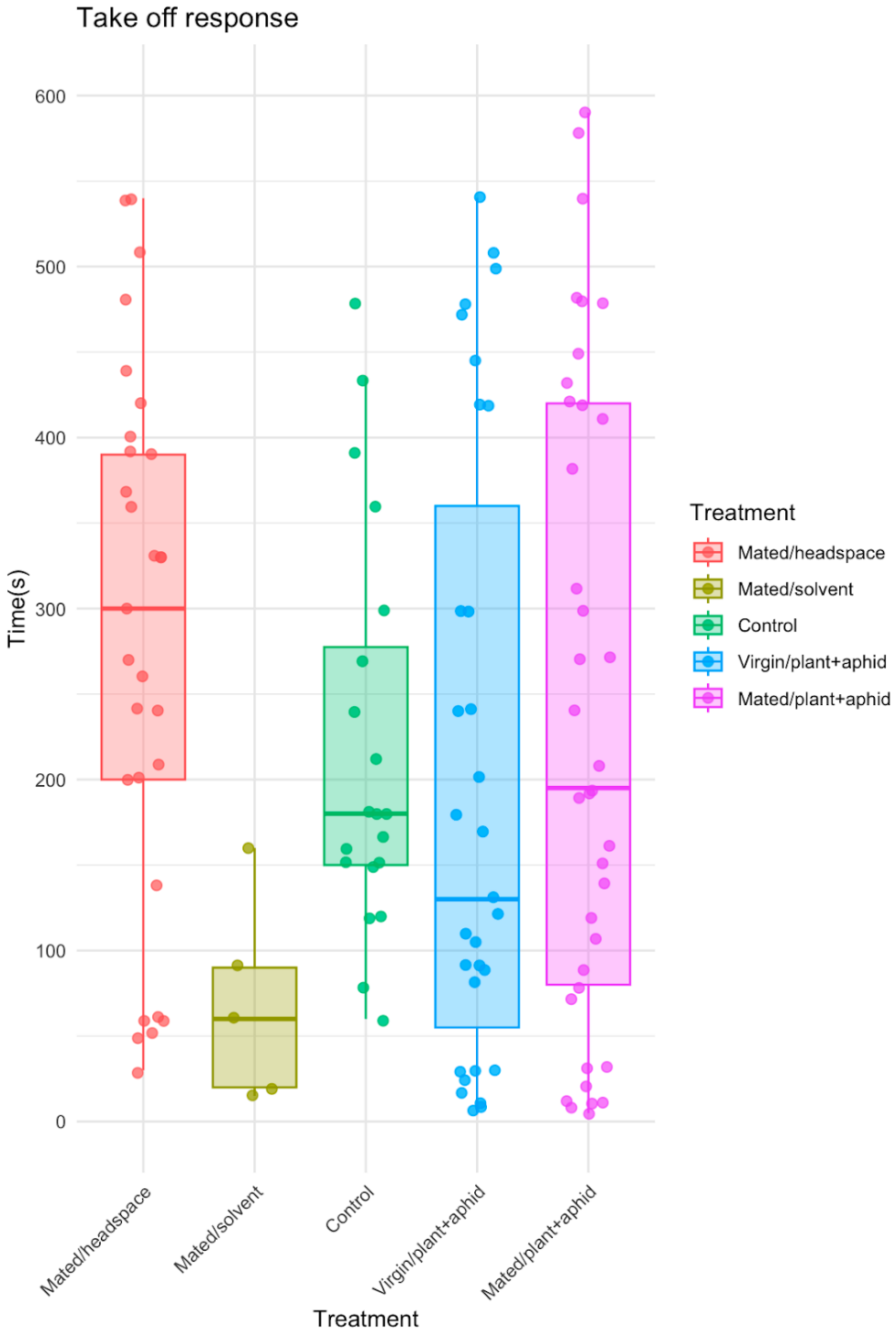

Figure 3. Boxplot visualizing time recorded before hoverflies take off after being placed in the wind tunnel.

3.2.3 Oviposition behavior

The response to chemical cue was recorded in an oviposition behavior assay. Biomass from infested leaf material along with aphids were solved in methanol, dichloromethane and hexane were tested as well headspace extract in organic solvent (hexane). None of the solutions promoted oviposition in gravid hoverfly.

4. Discussion and conclusion

Though chemical analysis of headspace volatiles eluted in hexane showed non or very small amount of EBF. Other HIPVs such as Z-(3)-hexenyl-acetate Z-(3)-hexenyl-valerate were also recorded in low amounts. Factors that might affect the lack of trapped compounds remain unclear.

When offered together with an uninfested radish plant, headspace volatiles from an aphid infested radish plant were sufficiently attractive to promote upwind flight of *E.corollae.* As recorded the attractive chemical compounds emitted serve as attractants for hoverflies searching for an oviposition site. 37% of hoverflies that were either exposed to an infested radish plant or the headspace volatiles took off, flew upwind and landed on- or within a 20 cm distance to the odor source. In contrast, uninfested plants induced upwind flight with landing in less than 10 % of tested flies . Our previous studies have shown that mating status determines attraction and further implies that virgin female *E.corollae* are not attracted towards aphid infested plants (Lundin 2025, unpublished project report).

When hoverflies' flight response was tested against headspace samples from aphid -infested plants dissolved in hexane a fleeing behavior was recorded suggesting that the solvent acted as a repellent for the hoverflies. From the results we can see that the time before taking off (62s) was shorter than for any other treatment. Additionally escaping from the windtunnel after take off was a common behavior shown by the 75% hoverflies tested against solvent extracts (n=8). Future testing of semiochemicals for understanding their role in attracting natural enemies should take the organic solvent applied to the headspace volatile collection into consideration.

Headspace volatiles applied in organic solvent were not sufficient to induce oviposition. Z-(3)-hexenyl-acetate was tested (1 µg/µl) in ethanol (n=3); methyl salicylate (100ng/µl) in ethanol (n=4) and EBF (100ng/µl) in ethanol (n=3). Biomass from plant material together with aphids were also tested, biomass:solvent = 1µg/µl (heptane) (n=5) was also tested. As a control treatment in the oviposition assay a female gravid hoverfly was placed together with aphid infested plant material and the presence of aphid did promote oviposition. This suggests that the amount of volatiles collected was not enough to induce oviposition and/or that the mixture of compounds was at the wrong ratio.

The difference in presence of aphids suggest that olfactory cues alone attract gravid female hoverflies but when finding the source of the volatiles the hoverfly

need other conformation of infestation than olfaction alone. The last step of accepting the odor source as an oviposition site may need confirmation from other sensory cues, gustatory and visual, and therefore odor alone does not induce oviposition. In near related species *Episyrphus balteus* (Syrphidae) Verheggen et al., (2008) showed that applied EBF to non-infested plant induced oviposition. With that knowledge further studies are needed to clarify the nature of behavioural active compounds and their blends to understand exactly why the attraction arises for *E.corollae*. Moreover the factors that induce oviposition should be further studied since extracts in our experiments could not induce oviposition.

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Acknowledgements

Thank you to my supervisors Associate Professor Paul G. Becher and Postdoctor Guillermo Rehemann for the support and guidance throughout this project.