RESEARCH ARTICLE

Olfactory responses of *Theocolax elegans* (Hymenoptera, Pteromalidae) females to volatile signals derived from host habitats

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Abstract

The responses of female *Theocolax elegans* (Hymenoptera: Pteromalidae) to volatile signals derived from its host habitats were investigated in a static four-chamber olfactometer. Our results demonstrated that *T. elegans* females, irrespective of experience, were apparently attracted by the odors released from the faeces of *Sitophilus zeamais* larvae and adults, which has never been investigated in previous researches. Moreover, we compared the responses of female parasitoids to odors released from grains of rice damaged by *S. zeamais* larvae, *S. zeamais* males, *S. zeamais* females, and mechanically. Artificially damaged grains do not emit large amounts of the volatiles that attract experienced parasitoid females to grains damaged by *S. zeamais* larvae. Further experiments revealed that experienced *T. elegans* females were more strongly attracted to rice grains which had been infused with extract from the heads and thoraxes of weevil larvae than to rice grains that had been infused only with sodium phosphate. The behavior of *T. elegans* females to odors released from pheromone-releasing *S. zeamais* males on healthy grains and unmated *S. zeamais* females on healthy grains were observed. The results revealed that *S. zeamais* aggregation pheromones are not useful signals for *T. elegans* females, irrespective of experience. Based on these observations, *T. elegans* females used faeces to detect potential hosts. Our results revealed that head and thorax of *S. zeamais* larvae induces rice grains to release volatiles attractive to *T. elegans* females, particularly after experience.

Introduction

Many parasitoid insects are orientated to the chemical cues released by their target or its environment. The successive steps of the orientation process, or host-finding, have been described as host habitat location, host location, host recognition and host acceptance (Vinson 1998). The behaviours and the cues involved in these steps have been examined in numerous parasitoids (Godfray 1994; Quicke 1997). Parasitoids may use stimuli from different odor sources to locate phytophagous hosts. The host as well as its food plant can be sources of stimuli. Volatile compounds emitted by the host's food can elicit long-range attraction in parasitoids (Vinson 1985; Nordlund et al. 1988; Lewis et al. 1990) and it has been assumed that they play a crucial role in mediating host-habitat location (Vinson 1976). However, host location in parasitoids has been examined mostly in systems with hosts feeding on fruits, leaves, or stems of plants or on fungi (Dorn et al. 2002; Suverkropp et al. 2008). The attractiveness of plant seeds to parasitoids of seed feeders has rarely been studied so far (Steidle et al. 2005). The success of parasitic wasps in suppressing pest populations depends on their ability to locate hosts and, consequently, understanding the mechanisms governing host searching behaviour is critical to the successful implementation of biological control programs (Gardner et al. 2007; Germinara et al. 2009; Li et al. 1992).

The present paper is devoted to a tritrophic system consisting of the parasitoid *Theocolax elegans* (Westwood) (Pteromalidae), the maize weevil *Sitophilus zeamais* (Motschulsky) (Curculionidae), and grains of rice *Oryza sativa* L. (Poaceae). The beetle *S. zeamais* is one of the most destructive insect pests of stored cereals in tropical and sub-tropical regions (Ribeiro et al. 2014). *Sitophilus zeamais* is regarded as an internal feeder of grains. Adult females of *S. zeamais* cause damage by boring into the kernel and laying eggs (ovipositing). Then, larvae eat the inner parts of the kernel, resulting in a damaged kernel and reduced grain weight (Tang et al. 2008). Apart from weight losses, the damaged kernels have low nutritional value, low rates of germination, low commercial value, and increased susceptibility to fungal infestation (Nwosu et al. 2015; Guedes et al. 2006). *Sitophilus zeamais* causes extensive losses in quality and quantity of the grain in the field as well as in storage (Carvalho et al. 2014). *Sitophilus zeamais* utilizes male produced aggregation pheromones that attract both males and females. Release and perhaps production of the pheromones by *S. zeamais* males is closely tied to feeding or contact with food: males locate food, produce pheromones, attract females and other males, and mate; females oviposit at that site, where larvae ultimately develop (Walgenbach et al. 1983; Phillips and Throne 2010). *Theocolax elegans* is a solitary ectoparasitoid that parasitizes larvae and pupae of *Rhyzopertha dominica* (F.) (Coleoptera: Bostrichidae), *Sitophilus* spp. (Coleoptera: Curculionidae), *Stegobium paniceum* (L.) (Coleoptera: Anobiidae), *Callosobruchus* spp. (Coleoptera: Bruchidae), and *Sitotroga cerealella* (Olivier) (Lepidoptera: Gelechiidae) which develop inside cereal grains or legume seeds (Flinn et al. 1996). Its wide host range of grain-damaging beetles means that *T. elegans* has been shown to have wide potential as a biocontrol agent effective in controlling insect pests of stored products and with a positive impact

on the quality of stored cereal products. However, the sources of volatiles that attract the parasitoid to grains infested with the weevils were still unknown.

The present study investigates the sources of olfactory cues that *T. elegans* uses to locate infested rice grains. The potential sources investigated were the weevil larvae, their faeces, aggregation pheromone, and the grain material. We also studied the effect of experience with a host on the attractiveness of host-related stimuli to adult females of *T. elegans*.

Materials and methods

Insect cultures

All insect cultures were kept at 26±2 °C, 70±5% relative humidity (r.h.) and a photo-period of L14: D10. To rear *T. elegans*, 50 newly emerged adult wasps were placed into Petri dishes (9 cm diameter, 1 cm high) with about 50g of rice grains infested by 3rd-4th instar larvae of *S. zeamais* and kept there until their death. After a developing time of 19–25 days, emerged parasitoids from the next generation were collected daily from each Petri dish. To rear *S. zeamais*, 30 adults were allowed to oviposit into 300ml of rice grains with about 14% moisture content in glass jars (8 cm diameter, 10 cm high). To obtain unmated males or females of *S. zeamais*, adults were separated by dimorphic rostral characteristics within 12 h of emergence (Halstead 1963).

Insects for bioassays

Parasitoids used in experiments were about 2 d old. To obtain experienced parasitoid females, recently emerged (< 24 hr old) wasps were placed in Petri dishes containing rice grains infested by weevil larvae and adults of *S. zeamais*. Females were allowed to mate and oviposit for 3 days. Subsequently they were removed and kept in Petri dishes with moistened filter paper until they were used in the experiments on the following day.

In accordance with Vet and Groenewold (1990), we define inexperienced parasitoids as insects which had no experience with the host beyond that which occurred during development within and eclosion from the host. To obtain naive parasitoids for bioassays, freshly emerged male and female parasitoids were collected from the infested grains within 1 hr of emergence and kept in Petri dishes on moistened filter paper in a climatic chamber without host odors under the same conditions as described above.

Static four-chamber olfactometer

The response of female parasitoids towards different odor samples was examined using a static four-chamber olfactometer as described by Ruther and Steidle (2000). The ol-
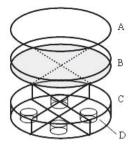
Figure 1. Static four-chamber-olfactometer used for all bioassays. For details see text.

factometer (Fig. 1) was made of acrylic glass and consisted of a cylinder (4 cm high, 19 cm diameter) divided by vertical plates into four chambers. On the top of the cylinder, a walking arena (1 cm high, 19 cm diameter) was placed consisting of plastic gauze (mesh 0.5 mm) with a rim of acrylic glass (0.9 cm high) and covered with a glass plate to prevent parasitoids from escaping. No airflow was generated. An odor sample was placed in a Petri dish (5.5 cm diameter) with brown filter paper (4 cm diameter) in one chamber or in two opposite chambers. Volatiles were allowed to diffuse through the gauze, resulting in an odor field in the walking arena above. The remaining chambers contained Petri dishes with brown filter paper only as controls.

General methods for bioassays

Evaluations were performed in a constant temperature and humidity room at 26±2 °C and 70±5% r.h., in darkness under red light to avoid distraction of parasitoids by light but to enable observations. Behavioural data were visually recorded using a stopwatch. To avoid biased results due to possible orientation preferences of the parasitoids, the position of the olfactometer was rotated clockwise by 90° after every insect. Contamination of the walking arena with sample odors or by possible pheromones of the parasitoids was avoided by cleaning the walking arenas and glass plates with ethanol and demineralized water before each insect. To avoid biased results due to possible human contamination of experimental material, disposable gloves were worn when carrying out the experiment. For all experiments, odor samples were renewed after five parasitoids each.

Fifty parasitoids were tested for each type of sample. Each individual parasitoid was used only once. At the start of each bioassay, the parasitoids were released individually in the center of the walking arena and their arrestment times in the four sectors above the arena were registered for 600 sec. The time the parasitoids spent walking in the areas directly above the Petri dishes with odor samples was compared to the areas with control Petri dishes and used to assess the arrestant effect of an odor sample. Parasitoids that walked for less than 50% of the total observation time were not included in the statistical analysis.

Responses of *T. elegans* females to *S. zeamais* faeces volatiles

Three different experiments were conducted using the static four-chamber olfactometer descried above. (1) 100 mg of faeces of *S. zeamais* larvae (LF) versus three empty Petri dishes (C); (2) 100 mg of faeces of adult *S. zeamais* males (MF) versus three empty Petri dishes (C); (3) 100 mg of faeces of adult *S. zeamais* females (FF) versus three empty Petri dishes (C).

Fifty parasitoids were tested for each experiment. Larval faeces from *S. zeamais* were obtained by sieving grain infested by 3rd-4th instar weevil larvae. Adult faeces from *S. zeamais* were obtained by sieving grain infested by unmated weevils.

Responses of *T. elegans* females to *S. zeamais* induced rice grains volatiles

We conducted a series of experiments to test the attraction of *T. elegans* females to herbivore-induced odors emitted from rice grains. (1) 50 grains infested by weevil larvae from which larvae, faeces, and egg plugs had been removed [infested grain only; (LIGO)] versus 50 healthy grains, which had been artificially damaged [artificially damaged grain; (AG)] and two empty petri dishes (C); (2) 50 grains infested by adult *S. zeamais* males from which weevils and faeces had been removed [infested grain only; (MIGO)] versus 50 healthy grains, which had been artificially damaged (AG) and two empty petri dishes (C); (3) 50 grains infested by unmated adult *S. zeamais* females from which weevils and faeces had been removed [infested grain only; (FIGO)] versus 50 healthy grains, which had been artificially damaged (AG) and two empty petri dishes (C).

Fifty parasitoids were tested for each experiment. The infested grain was obtained by dissecting grains infested by 3rd-4th instar weevil larvae, from which the larvae were removed, and removing faeces using a fine brush. Artificially damaged grains were cut with scissors, knives or needles in order to better mimic damage caused by the gnawing larvae or adults.

Responses of *T. elegans* females to extract from the heads and thoraxes of *S. zeamais* larvae and induced rice grains volatiles

Extract from the heads and thoraxes of *S. zeamais* larvae were prepared using the method described by Peiffer and Felton (2014) with slight modification: one hundred heads and thoraxes (3rd–4th instar weevil larvae) were ground with 5 ml of 0.05 M sodium phosphate (pH 8.0) (S) in order to maintain biological activity. The samples were centrifuged at 1000 r/min for 10 min. The resulting supernatant (E) was used for infusion (see below) within 12h.

A hole approximately 2 mm deep was drilled into the base of healthy rice grains with a 1 mm diameter drill. Some of the rice grains (EG) with dug holes were infused

with 2μl extract from the heads and thoraxes of *S. zeamais*, the remaining (SG) with dug holes were infused with 2μl of 0.05 M sodium phosphate (pH 8.0) alone. EG and SG were separately placed in petri dishes in humidifiers containing a saturated sodium chloride solution at 65%–70% r.h. for seven days before being used.

Two different experiments were conducted using the static four-chamber olfactometer descried above. (1) 1000 μl extract from the heads and thoraxes of 3rd-4th instar weevil larvae (E) versus 1000 μl sodium phosphate (S), and two empty petri dishes (C); (2) 50 EG that had been infused with 2 μl extract from the heads and thoraxes of 3rd-4th instar weevil larvae (LEG) versus 50 SG, and two empty petri dishes (C). Fifty parasitoids were tested for each experiment.

Responses of *T. elegans* females to *S. zeamais* aggregation pheromone

Fifty parasitoids were tested for each experiment. One experiment was conducted using the static four-chamber olfactometer to test the attraction of *T. elegans* females to aggregation pheromone of *S. zeamais*. (1) 20 pheromone-releasing *S. zeamais* males on 100 healthy grains (GM) versus 20 unmated *S. zeamais* females on 100 healthy grains (GF) and two empty petri dishes (C).

Statistical analysis

The Friedman ANOVA was used to test for differences between the four areas. In case of significant differences the Wilcoxon-Wilcox-test for multiple comparisons was used to determine which sectors are different from each other.
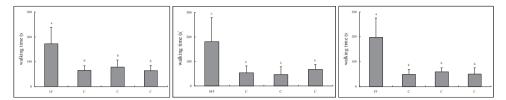
Results

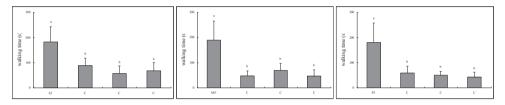### Responses of female parasitoids to faeces volatiles

Both naive and experienced parasitoid females spent significantly (Naive, LF, P=0.0009; Naive, MF, P=0.0015; Naive, FF, P=0.0006; Experienced, LF, P=0.0022; Experienced, MF, P=0.00016; Experienced, FF, P=0.0008) more time walking in the sector above the faeces than in the sectors with the control (Figs 2, 3). The results suggested that faeces of the host could be innately used as cues for habitat preference by *T. elegans*.

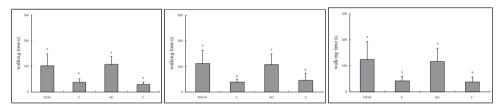### Responses of female parasitoids to *S. zeamais* induced rice grain volatiles

Both naive and experienced *T. elegans* females spent significantly more time in treatment odor fields compared to control odor fields in experiments involving either infested rice grains by *S. zeamais* from which weevil, faeces, and egg plugs had been

Figure 2. Mean walking time (± SD; n = 50) of naive females of *Theocolax elegans* in a four chamber olfactometer. LF: areas above Petri dishes with faeces of *S. zeamais* larvae, MF: areas above Petri dishes with faeces of *S. zeamais* males, FF: areas above Petri dishes with faeces of *S. zeamais* females, C: areas above control Petri dishes. Bars with different letters are significantly different at *P* < 0.05 (Friedman ANOVA followed by Wilcoxon-Wilcox-test for multiple comparisons).

Figure 3. Mean walking time (± SD; n = 50) of experienced females of *Theocolax elegans* in a four chamber olfactometer. LF: areas above Petri dishes with faeces of *S. zeamais* larvae, MF: areas above Petri dishes with faeces of *S. zeamais* males, FF: areas above Petri dishes with faeces of *S. zeamais* females, C: areas above control Petri dishes. Bars with different letters are significantly different at *P* < 0.05 (Friedman ANOVA followed by Wilcoxon-Wilcox-test for multiple comparisons).
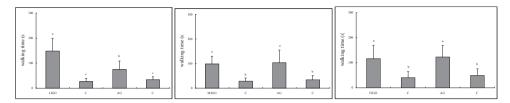

Figure 4. Mean walking time (± SD; n = 50) of naive females of *Theocolax elegans* in a four chamber olfactometer. LIGO: areas above Petri dishes with grains infested by weevil larvae from which larvae, faeces, and egg plugs had been removed, MIGO: areas above Petri dishes with grains infested by adult *S. zeamais* males from which weevils and faeces had been removed, FIGO: areas above Petri dishes with grains infested by unmated adult *S. zeamais* females from which weevils and faeces had been removed, AG: artificially damaged grains, C: areas above control Petri dishes. Bars with different letters are significantly different at *P* < 0.05 (Friedman ANOVA followed by Wilcoxon-Wilcox-test for multiple comparisons).

removed or artificially damaged rice grains (Figs 4, 5). The results suggested that *T. elegans* directs host location by using innate cues from host rice grains.

Naive *T. elegans* females showed no statistically significant difference (LIGO, P=0.2512; MIGO, P=0.1693; FIGO, P=0.2178) in choice between rice grains infested by *S. zeamais* and artificially damaged rice grains (Fig. 4). However, in contrast to inexperienced parasitoids, experienced *T. elegans* females were strongly (LIGO,

Figure 5. Mean walking time (± SD; n = 50) of experienced females of *Theocolax elegans* in a four chamber olfactometer. LIGO: areas above Petri dishes with grains infested by weevil larvae from which larvae, faeces, and egg plugs had been removed, MIGO: areas above Petri dishes with grains infested by adult *S. zeamais* males from which weevils and faeces had been removed, FIGO: areas above Petri dishes with grains infested by unmated adult *S. zeamais* females from which weevils and faeces had been removed, AG: artificially damaged grains, C: areas above control Petri dishes. Bars with different letters are significantly different at *P* < 0.05 (Friedman ANOVA followed by Wilcoxon-Wilcox-test for multiple comparisons).

P=0.0276) attracted to the rice grains infested by weevil larvae from which weevil, faeces, and egg plugs had been removed over the artificially damaged rice grains (Fig. 5). Experienced *T. elegans* females showed no statistically significant difference (MIGO, P=0.3729; FIGO, P=0.4745) in choice between rice grains infested by adult *S. zeamais* from which faeces had been removed and artificially damaged rice grains (Fig. 5). The results strongly suggested that experienced *T. elegans* females were attracted by the chemicals released from infested grains.
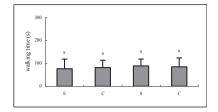
Responses of female parasitoids to extracts from heads and thoraxes of *S. zeamais* induced rice grain volatiles

Experienced *T. elegans* females showed no statistically significant difference (P=0.4659) in choice between areas containing extract from the heads and thoraxes of 3rd-4th instar weevil larvae (E) and sodium phosphate alone (S) (Fig. 6). This result confirmed that the extract from the heads and thoraxes of 3rd-4th instar weevil larvae was not directly responsible for attracting experienced *T. elegans* females.
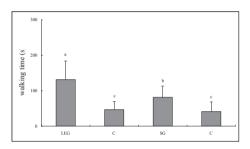
Experienced *T. elegans* females were more strongly (LEG, P=0.0381) attracted to the rice grains which had been infused with 2μl extract from the heads and thoraxes of weevil larvae over the rice grains which had been infused only with 2μl sodium phosphate (Fig. 7). Based on these observations, it appeared that the extract from the heads and thoraxes of *S. zeamais* larvae induced the wounded rice grains to release volatile chemicals for attracting *T. elegans*.

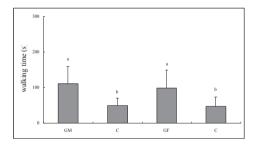## Responses of female parasitoids to aggregation pheromone

Naive and experienced *T. elegans* females spent significantly more (P < 0.05) time in treatment odor fields compared to control fields of the olfactometer in all experiments (Figs 8, 9). Naive and experienced *T. elegans* females showed no statistically significant

Figure 6. Mean walking time (± SD; n = 50) of experienced females of *Theocolax elegans* in a four chamber olfactometer. E: areas above Petri dishes with 1000μl extract from the heads and thoraxes of 3rd–4th instar weevil larvae, S: areas above Petri dishes with 1000μl of sodium phosphate alone, C: areas above control Petri dishes. Bars with different letters are significantly different at $P < 0.05$ (Friedman ANOVA followed by Wilcoxon-Wilcox-test for multiple comparisons).

Figure 7. Mean walking time (± SD; n = 50) of experienced females of *Theocolax elegans* in a four chamber olfactometer. LEG: areas above Petri dishes with grains which had been infused 2μl extract from the heads and thoraxes of 3rd-4th instar weevil larvae, SG: areas above Petri dishes with grains which had been infused 2μl of sodium phosphate alone, C: areas above control Petri dishes. Bars with different letters are significantly different at $P < 0.05$ (Friedman ANOVA followed by Wilcoxon-Wilcox-test for multiple comparisons).
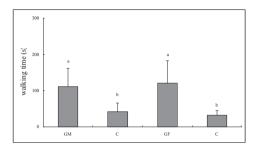

Figure 8. Mean walking time (± SD; n = 50) of naive females of *Theocolax elegans* in a four chamber olfactometer. GM: areas above Petri dishes with 20 pheromone-releasing *S. zeamais* males on 100 healthy grains, GF: areas above Petri dishes with 20 unmated *S. zeamais* females on 100 healthy grains, C: areas above control Petri dishes. Bars with different letters are significantly different at $P < 0.05$ (Friedman ANOVA followed by Wilcoxon-Wilcox-test for multiple comparisons).

difference (Naive, P=0.4879; Experienced, P=0.5127) in choice between 20 pheromone-releasing *S. zeamais* males on 100 healthy grains and 20 unmated *S. zeamais* females on 100 healthy grains (Figs 8, 9). The results suggested that *T. elegans* female response may not be mediated by aggregation pheromone.

Figure 9. Mean walking time (± SD; n = 50) of experienced females of *Theocolax elegans* in a four chamber olfactometer. GM: areas above Petri dishes with 20 pheromone-releasing *S. zeamais* males on 100 healthy grains, GF: areas above Petri dishes with 20 unmated *S. zeamais* females on 100 healthy grains, C: areas above control Petri dishes. Bars with different letters are significantly different at $P < 0.05$ (Friedman ANOVA followed by Wilcoxon-Wilcox-test for multiple comparisons).

Discussion

The results show clearly that the naive and experienced *T. elegans* females can be attracted by faeces of host *S. zeamais*. Results similar to those for *S. zeamais* faeces are responsible for attracting female *Lariophagus distinguendus* (Tang et al. 2009). Faeces, as a reliable indicator of host presence, has been described as a widespread foraging cue for parasitoids (Hendry et al. 1973; Henson et al. 1977; Gross et al. 1975; Takabayashi and Takahashi 1989; Turlings et al. 1991; Agelopoulos et al. 1995; Alborn et al. 1995; Chiu-Alvarado et al. 2010). The present results suggest that faeces could be innately used as cues for habitat preference by *T. elegans* females. This aspect was neglected in earlier work on the innate use of kairomones for host-location in *T. elegans* (Germinara et al. 2009; 2016).

Parasitoids of phytophagous hosts can be attracted directly by infested host plants (Tumlinson et al. 1992; Godfray 1994; Kennedy 2003; Ode 2006; Hilker & Fatouros 2015). However, so far, little experimental evidence has been reported to support this, and the release of herbivore-induced synomones (HIS) has almost exclusively been demonstrated in somatic plant tissues. In 2005, Steidle *et al* concluded that grains have the ability to 'whisper for help' from the parasitoid *L. distinguendus*. Here, I provide the experimental data to demonstrate that experienced females of the parasitoid *T. elegans* are able to discriminate between artificially damaged grains and grains infested by *S. zeamais* from which weevil, faeces, and egg plugs had been removed. The attractiveness could be caused by emission of volatiles from the seeds due to phytochemical induction caused by the host. There is considerable evidence that the volatile "alarm signals" are induced by interactions of substances from the herbivore with the damaged plant tissue (Steowe et al. 1995). As demonstrated by Turlings et al. (1991) for the parasitoid *Cotesia marginiventris* (Cresson) and its host *Spodoptera exigua* (Hübner) and by Mattiacci et al. (2001) for *Cotesia glomerata*

(L.) and its host *Pieris brassicae* (L.), plant volatiles can be induced by the saliva of caterpillars. Experienced *L. distinguendus* females have been shown to be strongly attracted to grains to which had been applied protein substances from original regurgitants (Tang et al. 2013).

The experimental data provided here demonstrates that the behavior of experienced *T. elegans* females is not affected simply by extract from the heads and thoraxes of 3rd-4th instar weevil larvae or sodium phosphate. More interestingly, when the extracts from the heads and thoraxes of *S. zeamais* were applied to the artificially damaged grains, the *T. elegans* females can be strongly attracted, which apparently indicated that the specific defense chemical cues attracting *T. elegans* females were released as a result of the presence of *S. zeamais* extract. It appears that the extract from the heads and thoraxes of *S. zeamais* larvae induced the wounded grains to release volatile chemicals for attracting *T. elegans*. Thus it seems likely that specific parasitoid attracting volatiles are also induced by the saliva of the feeding weevil larvae.

It appears that many predatory and parasitoid arthropods are able to intercept the sex pheromone signals of their prey or hosts. For example, Bedard (as cited in Wood 1982) first reported the attraction of a parasitoid wasp, the pteromalid *Tomicobia tibialis* Ashmead, to volatiles produced by males of the bark beetle *Ips paraconfusus* (Le Conte) boring in ponderosa pine in 1965. Later, several hymenopterous parasitoids of the elm bark beetle, *Scolytus multistriatus* (Marsham), were found to be attracted to pheromones (Steowe et al. 1995). However, the *S. zeamais* aggregation pheromones are not useful signals for *T. elegans* females. Maybe it is because *T. elegans* is a broad spectrum ectoparasitoid that parasitizes larvae and pupae.

Under conditions whereby specific pest-derived chemical cues are used by natural enemies (Cox 2004), the strategy has been considered of applying semiochemicals during biological control to attract parasitoids or predators into a crop or to increase the amount of time they spend in a field. Therefore, increased understanding of the chemically mediated interactions between arthropod hunters and their victims will be very useful for the biological controls of pests on a crop. Our ultimate goal is to be able to develop an environmentally friendly method to control pest resurgence on a crop and reduce the currently heavy dependence on pesticides.

Acknowledgements

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