

# Innate and Learned Olfactory Responses in a Wild Population of the Egg Parasitoid *Trichogramma* (Hymenoptera: Trichogrammatidae)

J. Keaton Wilson<sup>1,3</sup> and H. Arthur Woods<sup>2</sup>

<sup>1</sup>Center for Insect Science, University of Arizona, 1007 E Lowell Street, P.O. Box 210106, Tucson, AZ 85721, <sup>2</sup>Organismal Biology, Ecology and Evolution, Division of Biological Sciences, University of Montana, 32 Campus Drive HS104, Missoula, MT 59812, and

<sup>3</sup>Corresponding author, e-mail: keatonwilson@email.arizona.edu

Subject Editor: Paul Ode

Received 20 September 2016; Editorial decision 7 November 2016

## Abstract

Parasitoid insects face the fundamental problem of finding a suitable host in environments filled with competing stimuli. Many are deft sensors of olfactory cues emitted by other insects and the plants they live on, and use these cues to find hosts. Using olfactory cues from host-plants is effective because plants release volatile organic compounds (VOCs), in response to herbivory or oviposition, that contain information about the presence of hosts. However, plant-produced cues can also be misleading because they are influenced by a variety of stimuli (abiotic variation, infection and multiple sources of induction via herbivory or oviposition). Flexible behavior is one strategy that parasitoids may use to cope with variation in olfactory cues. We examine the innate and learned responses of a natural population of wasp egg parasitoids (*Trichogramma deion* and *Trichogramma sathon*) using a series of laboratory and field Y-olfactometer experiments. Wasps typically attack eggs of the hawkmoth *Manduca sexta* and *Manduca quinquemaculata* on native *Datura wrightii* plants in the southwestern United States. We show that *Trichogramma* wasps responded innately to VOCs produced by *D. wrightii* and could distinguish plants recently attacked by *M. sexta* from non-attacked plants. Furthermore, adult *Trichogramma* wasps were able to learn components of the VOC blend given off by *D. wrightii*, though they did not learn during exposure as pupae. By further exploring the behavioral ecology of a natural population of *Trichogramma*, we gain greater insight into how egg parasitoids function in tri-trophic systems.

**Key words:** learning, *Manduca*, plant VOC, parasitoid, *Trichogramma*.

How do small parasitoid insects find suitable host insects in complex environments? One strategy, adopted by many parasitoids of herbivorous insects, is to use odors emitted by plants (volatile organic compounds—VOCs) as cues to find hosts (Hare 2011, McCormick et al. 2014). Compared with host insects, plants are large and produce conspicuous olfactory signals, and they also have an evolutionary interest in alerting third trophic-level members to the presence of herbivores (Heil 2008). By using plant cues, however, parasitoids face a new problem—any packet of air may contain a multitude of plant cues, some containing useful information and others just background noise (Wilson et al. 2015). Even among plants that may provide useful information, there can be substantial variation in cues. Many parasitoids are generalists and attack multiple hosts, while many herbivorous insects feed on a variety of plants (Ali and Agrawal 2012); this is problematic when coupled with the fact that some plants release odors that are specific to the type of herbivore attacking them (De Moraes et al. 1998). Additionally, there is variation in VOC production even among individuals in populations of

plants (Dicke and Baldwin 2010, Wason and Hunter 2013). Considering all of this variability together, how do parasitoids ever find hosts?

One way may be flexible behavior, and in particular, associative learning or sensitization. In the case of parasitoid insects searching for hosts, associative learning is when an insect makes a connection between two stimuli—the host and a particular olfactory cue related to that host. In contrast, sensitization is when an insect is exposed to an olfactory stimulus and the response gradually increases with repeated exposure (Papaj and Prokopy 1989). These two processes can be hard to distinguish (Papaj and Prokopy 1989, Mackintosh 1983), but may both be beneficial strategies of flexible behavior by insect parasitoids. By learning that particular odors occur near viable hosts, parasitoids may later find other hosts more easily; in effect, the parasitoids are acting as insect statisticians, using prior correlations to predict future host locations. Insects from a variety of taxonomic groups and lifestyles are capable of learning cues in virtually all sensory modalities (Dukas 2008, Papaj and Lewis

2012). However, the evolutionary explanations for learning are still under debate. Two general adaptive explanations are posited: the neural economy hypothesis and the environmental unpredictability hypothesis. The neural economy hypothesis suggests that there is far too much complexity in the natural world for an insect to evolve reasonable innate responses for every conceivable situation and that learning is a way around this neural shortcoming (Papaj and Prokopy 1989; though see arguments against brain size being limiting in insects [Dukas 2008]). In contrast, the environmental unpredictability hypothesis posits that there is too much environmental variability over the course of the lifetime of an individual or over generations for natural selection to produce strategies for all possible scenarios (Papaj and Prokopy 1989). Although the evolutionary mechanism by which learning is favored differs between strategies, one pattern is constant: the environment is complex, and learning may help insects to find hosts.

Furthermore, although learning is often examined in adult stages, it can occur earlier and be retained into adulthood and can even persist through the complex transformations of metamorphosis (Corbet 1985, Gandolfi et al. 2003, Blackiston et al. 2008). This type of persistence may help in situations where insects would be privy to information during development that they could not obtain during adulthood—such as egg parasitoids in tri-trophic systems that are exposed to plant VOCs during development. In this scenario, a developing egg parasitoid can be certain that at least when its mother found a host, the smell of the host plant indicated the presence of a suitable host. Furthermore, some insects may only be able to learn effectively during pupal and larval development, because they have relatively short adult stages.

Although VOC induction in plants has been examined mostly in response to attack by herbivores (herbivory-induced volatile compounds or HIVOCs), VOCs can also be induced by oviposition and subsequently attract egg parasitoids (Colazza et al. 2004a,b; Pashalidou et al. 2010; Hilker and Fatouros 2015). This means that at least some cues may indicate the presence of eggs. We do not yet know whether all plants emit VOCs in response to oviposition (though evidence that induction happens across a variety of plant and insect taxa points towards some generality; Hilker and Fatouros 2015), and how such induction interacts with other factors (i.e. above- and below-ground herbivores, infection, and abiotic stress). These interactions are potentially problematic for egg parasitoids. How does a parasitoid determine whether the appropriate life-stage of its host resides on the plant if plants emit odors in response to many stimuli? They probably cope by two main routes: either by processing the complex information in VOCs induced from plants that have a myriad of induction sources, or by using past experience to guide them. Many egg parasitoids pupate inside their host eggs, which reside inside the leaf boundary layer (Woods 2010) where VOCs are especially concentrated. If the presence of a host on a particular plant in the past (when a parasitoid's mother found a host) predicts more hosts on the same species of plant, then learning the olfactory signature should be beneficial. All of this suggests that egg parasitoids should exploit plant-derived olfactory cues and that learning, even during the pupal or larval stages, may help them to find egg hosts as adults.

*Trichogramma* are well studied and widely used as biological control agents, and therefore much is known about the behavior and ecology of commercially raised species (Smith 1996, Fatouros et al. 2008, Consoli et al. 2010). There are many examples of *Trichogramma* using plant volatiles as host-finding cues (Romeis et al. 2005) and some *Trichogramma* species modify host-searching behavior in response to learned olfactory cues (Bjorksten and

Hoffmann 1998a, Ngumbi et al. 2012). Also, learned behaviors can persist for relatively long periods of time (Bjorksten and Hoffmann 1998b, Farahani et al. 2014). Here we focus on wild populations of two morphologically indistinguishable species of *Trichogramma* (*T. deion* and *T. sathon*; Potter and Woods 2012) near Portal, Arizona that attack eggs of the hawkmoths *Manduca sexta* and *Manduca quinquemaculata*. Females search for and oviposit into *M. sexta* eggs laid on the underside of *Datura wrightii* leaves. We address three main questions using a series of Y-olfactometer experiments in both the field and laboratory: 1) do wasps use olfactory cues from plants and hosts, 2) how do olfactory stimuli associated with the presence of eggs shape future behavior, and 3) how does the olfactory environment experienced during development inside the host egg shape future olfactory preference?

## Materials and Methods

### Collection and Rearing of Wasps

*Trichogramma* were collected from wild, parasitized eggs of *M. sexta* or *M. quinquemaculata* in the field near Portal, AZ. Parasitized eggs are easily recognized because of their darkened appearance. Groups of parasitized eggs were placed in 2-liter plastic containers and, once wasps emerged, were fed daily by placing balls of cotton moistened with honey water in the container. Wasps in this rearing chamber were then given *Manduca* spp. eggs (*M. sexta* and *M. quinquemaculata* are both present at our field site, and are visually indistinguishable in the egg stage) from the field to parasitize. The wasps from this F1 generation of parasitoids were used in subsequent experiments. This rearing method allowed us to generate groups of wasps that had no prior experience with leaf volatiles or odors from the field. These parasitized eggs were separated into ~2 cm lengths of one-eighth inch inner-diameter polyvinyl tubing with cotton plugs at each end. Once groups of adult wasps (hereafter termed “families”) emerged from eggs, they were fed honey water daily by moistening the cotton plug at one end. At our field site, seven species of wasps have been identified from *Manduca* eggs (Potter and Woods 2012). Here, we separated wasps by morphotype and used only those in the genus *Trichogramma*, as the two species at our field site (*Trichogramma deion* and *Trichogramma sathon*) can only be separated by genotype (Stouthamer et al. 1999). The previous work has shown that *Manduca* eggs are parasitized by either *T. deion* or *T. sathon*, and that multiple species do not frequently emerge from a single egg (Potter and Woods 2012). Family size ranged from 1 to 21 wasps in our experiments.

Across two field seasons and all experiments, we observed an approximate sex ratio of 84% female (the sexes of some individuals were unclear, and these were excluded from analysis). This sex ratio falls within the known range of sex ratios in other *Trichogramma* species (Oliveira et al. 2003).

### Wasp Behavioral Responses to Excised *D. wrightii* Leaves and *Manduca* Eggs

In total, 45 wasp families and 379 individuals were tested in series of Y-olfactometer experiments in July and August of 2013 between 8 a.m. and 9 p.m. in laboratories at the Southwestern Research Station near Portal, AZ. Temperatures in the laboratory typically varied between 19 and 27°C (Woods unpublished data). Y-olfactometers (glass arms 5 cm in length with an inner diameter of approximately 4.5 mm), connected via a drilled circular piece of transparent Plexiglas were attached to different stimuli present in small plastic cups via 40 cm of one-sixteenth inch inner diameter polyvinyl tubing.

One side contained one of three stimuli (a recently excised *D. wrightii* leaf, three recently field-collected wild *Manduca* eggs, or both a leaf and egg) with the other side acting as a control with room air. Each Y-olfactometer was taped horizontally on a table and primed by pulling air through the setup for two minutes before wasps were introduced. To generate low flow rates that would not disturb wasp movement, we used a water displacement method where water was pulled from a large plastic container at a constant rate (5 ml/s) by a Gilson Minipuls-3 (Gilson, Inc., Middleton, WI) peristaltic pump and replaced by air flowing through 95 cm of 1 per 16 in. diameter polyvinyl tube connected to the bottom of each Y-olfactometer. The total flow of air through the olfactometer was therefore 5 ml/s. In this set of experiments, and in subsequent field and laboratory experiments, wasp families were introduced at the bottom of the Y-olfactometer and the entire apparatus was covered in aluminum foil to minimize directional biases due to light. Additionally, the arm presenting the stimulus was also randomized between runs. Olfactometers were connected via a glass pipette at the end of each arm into a collection tube. Once the wasp exited the pipette tip into the tube, it could not move back into the apparatus. Collection tubes were removed and individuals counted. Between trials, Y-olfactometers were rinsed with water and dried in a drying oven at 50°C.

Families of wasps were run simultaneously in three separate Y-olfactometers in lab trials, and in two Y-olfactometers in field trials. Trials lasted until either all wasps had entered the collection tubes at the end of the Y-olfactometer or until water in the pump reservoir ran out (after ~1.5 h). Wasps in each collection tube were then counted and sex was identified later using a dissecting microscope. Any wasps that did not emerge from either arm (either because they died during the experiment or were still in the tube after 1.5 h) were not counted in the analysis.

#### Wasp Behavioral Responses to VOCs from *D. wrightii* Plants in the Field

All herbivores were removed by hand from 10 *D. wrightii* plants (any *M. sexta* or *M. quinquemaculata* larvae and eggs, as well as other common herbivorous insects) and two fifth-instar *M. sexta* larvae were placed on plant and allowing them to feed for 48 h, inducing VOC production in *D. wrightii* (Wilson and Woods 2015). *Datura wrightii* plants were from four different patches at our field site, spanning 8 km. Similarly sized plants were used to standardize the amount of leaf damage by the experimental caterpillars. After the 48-h induction period, caterpillars were removed from the plant, and intact, uneaten leaves were used as the stimulus in olfactory tests.

Sixteen naïve families of wasps (101 individual wasps) were transported to the field plants in a cooler. An identical setup to that in the laboratory trials was used, except that the pump was powered by a deep cycle marine battery connected to a power inverter, and all trials were done between 10 a.m. and 5 p.m. Y-olfactometers were covered with aluminum foil, and each trial was conducted with the entire apparatus facing the sun to control for additional light biases. Temperatures in the field (25–40°C) were much warmer than in the lab. Collection, counting and sexing methods were identical to those in lab experiments.

Additionally, four pairs of *D. wrightii* plants were used to determine whether wasps preferred olfactory cues from damaged versus undamaged plants. Paired *D. wrightii* plants of similar size and natural herbivory levels were located, and we removed all insect herbivores from them. VOC production was induced in one of the paired plants using methods described above. Instead of testing an

olfactory stimulus against background air, a comparison between the induced and un-induced plant was made by connecting each end of the Y-olfactometer to tubing attached to intact, uneaten leaves from each plant. In this series of experiments, 22 families of wasps were tested (218 individuals).

#### Learning of Olfactory Cues

In total, 47 families of wasps (387 individuals) were exposed to 1 of the 9 experimental treatments – each to one combination of 3 olfactory treatments and 3 developmental treatments. For olfactory cues, we used orange extract, decanal and z-3-hexenyl acetate (both obtained from Sigma Aldrich (St. Louis, MO, USA); decanal and z-3-hexenyl acetate are two plant VOCs often found in *D. wrightii* emissions; Hare 2007, Wilson unpublished data). A wasp family was exposed to each 1) as pupae inside the host egg, or 2) as adults after emergence from the egg, or 3) for the first time in the Y-olfactometer as adults. Pupal exposure was conducted by using parasitized eggs containing naïve families of wasps and placing a drop of scent on one end of the cotton plug on one side of the housing tube. This procedure was repeated daily until the wasps emerged as adults, at which point they were then tested in the Y-olfactometer (this exposure period varied between families, but typically lasted between 3 and 6 d after blackening of eggs – indicating development of the wasp pupae). For adult exposure, upon emergence, wasp families were given three unparasitized eggs along with a drop of each scent applied to one side of the cotton plug. Adult wasps were exposed to eggs and scent for 24 h before being tested in the Y-olfactometer. Families of naïve adult wasps were used as a control to test whether there was intrinsic attraction to or repulsion from each scent. Adult wasps were fed honey water during the course of exposure. Y-olfactometer tests, as described above, were used to determine wasp preference for each scent compared to background air.

#### Data and Statistical Analyses

All Y-olfactometer data were analyzed using repeated G-tests of goodness-of-fit. We used this approach to assess 1) whether ratios of wasps moving toward olfactory stimuli within individual families was greater than 0.5, 2) whether heterogeneity was low enough to allow us to pool families within olfactory treatments together, and 3) whether pooled ratios of wasps moving towards olfactory stimuli were greater than 0.5 (McDonald 2014). All analyses were performed in R (Version 3.1.1 – www.r-project.org).

#### Y-Olfactometers

Many tests of olfactory sensing in insects use Y-olfactometers in controlled laboratory settings. By minimizing many potential confounding factors, these types of experiments can show clear patterns of behavior. Nevertheless, they have come under recent criticism as not always being ecologically applicable (Ballhorn and Kautz 2013). In some instances, incorporating ecological variation into Y-olfactometer experiments can generate alternative conclusions (Wilson and Woods 2015). Here, we use a combination approach that employs Y-olfactometers in the laboratory and in the field, where we attempt to capture ecological variance (differences among plants, levels of herbivory, induction, and abiotic variation) that might modify insect behavior in important ways.

## Results

### Wasp Behavioral Responses to *D. wrightii* Leaves and *Manduca* Eggs in the Laboratory

Wasps moved more frequently toward odors from excised *D. wrightii* leaves ( $G = 8.194$ ,  $df = 1$ ,  $P = 0.004$ ), but not odors from *Manduca* eggs ( $G = 1.72$ ,  $df = 1$ ,  $P = 0.19$ ) or a combination of eggs and *D. wrightii* leaves ( $G = 0.248$ ,  $P = 0.619$ ). The values above are from pooled G-tests of goodness-of-fit that incorporate choices from multiple families that were subjected to each treatment (excised *D. wrightii* leaves, *Manduca* eggs, or leaves and eggs). There was significant heterogeneity among families of wasps within all treatments (*D. wrightii* leaves:  $G = 112.944$ ,  $df = 25$ ,  $P < 0.0001$ ; *Manduca* eggs:  $G = 81.464$ ,  $df = 12$ ,  $P < 0.0001$ ; eggs and leaves:  $G = 69.397$ ,  $df = 10$ ,  $P < 0.0001$ ).

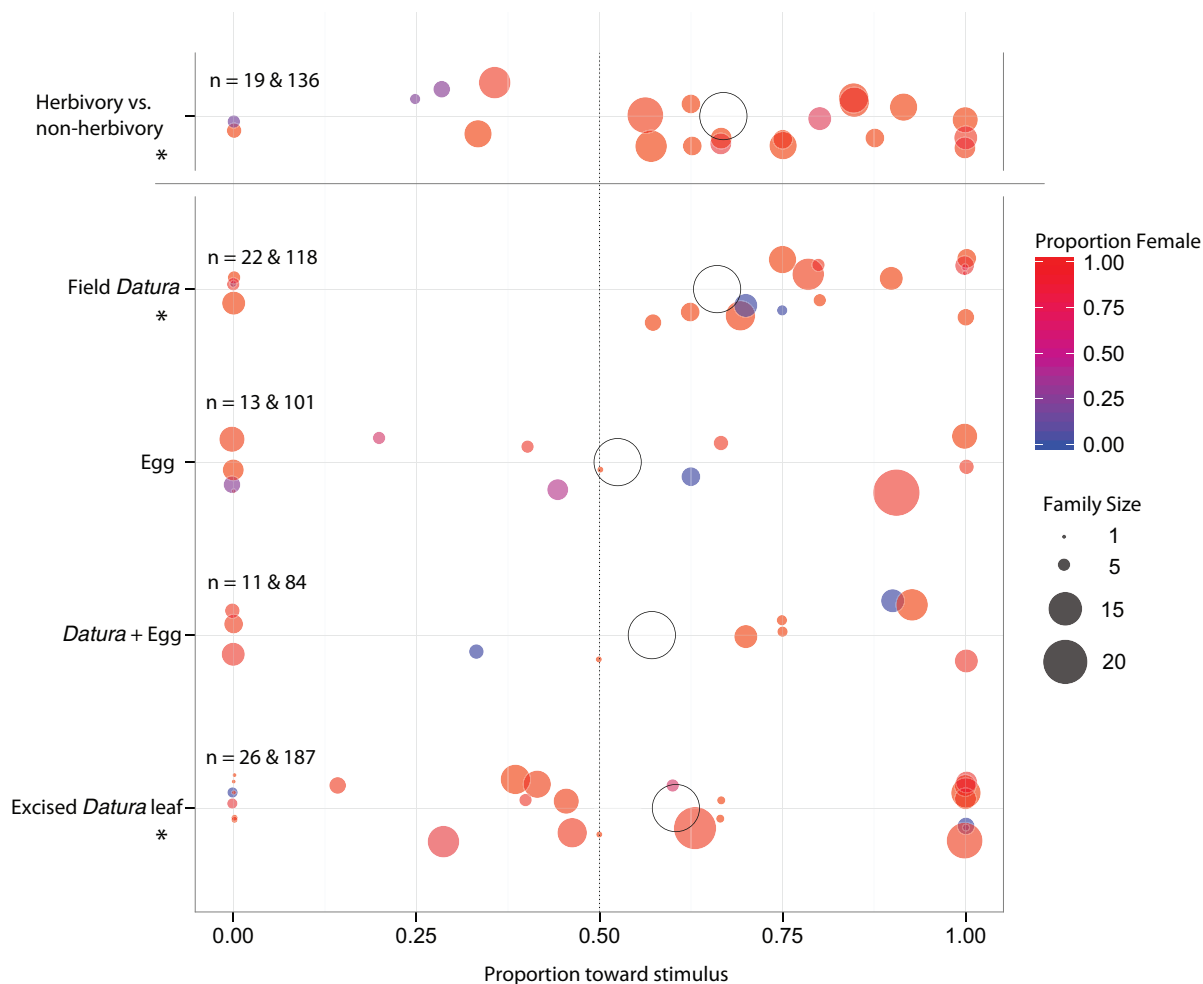
### Wasp Behavioral Responses to *D. wrightii* Leaves in the Field

Overall, wasps moved more frequently into arms of the Y-olfactometer containing odors from plants that had been attacked by *M. sexta* caterpillars compared to background air ( $G = 15.87$ ,  $df = 1$ ,  $P < 0.0001$ ). However, there was significant heterogeneity among families of wasps ( $G = 75.202$ ,  $df = 15$ ,  $P < 0.0001$ ), where some

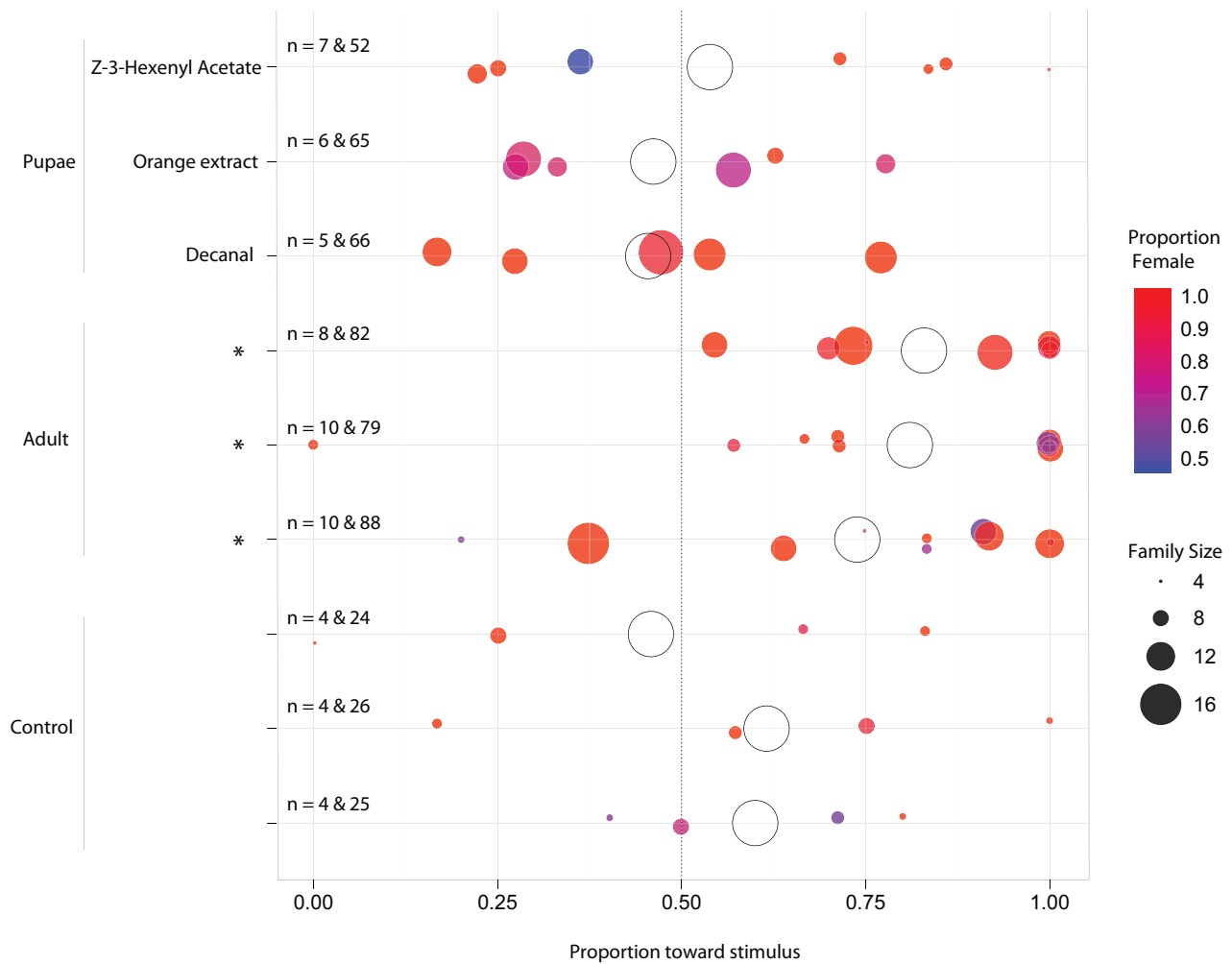
families moved toward odors emitted by *D. wrightii* but others did not (Fig. 1). Wasps also preferred odors from *D. wrightii* plants that were attacked by *M. sexta* compared to plants that were uneaten ( $G = 22.88$ ,  $df = 1$ ,  $P < 0.0001$ ). There was significant heterogeneity in this experiment as well ( $G = 80.057$ ,  $df = 21$ ,  $P < 0.0001$ ), with some families moving towards attacked plants and others moving towards un-attacked plants (Fig. 1).

### Learning of Olfactory Cues

Three statistical tests were performed on the results from each treatment: a G-test of independence, a G-test of goodness-of-fit on the summed choices of all families in a treatment, and a calculation of the total G-value (McDonald 2014). G-tests of independence showed that there was significant heterogeneity among families (i.e. some families responded positively to olfactory cues after exposure, while others did not) particularly in the adult-exposure treatment (Fig. 2). G-tests of goodness-of-fit showed that, overall, wasps responded positively to all olfactory cues (orange extract, decanal and z-3-hexenyl-acetate) after being exposed as adults to cues and moth eggs (Table 1). In contrast, wasps had no intrinsic attraction overall to olfactory cues (control treatment) without prior



**Fig. 1.** Innate responses of *Trichogramma* to natural VOCs from *D. wrightii* and *Manduca* eggs. Different experimental treatments are arranged vertically. Closed circles represent the proportion of a given wasp family that moved toward a stimulus. The number of wasps in a given family is represented by circle size and the proportion of a given family that was female is represented by the color of each circle. Open circles are the average proportions toward a given stimulus within each treatment. Asterisks represent treatments where wasps moved on average towards the stimulus greater than 50% of the time, according to performed G-tests. The number of families and individual wasps in a given treatment are also shown.



**Fig. 2.** Learned responses of *Trichogramma* to synthesized VOCs (z-3-hexenyl acetate, decanal and orange extract). Closed circles represent the proportion of a given wasp family that moved toward an olfactory stimulus. The number of wasps in a given family is represented by circle size and the proportion of a given family that was female is represented by the color of each circle. Open circles are the average proportions toward a given stimulus within each treatment. Asterisks represent treatments where wasps moved on average towards the stimulus greater than 50% of the time, according to the performed *G*-tests. The number of families and individual wasps within a given treatment are also shown.

**Table 1.** Repeated *G*-tests of goodness-of-fit for *Trichogramma* learning trials

Treatment	Odor	<i>G</i> -test of heterogeneity			Summed <i>G</i> -test of independence			Total <i>G</i>		
		<i>G</i>	df	<i>P</i>	<i>G</i>	df	<i>P</i>	<i>G</i>	df	<i>P</i>
Control	Decanal	2.45	3	0.484	1.007	1	0.316	3.456	3	0.327
	z-3-Hexenyl Acetate	11.062	3	0.011	0.167	1	0.683	11.229	3	<b>0.011</b>
	Orange Extract	10.682	3	<b>0.014</b>	1.397	1	0.237	12.078	3	<b>0.007</b>
Adult	Decanal	31.516	9	<b>0.0002</b>	20.886	1	<0.0001	52.5	9	<0.0001
	z-3-Hexenyl Acetate	18.479	7	<b>0.010</b>	38.721	1	<0.0001	57.199	7	<0.0001
	Orange Extract	42.844	9	<0.0001	32.723	1	<0.0001	75.566	9	<0.0001
Pupae	Decanal	11.747	4	<b>0.019</b>	0.546	1	0.46	12.293	4	0.015
	z-3-Hexenyl Acetate	19.515	7	<b>0.007</b>	0.308	1	0.579	19.61	7	<b>0.003</b>
	Orange Extract	9.383	5	0.095	0.385	1	0.535	9.768	5	0.082

Bold values indicate significant (<0.05) *P*-values.

experience, or when exposed to cues during pupal development (Table 1). Finally, calculations of total *G* suggest that in many treatments, there is evidence that some families responded to olfactory stimuli innately or after exposure during the pupae stage, even though the overall trend was that these treatments had no effect.

**Sex Effects**

In the analyses above, we did not discriminate between male and female wasps, primarily because of the highly biased sex ratio (~84% female) and consequent small numbers of males. When sexes were analyzed separately, male wasps did not appear to be

attracted to VOCs from attacked plants over un-attacked plants ( $G=0.43$ ,  $df=1$ ,  $P=0.512$ ), whereas females were attracted ( $G=23.836$ ,  $df=1$ ,  $P<0.0001$ ). In field Y-olfactometer experiments that compared attacked plants against background air, males were attracted to plant odors ( $G=4.86$ ,  $df=1$ ,  $P=0.027$ ) whereas females were not ( $G=1.395$ ,  $df=1$ ,  $P=0.238$ ). Laboratory Y-olfactometer experiments showed that females were attracted to *D. wrightii* odors from excised leaves ( $G=13.246$ ,  $df=1$ ,  $P=0.0032$ ) but males were not ( $G=2.346$ ,  $df=1$ ,  $P=0.125$ ). Neither males nor females were differentially attracted to either egg or egg and leaf stimuli, following the overall pattern for families. Sample sizes for the learning experiments were smaller than for other treatments, and did not provide enough power to determine whether there were sex-specific differences.

## Discussion

Wasps responded strongly and positively to VOCs produced by *D. wrightii* but not to odors produced by *Manduca* eggs (Fig. 1). This supports the hypothesis that host insects do not always produce good cues (Heil 2008), although other parasitoid species are known to use host cues directly (Fatouros et al. 2008). Compared with caterpillars, eggs are small, respire relatively little, and likely produce faint olfactory cues. However, parasitoid insects can use olfaction to detect subtle differences among hosts and often prefer hosts of a particular age. We used wild-collected *Manduca* spp. eggs of unknown age and parasitization state, and differences in age might explain some of the variability in wasp responses to eggs. Additionally, because parasitization cannot be determined in eggs until they darken from the pupation of wasp larvae inside them, some of the eggs provided as olfactory cues may have been parasitized, potentially altering wasp responses. Though wasps were attracted to *D. wrightii* VOCs in most forms presented to them, they responded most strongly to intact plants in the field (Fig. 1). Wasps were also able to distinguish between plants that had been recently attacked by *M. sexta* and those that had not (Fig. 1). Together, these results suggest that wasps at our field site respond to both constitutively emitted and induced VOCs from *D. wrightii*.

Though wasps oriented positively toward odors of *D. wrightii*, groups of wasps, groups of wasps showed high variability (Figs. 1 and 2). Such high variation has been demonstrated in other olfactory assays of *Trichogramma* species (Colazza et al. 1997, Reddy et al. 2002, Fatouros et al. 2005), and may have occurred in our work for two reasons. First, we examined the behavior of two morphologically indistinguishable species (*T. deion* and *T. sathon*). The methodological challenge of separating these species and conducting behavioral assays in the field at a remote field station are substantial. Second, both constitutive and induced VOCs can vary between individual plants (Schuman et al. 2009), so some behavioral variation may stem from differences among plants in cues emitted. Despite this ecologically relevant variability, the data still showed that *D. wrightii* plants in the field emit strong cues for egg parasitoids that attack *M. sexta* and *M. quinquemaculata* in southeastern Arizona.

In addition to innate responses, wasps also learned and responded to specific plant VOCs after exposure during adulthood (Fig. 2), meaning that wasps possess both innate and learned responses to *D. wrightii* odors. This dual approach to finding hosts may be common, and other studies have demonstrated similar patterns (Stireman 2002, Papaj and Lewis 2012). The cues emitted by plants and received by parasitoids may be responsible in part for this duality. Plant VOCs can be broadly grouped into two categories: 1)

induced volatiles that are actively synthesized and emitted by the plant following a stimulus and 2) green leaf volatiles that are released immediately from sites wounded by herbivory (Holopainen and Blande 2013). Although induced plant VOCs are highly variable across taxa, green leaf volatiles are more conserved. Thus, green leaf volatiles indicate herbivory, and the potential presence of hosts. This information can then be further refined by associatively learned responses to plant VOCs. Unfortunately, our experiments do not discriminate between the precise behavioral strategy used by wasps (associative learning or sensitization), but this is an area that would be ripe for further work in this system.

*Trichogramma* are often generalists. If they were to rely only on innate behavior for host finding, they would have to evolve recognition machinery (physiological systems to intercept and perceive odors) and response behaviors that cover the entire suite of chemicals emitted by different plants and hosts. Odor presence depends on ecological conditions (e.g., phenology, demographic cycles, abiotic variation), and varies in time and space both within the lifetime of an individual and across generations. Thus, it might be difficult for wasps to evolve a full suite of innate responses to host and plant odors. The environmental unpredictability hypothesis suggests that it is best to learn odor cues that are unique to a certain time and place (Dukas 2008), rather than evolve innate responses (Papaj and Prokopy 1989). However, a pure learning approach also has pitfalls. For example, fruit flies that were artificially selected for their ability to learn had larvae that competed poorly (Mery and Kawecki 2003) and adults that laid eggs at lower rates and died sooner (Mery and Kawecki 2004, 2005). In our system, a short-lived female parasitoid has only a brief period during her adult life to find hosts and oviposit. If she only uses learning and has no innate preferences, this may decrease her chances of finding a first host with which to associate olfactory cues. Innate and learned responses pose problems when used in isolation, which may be why many insect parasitoids employ both strategies – learning exploits novel patterns of host availability in the field whereas innate responses raise the chance of initial contact.

Wasps from the two species at our field site learned and responded to olfactory cues presented in adulthood but not in the pupal stage. We suggest two explanations for this pattern. First, although some parasitoids can learn olfactory cues during early life-stages (Gandolfi et al. 2003), wasps in our system may not have done so because they are physiologically incapable (a proximate reason) or because it is not evolutionarily beneficial (an ultimate reason); perhaps the VOCs emitted by *D. wrightii* plants do not provide good information about the presence or absence of future hosts). *Trichogramma* are generalists that attack a variety of lepidopteran hosts (Kuske et al. 2003). With a variety of available hosts, perhaps restricting searching patterns to a single host plant is not the best strategy. Second, it may be that we presented wasps with compounds outside of the context of an odor blend. They were exposed to only a few compounds (two known to be emitted from *D. wrightii* – *z*-3-hexenyl acetate and decanal and one that is not produced by *D. wrightii*, orange extract) in isolation. However, insects experience odor blends, and the information contained in chemical cues can be tied to the relative ratio of compounds to each other (Bruce et al. 2005, Wilson et al. 2015). Induced VOCs from *D. wrightii* are complex, with over 17 identified compounds in some plants (Hare 2007, Wilson unpublished data), so it may be that using only a few main components from the blend does not induce the responses produced by plants.

For parasitoids, plant VOCs can be a double-edged sword. Plants can produce large signals and may be able to improve their fitness by attracting insect carnivores. However, the signals they

produce can be noisy (Wilson et al. 2015). Because plants emit VOCs that are stimulus-specific, multiple induction stimuli (attack by below and aboveground herbivores, pathogen infection, and abiotic stress) can produce mixed signals. The VOC blend experienced by a parasitoid is the result of the combination of these factors, and contains irrelevant information that may confuse parasitoids if they are unable to filter it out or to ignore it. This scenario is further complicated for wasps in our system. Do VOCs induced by larvae reliably indicate the presence of eggs, or are oviposition-specific cues required? How much overlap is there between these two cues? Here, we show that adult wasps respond innately to the VOCs induced by larval attack (Fig. 1), but we do not yet know the details about oviposition-induced VOC production in this system. Oviposition-induced VOC production has been demonstrated in a variety of plant–insect systems (Hilker and Meiners 2002, Colazza et al. 2004b, Hilker et al. 2005, Hilker and Fatouros 2015), but to our knowledge has not been demonstrated in Solanaceae (Hilker and Fatouros 2015), the family containing *Datura* spp. and other plants typically attacked by *M. sexta* and *M. quinquemaculata*. If *D. wrightii* does not produce VOCs in response to oviposition by *Manduca*, perhaps *T. deion* and *T. sathon* use olfactory cues from larvae to make the best of a bad situation, whereby larval cues are better than no cue at all. Here, we take the first steps in determining the behavior of parasitoids that attack *Manduca* spp. eggs, but the system would benefit from further work examining oviposition-induced VOCs and the responses of wasps.

Our study is one of few to examine a natural population of two *Trichogramma* species in the field. We show that adult wasps can learn olfactory cues, and that cues from *D. wrightii* attract wasps. These results highlight the importance of egg parasitoids in tri-trophic interactions – wasps use plant VOCs to find hosts, and likely benefit plants by killing herbivores before they consume any plant tissue (unlike larval parasitoids or predatory insects). This mixture of innate and learned responses may be a product of competing problems for generalist egg parasitoids, which face a multitude of cues from different hosts and associated plants, all which vary in time and space.

## Acknowledgments

We thank to Steve Lane and Nikita Cooley for feedback on data analysis and preparation, and also thank to the director and staff of the Southwestern Research Station and to Erin McCullough for her help with field and laboratory experiments.

## Funding

This work was supported by the National Science Foundation (IOS 0844916) to H.A.W. and the University of Montana.

## References Cited

Ali, J. G., and A. A. Agrawal. 2012. Specialist versus generalist insect herbivores and plant defense. *Trends Plant Sci.* 17: 293–302.

Ballhorn, D. J., and S. Kautz. 2013. How useful are olfactometer experiments in chemical ecology research? *Commun. Integr. Biol.* 6: 1–3.

Bjorksten, T. A., and A. A. Hoffmann. 1998a. Plant cues influence searching behaviour and parasitism in the egg parasitoid *Trichogramma nr. brassicae*. *Ecol. Entomol.* 23: 355–362.

Bjorksten, T. A., and A. A. Hoffmann. 1998b. Persistence of experience effects in the parasitoid *Trichogramma nr. brassicae*. *Ecol. Entomol.* 23: 110–117.

Blackiston, D. J., E. S. Casey, and M. R. Weiss. 2008. Retention of memory through metamorphosis: Can a moth remember what it learned as a caterpillar? *PLoS ONE.* 3: e1736.

Bruce, T. J. A., L. J. Wadhams, and C. M. Woodcock. 2005. Insect host location: a volatile situation. *Trends Plant Sci.* 10: 269–274.

Colazza, S., A. Fucarino, E. Peri, G. Salerno, E. Conti, and F. Bin. 2004a. Insect oviposition induces volatile emission in herbaceous plants that attracts egg parasitoids. *J. Exp. Biol.* 207: 47–53.

Colazza, S., J. S. McElfresh, and J. G. Millar. 2004b. Identification of volatile synonemes, induced by *Nezara viridula* feeding and oviposition on bean spp., that attract the egg parasitoid *Trissolcus basalidis*. *J. Chem. Ecol.* 30: 945–964.

Colazza, S., M. C. Rosi, and A. Clemente. 1997. Response of egg parasitoid *Telenomus busseolae* to sex pheromone of *Sesamia nonagrioides*. *J. Chem. Ecol.* 23: 2437–2444.

Corbet, S. A. 1985. Insect chemosensory responses: a chemical legacy hypothesis. *Ecol. Entomol.* 10: 143–153.

Consoli, F. L., J. R. P. Parra, and R. A. Zucchi (eds.). 2010. Progress in biological control: egg parasitoids in agroecosystems with emphasis on *Trichogramma*. Springer Science and Business Media. New York, NY.

De Moraes, C. M., W. J. Lewis, P. W. Pare, H. T. Alborn, and J. H. Tumlinson. 1998. Herbivore-infested plants selectively attract parasitoids. *Nature.* 393: 570–573.

Dicke, M., and I. T. Baldwin. 2010. The evolutionary context for herbivore-induced plant volatiles: beyond the “cry for help”. *Trends Plant Sci.* 15: 167–175.

Dukas, R. 2008. Evolutionary biology of insect learning. *Annu. Rev. Entomol.* 53: 145–160.

Farahani, H. K., A. Ashouri, S. H. Goldansaz, M. S. Shapiro, A. Golshani, and P. Abrun. 2014. Associative learning and memory duration of *Trichogramma brassicae*. *Progr. Biol. Sci.* 4: 87–96.

Fatouros, N. E., G. Bukovinszkine’Kiss, L. A. Kalkers, R. S. Gamborena, M. Dicke, and M. Hilker. 2005. Oviposition-induced plant cues: do they arrest *Trichogramma* wasps during host location? *Entomol. Exp. Appl.* 115: 207–215.

Fatouros, N. E., M. Dicke, R. Mumm, T. Meiners, and M. Hilker. 2008. Foraging behavior of egg parasitoids exploiting chemical information. *Behav. Ecol.* 19: 677–689.

Gandolfi, M., L. Mattiacci, and S. Dorn. 2003. Preimaginal learning determines adult response to chemical stimuli in a parasitic wasp. *Proc. Biol. Sci./R Soc.* 270: 2623–2629.

Hare, J. D. 2007. Variation in herbivore and methyl jasmonate-induced volatiles among genetic lines of *Datura wrightii*. *J. Chem. Ecol.* 33: 2028–2043.

Hare, J. D. 2011. Ecological role of volatiles produced by plants in response to damage by herbivorous insects. *Annu. Rev. Entomol.* 56: 161–180.

Heil, M. 2008. Indirect defence via tritrophic interactions. *New Phytol.* 178: 41–61.

Hilker, M., and N. E. Fatouros. 2015. Plant responses to insect egg deposition. *Annu. Rev. Entomol.* 60: 493–515.

Hilker, M., and T. Meiners. 2002. Induction of plant responses to oviposition and feeding by herbivorous arthropods: a comparison. *Entomol. Expe. Appl.* 104: 181–192.

Hilker, M., C. Stein, R. Schröder, M. Varama, and R. Mumm. 2005. Insect egg deposition induces defence responses in *Pinus sylvestris*: characterisation of the elicitor. *J. Exp. Biol.* 208: 1849–1854.

Holopainen, J. K., and J. D. Blande. 2013. Where do herbivore-induced plant volatiles go? 4: 1–13.

Kuske, S., F. Widmer, P. J. Edwards, T. C. J. Turlings, D. Babendreier, and F. Bigler. 2003. Dispersal and persistence of mass released *Trichogramma brassicae* (Hymenoptera: Trichogrammatidae) in non-target habitats. *Biol. Control.* 27: 181–193.

Mackintosh, N. J. 1983. General principles of learning. *Anim. Behav.* 3: 149–177.

McCormick, A. C., J. Gershenzon, and S. B. Unsicker. 2014. Little peaks with big effects: Establishing the role of minor plant volatiles in plant-insect interactions. *Plant Cell Environ.* 37: 1836–1844.

McDonald, J. H. 2014. Handbook of biological statistics, 3rd ed. Sparky House Publishing, Baltimore, MD.

Mery, F., and T. J. Kawecki. 2003. A fitness cost of learning ability in *Drosophila melanogaster*. *Proc. Biol. Sci./R. Soc.* 270: 2465–2469.

- Mery, F., and T. J. Kawecki. 2004. An operating cost of learning in *Drosophila melanogaster*. *Anim. Behav.* 68: 589–598.
- Mery, F., and T. J. Kawecki. 2005. A cost of long-term memory in *Drosophila*. *Science (New York, NY)*. 308: 1148.
- Ngumbi, E., M. Jordan, and H. Fadamiro. 2012. Comparison of associative learning of host-related plant volatiles in two parasitoids with different degrees of host specificity, *Cotesia marginiventris* and *Microplitis croceipes*. *Chemoecology*. 22: 207–215.
- Oliveira, H. N., J. C. Zanuncio, D. Pratisoli, and M. C. Picanço. 2003. Biological characteristics of *Trichogramma maxacalii* (Hymenoptera: Trichogrammatidae) on eggs of *Anagasta kuehniella* (Lepidoptera: Pyralidae). *Braz. J. Biol.* 63: 647–653.
- Papaj, D. R., and R. J. Prokopy. 1989. Ecological and evolutionary aspects of learning in phytophagous insects. *Annu. Rev. Entomol.* 34: 315–350.
- Papaj, D.R., and A.C. Lewis (eds.). 2012. *Insect learning: ecology and evolutionary perspectives*. Springer Science & Business Media. New York, NY.
- Pashalidou, F. G., M. E. Huigens, M. Dicke, and N. E. Fatouros. 2010. The use of oviposition-induced plant cues by *Trichogramma* egg parasitoids. *Ecol. Entomol.* 35: 748–753.
- Potter, K. A., and H. A. Woods. 2012. *Trichogramma* parasitoids alter the metabolic physiology of *Manduca* eggs. *Proc. R. Soc. B: Biol. Sci.* 279: 3572–3576.
- Reddy, G. V. P., J. K. Holopainen, and A. Guerrero. 2002. Olfactory responses of *Plutella xylostella* natural enemies to host pheromone, larval frass, and green leaf cabbage volatiles. *J. Chem. Ecol.* 28: 131–143.
- Romeis, J., D. Babendreier, F. L. Wäckers, and T. G. Shanower. 2005. Habitat and plant specificity of *Trichogramma* egg parasitoids—underlying mechanisms and implications. *Basic Appl. Ecol.* 6: 215–236.
- Schuman, M. C., N. Heinzel, E. Gaquerel, A. Svatos, and I. T. Baldwin. 2009. Polymorphism in jasmonate signaling partially accounts for the variety of volatiles produced by *Nicotiana attenuata* plants in a native population. *New Phytol.* 183: 1134–1148.
- Smith, S. M. 1996. Biological control with *Trichogramma*: advances, successes, and potential of their use. *Annu. Rev. Entomol.* 41: 375–406.
- Stireman, J. O. iii. 2002. Host location and selection cues in a generalist tachinid parasitoid. *Entomol. Exp. Appl.* 103: 23–34.
- Stouthamer, R., J. Hu, F. J. P. M. Van Kan, G. R. Platner, and J. D. Pinto. 1999. The utility of internally transcribed spacer 2 DNA sequences of the nuclear ribosomal gene for distinguishing sibling species of *Trichogramma*. *BioControl*. 43: 421–440.
- Wason, E. L., and M. D. Hunter. 2013. Genetic variation in plant volatile emission does not result in differential attraction of natural enemies in the field. *Oecologia*. 174: 479–491.
- Wilson, J. K., A. Kessler, and H. A. Woods. 2015. Noisy Communication via Airborne Infochemicals. *BioScience*. 65: 667–677.
- Wilson, J. K., and H. A. Woods. 2015. Protection via parasitism: *Datura odors* attract parasitoid flies, which inhibit *Manduca* larvae from feeding and growing but may not help plants. *Oecologia*. 179: 1159–1171.
- Woods, H. A. 2010. Water loss and gas exchange by eggs of *Manduca sexta*: trading off costs and benefits. *J. Insect Physiol.* 56: 480–487.