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Odor learning in *Microplitis mediator* (Hymenoptera: Braconidae) is mediated by sugar type and physiological state

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ABSTRACT

Parasitoids can be conditioned to respond to novel odors through associative learning, and learning can be sensitive to physiological state. This study examined the effects of various types of sugar, and two physiological factors, mating status and oviposition experience, on odor learning in the parasitoid *Microplitis mediator* Haliday. Female *M. mediator* exhibited prolonged feeding periods on fructose, glucose and sucrose, whereas periods of feeding on raffinose, mannose and galactose were not different from water. Mating status did not affect feeding time on any sugars, but the conditioned response to eucalyptol was stronger in mated females than in virgins when the unconditioned stimulus was glucose. When females were conditioned to both food- and host-associated odors and then given a choice between them in a Y-tube olfactometer, hungry females prefered the former and satiated females, the latter, regardless of whether they had prior oviposition experience. However, oviposition experience shifted the preference of partially fed females in favor of the host-associated odor, whereas those without such experience preferred the food-associated odor. This finding suggests that parasitoid females in intermediate hunger states might be more responsive in tests of other experience effects than either starved or fully fed ones.

Key words: Gustatory response; sugar type; physiological state; learning; hunger state; ovipositional experience; parasitoid

1. Introduction

Many parasitoids play an important role in sustainable agriculture due to their ability to regulate herbivorous insect pests. However, parasitoid effectiveness against pests is often dependent on their ability to access nectar or honeydew, as sugar feeding is typically a prerequisite for survival, dispersal, and successful reproduction (e.g. Winkler et al., 2006). Many adult parasitoids exploit diverse sugar sources such as flower nectar, honeydew and pollen. Sugar feeding by parasitoids can accelerate the rate of egg maturation (Irvin and Hoddle, 2009) and substantially increase fecundity (Luo et al., 2010) and longevity (Lee and Heimpel, 2008). Although maximal carbohydrate intake may not maximize parasitoid longevity (Ellers et al., 2010), sugar deprivation reduces the efficiency of host searching (Wäckers, 1994; Takasu and Lewis, 1995). Since these factors can have a cumulative effect on parasitism rates in the field, the availability of non-prey food can be crucial to the efficacy of parasitoids in biological control (Wäckers et al., 2008).

Many parasitoid species adjust their responses to relevant chemical and visual stimuli through the pairing of a conditioned stimulus (CS) with an unconditioned stimulus (US) (Vet et al., 1995). Under natural conditions, most important resources (food or hosts) are encountered in association with specific olfactory stimuli (Wäckers and Lewis, 1994). Parasitoids employ associative learning during habitat selection (Segura et al., 2007), host searching (Darwish et al., 2003), and sugar foraging (Takasu and Lewis, 1993; 1996). Associative learning enables parasitoids to recognize and utilize profitable cues that may have only temporary associations with resources critical for survival and reproductive success in a complex and dynamic environment. However, the process of parasitoid learning is species-specific and may depend on the nature of the stimulus (Wäckers and Lewis, 1994; van Baaren et al., 2005) as well as the physiological state of the parasitoid. For example, developmental stage (Honda and Kainoh, 1998), mating status (Pérez-Maluf and Kaiser, 1998), oviposition experience (Kaiser et al., 2003) and hunger level (Lewis and Takasu, 1990) have all been shown to influence learning. Learning is also sensitive to parasitoid age. Females of *Ascogaster reticulatus* Watanabe can learn to

respond to tea leaf extract within 24 h of emergence, but older females demonstrate stronger learning ability (Honda and Kainoh, 1998).

The present study examined associative learning in *Microplitis mediator* (Haliday) (Hymenoptera: Braconidae), a larval endoparasitoid of two serious agricultural pests, the cotton bollworm, *Helicoverpa armigera* (Hübner), and the oriental armyworm, *Mythimna separata* (Walker) (Lepidoptera: Noctuidae). The objectives were to determine whether associative learning of odors by *M. mediator* females is influenced by the type of sugar provided as the unconditioned response, and whether conditioned responses to food- and host-associated odors were sensitive to female physiological states (mating status, hunger level).

2. Materials and methods

2.1. Insects

A colony of *M. mediator* was extablished from material obtained from the Institute of Plant Protection, Hebei Academy of Agriculture and Forestry in China. All insects were held at 26 ± 1 ℃, 60–80% RH and a photoperiod of 14 L:10 D, unless otherwise specified. Females parasitoids were offered second instar larvae of *M. separata* feeding on corn leaves for 24h, whereupon leaves with parasitized hosts were transferred to mesh rearing bags ($30 \times 40 \times 25$ cm) until parasitoids pupated. Cocoons were isolated in glass vials (7 cm $\times$ 2 cm diam.) covered with a piece of gauze until adult emergence.

2.2. Sugar treatments

Six types of sugars were tested in gustatory response and appetitive conditioning experiments that all occur naturally in nectar and/or honeydew, including D(-) fructose (Sigma), D(-) glucose (Sinopharm Chemical Reagent), D(+) galactose (Sinopharm Chemical Reagent), D(+) mannose (Sinopharm Chemical Reagent), D(+) sucrose (Sinopharm Chemical Reagent), and D(+) raffinose pentahydrate (Sigma). All sugars were >99% pure and dissolved in distilled water to make 1 M concentration solutions.

One M glucose solutions were used in hunger level and oviposition experience tests.

2.3. Gustatory response assay

Newly emerged adult wasps were maintained in plexiglass cages (30×30×45 cm) in a rearing room at a constant temperature of 19 ± 1 , 60–80% RH and photoperiod 14L : 10D conditions and provided only with water on a cotton wick. Females for the mated treatment (n ~ 40) were provided with equal numbers of males (n ~ 40) in the cage, whereas virgin females (n ~ 80) were held in same-sex groups. Male wasps were removed from the mating treatment after two days and all females were assumed to be mated. All the wasps were used in the experiment at three days of age. Previous studies (Luo et al., 2010) indicated that food-deprivation for this period results in wasps that are motivated to feed without any perceptible negative effects on behavior.

Individual females (virgin or mated) were offered 1M solution of each of the six type of sugars, or water as a control. Female parasitoids were collected from cages in small glass vials (8cm x 2 cm diam.) and introduced individually into glass Petri dishes (9 cm diam) that had been prepared with a 10 μl droplet of each test solution on the bottom of Petri dish. Each vial was then inverted over a droplet of solution until the parasitoid within contacted the droplet with its antennae. Contact invariably elicited feeding and each wasp was allowed to feed until satiation. Gustatory response was estimated by quantifying the time that parasitoid mouthparts remained in contact with the droplet (Wäckers, 2001). Each treatment was replicated 25 times and each parasitoid was tested only once. Data were checked for normality and homoscedasticity as appropriate and, if needed, were arcsine square root or log-transformed before being subjected to a one-way ANOVA (SAS Institute, 2004). Fisher's protected LSD test ($\alpha = 0.05$) was used to separate treatment means.

2.4. Associative learning assay

In this experiment, both virgin and mated wasps were exposed to an unconditioned stimulus (sugar) in association with a conditioned stimulus (eucalyptol odor). The same

series of sugars (as above) were tested for suitability as an unconditioned stimulus in appetitive conditioning. A 1:10 solution of eucalyptol (as 1,8 – cineol, Sigma, St Louis, MO) in hexane was used as the conditioned stimulus in association with a sugar solution, with control groups offered eucalyptol with water or eucalyptol alone. Wasps were exposed to stimuli, one at a time, in the bottom of a glass Petri dish (9 cm diam.) with the lid removed, as described by Wäckers et al. (2006). Twenty µl of the cineol-hexane solution was added to a square piece of filter paper (2 x 2 cm) which was then placed into the open Petri dish and left for 5 min to evaporate the hexane carrier. A glass microscope cover slip (6 x 6 mm) was placed on the treated filter paper to prevent any direct contact between the food and odor sources. A second smaller piece of filter paper (4 x 4 mm) was soaked in one of the test solutions (sugar, or water as control) and placed on the top of the cover slip.

Glass vials containing individual parasitoids were each inverted on a small filter paper until the wasp contacted the sugar solution with its antennae and began feeding. Each wasp was allowed to feed for 10 s before it was gently removed with the vial. This procedure was repeated three times for each wasp with a 30 s interval between feeding bouts. Control parasitoids were exposed three times to a water-soaked filter paper. Following the three conditioning bouts, parasitoids were isolated in a ventilated Plexiglass cylinder (7.0 x 3.5 cm diam) without food or water for 2 h before testing. Only parasitoids that accepted the food source during all three feeding bouts were tested in the olfactory assay. The short feeding bouts combined with the 2 h rest period prior to testing were designed to ensure that parasitoids were not satiated during the olfactory assay.

For each assay, two glass Petri dishes (9 cm diam) were prepared with filter paper circles (8 cm diam) in each. A droplet of 2 µl of the eucalyptol-hexane solution was applied to the middle of one filter paper (+) impregnating a surface ca. 4 mm diam and left to evaporate; the other was treated with hexane alone as a control (-). Each conditioned wasp (n = 25 virgin, 25 mated) was first placed on the control paper and its behavior recorded for one min. Subsequently, the insect was gently removed with the glass vial and placed on the eucalyptol-treated paper and its behavior again recorded for one min. Each treatment was replicated with 25 wasps and each filter paper was renewed

after exposure to five parasitoids.

Wasps showed two clearly distinguishable responses within the arenas (Wåckers et al., 2002; Wäckers et al., 2006): (1) foraging behaviors; these included an area-restricted search, antennal tapping of the substrate, increased turning frequency, and frequent extension of the proboscis and scraping of the mouthparts over the substrate; these behaviors were considered indicative of appetitive learning; (2) alternative behaviors associated with grooming or dispersal; these included preening, resting, walking and flying. Each parasitoid was scored for time spent in the feeding-specific behaviors described in (1) during a one min period of direct observation. Data were checked for normality and homoscedasticity as appropriate and, if needed, were arcsine square root or log-transformed before being subjected to a one-way ANOVA (SAS Institute, 2004). Fisher's protected LSD test ($\alpha = 0.05$) was used to separate treatment means.

2.5. Conditioning with host- and food-associated odors

This experiment was designed to determine whether a parasitoid's hunger level would affect associative learning of odors associated with food or hosts. Groups of virgin female wasps (n = 20) were provided only water for two days post-emergence in Plexiglass cylinders (as above). Individual females were then offered 1M glucose for either five min (well fed) or two min (partially fed) or nothing (hungry) before being conditioned individually to respond to host- and food-associated odors.

As demonstrated by Lewis and Tumlinson (1988), antennation of host frass by parasitoids results in their volatile odors becoming associatively linked to hosts. Experimental females were allowed to antennate fresh frass (ca. 25 mg) obtained from cotton-fed *M. separata* second instar larvae on a filter paper for 30 sec, following the procedure of Lewis and Takasu (1990). Females were then exposed for five sec to 10 µl droplets of 1 M glucose solution and a 1:10 mixture of 1,8–cineol and hexane separated by five mm on the bottom of a glass Petri dish (8 × 1.5 cm). This experience was repeated five times at 30 sec intervals, alternating the sequence of encounter with food and host odors.

Hungry, partially fed, and well fed females (as described above) were each allowed to

antennate host frass for 30 sec before ovipositing in three second instar *M. separata* larvae. Females were then tested in the olfactometer for their response to hexane extract of the frass versus eucalyptol. For each treatment, ten females were tested each day for 5 days (replication) (a total of 50 females each treatment).

Females were tested in a Y-tube glass olfactometer (6 cm diam. tubing, 30 cm main arm length with 20 cm test arms angled at 45°) ca. 20 minutes after their last conditioning experience. Air flow through the apparatus was created by a vacuum pump (Guangdong Haili Corporation, AOC-5504) and monitored with air flow meters (Fushan, 701HB) to maintain 600 ml/min (300 ml/min in each arm). One test arm connected to a glass chamber containing the host odor source and another test arm connected to another glass chamber containing the food odor source. A 10 μl sample of hexane extract of the frass served as the host odor source and a 10 μl cineol and hexane (1:10 mixture) sample served as the food odor source. Air was pumped through activated charcoal and cork and then humidified in a container of distilled water before flowing into the sample chambers. The system was calibrated at the beginning of each experiment and illuminated with a single overhead 40W incandescent light bulb. All wasps were tested individually.

A wasp was considered to have made an odor selection when she entered a test arm to a depth > 2 cm within five min of introduction into the apparatus and remained there for 30 sec or longer; otherwise, no response was recorded. The olfactometer was cleaned with 75% ethanol followed by distilled water after every 10 trials. The apparatus was then dried and the test arms reversed to control for any inherent bias in the apparatus. Ten females in each treatment were tested each day for 5 days, for a total of 50 per treatment. Proportions of females responding to different odor sources in the olfactomer were subject to a Chi-square, Goodness-of-fit test with one df.

3. Results

3.1. Gustatory response assay

The two-way ANOVA was significant overall with significant effects of sugar type

but not mating status, and without a significant interaction between these independent variables (Table 1). Therefore, mating status was ignored for comparison of sugars. *Microplitis mediator* feeding times on fructose, sucrose and glucose did not differ, but exceeded those on raffinose, mannose, galactose and water control that also did not differ (Fig. 1).

3.2. Associative learning assay

Both type of sugar and mating status affected associative learning by adult *M. mediator* but the interaction between these two independent variables was not quite significant (Table 1). Training with glucose, fructose and sucrose all conditioned a feeding response to eucalyptol, but the tendency for mated females to respond more strongly than virgins was significant only for glucose (Fig. 2).

3.3. Conditioning with host- and food-associated odors

When virgin females had no prior experience with hosts, both hungry ($\chi^2 = 15.21$, $P < 0.001$) and partially fed females ($\chi^2 = 4.55$, $P < 0.05$) responded more strongly to the food-associated odor than to the host-associated odor, whereas satiated females responded more strongly to the host-associated odor ($\chi^2 = 13.14$, $P < 0.001$; Fig. 3A). Females that had opportunities to oviposit prior to testing also preferred food-associated odor to host-associated odor when they were hungry ($\chi^2 = 12.10$, $P < 0.001$), and satiated females still preferred host-associated odor ($\chi^2 = 13.50$, $P < 0.001$), but partially fed females now preferred host-associated odor instead of food-associated odor ($\chi^2 = 8.52$, $P < 0.005$; Fig. 3B).

4. Discussion

The gustatory response of parasitoids depends on the food source and may vary with the type and concentration of sugars (Wäckers, 1999; Beach et al., 2003). Previous work showed that *M. mediator* accepted and utilized a broad range of sugars that occur

naturally in nectar and honeydew (Luo et al., 2010). The present study demonstrates that fructose, glucose and sucrose are feeding stimulants and that galactose, mannose and raffinose are neither feeding stimulants nor deterrents because they do not elicit a response any different from water. Thus, *M. mediator* exhibits gustatory discrimination; sugars which presumably provide a relatively high nutritional benefit (Stepphun and Wäckers, 2004) elicited a strong feeding response, whereas others did not. The exceptions were mannose and galactose, which elicited a relatively low-gustatory response but still provide some nutritional benefits for females (Luo et al., 2010). Williams and Roane (2007) reported similar results for fructose and sucrose, but not glucose, with *Anaphes iole* Girault; the latter elicited only a weak feeding response despite its nutritional value. It is not uncommon for parasitoids to exhibit a weak feeding response to certain sugars despite their nutritional suitability (Nettles and Burks, 1971; Wäckers, 2001; Winkler et al., 2005) and they may also exhibit strong feeding responses to sugars that are unsuitable (Romeis and Wäckers, 2002; Williams and Roane, 2007).

Those sugars stimulating a gustatory response in *M. mediator* females also resulted in a strong response to eucalyptol following appetitive conditioning, whereas sugars without phagostimulatory activity did not. Thus, sugar composition is key to appetitive learning by *M. mediator* in the course of foraging for food; exposure to low quality food sources did not lead to successful odor acquisition. These findings are comparable to those of Wäckers et al. (2006) for the related species *Microplitis croceipes* Cresson. Although mating status did not affect the gustatory response, there was tendency for mated females to react to conditioning with a longer duration of response than virgin females when phagostimulatory sugars were used, although the difference was only significant in the case of glucose (Fig. 2). This effect may occur because virgin females remain primed to respond to cues involved in mate location, which affects their predisposition to allocate time to other behaviors.

Learning is a form of phenotypic flexibility that enables adaptive behavioral responses in complex and dynamic habitats. Many behaviors critical to parasitoid fitness are guided by olfactory cues, such as those involved with foraging for food (Takasu and Lewis, 1993; 1996), mate location (Collatz et al., 2009; Sivinski and Aluja, 2012), and host selection (Turlings et al., 1993; Powell et al., 1998) and it follows that odors are

among the more reliable and useful stimuli insects can learn to associate with these biological goals. However, the magnitude of the response to a particular conditioned stimulus can be diminished when the unconditioned stimulus is a low priority due to current physiological state (Lewis and Takasu, 1990; Takasu and Lewis, 1993), and prioritization can be influenced by experience. Oviposition experiences did not affect the response of females to food vs host odor in the olfactometer when they were either starved or fully satiated; the former chose food and the latter hosts in > 85% of cases (Fig. 3). However, when only partially fed, naïve females responded more strongly to food odor, whereas oviposition experience shifted the response in favor of host odor. Similarly, Lightle et al. (2010) observed that well-fed females of another braconid, *Apanteles aristoteliae* Viereck, were less likely to make any choice in an olfactometer, whereas partially sated females responded best to host odors, and hunger shifted choices toward food odors. Our results illustrate that physiological state may influence the conditioned responses of females only within a narrow range of condition, and not necessarily across the full range of condition states. Clearly, feeding is a priority when wasps are starving, and host location is a priority when they are satiated, but in intermediate hunger states, oviposition experience shifts the balance in favor of hosts, whereas food is still prioritized in the absence of such experience. These findings underscore the importance of testing the behavioral responses of parasitoids in various hunger states even when responses to food resources are not the experimental focus.

In conclusion, these results illustrate clearly that sugar type mediates the strength of associative odor learning in *M. mediator*, and that prior oviposition experience can modify sensitivity to food- versus host-associated odors, but only within a range of intermediate hunger states. The findings are worthy of consideration by workers seeking ways to improve the performance of parasitoids reared and released in biological control programs.

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Table 1. Effects of sugar type and mating status on the gustatory response and associative learning of two day-old *M. mediator* females.

| Factor	Gustatory response (feeding time in sec)			Associative learning (foraging time in sec)		
	F	df	*P*	*F*	df	*P*
Corrected model	40.69	13,336	< 0.001	67.93	13,336	< 0.001
Sugar type	86.85	6,336	< 0.001	143.32	6,336	< 0.001
Mating status	1.50	1,336	0.222	10.85	1,336	0.001
Sugar type*mating status	1.07	6,336	0.381	2.07	6,336	0.056

1 **Figures**
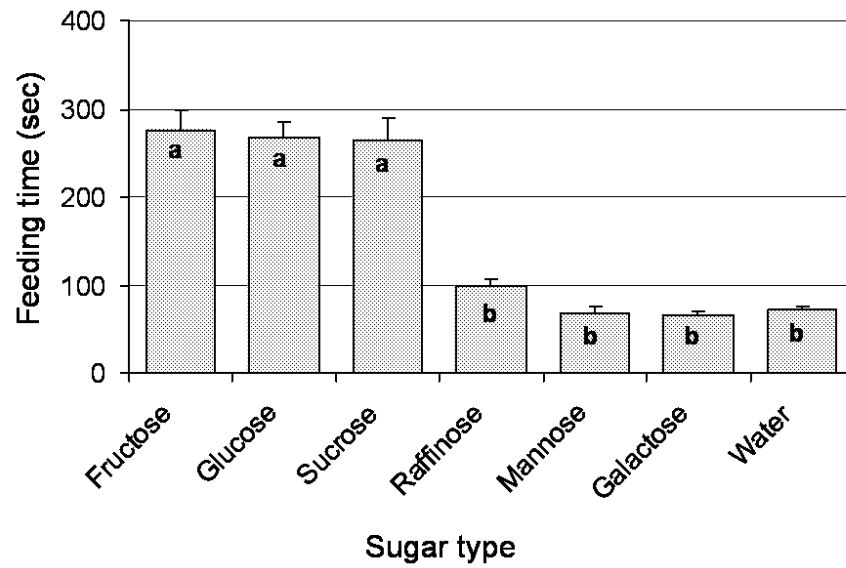
2
3

4 **Figure 1.** Mean + SE feeding times in the gustatory response assay of three day-old
5 *M. mediator* females on different sugar types (with water as control) when presented
6 with 10 µl droplets of 1M solutions in glass Petri dishes. Means bearing the same
7 letters were not significantly different (LSD test, $\alpha = 0.05$).
8
9
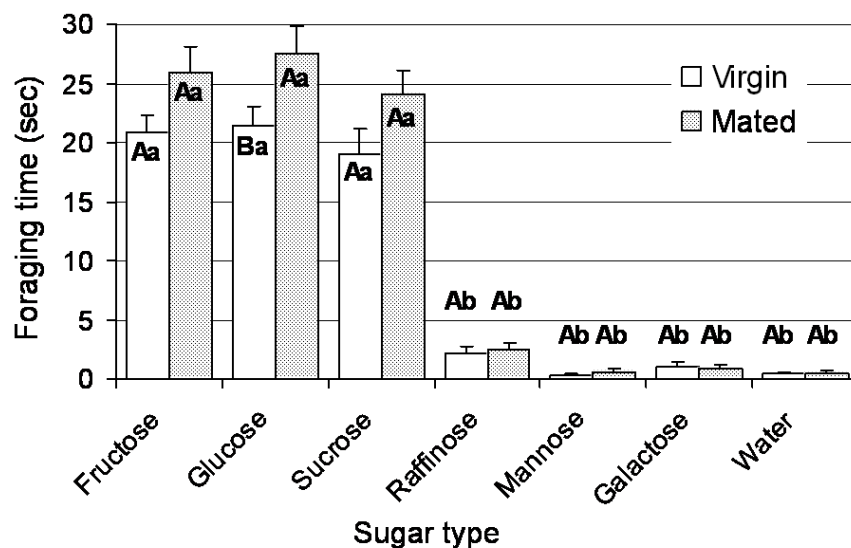
Figure 2. Mean + SE duration of foraging behaviors in the associative learning assay for virgin (open columns) and mated (shaded columns) *M. mediator* females during one min periods of exposure to 2 μl of 10% eucalyptol adsorbed into filter paper two hours after being conditioned to eucalyptol with different sugar types. Means bearing the same upper case letters were not significantly different between virgin and mated treatments; means bearing the same lower case letters were not significantly different among sugar types for a given mating status (LSD test, α = 0.05).
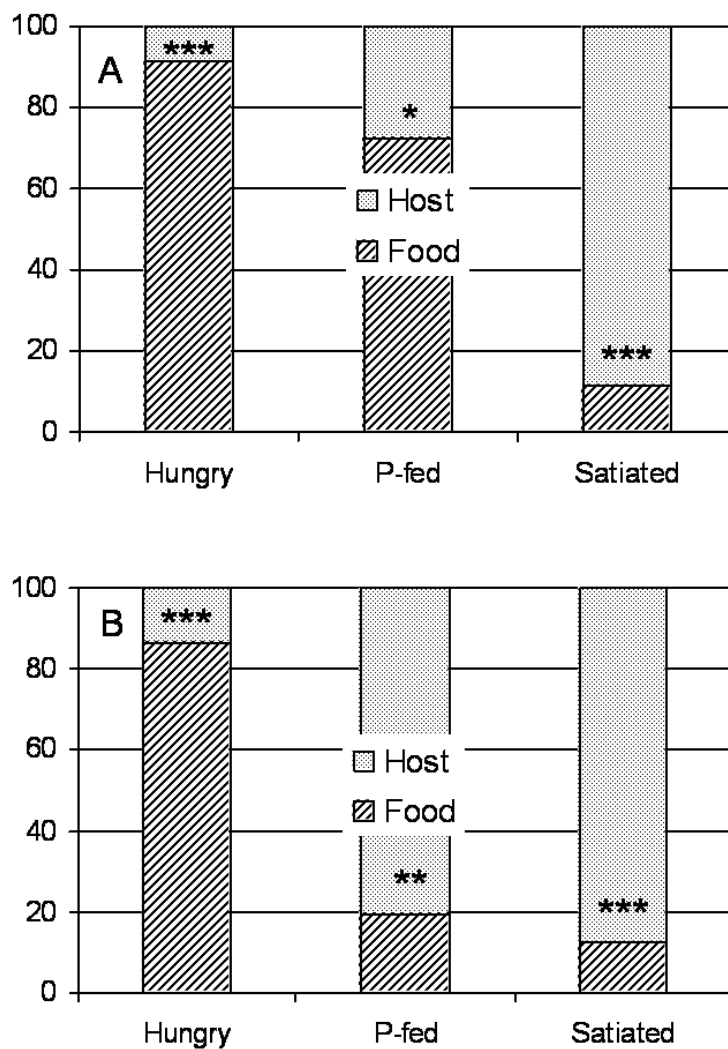
20

21 **Figure 3.** Mean proportion of responding females in the odor conditioning assay that

22 oriented to food-associated odor (eucalyptol conditioned with glucose) versus host-

23 associated odor (frass of *M. separata* larvae) when hungry, partially fed, or satiated

24 and without (A) or with (B) prior oviposition experience. Asterisks indicate level of

25 significance (*, P < 0.05, ** P < 0.01, ***, P < 0.001).

26