Animal Behaviour 83 (2012) 1491–1496

Contents lists available at SciVerse ScienceDirect

Animal Behaviour

journal homepage: www.elsevier.com/locate/anbehav

Two-step learning involved in acquiring olfactory preferences for plant volatiles by parasitic wasps

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ARTICLE INFO

Article history:
Received 28 October 2011
Initial acceptance 29 November 2011
Final acceptance 2 March 2012
Available online 27 April 2012
MS. number: 11-00866R

Keywords:
Aphidius ervi
herbivore-induced plant volatile
host-searching behaviour
olfactory learning
parasitic wasp

Aphidius ervi is a parasitic wasp of several aphid species, including *Acyrthosiphon pisum*. This wasp is used as a biological control agent of its host aphid species in many regions of the world. Compared with responses to volatiles from intact plants, *A. ervi* females respond to host-infested plant volatiles but not to nonhost-infested plant volatiles. Furthermore, we previously demonstrated that *A. ervi* preferred host aphid-infested plant volatiles to volatiles from intact plants only when they had been exposed to the host aphid-infested plant volatiles during their developmental stages (larval to emergence stages). The results suggested that *A. ervi* females learn the host-infested volatiles during their development and we tested this hypothesis in this study. Learning occurred in two steps: during the late larval to prepupal stages (the first learning stage) and during adult emergence (the second learning stage). Furthermore, we observed specificity to the host plant volatiles in the two-step learning. The preference for host-infested plant volatiles was modified when the wasps had been exposed to host aphid-infested plant volatiles in the first stage and then exposed to nonhost aphid-infested plant volatiles in the second stage. When they were exposed to nonhost aphid-infested plant volatiles in the first stage, wasp preference for volatiles was not detected, irrespective of their exposure to either host or nonhost aphid-infested plants in the second stage. The ecological functions of the two-step learning are discussed.

In response to damage caused by herbivorous arthropods, plants emit volatile organic compounds that are not, or only in trace amounts, emitted from intact plants (e.g. Dicke et al. 1990; Turlings et al. 1990; Takabayashi & Dicke 1996). Carnivorous natural enemies of herbivores, such as parasitic wasps, exploit such volatiles as chemical cues indicating the presence of their prey to increase their prey-searching efficiency. For plants, the emission of volatiles that attract natural enemies of herbivores is regarded as an induced indirect defence strategy when the attracted carnivores reduce the damage caused by a current herbivore infestation (Takabayashi & Dicke 1996; Dicke et al. 2003; Arimura et al. 2005; Sabelis et al. 2007; Arimura et al. 2009). An intriguing question is how carnivores respond to volatiles emitted from plants infested by their victims.

One of the answers to the above question is the specificity of infested plant volatiles: the blends of volatiles emitted by herbivore-infested plants are qualitatively and/or quantitatively unique in terms of plant species, plant cultivars, plant developmental stages, herbivore species and herbivore developmental stages (Takabayashi & Dicke 1996; Dicke 1999; Sabelis et al. 2007), and natural enemies prefer volatiles of prey-infested plants to those of nonprey-infested plants (Du et al. 1996; De Moraes et al. 1998; De Boer et al. 2004). These plant-specific responses by carnivores may be caused by their innate olfactory preferences (e.g. Shiojiri et al. 2000, 2001) or olfactory learning of prey-infested plant volatiles (Turlings et al. 1993). Learning is widespread among insects, which rely on learning for all major activities. Parasitic wasps are established as model systems for research on insect learning (Dukas 2008). Olfactory learning in parasitic wasps has been shown to occur mostly at adult emergence (reviewed in van Emden et al. 2008; Takemoto et al. 2011), but it may occur at earlier stages in their ontogeny. Blande et al. (2007) reported that the learning responses of *Diaeretiella rapae*, a parasitoid of aphids that attack cruciferous plants, was affected by the plant species on which their hosts had developed. In this study we focused on the role of olfactory learning in parasitic wasps. The objective of this study was to clarify the mechanisms involved in the specific responses of parasitic wasps to host-infested plant volatiles by considering the developmental conditions of the parasitoid larvae in their hosts.

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0003-3472/$38.00
doi:10.1016/j.anbehav.2012.03.023

Aphidius ervi is a solitary endoparasitoid of several aphid species including *Acyrthosiphon pisum*, *Macrosiphum euphorbiae*, *Aulacorthum solani* and *Sitobion avenae* (Takada & Tada 2000). Females of *A. ervi* prefer volatiles from host (*A. pisum*)-infested broad bean, *Vicia faba*, plants over those from intact plants, but they do not show such preference when offered nonhost (*Aphis fabae*)-infested plants versus intact plants (Du et al. 1996). *Aphidius ervi* is reported to learn host-related cues during the larval to prepupal development periods (Gutiérrez-Ibáñez et al. 2007). We previously showed that *A. ervi* preferred volatiles from host (*A. pisum*)-infested broad bean plants over those from intact plants when the wasps were allowed to emerge on the host aphid-infested plants, but wasps that had emerged in a clean petri dish showed no significant preference between the two volatile sources (Takemoto et al. 2009). In this study, we compared the responses of *A. ervi* to two types of plants: those infested with host aphids and those infested with *Aphis craccivora*, a nonhost aphid.

METHODS

Plants and Insects

Three broad bean plants (*V. faba* var. Nintoku Issun) were grown in a vinyl cup (ca. 300 ml) under laboratory conditions (20 ± 2 °C, 16:8 h light:dark). Seedlings ca. 20 cm high with three or four pairs of leaves (ca. 3 weeks after germination) were used for experiments and rearing of aphids (20 ± 2 °C, 16:8 h light:dark, 50–70% relative humidity). *Acyrthosiphon pisum* was obtained from the culture maintained by Y. Nakashima (Obihiro University of Agriculture and Veterinary Medicine) in April 2006. They were reared on broad bean plants in the laboratory. The nonhost aphid *A. craccivora* was obtained from experimental fields of the Center for Ecological Research, Kyoto University, Otsu, Shiga, Japan. Under laboratory conditions, *A. ervi* did not attack *A. craccivora* adults. *Aphis craccivora* were reared under the same conditions as *A. pisum* (20 ± 2 °C, 16:8 h light:dark, 50–70% relative humidity). *Acyrthosiphon pisum* and *A. craccivora* often coexist naturally on the same plant.

The parasitic wasp *A. ervi* was obtained from the culture maintained by Y. Nakashima in April 2008. Wasps were kept in an acrylic resin cage (30 × 25 cm and 35 cm high) under laboratory conditions (20 ± 2 °C, 16:8 h light:dark, 50–70% relative humidity) on broad bean plants with *A. pisum* as hosts. Newly emerged male and female wasps mate within 24 h in laboratory conditions. Wasps used in all treatments were 1–4 days old.

Conditioning of Wasps

For preparation of wasps, 200 *A. pisum* (instars 1–2) were exposed to parasitism by 15–20 *A. ervi* females (mated, 1–4 days old), transferred to intact *V. faba*, and reared under laboratory conditions (20 ± 2 °C, 16:8 h light:dark, 50–70% relative humidity). We conducted two experiments on volatile preference in *A. ervi*. Experiment 1 tested the effects of conditioning at emergence (either clean air, volatiles from host aphid-infested plants (host-infested plant volatiles) or volatiles from nonhost aphid-infested plants (nonhost-infested plant volatiles)). Experiment 2 tested the combined effects of conditioning at the larval to prepupal stages (clean air, volatiles from host aphid-infested plants or volatiles from nonhost aphid-infested plants) and at emergence of *A. ervi* (volatiles from either host aphid-infested plants or nonhost aphid-infested plants).

In experiment 1, 200 parasitized aphids were placed on three intact *V. faba* plants for 12 days and allowed to mummify on the plants (Fig. 1a). Thus, the parasitized aphids were exposed to volatiles from the infested plants, and the *A. ervi* parasitoid wasps were conditioned at the larval to prepupal stages. About 95% of the aphids were parasitized. After 7 days, only two or three unparasitized aphids and ca. 120 parasitized aphids were observed on a plant; the mortality factor(s) of the other aphids was unknown.

To condition wasps at late larval to prepupal stages in experiment 2, parasitized aphids were first placed on an intact plant for 7 days. After 7 days, the parasitized aphids, which had already stopped feeding on the plants, were transferred to petri dishes with purified air (experiment 2a), onto leaves of *A. pisum*-infested *V. faba* (experiment 2b) or onto leaves of *A. craccivora*-infested *V. faba* (experiment 2c). To prepare the host aphid-infested plants for experiment 2b, we inoculated 30 unparasitized *A. pisum* onto a bean plant with a fine brush and let them infest it for 3 days. To prepare the nonhost aphid-infested plants for experiment 2c, we inoculated 200 *A. craccivora* onto a bean plant with an insect aspirator, and let them infest it for 3 days so that the infestation level was the same as on the host aphid-infested plants (*A. craccivora* increased less than *A. pisum*). Note that the conditions of the infested plants at the late larval to prepupal stages of *A. ervi* were different between experiments 1 and 2.

For the conditioning of *A. ervi* at adult emergence, aphid mummies were collected on the 12th day after the parasitism and kept in a small polyethylene terephthalate (PET) cage (6 cm diameter, 15 cm long, with the openings covered with gauze). Aphid mummies and newly emerged wasp adults were exposed to plant volatiles for 3 days by placing the PET cage in a rearing cage containing plants that had been infested by either *A. pisum* (host) or *A. craccivora* (nonhost). The PET cages were loosely covered with aluminium foil to prevent wasps from coming into contact with aphid honeydew and exuviae. For the control, mummies were transferred to petri dishes filled with purified air. Plants used for conditioning at emergence were inoculated with either 30 *A. pisum* or 200 *A. craccivora* on each plant and maintained for 3–5 days prior to the wasp conditioning exposures.

The wasps emerging in the PET cage (0–2 days old) were transferred to another acrylic resin rearing cage (30 × 25 cm and 35 cm high) with access to 50% honey solution. In the following 2 days, adult wasps (1–4 days old) were sexed, and females were used for choice tests in a Y-tube olfactometer.

Y-tube Olfactometer

For the preparation of volatile sources, 100 *A. pisum*, 200 *A. craccivora* adults (ca. 100 mg equivalent) or no aphids (for the preparation of uninfested plants) were transferred from stock cultures to clean *V. faba* plants, and kept in laboratory conditions for 3 days (20 ± 2 °C, 16:8 h light:dark, 50–70% relative humidity). A Y-tube olfactometer (3.5 cm inner diameter, 13 cm long for each branch) was used to test the olfactory responses of *A. ervi* females in a climate-controlled room (20 ± 2 °C, 50–70% relative humidity). Air was passed through activated charcoal and volatile source bottles, in which either infested or intact plants were placed, to a branch of the olfactometer at the rate of 400 ml/min. A small vial with one *A. ervi* was placed at the base of the Y-tube with its opening facing upwind to release the wasp. When the wasp walked across a line marked in each branch, 7 cm from the Y junction, and stayed there for at least 30 s, we judged that the wasp had made a choice. A wasp that did not make a choice within 5 min was recorded as a nonresponder. The control and experimental ends of the olfactometer were alternated every six bioassays. Volatile sources were replaced every 12 bioassays. For each comparison, 60 wasps were used. A replicated *G* test (Sokal & Rohlf 1995) was used to test the statistical significance of the difference between the distribution of wasps that made choices of either of the two volatile