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Short Communication

Homopteran chemical signatures reduce aggression of tending ants

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Summary. Because generalist ants are aggressive towards foreign insects, the recognition of homopterans by tending ants is critical in ant/homopteran trophobiosis. Herein we report experimental evidence indicating that Argentine ant, *Linepithema humile* (Mayr) (Hymenoptera: Formicidae) learn to associate the production of honeydew with the chemical characteristics of homopteran cuticle, suppressing ant aggression and allowing the ants to tend homopterans. Although chemically-mediated associative learning is well understood in honeybee foraging, to our knowledge, it has not been reported before in ant / homopteran trophobiosis.

Key words. Ant / homopteran mutualism – trophobiosis – aggression – Argentine ant – *Linepithema humile* – brown soft scale – *Coccus hesperidum* – associative learning

Introduction

The tramp ant species, the Argentine ant *Linepithema humile* (Mayr) (Hymenoptera: Formicidae), is extremely aggressive and frequently displaces native arthropods (Hölldobler & Wilson 1990; Vega & Rust 2001), but it also exhibits trophobiosis with morphologically and systematically diverse honeydew-producing homopterans (Smith 1965; Markin 1967; Moreno *et al.* 1987; Thompson 1990; Klotz *et al.* 2004), often enhancing the pest status of both the ants and the homopterans (Gullan 1997).

When tending homopterans, *L. humile* deliberately antennate them, a behavior referred to as solicitation (Way 1963). Ants are generally aggressive towards other insect species, recognizing them as non-nestmates by their movements or cuticular chemical cues (Way 1963; Hölldobler & Wilson 1990). Thus, non-aggressive solicitation towards various homopteran insects is considered as a distinctive behavioral modification. Preliminary observation indicated that a laboratory *L. humile* colony which had not tended any homopterans for at least 3 months did not immediately solicit honeydew-producing homopterans, and even aggressively seized them with their mandibles. However, after trophobiosis was established, scale-tending *L. humile* displayed solicitation even to the flattened dead scale which did not produce honeydew in response to the ant's antennation. Hexane-soluble cues of formerly-tended homopteran cuticle seem to be most responsible for making ants less aggressive to the models which otherwise elicit a higher attack rate by the ants (Glinwood *et al.* 2003). In this paper, we hypothesized that *L. humile* associate cuticular chemistry of the trophobiotic homopterans with the presence of honeydew, leading to suppressed aggression and proper solicitation when they visit them again. We tested this hypothesis with honeydew-producing brown soft scale, *Coccus hesperidum* L. (Homoptera: Coccidae) in laboratory settings.

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Materials and methods

Test insects

Linepithema humile were collected from the biological control groves on the University of California, Riverside campus. Ant nests were dug up from the base of trees and transported to a laboratory chamber where they were extracted from soil in a process described by Hooper-Bui and Rust (2000). Ant colonies were maintained in plastic boxes (26.5 by 30 by 10 cm, Spectrum Containers Inc., Evansville, IN) with the inner sides coated with fluoropolymer resin to prevent ants from escaping. In the plastic boxes, the ant colony occupied two or three Petri dishes filled with plaster for providing a moist environment.

Another fluoropolymer resin-coated plastic box connected with the colony box served as a 'foraging arena.' To connect the colony box to the foraging arena, the lower center of the end of each box was drilled and plastic micropipette tips were attached using hot melt glue (Glue Sticks, Ace Hardware Corp., Oak Brook, IL). The micropipette tips were cut to permit ants access and the tips were connected with a piece of Tygon tube (0.6 cm diam). Each foraging arena was provisioned with two polystyrene weighing dishes, one filled with 25% sugar water, and the other with pieces of fresh American cockroach, *Periplaneta americana* (L.).

The colony used was collected on 11 August 2004, and was maintained in laboratory condition for at least three months in the absence of scale insects before being used in experiments. The laboratory colony consisted of 10-20 queens, brood, and 2000-5000 workers. *L. humile* workers may live as long as 9 months (Newell & Barber 1913), but in smaller laboratory colonies the mortality rate ranged from 15 to 25 % each month. Consequently, the large proportion of the laboratory colony consisted of foragers which had never tended homopterans. Thus, the laboratory colony was considered to be 'naïve' with respect to tending scale insects.

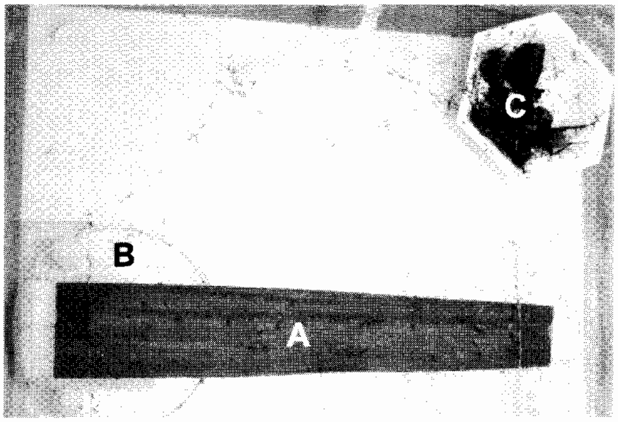

Fig. 1 Tending colony setup. Argentine ants, *L. humile* were allowed to access to a yucca leaf (A) maintained between water containers (B). Fresh dead cockroaches were supplied in a weighing dish (C)

Brown soft scale populations were maintained on excised *Yucca sp.* (Agavaceae) leaves for several generations in the insectary of University of California, Riverside (Bernal *et al.* 1999).

Cuticular extracts

Hexane extracts of external cuticular surfaces were made from brown soft scales. The scales were removed from the yucca leaf without damaging their covers. The scales were washed in distilled water before extracting them to rid them of adhering honeydew. About 10 scales of medium size (3-4 mm in length) were collected and placed in a clean extraction tube containing 100 µl hexane. The tube was shaken gently by hand for 1 minute, and the hexane extract was transferred to a clean tube leaving the intact bodies of the scales in the first tube. The extracts were used for behavioral bioassays within 10 minutes.

Ants with different tending experience

The laboratory colony was divided into two colonies with similar numbers of queens and workers, and put into separate colony boxes. The ant colony, referred to as 'tending' colony was allowed to tend brown soft scales by providing one yucca leaf(4 by 20 cm) infested with approximately 150 scales into the foraging arena (Fig. 1). Two petri dishes (9 cm diam) were attached on the bottom of the foraging arena, and served as water containers for maintaining yucca leaf quality. One end of a piece of dental cotton wick was placed in the water container, and the other end was placed between two plastic mounts (9 by 2.5 by 0.3 cm). The wick served to provide water to the yucca leaf held between the plastic mounts. No other carbohydrate source was provided for the tending colony except the honeydew produced by the scales. The other colony, referred to as the 'naïve' colony, was not allowed to tend brown soft scales on yucca leaf. In the foraging arena of the 'naïve' colony, 25 % sugar water was provided as a carbohydrate source in a polystyrene weighing dish. The foraging arenas of both colonies (i.e., 'tending' colony and 'naïve' colony) were provisioned with a polystyrene weighing dish filled with pieces of fresh American cockroach. At the time of the experiments, each colony contained 5~10 queens, brood, and 1000-2500 workers.

Arena bioassay

The responses of *L. humile* workers to cuticular extracts of brown soft scales were determined by coating fruit flies with the extracts and placing the flies in the foraging arena. Fruit flies were killed by freezing for 5 minutes at -50 °C, then washed with hexane to remove their cuticular lipids and allowed to dry for 10 minutes. The cuticular extract prepared from scales was transferred to 10 hexane-washed fruit flies by wiretrol capillary tubes (Drummond Scientific Company, Broomall, PA) and the hexane allowed to volatilize before additional extract was applied. Approximately 8 µl of extract (~ one scale equivalent) was applied to each fruit fly.

Using clean forceps, a single treated fruit fly was introduced into the bottom of the foraging arena. The positions where the fruit flies were introduced were selected randomly around the trail of colonies foraging on sugar water ('naïve' colony) or scale insects honeydew ('tending' colony.) The entire behavioral response of ants was recorded using a digital camcorder (XL-1, Canon Inc., Lake Success, NY) equipped with a 100 mm macro lens, from the first encounter of a foraging ant with the treated fruit fly carcass until the fruit fly was removed from its original place by an ant.

Ants reacted either aggressively or passively toward the treated fruit fly. Each encounter of the ants with the treated fruit flies was categorized as follows: (a) Aggressive encounter (the ants attacked the fly and tried to seize it with open mandibles) (Fig. 2a). (b) Non-aggressive encounter (the ants passed the fly within a distance of < 2 mm but did not physically contact it; or the ant made contact but did not attack it) (Fig. 2b).

Ant colonies were tested three times to determine whether ants recognized brown soft scale extracts and whether they changed the level of aggression in response to experience with tending scale. The first test was conducted with original laboratory colony foragers on 10 November 2004, before dividing the colony. After this test, the colony was divided into two equal colonies: 'tending' and 'naïve' colonies. The second and third trials were conducted with the 'tending' and 'naïve' colonies on 12 and 22 November 2004, respectively. In each test, the numbers of aggressive encounters and total encounters were counted.

Statistical analysis

We compared the behavioral responses of foraging workers between the 'naïve' and 'tending' colonies by examining the proportions of foragers responding aggressively towards the treated fruit fly. The data were square-root transformed prior to analysis to meet assumptions of normality (Sokal & Rohlf 1981). Because the same experimental unit (colony) was examined repeatedly, and we observed ten groups of foragers randomly chosen within each colony, the data were analyzed by a repeated measures two-factor nested analysis of variance (ANOVA) where day and colony were completely crossed as main fixed effects and ten random observations were nested in colony (PROC MIXED; SAS 8.0 for Windows).

Results

Experience with scale-tending significantly changed the behavioral responses of the ant colony towards cuticular extract of scales (Fig. 2c). Overall aggression level in ant colonies was significantly decreased over time (Repeated measures ANOVA, $F_{2,36} = 12.57$, $\boldsymbol{P} < 0.0001$). However, a significant interaction occurred between day and colony indicating that 'naïve' and 'tending' colonies were significantly different in terms of their aggression level change throughout experimental period (Repeated measures ANOVA, $F_{2,36} = 4.21$, $\boldsymbol{P} = 0.023$). Aggression levels of the 'naïve'colony were significantly higher than those of 'tending' colony at day 2 and day 12 tests (Repeated measures ANOVA, $F_{1,36} = 6.77$, $\boldsymbol{P} = 0.013$, and $F_{1,36} = 16.40$, $\boldsymbol{P} < 0.001$, respectively).

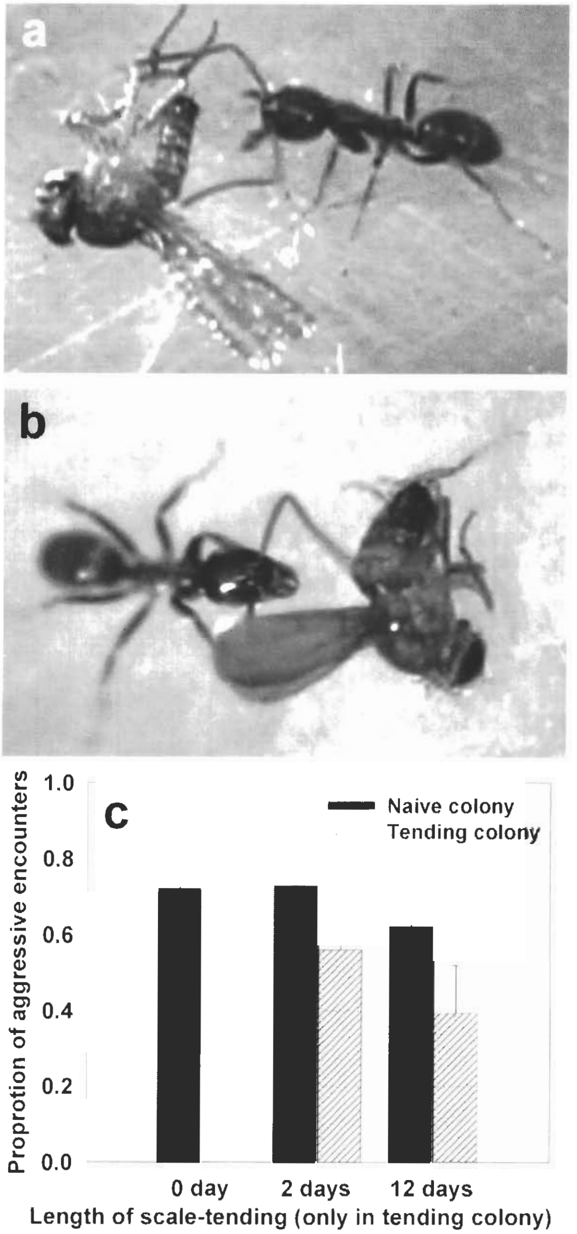

Fig. 2 Behavioral response of Argentine ant L. humile towards dead fruit fly bodies treated with brown soft scale cuticular extracts. a. Aggressive encounter. b. Non-aggressive encounter. c. Aggression levels (aggressive encounters / total encounters) of 'naïve' and 'tending' colonies. Bars represent mean ± SD. See text for details

Discussion

Homopterans produce a food reward (honeydew) in response to antennation or aggression of ants. In many (but not all) myrmecophilous aphid species, the honeydew production can be induced by a simple, mechanical stimulus such as brushing the abdomen with a delicate object (Hölldobler & Wilson 1990). Brown soft scales also produce honeydew as a response to non-specific stimuli such as tapping with fine paint brush hairs, mimicking antennation by *L. humile* (Choe, personal observation). Sakata (1994) suggested that a myrmecophilous aphid, *Lachnus tropicalis* (van der Goot) (Homoptera: Aphididae), often excreted honeydew in response to aggression by *Lasius niger* (L.) (Hymenoptera: Formicidae).

Ants' interspecific aggression is significantly suppressed while they are ingesting liquid food droplets presented by other insects. Reducing aggression by giving regurgitated liquid food droplets, so-called trophallactic appeasement (Bhatkar & Kloft 1977; Bhatkar 1979; Hölldobler & Wilson 1990) is a well known phenomenon in the aggressive interaction between two different ant species. Trophallactic appeasement is also probably one mechanism which allows ants to initiate tending of homopterans by suppressing aggression. Sakata (1994) reported that if an aphid produces honeydew in response to aggressive ants and the honeydew is taken up by the ants, the predation rate by the ants was lower than it was in other cases in which aphids did not excrete honeydew, or failed to give honeydew to the ants.

Once the ants associate particular cues of homopterans and their production of honeydew, the cues (i.e., controlled stimulus) may serve as a predictor for the honeydew acquisition (i.e., uncontrolled stimulus) to the ants, allowing them antennate homopterans with reduced aggression (i.e., operant behavior). Our results clearly showed that hexane-soluble chemicals on brown soft scale cuticle are important cues for the *L. humile*'s recognition. Unlike some ants which have obligate mutualism with a limited number of homopteran species, *L. humile* tends a broad diversity of homopterans. The learning ability of ants to recognize various honeydew-producing homopterans would predispose ants to quickly adapt to new honeydew sources with great flexibility. This phenomenon may be important for a tramp ant species, like *L. humile*, that may encounter many new honeydew sources (i.e., different homopteran species) and potential competitors in their non-native habitats. We suggest that the ant / homopteran trophobiosis can be mediated not only by the strong preference for honeydew by ants, but also by sophisticated learning processes related to honeydew acquisition and associated cuticular chemical cues.

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