The Journal of Experimental Biology 213, 386-392

doi:10.1242/jeb.035642

Use of an alarm pheromone against ants for gaining access to aphid/scale prey by the red velvet mite *Balaustium* sp. (Erythraeidae) in a honeydew-rich environment

Jay A. Yoder[1], Michael R. Condon[1], Chloé E. Hart[1], Matthew H. Collier[1,*], Kevin R. Patrick[2] and Joshua B. Benoit[2]

[1]Department of Biology, Wittenberg University, Springfield, OH 45501, USA and [2]Department of Entomology, The Ohio State University, Columbus, OH 43210, USA

*Author for correspondence (mcollier@wittenberg.edu)

Accepted 25 October 2009

SUMMARY

This study shows that honeydew prompts arrestment and reduced activity, but not attraction, by the mite *Balaustium* sp. nr. *putmani*. When presented with short-range, two-choice bioassays, mites ceased their characteristic rapid crawling activity when they encountered honeydew-treated surfaces, resulting in them clustering around the honeydew. Approximately 80% of mites were retained by honeydew, with responses being independent of both mite life-history stage and source of honeydew (coccid scale insect or aphid). No obvious crawling movements or redirection of running path were made to the honeydew by the mites, implying the lack of any kind of attractant. Response of mites to single-sugar presentations of the main honeydew components – glucose, sucrose, fructose and trehalose – (0.001–0.1 mmol l^{-1}) were inconsistent and failed to reproduce the arrestment/clustering associated with raw honeydew, suggesting that none of these sugars is an active arrestant ingredient. Formation of feeding clusters on honeydew does not contribute to enhancing water conservation by suppressing net transpiration (water loss) rates of individual mites as group size increases, indicating that the clustering is an artifact of arrestment. We hypothesize that release of neryl formate by the mites reduces negative interactions with the local ant species commonly associated with honeydew. We hypothesize that honeydew serves as: (1) a cue that facilitates discovery of scale/aphid prey; (2) a retainer on plants where these prey are present, signaling abundance and quality; and (3) an alternative and supplemental food source like that noted for other plant-inhabiting predatory mites. Neryl formate serves as an alarm pheromone and foul-tasting allomonal defense secretion that prevents predation of mites by ants that co-exist with aphid/scale insects in these honeydew-rich habitats.

Key words: *Balaustium*, neryl formate, tritrophic interaction, water balance.

INTRODUCTION

Observing red velvet mites, *Balaustium* spp. (terrestrial Parasitengona: Erythraeidae), in an inactive condition, whether solitary or grouped, is highly unusual because of their typical rapid scurrying activity where they look for food or sites for oviposition (Putman, 1970; Childers and Rock, 1980; Welbourn, 1983; Welbourn and Jennings, 1991; Gabrys, 2000; Halliday, 2001). For the *Balaustium* species we worked with in Central Ohio, *B*. sp. nr. *putmani*, one of the few times they are found aggregated, or slowed (typically in small fissures, cracks and crevices), is in response to low temperature (7–12°C). Small groups of these mites can also be found on occasion in similar hidden retreats under tree bark and rocks, with the mites grouped in small numbers. Grandjean (Grandjean, 1947; Grandjean, 1957; Grandjean, 1959) also reports similar behavior in *Balaustium* mites. During our work, we noted patchy clusters of *Balaustium* mites on plant leaf surfaces covered with honeydew and an absence of these aggregations in areas where honeydew was not present. Mites that were initially rapidly crawling through these honeydew-covered areas typically became less active and sometimes joined a group and stopped walking. Group size ranged from 4–12 loosely aggregated individuals of mixed developmental stages (e.g. deutonymphs and adult females and males), with a few individuals in direct contact with each other at any given time. Cluster size tended to fluctuate every few minutes, when mites rapidly departing the aggregation were replaced by single or multiple individuals. Clusters quickly disbanded when the mites or leaves were disturbed – sometimes for what appeared to be no apparent reason. This aggregation behavior is similar to that seen in *Balaustium* when the temperature is low (Yoder et al., 2008) but is rarely observed in mites exposed to full sun, where the temperature is higher (23–28°C). There are two possible explanations for this behavior. First, mites often form aggregations as a behavioral mechanism to help reduce water stress (Benoit et al., 2008 and references therein). Second, it is also possible that these aggregations are feeding clusters formed in response to honeydew being present as an alternative food source, a behavior (clustering around a food source) reminiscent of what is seen in other plant-inhabiting predatory mites (Nomikou et al., 2003). All mobile stages (larva, deutonymph, female and male adult) of *Balaustium* spp. are voracious predators of a range of soft-bodied arthropods and their eggs, including scale insects, phytophagous mites and adult aphids (Putman, 1970), which makes the observation that they are arrested on surfaces covered with honeydew rather than an actual prey item distinctive (these mites do arrest on prey because they are feeding on them). They thrive in semi-arid to arid biotopes (Wohltmann, 1998) and have a particular affinity for hot surfaces (≥50°C) exposed to direct sun (Yoder et al., 2007a). All *Balaustium* life-stages share the same habitat of soil, leaf litter, plants and organic debris and can be found under bark and rocks (Grandjean, 1947; Grandjean, 1957; Grandjean, 1959). A large pair of protrusible glands, known as urnulae, is present in deutonymphs and adults (Southcott, 1961; Newell, 1963; Gabrys, 2000; Halliday, 2001). These glands release

a secretion that protects the mite from heat injury and excessive desiccation by providing additional water-proofing (Yoder et al., 2008), while also functioning as an alarm and a defense from predators (Yoder et al., 2006b). One of the main components of the urnulae secretion is the terpene neryl formate (Yoder et al., 2007b). *Balaustium* spp. larvae lack urnulae (Southcott, 1961; Newell, 1963; Gabrys, 2000; Halliday, 2001) and thus are conceivably not protected in this way against dehydration and predation, although they are exposed and share the same habitat as postlarval stages. These mites are soft-bodied and thus would appear to be fairly easy targets for predators. Despite this, *Balaustium* spp. mites are noted wherever ants are abundant, crawling freely among each other (Grandjean, 1957; Grandjean, 1959), and the *Balaustium* mites used in our study have a co-occurrence with ants; indeed, ants very frequently occur abundantly in the absence of *Balaustium*. Particularly, we see mite–ant interactions in regions with copious amounts of honeydew, but rarely, if ever, do the ants attack the *Balaustium* mites.

The first goal of this paper was to use short-range, two-choice bioassays to determine whether raw honeydew acts as an arrestant that leads to clustering of *Balaustium* mites. Additionally, Evans blue dye was used to stain honeydew and offered to mites to test for honeydew feeding based on evidence of the presence of the blue tracer in the gut. Glucose, fructose, sucrose and trehalose, principal components of aphid and coccid honeydew (Strong, 1965; Brown, 1975; Bogo and Mantle, 2000; Bogo, 2003), were each tested individually to determine the potential of these sugars to elicit the response observed in the presence of honeydew. Experiments were conducted using larvae, deutonymphs and adults to determine whether the aggregation observed on honeydew is restricted to specific stages or is consistently expressed throughout the life cycle. A second goal was to determine whether this clustering effect improves water conservation by measuring net transpiration (water loss) rates in isolated adult females in comparison with adult females in a group. The third goal of this study was to propose a relationship that accounts for the observed co-existence of ants, red velvet mites, scale/aphids and the presence of honeydew, particularly if the compound (neryl formate) released by the mites were to prevent ant predation.

MATERIALS AND METHODS

Balaustium mites

Mite taxonomists have informed us that the *Balaustium* sp. used in this study is a new species and have advised us to refer to it as *B*. sp. nr. *putmani* until it has been described to the species-level (Anonymous, 2009; the taxonomists who have provided the species identification to us have requested to remain anonymous, and we are honoring their request). The taxonomists provided the following information to us for inclusion in this manuscript, and this is the only taxonomic information provided to us at this time. The one described species in eastern North America is *Balaustium putmani* Smiley (Smiley, 1968), described from Ontario, Canada, but also recorded in North Carolina by Childers and Rock (Childers and Rock, 1980). Voucher specimens of this mite are in the Acarology Laboratory, The Ohio State University, Columbus (specimen lot number OSAL 013113). In our area (Clark Co., Springfield, OH, USA) the peak *B*. sp. nr. *putmani* season is in May–June, and in our survey work no other *Balaustium* species appear to be present (exemplar mites were taken throughout the test period, slide-mounted and examined by mite taxonomists). Regarding the mobile mite stages used in this experiment, larvae generally appear in early April, adults are present until early July, and deutonymphs overlap in between. The mites were collected using an aspirator, placed into 1.5 ml polypropylene microfuge tubes and brought into the laboratory, where they were identified to life-history stage [larvae by having six legs, and nymphs and adults by illustrations of related *Balaustium* mites figured by Newell (Newell, 1963) and Gabrys (Gabrys, 2000)]. Mites were used for an experiment within 30 min of collection and none of the mites was used twice.

Examination of clustering/feeding on honeydew

Honeydew was collected from two sources, leaves of trees/plants from field sites (from aphids), where *Balaustium* sp. mites were present (mites were present at the field sites), and from plants in the Wittenberg University greenhouse (from coccid scale insects and aphids), where *Balaustium* sp. mites are not present. Forceps and glass capillaries were used to collect the honeydew and place it into 10 ml glass vials. All honeydew samples were used on the day they were collected and were standardized for size (1 mg quantity) by weighing on an electrobalance (s.d. ±0.2 μg precision and accuracy of ±6 μg at 1 mg; CAHN, Ventron Co.; Cerritos, CA, USA). Honeydew was presented to the mites in hardened form and in liquid preparations of various concentrations (1.0 mg ml^{-1}, 0.5 mg ml^{-1}, 0.01 mg ml^{-1}, 0.05 mg ml^{-1}) made in deionized, double-distilled (DI) water. Glucose, fructose, sucrose and trehalose (Sigma Chemical Co., St Louis, MO, USA; Fisher Scientific, Philadelphia, PA, USA) were diluted in DI water (in concentrations of 0.001 mol l^{-1}, 0.01 mol l^{-1} and 0.1 mol l^{-1}). These sugars were also tested in solid form (i.e. not dissolved; 1 mg quantity). DI water served as a control. Evans blue dye (0.1%; Sigma) was added to the honeydew as a tracer (modified from Yoder, 1996; Kahl and Alidousti, 1997).

Basic observations were conducted in an environmental room at 25±1°C, a light:dark cycle of 14 h:10 h, and 75% relative humidity (RH) provided by saturated NaCl (Winston and Bates, 1960). All relative humidities are dehydrating for this mite [i.e. the critical equilibrium humidity is >100% RH (Yoder et al., 2006a)]. Relative humidity was measured with a Taylor hygrometer (s.e.m. ±3% RH; Thomas Scientific, Philadelphia, PA). Tests were conducted in Petri dishes (diameter 100 mm, height 15 mm; Fisher) fitted with a No. 3 filter paper disk (diameter 9 cm; Whatman, Hillsboro, OR, USA) that had been scored into quadrants using a No. 2 pencil (Pentel, Palm Desert, CA, USA). This is a routinely used, short-range, two-choice bioassay for mite pheromone/attractant work (Arlian and Vyszenski-Moher, 1995). Application of liquid test materials onto the filter paper disk was achieved using calibrated glass microcapillary tubes (±0.25%, Fisher). Twenty microliters of test material was applied in opposite quadrants, alternating with untreated or water-treated controls; thus, test, control, test, control [test material was applied in the center of each quadrant; a diagram of this layout is given in Arlian and Vyszenski-Moher (Arlian and Vyszenski-Moher, 1995)]. Solid honeydew (1 mg quantity) and the various test sugars were placed into a pile in the middle of the designated treatment quadrant. Compounds were tested one at a time, and only one concentration was tested per Petri dish. The filter paper disk was air-dried before use. Mites were introduced, 20 at a time, into the center of the bioassay arena and allowed to crawl around the dish. Counts of mites in the various quadrants were made when 30 min had elapsed. During the first 15 min of the 30 min test period, behavioral observations were made under a stereomicroscope (40×). Untreated, blank filter-paper disks were used to rule out left- and right-hand bias.

Each test involved the response by a total of 100 mites: 20 mites per plate at a time and replicated five times using different sets of unconditioned (not previously used in an experiment) mites for each replicate (statistical analyses demonstrated differences between

replicates; thus data were pooled). Data were expressed as percentages (response/100) for statistical analysis using chi-square test (χ^2) to compare observed (O) and expected (E) values (Sokal and Rohlf, 1995), with E value=50; level of significance α=0.05, and d.f.=1. To compare the attraction potential with that of other attractants in the pheromone literature, data were also expressed as relative efficacy of attraction [REA (Yunker et al., 1992)]: REA=100 × [(%unattracted to control)–(% unattracted to test)]/(% unattracted to control); this is the form and exact wording of the equation specified by Yunker et al. (Yunker et al., 1992). Counts of mites on untreated filter paper (used to rule out left-/right-hand bias) and water-only controls were combined and served as the 'control' for use in the REA calculation (this is a standard control used in the REA calculation).

Examination of mite clustering to reduce water loss

Net transpiration (water loss) rates were determined at 25°C based on hourly weighing intervals for a total of four successive readings of mass with the electrobalance (source and sensitivity are described above). Mites were weighed and monitored singly without an enclosure and without anesthesia. An aspirator was used to transfer the mite to the weighing pan of the balance. Mites were kept in 5 ml mesh-covered chambers. There were four different sets of chambers: (1) a chamber that contained a single mite; (2) a chamber that contained ten mites; (3) a chamber that contained 1 mg honey dew and a single mite; and (4) a chamber that contained 1 mg honeydew and ten mites. A spot of white paint (Pactra Industries, Van Nuys, CA, USA) was put on the focal mite using an 'eyelash brush' so that it could be identified for re-weighing (paint had no effect on mass changes; data not shown). The mite was removed from its enclosure with an aspirator, transferred to the weighing pan of the balance, weighed (the mite was out of test conditions for less than a minute) and then returned to its enclosure. Measurements from mites where the group disbanded before the end of the experiment were not included in data sets. The first mass measurement was taken as the fresh mass (f). At the end of the experiment, the mite was dried [90°C drying oven for 48 h (Hadley, 1994)] and this was taken as the dry mass (d). The dry mass was subtracted from each mass measurement to convert it into a value that reflected the amount of water in the body of the mite.

To determine net transpiration (water loss) rate (integumental plus respiratory water loss), the relative humidity that the mites were exposed to was 0% RH [provided by anhydrous $CaSO_4$; 1.5×10^{-2}% RH; Toolson (Toolson, 1978); measured with a hygrometer ±3% RH; Fisher] in a 3000 ml (l×w×h) sealed glass desiccator. Thus, chambers holding the mites were placed at 0% RH, 25°C, on a porcelain plate such that they were above the $CaSO_4$ placed in the base of the desiccator. Selection of 25°C was used to permit comparison with the previous literature on water balance (Hadley, 1994). The 0% RH is used because this is the only relative humidity where water loss is exponential that permits calculation of net transpiration rate based on the following equation: $m_t=m_0e^{-kt}$, where m_t is the water mass at any time, t, m_0 is the initial water mass and $-kt$ is the net transpiration rate (Wharton, 1985). Adult females of *B*. sp. nr. *putmani* survive longer than a day at 0% RH and 25°C; thus the exposure for 4 h to this arid environment for measuring the net transpiration rate is unlikely to cause water stress sufficient to interfere with the regular behavior of this mite (Yoder et al., 2006a). Calculation of the net transpiration rate is based on the slope of a regression line on a plot of $\ln(m_t/m_0)$ against time and is expressed as %/h. Percentage body water content was determined using the first measurement as the fresh mass (f) and substituting in the equation: percentage m=100% (f–d)/f, as described by Wharton (Wharton, 1985).

Each net transpiration rate was based on a mean (±s.e.m.) of 45 mites for each of the individual treatments. A different lot of 45 mites was used to determine the percentage body water content and was also expressed as the mean (±s.e.m). The entire set of 45 mites was split into three replicates, with each replicate (set of 15) coming from different days of collection (each day=one replicate). These water balance data were compared with an ANOVA (level of significance α=0.05) using an arcsin transformation in the case of percentages and tested for the equality of slopes of several regressions (Sokal and Rohlf, 1995).

Examination of neryl formate as the active defensive allomone in mite secretion

A standard mealworm (*Tenebrio molitor*) predation assay (Yoder and Brunet, 1997; Yoder et al., 2006b) was used to test the response of ants to mite-based secretions and to the main component of this secretion (neryl formate; Sigma). Worker ants (cornfield ants, *Lasius alienus*, and black carpenter ants, *Camponotus pennsylvanicus*) were collected near Columbus, OH, USA. Groups of 15 ants (single species) were introduced into a bioassay arena and allowed to move freely for 15 min. Groups of three mealworms (third instar; mean length=23±2 mm to standardize for surface area) were then introduced into the arena. Control mealworms consisted of untreated individuals and those treated with acetone (HPLC grade; Sigma). Acetone extracts from ground whole-body mites, secretion-only (mites immersed into acetone and removed after urnulae secretion) and from those that did not secrete (mites were frozen before immersion into acetone to prevent urnulae secretion; no secretion was observed) were prepared according to Yoder et al. (Yoder et al., 2006b). Neryl formate was tested at the concentrations of 0.1 mol l^{-1}, 0.01 mol l^{-1} and 0.001 mol l^{-1} dissolved in acetone. These extracts were applied (10 µl) to the mealworms and allowed to air dry before individual mealworms were introduced into the bioassay arena. After 24 h, the mealworm larvae were examined for evidence of predation. Each extract was tested 15 times to measure the responses of 15 groups of three mealworms exposed to ants. An ANOVA was used to compare the data.

RESULTS

Observations on mite searching behavior

Observations are consistent for *B*. sp. nr. *putmani* in each of the mobile stages – larvae, deutonymphs and adults. When released into the bioassay arena, mites quickly dispersed and spread throughout the Petri dish, exhibiting a behavior that was characterized by rapid crawling, regular tapping with the front pair of legs ('legs I', functional equivalent of insect antennae) and probing with the mouthparts (gnathosoma). Movement by mites within the arena was relatively continuous; rarely did the mites pause and cease ambulatory activity. When mites encountered other mites, they briefly tapped the other mite with legs I and then continued crawling without changing direction. Mites were not observed to form clusters at the edge of the dish. Treatment areas on the filter paper were encountered passively. No slowing of movement or redirection in walking path was made towards, or away from, control or test material that was indicative of any kind of attractant or repellent. Throughout the test period, the mites were extremely active, scurrying about, making it difficult to contain them within the dish; keeping the number of mites low by introducing only 20 mites at a time per replicate facilitated making counts of mites in the various quadrants.

All stages of this mite expressed a different set of behaviors when raw honeydew was encountered in the arena. Honeydew prompted the mite to stop crawling and remain on top of the spot of filter paper treated with honeydew. While on the honeydew, the mites moved slowly about the surface of the honeydew, continuing to tap the surface with legs I and probe (although less vigorously), pausing on occasion for 2–3 min, with their mouthparts making direct contact with the honeydew. All mite stages (each N=100) offered samples of honeydew stained with 0.1% Evans blue were observed with their mouthparts making contact with the honeydew surface, and dye was observed filling the gut diverticula. Dissection of the mites under oil (1000×) revealed blue coloration as evidence of incorporation of the dye. At 75% RH and 25°C, mites in all stages that were observed to feed on honeydew were thriving the next day (>50% of mites were alive), whereas those mites not exposed to honeydew were dead (N=100 per stage). In our experiments, more than one mite was observed feeding on a single portion of honeydew at any given time, and none of them displayed any aggressive tendencies towards obtrusive mites that attempted to feed at the same site. Our observations are restricted to same stage interactions and did not include encounters involving mixed stages.

Quantitative assessment of honeydew detection and clustering

The responses by mites to honeydew are shown in Table 1 (±s.e.m. ≤5.7; replicates of 20; N=5; χ^2; P<0.05; d.f.=1). The observations apply to all stages in that reactions to test materials were nearly identical. None of the samples acted as attractants, as evidenced by the lack of change of direction and crawling activity to treatment areas. The greatest retention was exhibited by raw honeydew, resulting in 89% of adult females remaining on the honeydew, 82% of deutonymphs and 78% of larvae from honeydew collections originating from aphids. Corresponding counts of mites not leaving water-treated controls were 44%, 53% and 56%, respectively. As expected, high percentage retention values were associated with high REA values [values in the range 20–40 are considered good attractants (Yunker et al., 1992)]. Raw honeydew sampled from scales had a similar percentage retention, and high REAs, when exposed to the mites (χ^2; P<0.05; d.f.=1), with the exception of adult females that showed 15% fewer mites on honeydew from scale (76%) than on that from aphids (89%) (Table 1; χ^2; P>0.05; d.f.=1). The retention effect that was exhibited towards raw honeydew could be elicited by 1.0 mg honeydew ml^{-1}, but not diluted preparations of 0.1 mg ml^{-1} or 0.001 mg ml^{-1}, from either aphid or scale. The results showed a regular dose–response relationship: the greater the concentration of honeydew, the greater the retention effect by the mites [y=58.7x, R=94 for larvae (R=correlation coefficient on a plot where the equation of the line is y=mx); y=50.5x, R=95 for deutonymphs; y=52.9x, R=68.9 for adult females; two-way ANOVA; P<0.001]. Adult females responded to 0.1 mg honeydew ml^{-1} with slight, but significant, retention (Table 1; χ^2; P<0.05; d.f.=1), but this concentration prompted no retention in any of the other stages. Thus, honeydew has a retaining effect on all stages of the mite but requires concentrations close to natural to be effective.

Glucose, fructose, sucrose and trehalose did not induce arrestment of any of the stages, even at the highest concentration of 0.1 mol l^{-1} (Table 1). The only difference among these compounds where a significant retention effect was demonstrated was at 0.01 mol l^{-1} glucose (72%, REA of 26.4) and 0.01 mol l^{-1} sucrose (63%, REA of 15.8) for deutonymphs, and at 0.1 mol l^{-1} trehalose (70%, REA of 37.1) for adult females. Glucose at concentrations of 0.01 mol l^{-1} (43%, REA=–30.2) appeared to act as a repellent to larvae (χ^2; P<0.05; d.f.=1), as evidenced by a highly negative REA value. Unlike the response to diluted preparations of honeydew, no dose–response was evident when comparing the number of mites in treated quadrants with their respective concentration of mono- and disaccharides (R≤0.57; ANOVA; P>0.001). No mite stages were

Table 1. Arrestment by red velvet mites, *Balaustium* sp. nr. *putmani*, on honeydew, but not simple sugars, in short-range bioassays

	Larvae		Deutonymphs		Adult females	
Treatment	%	REA	%	REA	%	REA
Control						
(Combined)	56		53		44	
Honeydew (raw)						
Aphid	78*	28.2	82*	35.4	89*	50.6
Scale	84*	33.3	91*	41.8	76*	42.1
Honeydew (diluted)						
1.0 mg ml^{-1}	72*	22.2	83*	36.1	81*	45.7
0.1 mg ml^{-1}	62	9.7	58	8.6	67*	34.3
0.01 mg ml^{-1}	57	1.8	47	–12.8	55	20.0
Glucose†						
0.1 mol l^{-1}	55	–1.8	57	7.0	46	4.3
0.01 mol l^{-1}	43	–30.2	72*	26.4	58	24.1
0.001 mol l^{-1}	57	1.8	54	1.9	52	15.4
Sucrose						
0.1 mol l^{-1}	49	–14.3	47	–12.8	55	20.0
0.01 mol l^{-1}	58	3.4	63*	15.8	40	–10.0
0.001 mol l^{-1}	52	–7.7	55	3.6	51	13.7
Trehalose						
0.1 mol l^{-1}	48	–16.7	52	–1.9	70*	37.1
0.01 mol l^{-1}	55	–1.8	55	3.6	39	–12.8
0.001 mol l^{-1}	59	5.1	49	–8.2	43	–2.3

Arrestment by red velvet mites, *Balaustium* sp. nr. *putmani*, on honeydew, but not simple sugars, in short-range bioassays (25°C, 75% RH) after 30 min (similar results were obtained after 1 h). Results are shown as no./100(%) mites on treated areas after 30 min.

REA, relative efficacy of attraction; combined: untreated plus water-only controls.

†Fructose yielded nearly identical results; *significant difference compared with 50% attraction (replicates of 20; N=5; χ^2; P<0.05; d.f.=1).

Table 2. Effect of honeydew on clustering and resultant water balance characteristics for adult females of the red velvet mite *Balaustium* sp. nr. *putmani*

	Empty chamber		Chamber containing honeydew	
Characteristic	1 mite (isolated)	5 mites	1 mite (isolated)	5 mites
Behavior				
Heightened activity	+	+	–	–
Clustering	–	–	–	+
Water content				
Initial mass, *f* (mg)	0.139±0.0007	0.146±0.013	0.151±0.009	0.142±0.011
Dry mass, *d* (mg)	0.038±0.005	0.043±0.004	0.046±0.006	0.041±0.006
Water mass, *m* (mg)	0.101±0.005	0.103±0.007	0.105±0.004	0.101±0.002
Body water (%)	72.66±0.82	70.43±0.58	69.54±0.63	71.13±0.74
Water loss (see Fig. 1)				
NTR (%/h, 25°C)	2.56±0.06	2.71±0.05	1.74±0.07	1.69±0.08

Effect of honeydew on clustering and resultant water balance characteristics (means±s.e.m.; *N*=45) for adult females of the red velvet mite *Balaustium* sp. nr. *putmani*.
Heightened activity: regular scurrying crawling; NTR, net transpiration rate (integumental and respiratory water loss); +, observed; –, not observed.

attracted or retained when exposed to raw glucose, fructose, sucrose and trehalose (data not shown), suggesting that the physical presence of an object is not responsible for the retention effect observed with raw honeydew. Responses by mites to untreated and water-only controls were fairly uniform and split evenly among the 100 mites, ruling out left- and right-hand bias in the experiment, and approaching the 50% expected (E) value used in the chi-square calculation. Our conclusion is that retention prompted by honeydew is unlikely to be attributable to simple sugars acting as active ingredients for any of the stages of *B*. sp. nr. *putmani*; the effects of these sugars in combination have not been examined.

Net transpiration rates for mites in a cluster

Isolated mites contained 72.66% body water (fresh mass=0.139 mg; dry mass=0.038 mg; water mass=0.101 mg), and this percentage body water was similar for mites that had been exposed to ten other mites and for mites isolated and in a group setting with ten other mites that had been exposed to honeydew (Table 2; ANOVA; $P>0.05$). Corresponding water mass to dry mass ratios (m/d) are 2.6 (isolated mite with no honeydew exposure), 2.4 (mite in group of ten with no honeydew exposure), 2.3 (isolated mite on honeydew) and 2.5 (mite in group of ten on honeydew). In all cases, the water mass was a positive correlate of the dry mass (mites having no honeydew exposure: $R>0.89$ for isolated mites and $R>0.94$ for mites in a group of ten; mites having honeydew exposure: $R>0.96$ for isolated mites and $R>0.92$ for mites in a group of ten; the slope of a line describing this relationship was significantly different from zero; ANOVA; $P<0.001$). Our conclusion is that the adult female mites used in the experiment are similar in size, shape and water content. Thus, any observed differences in net transpiration rate are due to the impact of the group and not to differences in surface-area-to-volume properties or honeydew feeding.

Without exposure to honeydew, isolated adult female mites lost 2.56% water per h (net transpiration rate=integumental plus respiratory water loss; Table 2). Mites were similarly active, displaying excited crawling activity, when they were housed in a chamber with other mites with no honeydew present and resulted in no major alteration in net transpiration rate compared with mites in isolation (2.71% per h, ANOVA; $P>0.05$). The fact that no clusters were observed in chambers containing the group of 10 mites at 0% RH used to determine net transpiration rate (consistently all mites were engaged in rigorous searching behavior) implies that clustering due to brief desiccation for 4 h did not occur in *Balaustium* in the experiment. By contrast, an isolated mite exposed to honeydew ceased the rigorous searching behavior and remained on the honeydew and was considerably less active and probed less aggressively. This reduced activity and arrestment on honeydew by mites resulted in a clustering effect when in the presence of ten mites (Table 2). Mites exposed to honeydew lost similar amounts of water whether they were isolated (1.74%/h), in a group of five (2.01% per h; Fig. 1), 10 (1.69% per h; Table 2) or 20 (1.83% per h; Fig. 1). Thus, mites did not experience a decrease in net transpiration rate as group size increased (Fig. 1). A significant, approximate 30% reduction in the net transpiration rates was measured for mites having been exposed to honeydew that lost less water than mites that were not exposed to honeydew: for an isolated mite, the net transpiration rate is 2.56% per h without honeydew present and 1.74% per h with honeydew present, and, for mites in a group of ten, the net

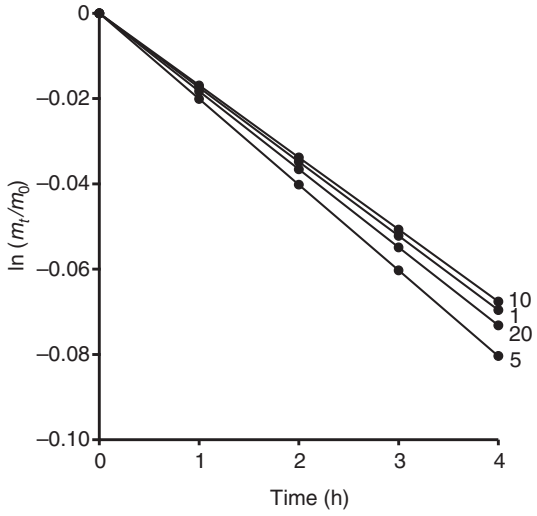

Fig. 1. Proportion of water mass lost by female adults of the red velvet mite *Balaustium* sp. nr. *putmani* at 25°C on raw honeydew exposed to mites in groups of different sizes (1, 5, 10 and 20 individuals). m_t, water mass at time t; m_0, initial water mass. Mass measurements were performed at 0% relative humidity so that the slope of the regression line is the net transpiration rate; each point on the graph is the mean of 45 individual mites, and error bars are within the symbols used on the graph (s.e.m. ±≤0.04).

transpiration rate is 2.71% per h for a mite without honeydew present and 1.69% per h for a mite with honeydew present (two-way ANOVA; $P<0.05$). Thus, clustering by adult female *B*. sp. nr. *putmani* by arrestment on honeydew promotes water conservation, in part, by reducing activity (lower respiratory water loss) and not through a group effect.

Role of neryl formate in preventing ant predation

A coating from the body fluid extract or the urnula secretion from the mites reduced evidence of ant attacks by at least 60% compared with acetone-treated (control) mealworms (Fig. 2; ANOVA; $P<0.05$). The body fluid extract from the mites was approximately 10% more effective than the urnulae-based secretion. Non-secretion extracts from the mites (killed mites that do not secrete when immersed in solvent) only showed a minimal, but not significant, reduction in predation (ANOVA; $P>0.05$). When neryl formate was applied alone, there was a significant reduction in the predation of mealworms (Fig. 2; ANOVA; $P<0.05$). The protective capacity of neryl formate increases positively with increasing concentration (Fig. 2). Nearly all of the untreated and acetone-treated mealworms (controls) were damaged by the ants (>90% predation; Fig. 2). We emphasize that, in the no-ant treatments, none of the compounds caused significant mortality to the mealworms.

DISCUSSION

We propose a tritrophic interaction among red velvet mites, *Balaustium* sp. nr. *putmani*, aphids/scale insects and ants (Fig. 3). This relationship is linked through the use of plant-derived honeydew by both ants [as a food source (Stadler and Dixon, 2005; Styrsky and Eubanks, 2007)] and red velvet mites [as prey cue and supplemental food source, much like phytoseiid mites in this regard (Nomikou et al., 2003) (this study)]. Despite consumption by aphids and scale insects, host plants can benefit from their interactions with ants. Ants remove honeydew from leaf surfaces, helping to decrease the incidence of disease by fungal pathogens, while also minimizing plant damage by preying on herbivorous insects (Bach, 1991). In exchange for the nutrition afforded by honeydew feeding in the ant–sternorrhynchal mutualism, the ant, in turn, aggressively defends the aphid/scale insects, shielding them against attack by potential predators, such as coccinellid beetles, syrphid flies, lacewings, gall midges and predatory mites (Bach, 1991; Styrsky and Eubanks, 2007). The lack of antagonistic interaction between *Balaustium* sp. mites and ants [ants tend to ignore the mites (Grandjean, 1947; Grandjean,1957)] indicates that ants provide little, if any, protection for aphids and scales against predation by these mites. Of interest is that honeydew would not be a viable food option, nor would aphid/scale insects be readily accessible prey, if the mites did not have a mechanism to escape the attending ants if ants were present.

In this proposed model (Fig. 3), we hypothesize that *Balaustium* mites are not attacked by ants defending honeydew resources because they release an alarm pheromone, neryl formate (Yoder et al., 2007b), that bears a strong structural resemblance to natural terpenoid pheromones produced by ants. This is a common strategy used in many insects and acarines to evade ant predation (Whitman et al., 1990). The release of this alarm pheromone allows the mites to go undetected and permits them to delicately slip into the ant–aphid/scale insect mutualism where the mites can prey upon aphids and scale insects (Fig. 3). As a consequence, we hypothesize that ants view *Balaustium* sp. mites as other ants, and do not view them as a threat to the aphids and scale insects.

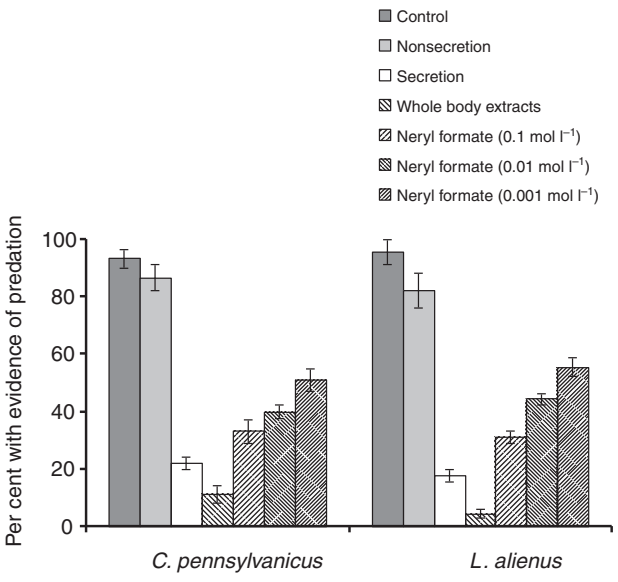

Fig. 2. Evidence of predation on mealworm (*Tenebrio molitor*) larvae by ants (carpenter ants, *Camponotus pennsylvanicus*, and cornfield ants, *Lasius alienus*) after coating individuals with *Balaustium* sp. nr. *putmani* secretions, body fluid extracts and neryl formate. Control: untreated and acetone-treated mealworm larvae; nonsecretion: mites frozen before immersion into acetone, thus preventing urnulae secretion (Yoder et al., 2006b); secretion: live mites immersed into acetone, resulting in urnulae secretion, (Yoder et al., 2006b); whole-body extracts: mites crushed in acetone to provide whole-mite extracts. In each test, three mealworms were exposed to 15 worker ants for 24 h, and each was replicated 15 times. Bars, s.e.m.

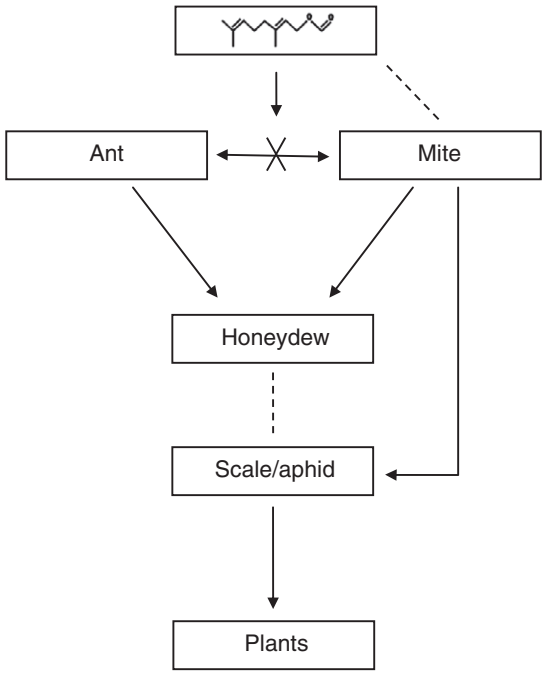

Fig. 3. Proposed tritrophic relationships and interactions among red mites (*Balaustium* sp. nr. *putmani*), scale/aphids and ants. Scales/aphids feed on plants and secrete excess carbohydrate as honeydew. Ants and red mites feed on honeydew, but no antagonistic relationship between these organisms is present owing to the release of a mite defense secretion (neryl formate, structure shown at top) that prevents recognition by ants and deters predation.

The mechanism of ant-avoidance used for gaining access to aphid/scale insect prey applies only to *Balaustium* deutonymphs and adults in that the alarm pheromone is not present in the larval stage because they lack the glands (urnulae) that produce this secretion (Southcott, 1961; Newell, 1963; Gabrys, 2000; Halliday, 2001; Yoder et al., 2006b). Thus, *Balaustium* larvae are seemingly not protected in this way against the ants. An additional feature that contributes to larval survival is their reduced stadial duration (around for briefer periods; Putman, 1970) that minimizes exposure time to the same risks as the postlarval stages because they are active under similar circumstances.

Honeydew detection has biological value for *B*. sp. nr. *putmani* by serving as a cue that aphid and scale insects are present, presumably signaling prey abundance and quality. The quality of honeydew, however, is expected to vary depending upon the species and stage of the aphid/scale insects and host plant. Such variation in honeydew composition has also been shown to alter the dynamics and effectiveness of the tending relationship by ants (Yao and Akimoto, 2001; Fisher et al., 2005), and therefore the relationships depicted in Fig. 3 are not rigidly fixed and are anticipated to be flexible. Evidence of feeding on honeydew by these red mites suggests that it can be used as an alternative food source when prey densities are low or as a supplemental food source. Predatory phytoseiid mites have also been shown to feed on honeydew and are able to sustain themselves on a honeydew diet, even enhancing reproductive rates (Nomikou et al., 2003). In our experiments, we did not test whether *B*. sp. nr. *putmani* are able to sustain themselves solely on a honeydew diet, and this aspect of their biology needs to be examined. The arrestment activity of honeydew has the significance of retaining *Balaustium* on plant surfaces where aphid/scale insect prey items are found, thereby increasing the likelihood of encountering prey. In other words, *B*. sp. nr. *putmani* appears to search randomly and concentrate on leaves covered with honeydew rather than on leaves lacking honeydew because, by feeding on it, they enhance their opportunity for locating an abundance of thriving prey and/or the honeydew provides an alternative source of energy.

REFERENCES

Arlian, L. G. and Vyszenski-Moher, D. L. (1995). Responses of *Sarcoptes scabiei* var. *canis* (Acari: Sarcoptidae) to lipids of mammalian skin. *J. Med. Entomol.* **32**, 34-41.

Bach, C. E. (1991). Direct and indirect interaction between ants (*Pheidole megacephala*), scales (*Coccus viridis*) and plants (*Pluchea indica*). *Oecologia* **87**, 233-239.

Benoit, J. B., Yoder, J. A., Lopez-Martinez, G., Elnitsky, M. A., Lee, R. E., Jr and Denlinger, D. L. (2008). Adaptations for the maintenance of water balance by three species of Antarctic mites. *Polar Biol.* **31**, 539-547.

Bogo, A. (2003). New group of oligosaccharides excreted in honeydew from scale insects *Stigmacoccus* sp. and *Coccus hesperidium* L. *Ciência Rural.* **33**, 593-599.

Bogo, A. and Mantle, P. (2000). Oligosaccharides in the honeydew of Coccoidea Scale Insects: *Coccus hesperidum* L. and a new *Stigmacoccus* sp. in Brazil. *An. Soc. Entomol. Brasil* **29**, 589-595.

Brown, K. S. (1975). The chemistry of aphids and scale insects. *Chem. Soc. Rev.* **4**, 263-288.

Childers, C. C. and Rock, G. C. (1980). Observations on the occurrence and feeding habits of *Balaustium putmani* (Acari: Erythraeidae) in North Carolina apple orchards. *Int. J. Acarol.* **7**, 63-68.

Fisher, M. K., Völkl, W. and Hoffman, K. H. (2005). Honeydew production and honeydew sugar composition of polyphagous black bean aphid, *Aphis fabae* (Hemiptera: Aphidae) on various host plants and implications for ant attendance. *Eur. J. Entomol.* **102**, 155-160.

Gabrys, G. (2000). *Balaustium xerothermicum* sp. nov. from Poland with remarks on all world species of the genus (Acari: Actinedida: Erythraeidae). *Ann. Zool.* **50**, 47-56.

Grandjean, F. (1947). Au sujet des Erythroides. *Bull. Mus. Nat. Hist. Nat. Paris*, *2e serie* **19**, 327-334.

Grandjean, F. (1957). Les stases due developpement ontogenetique chez *Balaustium florale* (Acarien, Erythroide), Premiere partie. *Ann. Soc. Entomol. France* **125**, 135-152.

Grandjean, F. (1959). Les stases du developpement ontogenetique chez *Balaustium florale* (Acarien, Erythroide), Deuxieme partie. *Ann. Soc. Entomol. France* **128**, 159-177.

Hadley, N. F. (1994). Water Relations of Terrestrial Arthropods. New York: Academic Press.

Halliday, R. B. (2001). Systematics and biology of the Australian species of *Balaustium* von Heyden (Acari: Erythraeidae). *Aus. J. Entomol.* **40**, 326-330.

Kahl, O. and Alidousti, I. (1997). Bodies of liquid water as a source of water gain for *Ixodes ricinus* ticks (Acari: Ixodidae). *Exp. Appl. Acarol.* **21**, 731-746.

Newell, I. M. (1963). Feeding habits in the genus *Balaustium* (Acarina, Erythraeidae), with special reference to attacks on man. *J. Parasitol.* **49**, 498-502.

Nomikou, M., Janssen, A. and Sabelis, M. W. (2003). Phytoseiid predators of whiteflies feed and reproduce on non-prey food sources. *Exp. Appl. Acarol.* **31**, 15-26.

Putman, W. L. (1970). Life history and behavior of *Balaustium putmani* (Acarina: Erythraeidae). *Ann. Entomol. Soc. Amer.* **63**, 76-81.

Smiley, R. L. (1968). A new genus and three new species of Erythraeoidea (Acarina: Erythraeidae and Smarididae). *Proc. Entomol. Soc. Wash.* **70**, 13-21.

Sokal, R. R. and Rohlf, F. J. (1995). Biometry: The Principles and Practice of Statistics in Biological Research. San Francisco: W. H. Freeman.

Southcott, R. V. (1961). Studies on the systematics and biology of the Erythraeoidea (Acarina), with a critical revision of the genera and subfamilies. *Aust. J. Zool.* **3**, 367-610.

Stadler, B. and Dixon, A. F. G. (2005). Ecology and evolution of aphid-ant interactions. *Ann. Rev. Ecol. Evol. Syst.* **36**, 345-372.

Strong, F. E. (1965). Detection of lipids in the honeydew of an aphid. *Nature* **205**, 1242.

Styrsky, J. D. and Eubanks, M. D. (2007). Ecological consequences of interactions between ants and honeydew-producing insects. *Proc. Biol. Sci.* **274**, 151-164.

Toolson, E. C. (1978). Diffusion of water through the arthropod cuticle: thermodynamic consideration of the transition phenomenon. *J. Therm. Biol.* **3**, 69-73.

Welbourn, W. C. (1983). Potential use of trombidioid and erythraeoid mites as biological control agents of insect pests. In Biological Control of Pests and Mites (ed. M. A. Hoy, G. L. Cunningham and L. Knutson), pp. 103-140, Berkeley: University of California Press.

Welbourn, W. C. and Jennings, D. T. (1991). Two new species of Erythraeidae (Acari: Prostigmata) associated with the Spruce Budworm, *Choristoneura fumiferana* (Clemens). (Lepidoptera: Tortricidae), in Maine. *Can. Entomol.* **123**, 567-580.

Wharton, G. W. (1985). Water balance of insects. In *Comprehensive Insect Physiology, Biochemistry and Pharmacology*, Vol. 4 (ed. G. A. Kerkut and L. I. Gilbert), pp. 565-603. Oxford: Pergamon Press.

Whitman, D. W., Blum, M. S. and Alsop, D. (1990). Allomones: chemicals for defense. In *Insect Defenses: Adaptive Mechanisms and Strategies of Prey and Predators* (ed. D. L. Evans and J. O. Schmidt), pp. 289-351. Albany: State University of New York Press.

Winston, P. W. and Bates, D. S. (1960). Saturated solutions for the control of humidity in biological research. *Ecology* **41**, 232-237.

Wohltmann, A. (1998). Water vapour uptake and drought resistance in immobile instars of Parasitengona (Acari: Prostigmata). *Can. J. Zool.* **76**, 1741-1754.

Yao, I. and Akimoto, S.-I. (2001). Ant attendance changes the sugar composition of the honeydew of the drepanosiphid aphid *Tuberculatus quercicola*. *Oecologia* **128**, 36-43.

Yoder, J. A. (1996). The Madagascar hissing-cockroach mite (*Gromphadorholaelaps schaeferi*): First observation of its larva and ptyalophagy in Acari. *Int. J. Acarol.* **22**, 141-148.

Yoder, J. A. and Brunet, M. E. (1997). Evaluation of squalene as a potential fire ant repellent. *Proc. La Acad. Sci.* **57**, 1-5.

Yoder, J. A., Ark, J. T., Benoit, J. B., Rellinger, E. J. and Gribbins, K. M. (2006a). Water balance components in adults of terrestrial red mite *Balaustium* sp. (Acarina: Erythraeidae). *Ann. Entomol. Soc. Amer.* **99**, 560-566.

Yoder, J. A., Benoit, J. B., Rellinger, E. J., Ark, J. T. and Gribbins, K. M. (2006b). Structure and function of the urnulae in *Balaustium* sp. (Parasitengona: Erythraeidae) featuring secretion of a defensive allomone and alarm pheromone. *Int. J. Acarol.* **32**, 3-12.

Yoder, J. A., Schumaker, L. K. and Tank, J. L. (2007a). High temperature resistance of the terrestrial red mite (*Balaustium* sp.) as a product of suppressed heat-induced water permeability. *Int. J. Acarol.* **33**, 275-281.

Yoder, J. A., Mowrey, D. D., Rellinger, E. J., Tank, J. L., Hanson, P. E. and York, R. W. (2007b). Detection of the mite alarm pheromone neryl formate in the velvet mite, *Balaustium* sp. (Parasitengona: Erythraeidae). *Int. J. Acarol.* **33**, 73-78.

Yoder, J. A., Rigsby, C. M. and Tank, J. L. (2008). Function of the urnulae in protecting the red velvet mite, *Balaustium* sp., against water loss and in enhancing its activity at high temperatures. *Int. J. Acarol.* **34**, 419-425.

Yunker, C. E., Peter, T., Norval, R. A. I., Sonenshine, D. E., Burridge, M. J. and Butler, J. F. (1992). Olfactory responses of *Amblyomma hebraeum* and *A. variegatum* adults to attractant chemicals in laboratory tests. *Exp. Appl. Acarol.* **13**, 295-301.