Popul Ecol (2012) 54:405–410
DOI 10.1007/s10144-012-0314-9

ORIGINAL ARTICLE

The aphid-tending ant *Lasius fuji* exhibits reduced aggression toward aphids marked with ant cuticular hydrocarbons

Shintaro Endo · Takao Itino

Received: 18 July 2011 / Accepted: 1 March 2012 / Published online: 17 March 2012

Abstract Some aphid species are attended by ants, which protect aphids against enemies, but ants sometimes prey on the aphids they are attending depending on the resource conditions. A previous study indicated that the ant *Lasius niger* preys less on the aphid individuals that experienced ant attendance than on those that did not. This observation leads to the hypothesis that ants transfer some substances to the aphids they attend and selectively prey on the aphids without the substances. In this study, we focus on cuticular hydrocarbons (CHCs), which are used by ants as nestmate recognition substances, and test whether ants discriminate the aphids on the basis of CHCs. We confirmed that the ant *Lasius fuji* preyed less on the aphids that were attended by their nestmates than those that were not attended. Glass dummies treated with CHCs from attended aphids were attacked less by ants than those treated with CHCs from non-attended aphids. The CHC profiles of ant attended aphids resembled those of the ants, suggesting that ants' CHCs are transferred to the aphids' body surface through ant attendance. These results support the hypothesis that ants "mark" their attended aphids with their CHCs and the CHCs reduce ant predation intensity.

S. Endo (✉)
Department of Mountain and Environmental Science, Shinshu University, 3-1-1 Asahi, Matsumoto, Nagano 390-8621, Japan
e-mail: s07t404@shinshu-u.ac.jp

T. Itino
Department of Biology, Faculty of Science, Shinshu University, 3-1-1 Asahi, Matsumoto, Nagano 390-8621, Japan

T. Itino
Institute of Mountain Science, Shinshu University, 3-1-1 Asahi, Matsumoto, Nagano 390-8621, Japan

Keywords Ant–aphid mutualism · Chemical discrimination · Cuticular hydrocarbon (CHC) · Selective predation · *Stomaphis yanonis*

Introduction

Ant–aphid interactions in which ants attend aphids for their honeydew and protect them against enemies are among the most famous examples of mutualism. However, ants also often prey on aphids they attend (Pontin 1958; Way 1963; Skinner and Whittaker 1981; Sakata 1994) when aphids are too abundant and provide honeydew in excess (Way 1954, 1963). As it is costly for ants to attend and protect too many aphid individuals (Stadler and Dixon 2005), it would be adaptive for ants to prey on extra aphids as a protein source rather than attending them to collect honeydew. Moreover, it would be adaptive for ants to protect the aphids providing ample honeydew and to prey on those providing less honeydew. For this, however, ants need to be able to discriminate the two kinds of aphids.

Sakata (1994) hypothesized that ants somehow mark aphids they attend to decide to prey on or not. Sakata (1994) observed that an ant stops attacking an aphid if it successfully provides honeydew to the ant, and that ants predate less frequently on aphids that previously provided honeydew to their nestmate ants. These observations indicate that ants may put some marks on their aphids when they receive honeydew and do not prey on well-marked aphids. Accordingly, aphids that produce much honeydew obtain more marks and are less likely to be preyed on by ants. However, the actual existence and physical nature of the postulated marking substance has yet to be explored.

Sakata (1994) showed that ants accept the aphids that were attended by their nestmates, whereas they tend to

attack the aphids that were attended by non-nestmate ants or that were not attended by any ants. This finding suggests that ants use their nestmate-recognition signal to mark aphids. Glinwood et al. (2003) conducted bioassays using a hexane extract from aphid cuticle and showed that ants can discriminate the extracts of ant attended aphids from those of non-attended aphids. Chemical substances, especially cuticular hydrocarbons (CHCs), which are hexane-soluble, are known to play a crucial role in nestmate recognition in various ant species (reviewed by Howard 1993; Blomquist et al. 1998; Vander Meer and Morel 1998). CHCs show species- and colony-specific profiles; workers from different ant colonies have different CHC profiles, and workers attack other workers with the CHC profile of a different colony (Yamaoka 1990; Akino and Yamaoka 2002). Hence, we hypothesize that ants use CHCs to mark and discriminate the aphids they attend.

This study aims to test the hypothesis that ants mark the aphids they attend with CHCs and predate on the aphids that are less marked. To test this hypothesis, we used the ant species *Lasius fuji* (formerly *L. fuliginosus* or *L. nipponensis*; Radchenko 2005) and the aphid *Stomaphis yanonis*. Both species are different from those Sakata (1994) used. First, we show that the ants are less likely to prey on the aphids that their nestmate ants attended than those that were not at all attended by ants. Next, we compare the intensity of ants' attack against glass dummies between those treated with the CHCs extracted from ant attended aphids and those treated with CHCs from non-attended aphids. Finally, we analyze the CHC profiles of aphid body surfaces to determine whether ants' CHCs are transferred to the aphid or not.

Materials and methods

Study site and insects

We conducted sample collection and field experiments at four *L. fuji* colonies in broad-leaved deciduous forests in Matsumoto, Nagano, Japan. In these forests, *L. fuji* nests in the soil around the trees of *Zelkova serrata* or *Celtis sinensis* which are colonized by *S. yanonis* aphids. *Stomaphis yanonis* forms a couple dozen colonies, each consisting of 5–20 aphids, on the trunk surface (up to 3 m above the ground). An aphid is consistently attended by several individual ant workers which collect the aphid's honeydew.

We collected *L. fuji* workers 1 week before the experiments. The workers from different colonies were separately reared in a plastic container (180 mm × 90 mm × 45 mm high) with a layer of plaster on the bottom of the container (10 mm thick) to retain moisture. The containers were covered with cardboard to keep the workers in the dark. Each container was bridged by a vinyl tube (300 mm long, 4 mm in diameter) to another plastic container (330 mm × 200 mm × 100 mm high) that served as the foraging area, where aqueous maple syrup was provided every 3 days.

Adult aphids were collected and individually reared in a small plastic cage (35 mm × 35 mm × 12 mm high) or in a plastic Petri dish (90 mm in diameter) with a moistured melamine sponge (5 mm thick) to retain moisture. The aphid rearing cages were placed in the foraging area of the ants. We conducted two treatments: in the "ant attendance" treatment, the ants were allowed to enter the aphid cages through a hole (5 mm in diameter) at the top of the cages; in the "non-ant attendance" treatment, the ants could not access the aphids because the aphid cages had no holes. The aphid cages were left in the foraging area for 3 days. Because of their extremely specialized mouthpart, the aphids could not be fed in the cage. Nonetheless, the aphids produced healthy offsprings and they survived for as long as 1 week. The first or second instar nymphs newly born during the 3 days were used as "ant attendance" or "non-ant attendance" aphids in the bioassay.

Bioassay

"Attendance" aphids or "non-attendance" aphids were individually introduced onto the ant- (and aphid-) inhabited tree in the field from which the aphids were initially collected. Within 10 min of observation, if an ant bit the aphid and carried it away, it was regarded as preyed on.

To assess the role of CHCs in aphid discrimination, we used glass dummies treated with aphids' CHCs. Four aphids from the ant attendance treatment or non-ant attendance treatment were immersed in 100 μl of hexane for 5 min. Then, the extract was applied to a 0.7 g silica gel column (Wakogel C-200, Wako), and the CHCs were eluted with 3 ml of hexane. The solutions were concentrated and one aphid equivalent of the concentrated solution was applied to a glass dummy (2.0 mm in diameter). Dummies were put on the ground near an ant trail within 2 m from the nest entrance, and the behavioral responses of the ant workers to the dummies were observed. We continued the observation for 300 s since the first encounter of a worker with a dummy, and the duration of "attack behavior" within the 300 s was recorded. When an ant worker bended its abdomen forward and opened its mandibles to bite the dummies, we defined it as attack behavior. All the aphids used for the bioassays were caught from the same host tree as the experiments were conducted.

Chemical analyses

We compared CHC profiles of *L. fuji* ant workers, ant attended aphids, non-attended aphids, and "field-collected

aphids". "Field-collected aphids" refers to the aphids that were attended by *L. fuji* in the field. The insects were individually immersed in 100 µl hexane for 5 min, the extract was applied to a 0.7 g silica gel column (Wakogel C-200, Wako), and then the CHCs were eluted with 3 ml hexane. The solutions were concentrated to 5 µl (aphids) or 15 µl (ants), and then analyzed by gas chromatography–mass spectrometry (GC–MS). GC–MS analyses were performed on an Agilent 5973MSD mass spectrometer interfaced with an Agilent 6890N gas chromatograph equipped with an HP-5MS capillary column (30 m long × 0.25 mm ID × 0.25 µm film thickness). Helium was used as the carrier gas with a constant flow rate of 0.9 ml/min. A split/splitless injector was set to splitless mode for 1 min at a temperature of 300 °C. The temperature program of the column oven was 40 °C for 3 min, 40–260 °C at 30 °C/min, 260–300 °C at 15 °C/min, followed by holding at the final temperature for 12 min. The electron impact mass spectrum was measured at 70 eV.

Statistical analyses

To assess overall similarity among hydrocarbon profiles, the area of each detectable peak in every chromatogram was converted to its proportional contribution to total peaks area of that sample and transformed to arcsine of the square root. Because some peaks contained more than one compound, peaks rather than individual chemicals were the units on which the statistical analyses were performed. Detected hydrocarbons were classified into one of three groups according to their chemical structure: normal alkanes, branched alkanes, and unsaturated hydrocarbons. Although most of the detected hydrocarbons were normal alkanes, recent investigations have shown that normal alkanes have little or no utility in nestmate recognition among social insects (Lohman et al. 2006). For this reason, all shared peaks of ants and aphids excluding normal alkanes were used in the analyses. Cluster analyses were performed on the data matrix using Ward's method with Euclidean distance. The statistical analyses were performed with R software ver. 2.12.1 (R Development Core Team 2010).

Results

Bioassay

The aphids that were attended by ants were less predated by the ants (17.7 %, $n = 30$) than those that were not attended (44.1 %, $n = 34$) (Fisher's exact test, $P = 0.03$). When an ant worker first came into contact with an ant attended aphid, the ant usually touched the aphid with its antennae and then began to attack the aphid by bending its abdomen or by biting the aphid. In response to this attack, the aphid tried to give honeydew to the ant. If the aphid successfully gave honeydew to the ant, then the ant stopped attacking and accepted the aphid. If the aphid failed to give honeydew, the ant often preyed on it. In contrast, when an ant worker met a non-attended aphid, the ant touched the aphid, often immediately bit it and carried it away.

When ant workers encountered the dummies treated with aphids' CHCs, they tried to attack them (biting or bending the abdomen). This attack behavior continued for a while. The duration of the attack was shorter to the dummies treated with attended aphids' CHCs than to those treated with non-attended aphids' CHCs (t test, $P = 0.006$; Fig. 1).

Cuticular hydrocarbon profiles

Hydrocarbon constituents from 27 chromatographic peaks with sufficiently strong signals were tentatively identified based on the retention index and mass spectra (Table 1; Fig. 2). CHC chromatogram of field-collected aphids had all the 27 peaks, of which 21 corresponded to chromatographic peaks of *L. fuji* ants (Fig. 2b; Table 1), while the other 6 (peaks 9, 15, 19, 23, 26, and 27) were exclusively detected in the aphids. In contrast, non-attended aphids' chromatogram had only 21 peaks, of which 15 peaks were shared with ants and the other 6 (peaks 9, 15, 19, 23, 26, and 27) were seen only in the aphids. The chromatogram lacked the 6 peaks (peaks 1, 3, 4, 16, 22, and 25) found in the field-collected aphids, and several peaks were smaller

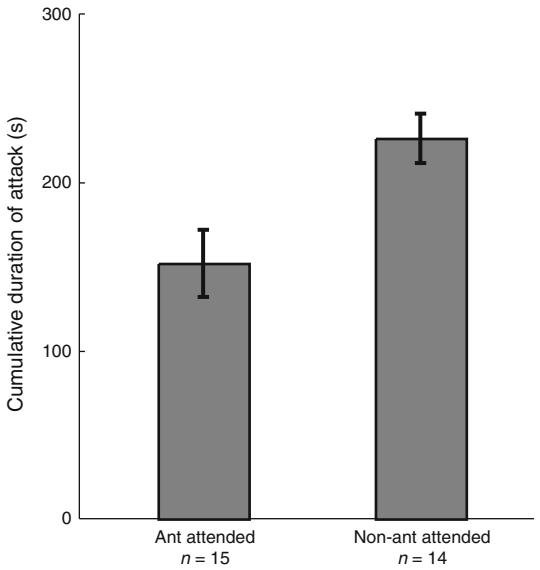

Fig. 1 Comparison of cumulative duration of ant attacks on dummies treated with ant attended and non-attended aphids' CHCs (mean ± SE; t test, $P < 0.01$)

Table 1 Comparison of cuticular hydrocarbon components between *Lasius fuji* and *Stomaphis yanonis* (field collected, ant attended in the cages, or non-ant attended in the cages)

Peak number	Compounds (abbreviation)	*L. fuji*	*S. yanonis*			
			Field collected	Ant attended		Non-ant attended
				(a)	(b)	
		$n = 20$	$n = 8$	$n = 3$	$n = 3$	$n = 6$
1	Pentacosene (C25:1)	t	t	t		
2	*n*-Pentacosan (nC25)	++	++	+	++	+
3	7-Methyl + 13-methyl pentacosanes (7Me + 13MeC25)	+	t	+		
4	5-Methyl pentacosane (5MeC25)	++	+	++	t	
5	3-Methyl pentacosane (3MeC25)	++	++	++	++	++
6	*n*-Hexacosane (nC26)	++	++	+	++	++
7	Heptacosene (C27:1)	+++	++	++	++	t
8	*n*-Heptacosane (nC27)	+++	+++	+++	+++	+++
9	Heptacosene (C27:1)		t	t		t
10	7-Methyl + 13-methyl heptacosanes (7Me + 13MeC27)	+++	+++	+++	+++	+++
11	5-Methyl heptacosane (5MeC27)	++	++	++	++	+
12	11,15-Dimethyl heptacosane (11,15diMeC27)	++	++	++	++	t
13	3-Methyl heptacosane (3MeC27)	+++	+++	+++	++	++
14	*n*-Octacosane (nC28)	++	++	+	++	++
15	3-Methyl octacosane (3MeC28)		++	++	++	++
16	Nonacosene (C29:1)	++	+	++	t	
17	Nonacosene + *n*-nonacosane (C29:1 + nC29)	+++	++	++	++	++
18	Nonacosadiene + 13-methyl nonacosane (C29:2 + 13MeC29)	+++	++	+++	++	++
19	7-Methyl nonacosane (7MeC29)		++	++	+++	+++
20	13,17-Dimethyl nonacosane (13,17diMeC29)	+++	+++	+++	+++	+++
21	3-Methyl nonacosane (3MeC29)	++	+	++		t
22	5,17-Dimethyl nonacosane (5,17diMeC29)	++	++	++		
23	3-Methyl triacontane (3MeC30)		++	++	++	++
24	15-Methyl hentriacontane (15MeC31)	++	+	++	t	t
25	13,17-Dimethyl hentriacontane (13,17diMeC31)	++	t	++		
26	9,19-Dimethyl hentriacontane (9,19diMeC31)		++	++	++	+++
27	3-Methyl dotriacontane (3MeC32)		+	t	t	+

Ant attended aphids comprise of the group (a), which has all the components of ants' CHCs, and the group (b), which lacks several of the ants' CHC peaks

t, <0.5 %; +, <1 %; ++, <5 %; +++, >5 %, relative to the total peak area on the chromatogram

(e.g., peaks 7, 11 and 12) than the corresponding peaks of the field-collected aphids. These absent or smaller peaks were those found in the ant chromatogram (Fig. 2d; Table 1). Attended aphids were divided into two groups based on their CHC profiles. The CHC chromatogram of group (a) resembled that of the field-collected aphids, and included all the 21 peaks that were shared with the ants (Fig. 2c; Table 1). The CHC chromatogram of group (b) had fewer peaks and resembled that of non-attended aphids (Table 1).

A cluster analysis of the 16 CHC peaks which were shared by the ants and aphids revealed that field-collected aphids and *L. fuji* ants clustered together (except for one nymphal aphid), while non-attended aphids were placed in another cluster (Fig. 3). Attended aphids of group (a) and the field-collected aphids clustered together, while attended aphids of group (b) and the non-attended aphids clustered together (Fig. 3).

Discussion

The ants preyed less on ant attended aphids than on non-attended ones, indicating that the *L. fuji* workers can recognize the aphids that were attended by the ant workers. The average duration of an ant attack on aphid-CHC treated dummies was shorter on those treated with attended aphids' CHCs than on those treated with non-attended

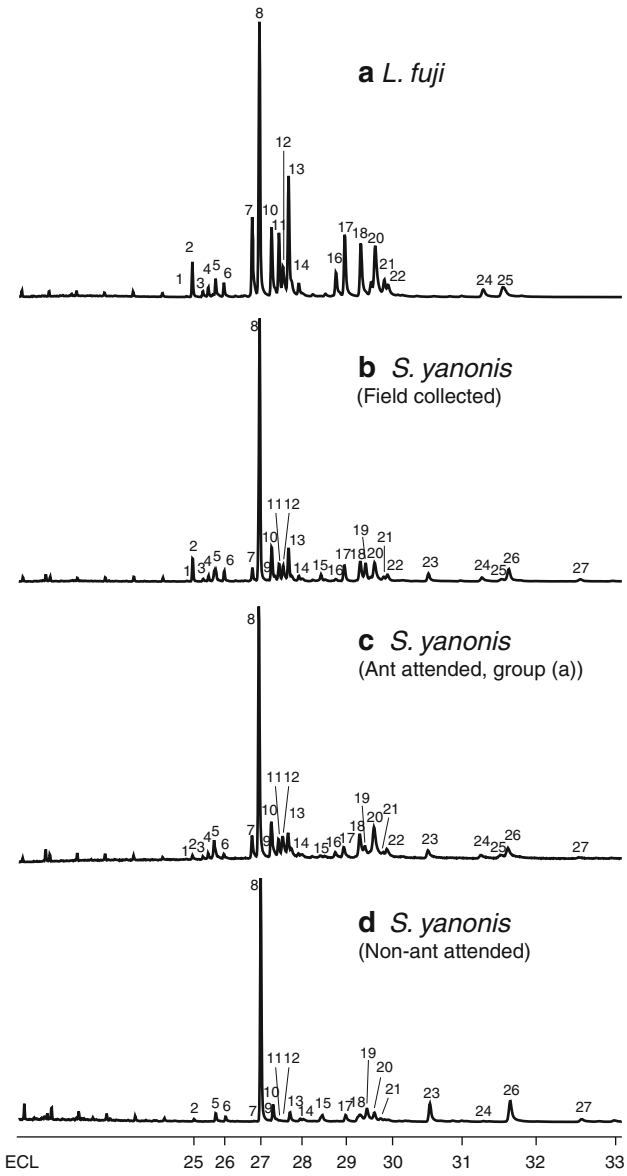

Fig. 2 Cuticular hydrocarbon profiles of **a** *Lasius fuji*, **b** *Stomaphis yanonis* (field collected), **c** *S. yanonis* (ant attended), and **d** *S. yanonis* (non-ant attended). Compounds corresponding to the peak numbers are listed in Table 1

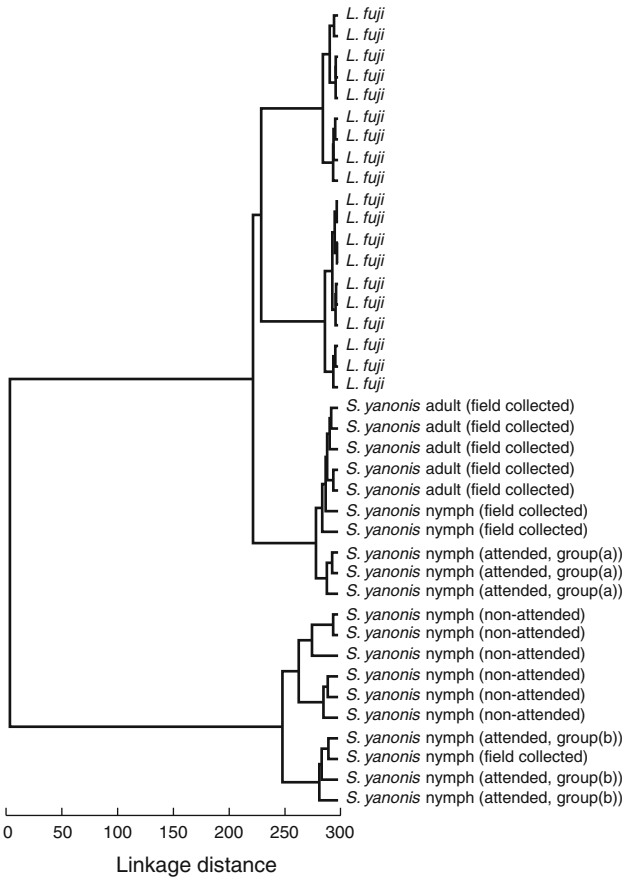

Fig. 3 The result of a cluster analysis (Ward's method, Euclidean distances) for the CHCs of the ant *Lasius fuji* and the aphid *Stomaphis yanonis*

aphids' CHCs (Fig. 1). Moreover, the CHC chromatogram of attended aphids resembled that of ants' CHCs (Table 1; Figs. 2, 3). These findings suggest that ants' CHCs are transferred to the aphids' body surfaces through ant attendance, and that the CHCs moderate the ants' attack behavior. These results support our hypothesis that ants mark the aphids they attend with their CHCs and the CHCs cause the ants to reduce their predation intensity.

Sakata (1994) suggested that, if ants "mark" the aphids, they can recognize aphid density by quantifying the marks, enabling them to regulate the aphid population by predation. Previously, the intensity of ant predation on ant attended aphids was reported to reflect aphid density (Edwards 1951; Sudd 1987). However, the mechanism with which individual ant workers recognize aphid density has been unknown. Sakata (1994) hypothesized that, when aphid density per ant is high, each aphid has little chance to provide honeydew to the ants and receive less "marking substance"; therefore, many aphids remain unmarked and ants prey on these unmarked aphids, leading to reduction of aphid population density. In contrast, when the density of aphids is low, many aphids are marked and fewer aphids are preyed on. For all the postulated functions of the hypothesized chemical "marks", concrete evidence of their existence has been lacking.

Here, we demonstrate that CHCs are used as marking substances in the *L. fuji–S. yanonis* system. Shared CHC profiles among *L. fuji* ant colony members have been known to be used as the nestmate recognition signal (Akino and Yamaoka 2002). In addition, the chemical nature of hydrocarbons is such that they can be easily transferred to substrates or other organisms by contact (Akino et al. 1996; Akino and Yamaoka 1998). Therefore, it is reasonable for *L. fuji* to use CHCs as marking substances.

It was not clear in this study whether the ants actively put CHCs on the aphids' body surface or incidentally transferred their CHCs to the aphids when they collect honeydew. Chemically camouflaged myrmecophilous wasps mount the petiole of a host ant and rub its body surface, before grooming themselves carefully (Akino and Yamaoka 1998). This behavior might enable them to incorporate their CHC profiles into the host ant colony (Akino and Yamaoka 1998). By contrast, neither *S. yanonis* nor *L. fuji* showed this typical grooming behavior, although several worker ants constantly surrounded and touched the aphid. Akino et al. (2005) observed that *L. fuji* footprint hydrocarbons resemble those of the ant CHC profiles, except that the footprints lack *n*-alkanes, and that the more an ant has walked on a glass plate, the more CHCs accumulate on the glass plate. Therefore, the CHCs may be incidentally transferred through ants' footprint to aphids as the ants collect honeydew.

The different profiles of the two groups of attended aphids may reflect different amounts of ants' CHCs accumulated on the aphid body surface. We divided the attended aphids into two groups: the one possessing all the CHC peaks of the ants (Table 1, group (a)), and the other having fewer CHC peaks, like non-attended aphids (Table 1, group (b)). Aphids of group (a) may obtain more ants' CHCs because they produce much honeydew and thus be in frequent contact with ants. Such productive aphids may thereby less likely to be preyed on. Consequently, the selective predation by ants may strongly select for aphids' honeydew productivity.

Acknowledgments We thank R. Yamaoka, T. Akino, N. Fujiwara-Tsujii, and M. K. Hojo for analytical advice and technical support, M. Maruyama for ant identification, and T. Akino, H. Kuzume and two anonymous reviewers for valuable comments on the earlier draft of the manuscript. This study was supported by a Grant-in-Aid for Scientific Research (C-22570015) and a Grant-in-Aid for Exploratory Research (18657008) from the Japan Society for the Promotion of Science.

References

Akino T, Yamaoka R (1998) Chemical mimicry in the root aphid parasitoid *Paralipsis eikoae* Yasumatsu (Hymenoptera: Aphidiidae) of the aphid-attending ant *Lasius sakagamii* Yamauchi & Hayashida (Hymenoptera: Formicidae). Chemoecology 8:153–161

Akino T, Yamaoka R (2002) Cuticular hydrocarbon profile as a critical cue candidate for nestmate recognition in *Lasius fuliginosus* (Hymenoptera: Formicidae). Entomol Sci 5:267–273

Akino T, Mochizuki R, Morimoto M, Yamaoka R (1996) Chemical camouflage of myrmecophilous cricket *Myrmecophilus* sp. to be integrated with several ant species. Jpn J Appl Entomol Zool 40:39–46

Akino T, Morimoto M, Yamaoka R (2005) The chemical basis for trail recognition in *Lasius nipponensis* (Hymenoptera: Formicidae). Chemoecology 15:13–20

Blomquist GJ, Tillman JA, Mpuru S, Seybold SJ (1998) The cuticle and cuticular hydrocarbons of insects: structure, function, and biochemistry. In: Vander Meer RK, Breed MD, Winston ML, Espelie KE (eds) Pheromone communication in social insects. Westview Press, Oxford, pp 34–54

Edwards RL (1951) Change in the foraging behavior of the garden ant *Lasius niger* L. Entomol Mon Mag 87:280

Glinwood R, Willekens J, Pettersson J (2003) Discrimination of aphid mutualists by an ant based on chemical cues. Acta Agric Scand B S P 53:177–182

Howard RW (1993) Cuticular hydrocarbons and chemical communication. In: Stanley-Samuelson DW, Nelson DR (eds) Insect lipids: chemistry, biochemistry, and biology. University of Nebraska Press, Lincoln, pp 179–226

Lohman DJ, Liao Q, Pierce NE (2006) Convergence of chemical mimicry in a guild of aphid predators. Ecol Entomol 31:41–51

Pontin AJ (1958) A preliminary note on the eating of aphids by ants of the genus *Lasius* (Hym., Formicidae). Entomol Mon Mag 94:9–11

R Development Core Team (2010) R: a language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. http://www.R-project.org/

Radchenko A (2005) A review of the ants of the genus *Lasius* Fabricius, 1804, subgenus *Dendrolasius* Ruzsky, 1912 (Hymenoptera: Formicidae) from East Palaearctic. Ann Zool 55:83–94

Sakata H (1994) How an ant decides to prey on or to attend aphids. Res Popul Ecol 36:45–51

Skinner GJ, Whittaker JB (1981) An experimental investigation of inter-relationships between the wood-ant (*Formica rufa*) and some tree-canopy herbivores. J Anim Ecol 50:313–326

Stadler B, Dixon AFG (2005) Ecology and evolution of aphid–ant interactions. Annu Rev Ecol Evol Syst 36:345–372

Sudd JH (1987) Individual behaviour and mixed diet strategy in ants. In: Pasteels JM, Deneubourg JL (eds) From individual to collective behavior in social insects. Birkhäuser, Basel, pp 81–92

Vander Meer RK, Morel L (1998) Nestmate recognition in ants. In: Breed MD, Winston ML, Espelie KE, Vander Meer RK (eds) Pheromone communication in social insects. Westview Press, Oxford, pp 79–103

Way MJ (1954) Studies on the association of the ant *Oecophylla longinoda* (Latr.) (Formicidae) with the scale insect *Saissetia zanzibarensis* Williams (Coccidae). Bull Entomol Res 45:113–134

Way MJ (1963) Mutualism between ants and honeydew-producing Homoptera. Annu Rev Entomol 8:307–344

Yamaoka R (1990) Chemical approach to understanding interactions among organisms. Physiol Ecol Jpn 27:31–52