Popul Ecol (2013) 55:27–34
DOI 10.1007/s10144-012-0355-0

THE SOCIETY
OF
POPULATION
ECOLOGY

ORIGINAL ARTICLE

Myrmecophilous aphids produce cuticular hydrocarbons that resemble those of their tending ants

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Received: 2 June 2012 / Accepted: 12 October 2012 / Published online: 24 November 2012

Abstract Aphid-tending ants protect aphids from natural enemies and collect honeydew secreted by the aphids. However, ants also often prey on the aphids they attend. Aphids, therefore, like social parasites of ants, may well have evolved chemical mimicry as an anti-predation strategy. In this study, we aimed to determine whether the aphid *Stomaphis yanonis* actively produces cuticular hydrocarbons (CHCs) that resemble those of the tending ant *Lasius fuji*. In the wild, ants put their CHCs on the aphids that they are tending, so in this study we analyzed "ant-free" aphids. Mature aphids that exuviated in the absence of ant attendance had almost all of the hydrocarbon components that the ants' CHCs had. Moreover, hydrocarbons artificially applied to the aphids' body surface were lost by exuviation. Taken together, these findings indicate that mature aphids actively produced ant-like CHCs, and they constitute the first documentation of a chemical resemblance between aphids and ants in a specific aphid–ant association.

Electronic supplementary material The online version of this article (doi:10.1007/s10144-012-0355-0) contains supplementary material, which is available to authorized users.

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Keywords Ant–aphid mutualism · Chemical mimicry · *Lasius fuji* · *Stomaphis yanonis*

Introduction

Ants are formidable predators of various animals so that they play important roles in terrestrial ecosystems. Their colonies are resource-rich and, though well-protected, are intruded by many social parasites (Hölldobler and Wilson 1990; Akino 2008). Because ants have complex communication systems that allow them to discriminate aliens from nestmates (Vander Meer and Morel 1998), social parasites have evolved morphological, physiological, behavioral, and chemical adaptations that allow them to integrate themselves into ant nests (Akino and Yamaoka 1998; Brandt et al. 2005). Chemical camouflage and chemical mimicry are especially effective for allowing integration into the nest because ants rely heavily on chemical signals to recognize their surroundings (Vander Meer et al. 1989; Hölldobler and Wilson 1990; Howard et al. 1990a, b; Dettner and Liepert 1994; Akino et al. 1999; Akino 2008).

The cuticular hydrocarbon profiles of ants serve as a nestmate recognition signals (Yamaoka 1990; Howard 1993; Vander Meer and Morel 1998), and a variety of social parasites has been reported to imitate cuticular hydrocarbons (CHCs) of their host ants to infiltrate host colony (Akino 2008). There are at least two possible ways for social parasites to imitate their host ants' CHC profiles. "Chemical camouflage" is the first way in which the parasites acquire CHCs from the host ants (sensu Howard et al. 1990a; e.g., Vander Meer and Wojcik 1982; Vander Meer et al. 1989; Akino et al. 1996; Akino 2002). For example, the myrmecophilous beetle *Myrmecaphodius* *excavaticollis* (Vander Meer and Wojcik 1982) and the myrmecophilous cricket *Myrmecophilus* sp. (Akino et al. 1996) acquire specific hydrocarbons of their host ants. The second way of imitation is "chemical mimicry" in which the parasites produce the CHCs by de novo biosynthesis (sensu Howard et al. 1990a; e.g., Howard et al. 1990a, 1990b; Akino et al. 1999). Howard et al. (1990b) revealed by radiolabeling experiments that the syrphid fly *Microdon albicomatus* larvae biosynthesizes the imitation CHCs. Akino et al. (1999) reported that the larvae of the myrmecophilous butterfly *Maculinea rebeli* have ant-like CHCs before they are transported to the host ants' nest by the ant workers, suggesting that they biosynthesize the specific chemicals of the host ants (Akino et al. 1999; Schlick-Steiner et al. 2004). For such chemical mimicry to evolve, the parasites must have high host specificity (Thomas and Elmes 1998; Akino et al. 1999; Elmes et al. 2002) because ant hydrocarbon profiles are generally species-specific (Howard 1993; Akino 2008).

Other than the social parasites of ants, many homopterans (aphids, coccids and membracids) and lycaenid butterflies have mutualistic association with ants. They provide sugar-rich secretion for ants and ants protect them from natural enemies such as predators. At the same time, however, ants also often prey on those insects they attend (Pontin 1958; Way 1963; Skinner and Whittaker 1981; Sakata 1994; Endo and Itino 2012). Thus, the sugar-rich secretion is sometimes considered to be an adaptation against ant predation (Sakata 2000; Stadler and Dixon 2005). Given such predation pressures by ants, those mutualists may well have evolved chemical mimicry as an anti-predation strategy, just as social parasites of ants have. The communication between ants and their mutualists has been intensively investigated (Pierce et al. 2002; Stadler and Dixon 2005). Nevertheless, unequivocal data supporting genuine chemical mimicry of the mutualists to the ants (in the sense that they biosynthesize the ant's recognition pheromone) have not yet been reported. Many species of mutualistic lycaenid butterflies use various chemicals to appease or attract ants, but not to mimic them (Pierce et al. 2002). Although chemical mimicry was reported in a few species of lycaenids, the butterflies are parasitic rather than mutualistic to the ants (Henning 1983, cited in Dettner and Liepert 1994; Akino et al. 1999). Silveira et al. (2010) reported that the mutualistic treehopper *Guayaquila xiphias* elude ant predation by chemical crypsis. However, the cuticular chemicals of the treehopper resemble not the ants but the host plants. As chemical mimicry would evolve only in a system with high host specificity (Akino et al. 1999; Elmes et al. 2002), it would be found, if at all, in a specific association.

The aphid *Stomaphis yanonis* feeds on the woody parts of host trees and is notable for its large body size and extremely long mouthparts, which can probe through the bark of trees. Because they cannot withdraw their mouthparts quickly to escape from enemies, they rely heavily on ants for protection. In addition, oviparous females of *S. yanonis* oviposit in autumn in the ant nests situated underground near the host tree, and those eggs are protected by the ants until next spring (S. Endo, personal observation). In Nagano, central Japan, three closely related species of *Lasius* (*Lasius fuji*, *L. nipponensis*, *L. orientalis*) belonging to the subgenus *Dendrolasius* are known to tend *S. yanonis* (S. Endo, personal observation).

Recently, Endo and Itino (2012) revealed that *L. fuji* (formerly *L. fuliginosus* or *L. nipponensis*; Radchenko 2005) worker ants put their CHCs on their partner *S. yanonis* aphids as "markers," and selectively prey on aphids without their CHCs. As a result, the CHC profiles of *S. yanonis* aphids resemble those of their tending ants. This resemblance may correspond to the "chemical camouflage" of the social parasites of ants. However, the ant CHCs may be lost after the aphids exuviate because they remain on the exuviae. In addition, less well marked aphids are more frequently preyed on; therefore, after exuviation, the aphids would need to acquire more ant CHCs if possible. These considerations lead us to hypothesize that aphids use not only chemical camouflage but also "chemical mimicry".

This study aimed to determine whether the aphid *S. yanonis* actively produces CHCs with a profile resembling that of the CHCs of their tending ants. First, we show that the CHC profiles of non-ant attended, newly exuviated aphids resemble those of the ants. Then, we confirm that a hydrocarbon artificially applied to the aphids' body surface is gone after exuviation, indicating that the ant CHCs are lost by exuviation.

Materials and methods

Cuticular hydrocarbons of *S. yanonis* aphids and *L. fuji* ants

Adults and nymphs of *S. yanonis* and workers of *L. fuji* were collected from the trunk surface of a *Zelkova serrata* tree on the campus of Shinshu University, Matsumoto, Nagano, Japan. On the *Z. serrata* tree, there were dozens of colonies of *S. yanonis*, each composed of up to 20 individuals. Several *L. fuji* workers were continuously tending each aphid, collecting the honeydew that it secreted.

The collected aphids were reared individually in small plastic cages (35 mm × 35 mm × 12 mm high), each containing a moistened melamine sponge (5 mm thick). Because of their extremely specialized mouthparts, the aphids could not feed in the cage. Nonetheless, they survived for about 1 week, and each either exuviated normally or produced healthy offspring in that time. They were not exposed to any ant workers after collection. Immediately after exuviation or birth, the newly exuviated or newborn aphids were individually placed in vials, which were stored at −30 °C until the CHC analyses. Both the exuviated and newborn aphids were assumed to have lost the ant CHCs that had previously been applied to them or their parent by the tending ants in the field, and they were categorized by life stage into three groups: third or fourth instar nymphs and adults (>3 mm in body length), second instar nymphs (<3 mm in body length), and first instar nymphs (newborn). We called these aphids "non-ant-attended aphids."

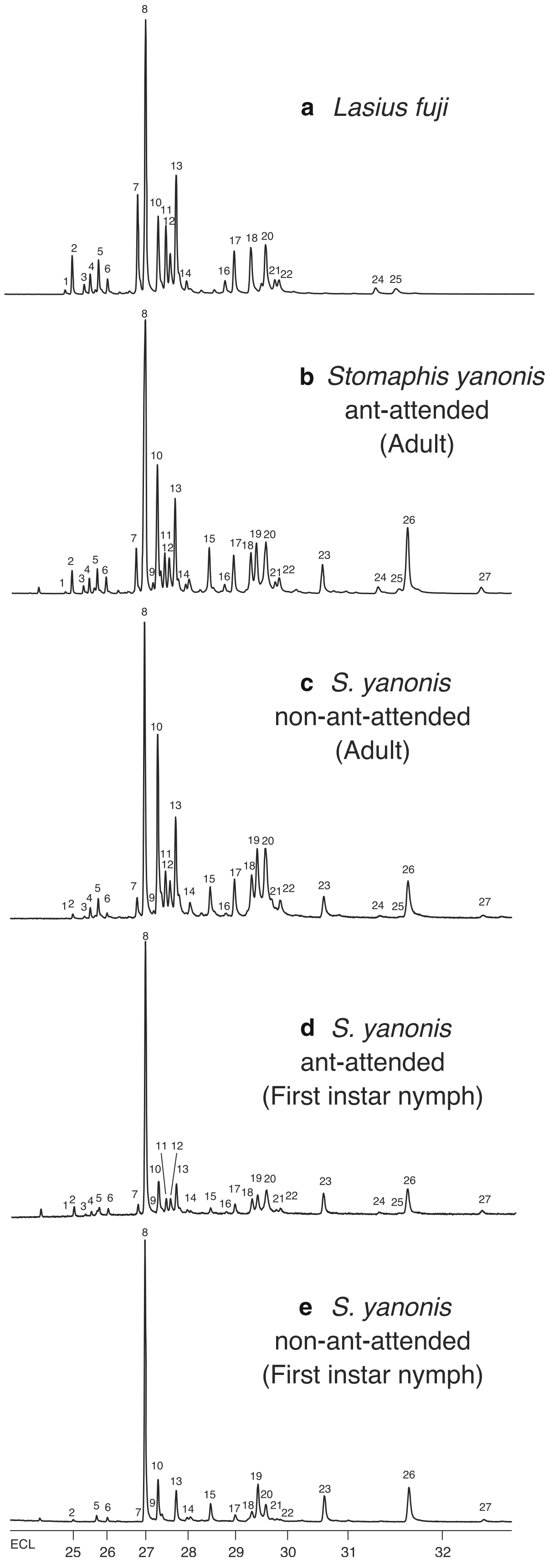
Field-collected, ant-attended aphids were termed "ant-attended aphids" and were stored individually in vials at −30 °C. They were categorized into two groups: third or fourth instar nymphs and adults (>3 mm in body length) and first or second instar nymphs (<3 mm in body length). *Lasius fuji* workers were collected haphazardly from the same tree as the aphids and stored at −30° until analyses.

The insects were individually immersed in 100 μl hexane for 5 min for CHC extraction. The extract was then applied to a 0.7 g silica gel column (Wakogel C-200, Wako Pure Chemical Industries), and then the CHCs were eluted with 3 ml hexane. The solutions were concentrated to 2–5 μl (aphids; adjusted according to body size) 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.

We compared the CHC profiles of non-ant-attended aphids, ant-attended aphids, and *L. fuji* ant workers. To assess the overall similarity among the hydrocarbon profiles, we determined the proportional contribution of the area of each detectable peak in every chromatogram to the total peak area of that sample and then transformed the value to the 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 because of their simple, linear structures that are less readily perceived (Dani et al. 2001; Lucas et al. 2005; Lohman et al. 2006; Guerrieri et al. 2009). On the other hand, however, some social insects use normal alkanes for signals of nestmate recognition (Akino et al. 2004; Greene and Gordon 2007). For this reason, statistical analyses were performed on two sets of data, the "all shared peaks" data matrix in which all shared peaks of ants and aphids are included, and a "reduced" data matrix from which all unbranched alkanes had been removed. The peak 17 was not treated as a shared peak because it consisted mainly of nonacosene in *S. yanonis* and of *n*-nonacosane in *L. fuji*. To visualize the similarity between samples, we performed non-metric multidimensional scaling (NMDS) using the Bray–Curtis dissimilarity index on the data matrix. This analysis has been fruitfully applied to interspecific comparisons of *Myrmica* ant CHC profiles (Elmes et al. 2002). The extent of any final lack of agreement was measured by a statistic called STRESS (standardized residual sum of squares). The lower the STRESS value, the better the NMDS plot represents the original Bray–Curtis dissimilarities (Elmes et al. 2002). The statistical analyses were performed with R software ver. 2.12.1 (R Development Core Team 2010).

Do hydrocarbons on an aphid's body surface disappear after exuviation?

We artificially applied a hydrocarbon to the aphid cuticle and checked whether it was gone after exuviation. Second, third, and fourth instar nymphs of *S. yanonis* were collected at the same site as for the first experiment. The dorsal side of each aphid was gently rubbed 20 times with a cotton swab that had been soaked in melted *n*-docosane (Wako Pure Chemical Industries). The aphids were individually reared for 3 days without ant attendance, and immediately after exuviation, each aphid's body and exuvia were separately collected in vials and stored at −30 °C until analyses. The aphids that did not exuviate within 3 days were also separately collected as control samples. The number of each instar in each of the two treatments were as follows. The aphids after exuviation (and the respective exuviae): three-third instar nymphs and three forth instar nymphs; the aphids before exuviation (control): two second instar nymphs, six third instar nymphs, and three forth instar nymphs. All samples were analyzed by GC–MS in the same manner as in the first experiment.



◀**Fig. 1** Cuticular hydrocarbon (CHC) profiles of **a***Lasius fuji*, **b***Stomaphis yanonis* (ant-attended adult), **c***S. yanonis* (non-ant-attended adult), **d***S. yanonis* (ant-attended first instar nymph), and **e***S. yanonis* (non-ant-attended first instar nymph). Compounds corresponding to the peak numbers are listed in Table 1

Results

Cuticular hydrocarbons of *S. yanonis* aphids and *L. fuji* ants

A total of 27 distinct chromatographic peaks were detectable in the ant and aphid cuticular extracts. The hydrocarbon constituents of the 27 peaks were tentatively identified by using the retention index values and mass spectra [Fig. 1; Table 1 and Table S1 in the Electronic supplementary material (ESM)]. The CHCs of *L. fuji* included 21 of the 27 constituents, and the other six (peaks 9, 15, 19, 23, 26, and 27) were detected exclusively in extracts from aphids. Extracts from ant-attended aphids (nymphs and adults) had almost the same peaks as those of the ants (Fig. 1b, d; Table 1). In contrast, the CHC chromatogram of first or second instar nymphs among the non-ant-attended aphids lacked some peaks of the ant chromatograms (i.e., peaks 3, 4, and 24), and several peaks were lower than the corresponding peaks of the ant-attended aphids (e.g., peaks 7, 11, and 12; Fig. 1e; Table 1). All of these “lacked” and “lower” peaks were mono- or di-methyl alkanes with the exception of peak 7 (alkene), and were detected in the ants (Table 1 and Table S1 in ESM). These results are consistent with the observation that *L. fuji* ants put their CHCs on their attended aphids (Endo and Itino 2012). On the other hand, chromatograms of adult non-ant-attended aphids shared almost all of the peaks of the ant chromatograms (Fig. 1c; Table 1).

NMDS ordination of 99 samples based on the Bray-Curtis dissimilarities of the arcsine-transformed relative abundance of cuticular hydrocarbons revealed the similarities and differences among the ants, ant-attended aphids, and non-ant-attended aphids (Fig. 2). The exclusion of normal alkanes had only minor effects on the pattern of NMDS plots (Fig. 2A, B). Ant-attended aphids were closely ordinated with the ants regardless of the aphid’s developmental stage (Fig. 2b, c). Likewise, third and fourth instar nymphs and adults of non-ant-attended aphids were closely ordinated with the ants (Fig. 2d). In contrast, non-ant-attended juveniles (first and second instar nymphs) plotted farther from the ants (Fig. 2e, f).

Do hydrocarbons on an aphid’s body surface disappear after exuviation?

The CHC chromatograms of the aphids after exuviation lacked the *n*-docosane peak ($n = 0/6$; Fig. 3b), whereas

Table 1 Cuticular hydrocarbon components of the ant *Lasius fuji* and the aphid *Stomaphis yanonis*

Peak number	Compounds (abbreviations)	*L. fuji*	*S. yanonis*			
			3rd and 4th instar nymphs, adults		1st and 2nd instar nymphs	
		$n = 11$	Ant-attended $n = 14$	Non-ant-attended $n = 25$	Ant-attended $n = 13$	Non-ant-attended $n = 36$
1	Pentacosene (C25:1)	t				
2	*n*-Pentacosan (nC25)	++	+	t	++	t
3	7-Methyl + 13-methyl pentacosanes (7Me + 13MeC25)	+	t	t	t	
4	5-Methyl pentacosane (5MeC25)	++	t	t	t	
5	3-Methyl pentacosane (3MeC25)	++	++	+	++	++
6	*n*-Hexacosane (nC26)	++	+	+	++	+
7	Heptacosene (C27:1)	+++	++	+	++	t
8	*n*-Heptacosane (nC27)	+++	+++	+++	+++	+++
9	Heptacosene (C27:1)		+	+	t	+
10	7-Methyl + 13-methyl heptacosanes (7Me + 13MeC27)	+++	+++	+++	+++	+++
11	5-Methyl heptacosane (5MeC27)	+++	++	++	++	t
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	++	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	t	t	t
22	5,17-Dimethyl nonacosane (5,17diMeC29)	++	++	++	++	+
23	3-Methyl triacontane (3MeC30)		++	++	++	++
24	15-Methyl hentriacontane (15MeC31)	++	t	t	t	
25	13,17-Dimethyl hentriacontane (13,17diMeC31)	++				
26	9,19-Dimethyl hentriacontane (9,19diMeC31)		+++	+++	+++	+++
27	3-Methyl dotriacontane (3MeC32)		t	+	t	+

Blank, <0.1 %; t, <0.5 %; +, <1 %; ++, <5 %; +++, >5 %, relative to the total peak area on the CHC chromatogram. Ant-attended aphids were collected in the natural habitat; non-ant-attended aphids were reared in isolation from ants

those of the control individuals included the peak ($n = 11/11$; Fig. 3a). In addition, the chromatograms of the exuviae of the exuviated individuals had the peak ($n = 6/6$; Fig. 3c). These results suggest that hydrocarbons attached to an aphid's body surface disappear after exuviation, and that the CHCs of aphids after exuviation are produced by the aphids themselves.

Discussion

The CHCs of third and fourth instar nymphs and adults of non-ant-attended aphids included almost all of component hydrocarbons of the ant CHCs (Fig. 1; Table 1). The CHC profile of these aphids resembled that of ant-attended aphids on which CHCs were put by the tending ants (Fig. 2). In addition, the hydrocarbon that was artificially applied to the aphids' body surface was lost by exuviation (Fig. 3). These results suggest that mature aphids produce ant-like CHCs by themselves.

Some social parasites penetrate into an ant colony by actively synthesizing the host's nestmate recognition signals (Howard et al. 1990a, b; Akino et al. 1999). In the same way, *S. yanonis* produces CHCs that resemble the CHCs of their *L. fuji* hosts. We assume that the function of the chemical resemblance between *S. yanonis* aphids and ants is avoidance by the aphids of predation by the ants. *Lasius fuji* worker ants put CHC markers on the aphids that

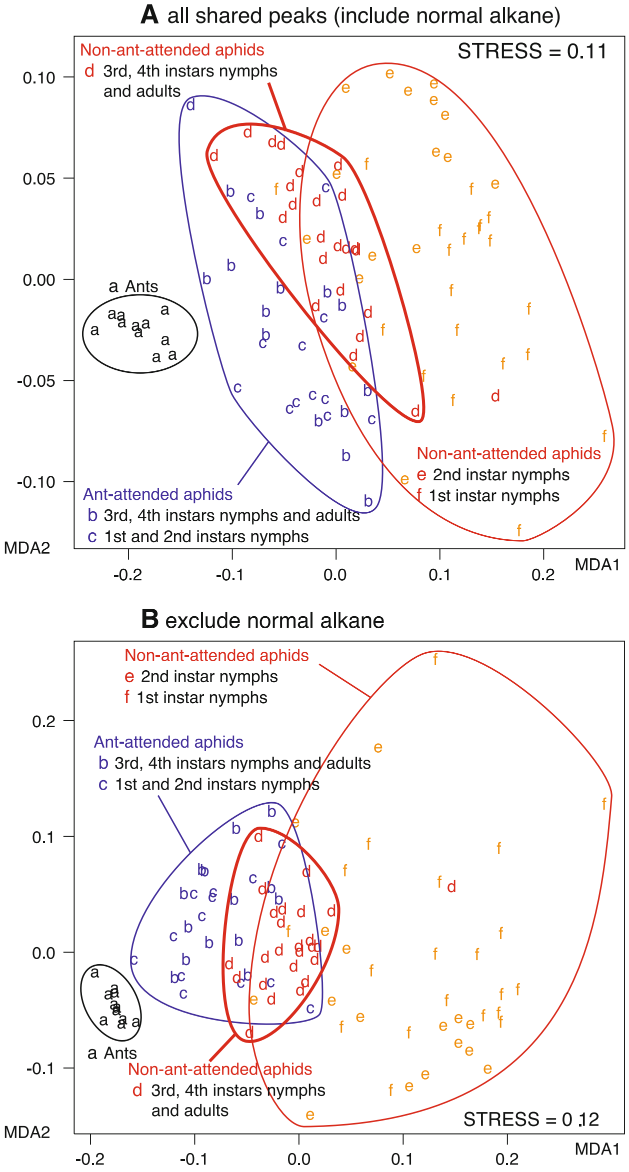


Fig. 2 Two-dimensional non-metric multidimensional scaling ordination of 99 samples, derived from the Bray–Curtis dissimilarities of the arcsine-transformed relative abundance of cuticular hydrocarbons, for a*Lasius fuji* ants, b*Stomaphis yanonis* aphids (ant-attended third and fourth instar nymphs and adults), c*S. yanonis* (ant-attended first and second instar nymphs), d*S. yanonis* (non-ant-attended third and fourth instar nymphs and adults), e*S. yanonis* (non-ant-attended second instar nymphs), and f*S. yanonis* (non-ant-attended first instar nymphs). **A** Analysis of all shared cuticular hydrocarbons; **B** analysis in which normal alkanes have been excluded. The scales are different between the panels

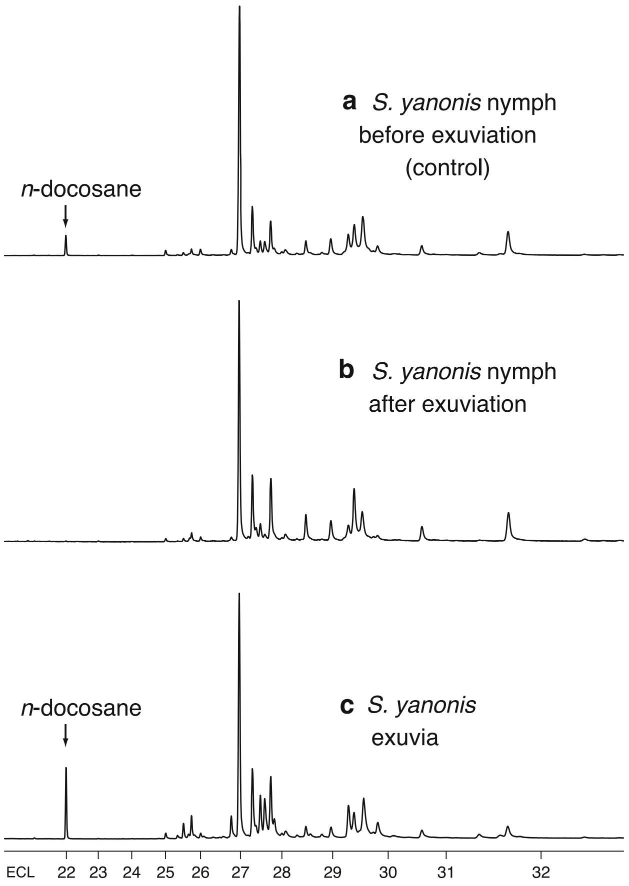


Fig. 3 Representative cuticular hydrocarbon (CHC) profiles of *S. yanonis* nymphs onto which *n*-docosane was artificially applied: **a** before exuviation (control) and **b** after exuviation. **c** CHC profile of an exuvia

provide them honeydew and prey less on the marked aphids (Endo and Itino 2012). Therefore, we can infer that *S. yanonis* provides more honeydew to reduce predation by ants. On the other hand, because the aphids lose the ants' CHC markers by exuviation, it is beneficial for them to produce the marker chemicals by themselves to avoid ant predation. The costs and benefits to the aphids of producing ant-like CHCs remain to be explored.

Predators or parasites of ants such as the syrphid fly *Microdon piperi*, the aphidiid wasp *Paralipsis eicoae*, and the lycaenid butterfly *Maculinea rebeli* deceive their host ants by chemical mimicry, which allows them to penetrate into the ants' nests, lay eggs, prey on the ants' brood, and/or exploit resources in nests (Howard et al. 1990a, b; Akino and Yamaoka 1998; Akino et al. 1999). Here, however, we discovered a chemical resemblance in a mutualistic interaction, not in a parasitic interaction. As far as we know, this is the first documentation of a chemical resemblance of mutualistic aphids to their tending ants.

The aphids' chemical resemblance to the ants may have evolved not only to reduce predation risk but also to reduce their honeydew production. Aphids are generally subject to predation risk by ants (Stadler and Dixon 2005), and even myrmecophilous aphids are often preyed on by ants (Pontin 1958; Way 1963; Skinner and Whittaker 1981; Sakata 1994; Endo and Itino 2012). However, if the aphids excrete honeydew and the ants take it, then the ants usually stop attacking those aphids (Sakata 1994), indicating the benefit of honeydew production. On the other hand, the production of a large amount of high-quality honeydew is known to have physiological cost for aphids (Nixon 1951; Yao et al. 2000; Yao and Akimoto 2001; Stadler and Dixon 2005). Thus, reducing their production of honeydew and instead secreting mimetic chemicals may be on balance beneficial for host-specific aphids.

The resemblance of the CHC profiles of the non-ant-attended juvenile aphids to that of the ants is incomplete, whereas the resemblance of the profiles of well-grown non-ant-attended aphids to that of the ants is close (Figs. 1, 2; Table 1). We propose two hypotheses to explain the developmental changes in the CHC profiles of the aphids. First, we hypothesize that the aphids may show phenotypic plasticity in CHC production. For example, the myrmecophilous butterfly *M. rebeli*, which parasitizes multiple host ant species, changes its CHC profile according to the host ant species (Schlick-Steiner et al. 2004). In the same way, because *S. yanonis* aphids can associate with several different ant species, they may adjust their CHCs to the host ant species in the course of their growth. The CHC profile of *M. rebeli* caterpillars has many peaks before the caterpillars are brought into the ant nest, whereas after they have been adopted into the nest, the number of peaks in the profile is gradually reduced until the caterpillar profile is similar to the host ant profile (Schlick-Steiner et al. 2004). On the other hand, the number of peaks (branched alkanes and an alkene) in the CHC profiles of *S. yanonis* is increased in the more mature aphids, thus causing the profiles to become similar to the ant profile (Fig. 1; Table 1). Perhaps branched alkanes and alkenes are relatively important in nestmate recognition in *L. fuji* like many social insects (Dani et al. 2001; Lucas et al. 2005; Guerrieri et al. 2009), while normal alkanes are not so because they have little effect on the NMDS ordinations (Fig. 2A, B). Second, we hypothesize the developmental change in CHC profiles can be explained by simply assuming that the juvenile aphids cannot produce the full set of mimetic CHCs owing to limitations of their biosynthetic pathways or to the cost of biosynthesis. Whichever hypothesis might be true, in their natural habitat, juveniles usually stay very near to mature aphids (S. Endo, personal observation) whose profiles closely resemble those of the ants and who excrete much honeydew, thus reducing their predation risk under the umbrella of mature aphids.

Taken together, our results suggest that the aphids deceive their tending ants by chemical resemblance. Is chemical resemblance, then, generally observed in natural aphid–ant associations? It might be observed only in host-specialist aphids because it usually evolves by species-specific interactions (Thomas and Elmes 1998; Akino et al. 1999). Further research on chemical resemblance in specific aphid–ant associations will deepen our understanding of coevolved mutualisms.

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 an earlier draft of this manuscript. This study was supported by a Grant-in-Aid for Scientific Research (C-22570015), a Grant-in-Aid for Exploratory Research (18657008) from the Japan Society for the Promotion of Science, and by Research and Education Funding for Japanese Alps Inter-Universities Cooperative Project, MEXT, Japan.

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