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Chemical tactic of facultative myrmecophilous lycaenid pupa to suppress ant aggression

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Abstract
Myrmecophilous lycaenid caterpillars have close relationships with their ant hosts by means of various myrmecophilous organs, most of which are usually lost after pupation. However, some lycaenid species, including *Lycaeides argyrognomon*, maintain such relationships at the pupal stage and go so far as to pupate in ant nests. This invokes the hypothesis that these myrmecophilous lycaenid pupae might have alternative tactics to retain myrmecophilous interactions without ant attacks. *Camponotus japonicus, Formica japonica*, and *Lasius japonicus* exhibited distinctive aggressive behaviors against ant cuticular hydrocarbons (CHCs) from different colonies of the same species but few attacks against the crude extract of *L. argyrognomon* pupae. GC–MS analysis revealed that the pupal cuticular lipids contain not only CHCs but also several long-chained aliphatic aldehydes, including 1-octacosanal and 1-triacontanal, which are absent from larval cuticular lipids. With the addition of synthesized 1-octacosanal and 1-triacontanal to ant CHCs from different colonies of the same species, the aggressive behavior decreased in *C. japonicus*, and the duration of physical contact shortened in *C. japonicus* and *F. japonica*. However, the behavior of *L. japonicus* remained unaffected after the addition of those aldehydes. These results suggest that the pupae-specific cuticular aldehydes of *L. argyrognomon* suppress ant aggression even after the loss of certain myrmecophilous organs, though the effects varied depending on the attending ant species. Since *L. argyrognomon* occasionally pupate in the nests of *C. japonicus* in the field, the lycaenids might be better adapted to associations with *C. japonicus* than with the other two ant species studied.

Keywords Ant-lycaenid mutualism · Pupal stage · Facultative myrmecophile · Aldehydes · *Lycaeides argyrognomon*

Introduction

Generally, ants are deadly predators of small insects and aggressively defend their colonies not only from aliens but also from conspecifics from other colonies. This exclusiveness is advantageous for myrmecophilous insects because by acceptance inside the ant colonies or by the establishment of mutual interaction with ants, they can escape from their predators and parasitoids. However, to be successfully adopted by ants, these myrmecophilous insects must avoid lethal ant attacks, as the ants often prey upon their symbionts (Way 1963; Pontin 1978; Sakata 1994, 1995; Offenberg 2001).

Many myrmecophilous insects adopt chemical tactics to establish beneficial interactions with their ant host as ants have a well-developed chemical communication system to maintain their society and rely mostly on chemical signals to recognize their nestmates, foreign ants, and other organisms (Hölldobler and Wilson 1990, 2008). It is well known that various myrmecophiles exhibit cuticular hydrocarbons (CHCs) that mimic those of their ant hosts to avoid ant attacks (e.g., crickets, Akino et al. 1996, 1999; aphids; Salazar et al. 2015; staphylinid beetles; Akino 2002; lycaenid caterpillars; Hojo et al. 2009; spiders; Allan et al. 2002) because the ant CHCs often serve as the key signals

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for nestmate discrimination (d'Ettorre and Lenoir 2010; Van Zweden and d'Ettorre 2010). Although several studies have focused on CHCs, a few myrmecophiles are known to use non-CHC chemicals to control the host ant behaviors (Thomas et al. 2002; Stoeffler et al. 2007).

Three-quarters of lycaenid butterfly immatures associate with ants in interactions ranging from parasitism to mutualism (Pierce et al. 2002). Some have obligate interactions which require the specific host ant species to survive, whereas others have facultative, loose relationships with several host ant species. Many interactions are well studied, including their behavioral and chemical communication, especially in the larval stage (Pierce et al. 2002; Hojo et al. 2014, 2015). Obligate types of caterpillars have CHCs that often mimic that of their ant hosts to be adopted as their nestmates and thereby enter the nests of the host ants (Akino et al. 1999; Hojo et al. 2009). On the other hand, the facultative types of caterpillars do not have CHCs that resemble those of their ant hosts (Omura et al. 2009) and establish a mutualistic relationship with ants outside their nests.

These caterpillars usually have myrmecophilous organs that play important roles in establishing and maintaining the mutual relationships with the ants. Pore cupola organs (PCOs) are single-celled epidermal glands that have been presumed to secrete substances capable of appeasing ants (Malicky 1970; Fiedler et al. 1992; Pierce et al. 2002). Tentacle organs (TOs) secrete volatile substances that attract and alert ants to avoid enemies (Axén et al. 1996; Fiedler et al. 1996; Pierce et al. 2002). The dorsal nectary organ (DNO) secretes nutritious droplets for ants and plays a critical role in the maintenance of associations with ants (Leimar and Axén 1993; Axén et al. 1996; Pierce et al. 2002; Hojo et al. 2014, 2015). These myrmecophilous organs are particular only to the larval stage in almost all myrmecophilous lycaenid species, but PCOs are often retained in the pupal stage. Nevertheless, pupae of some lycaenid species still keep close relationships with their host ant workers. Some lycaenid larvae and pupae are known to communicate with ants acoustically. In obligate association with ants, some lycaenid larvae and pupae attract and maintain host ant workers by acoustic signals (Travassos and Pierce 2000; Barbero et al. 2009a, b). Whereas in facultative association the function of the acoustic signal is unclear although the sound profile appears to relate to the strength of interaction with ants (Riva et al. 2017; Schönrogge et al. 2017).

Lycaeides argyrognomon praeterinsularis has a facultative association with various ant species, including *Camponotus japonicus, Formica japonica, F. yessensis, Pristomyrmex punctatus*, and *Lasius japonicus* (Omura et al. 2009). Similar to other myrmecophilous lycaenids, the caterpillars have PCOs, TOs, and a DNO, but the latter two organs disappear in the pupal stage. Some caterpillars pupate on their host plants, *Indigofera pseudotinctoria* (Fabales, Fabaceae), and others pupate in hollows in the ground which are occasionally ant nests. These caterpillars frequently pupate in *C. japonicus* nests (Watanabe and Hagiwara 2009) but sometimes use *F. yessensis* nests whenever *F. yessensis* is dominant. Pupation in the nest of *F. japonica* has been observed only once (Hagiwara personal observation). Although the lycaenid pupae do not mimic host ant CHCs (Omura et al. 2009) and do not have DNO and TOs, *C. japonicus* workers do not attack them even when present inside their nests (Watanabe and Hagiwara 2009). It is possible that the *L. argyrognomon* pupae have certain alternative tactics not to provoke or to suppress the aggression of their ant hosts.

This study aims to examine the hypothesis that the *L. argyrognomon praeterinsularis* pupae use chemical signaling to prevent the ant hosts from attacking them. We focused on the pupal cuticular lipids because those would be detected by the host ants through the first antennal contact when encountered. Suppression of ant attacks was evaluated to compare behavioral responses to conspecific foreign ant CHCs with and without the addition of the cuticular chemicals unique to the pupae.

Materials and methods

Capturing and rearing study insects

Three mated female butterflies of *L. argyrognomon* were collected from their natural habitat in Yamanashi prefecture in Japan. They were kept individually in net cages (20×20×30 cm) housing their host plant, *I. pseudotinctoria* for oviposition. Following that, all butterflies were released into their original habitat. We collected 10 to 15 eggs from the host plant leaves and collectively incubated them in a plastic petri dish (9 cm diam.) at 20 °C under an L14:D10 photoperiod. Newly hatched larvae were placed individually with host plant shoots in new petri dishes with a sheet of paper (KimWipes Kimberly-Clark Worldwide, Inc) spread on it and were kept at the same temperature and photoperiod. The host plants and the paper were exchanged with new ones and the feces were removed at an interval of 2 or 3 days. When the caterpillars grew up to the third instar, we introduced two *C. japonicus* workers into the dishes. After the caterpillars pupated either on the host plant or on and beneath the paper, the worker ants were removed.

As *L. argyrognomon* larvae are attended by *C. japonicus, F. japonica*, and *L. japonicus* in their natural habitat, for each ant species we collected about 100 foraging workers from three colonies from Kyoto prefecture. The ants were used either for extraction or for bioassays immediately after collection.

Extraction and fractionation of cuticular lipids

As the size of the worker differs between species, we collected three worker ants of *C. japonicus*, seven of *F. japonica*, and 18 of *L. japonicus* from each of the colonies and immersed the workers from the same colony together in 1 mL of *n*-hexane (Wako Pure Chemical Industries, Ltd.) for 2 min. We did this procedure for three colonies of each ant species for a total of nine extracts of ants (three extracts of each ant species). We used three-fourth-instar larvae and nine pupae of *L. argyrognomon*, which had been attended by *C. japonicus* workers, and immersed a single larva or pupa separately in 1 mL of *n*-hexane for 2 min. Before extraction, the lycaenid larvae and pupae, as well as the worker ants, were killed in a deep freezer at -30 °C. The three crude larval extracts and six of the nine crude pupal extracts were used for chemical analysis and the remaining three pupal extracts were used as samples for the bioassay.

For the chemical analyses of the crude larval and pupal extracts, half the quantity of each extract was used, and the remaining half was fractionated into a non-polar and polar fraction using liquid chromatography with ca. 300 mg of silica gel (230–400 mesh, Merck KGaA). It was successively eluted with 1.5 ml each of *n*-hexane and 5% diethyl ether-in-hexane (diethyl ether, Wako Pure Chemical Industries, Ltd.). The crude ant extracts were fractionated into a non-polar fraction with ca. 300 mg of silica gel (230–400 mesh) to separate the hydrocarbon components by elution with 1.5 ml *n*-hexane.

Gas chromatography-mass spectrometry analysis

Gas chromatography–mass spectrometry (GC–MS) analyses were performed on a Shimadzu GC-MS QP5000 equipped with GC-17A and a non-polar capillary column, DB-1HT (J&W, 15 m length, 0.25 mm in diam., 0.10 μm film thickness). Helium was used as the carrier gas and the column head pressure was maintained at 40 kPa. We injected 0.01 equivalent amount of each lycaenid sample (crude extracts, hexane fractions, and 5% diethyl ether-in-hexane fractions of fourth-instar larvae and pupae) into the column at the splitless mode for 1 min at 300 °C. The column oven temperature was held at 60 °C for 1 min, then increased from 60 °C to 300 °C at a rate of 10°C/min, and finally held at 300 °C for 10 min. The GC–MS interface was kept at 300 °C, and the El-mass spectrum was obtained at 70 eV.

Compositional ratios of the major larval and pupal cuticular lipids were estimated from the respective peak areas obtained from the individual samples on the basis of the peak areas in total ion chromatograms (TICs). As noticeable peaks were detected only between the Kováts retention index (Kováts 1958) of 2400 and 3600, we selected those peaks showing a relative abundance of more than 1% of the total abundances of all the peaks in the index range. The respective substances were estimated on the basis of each of their Kováts retention index and mass spectra. To calculate the Kováts retention index, we analyzed a series of *n*-alkanes (icosane, docosane, tetracosane, octacosane, tetratriacontane, and hexatriacontane; Nacalai Tesque Inc., hexacosane, triacontane, and dotriacontane; Sigma-Aldrich, Triacontane; Tokyo Chemical Industry Co., Ltd.) as well as the synthetic compounds described below in the same manner.

Synthesis of specific aldehydes of pupal cuticular lipids

We synthesized 1-octacosanal and 1-triacontanal by the oxidation of 1-octacosanol (Sigma-Aldrich) and 1-triacontanol (Wako Pure Chemical Industries, Ltd.), respectively, using pyridinium chlorochromate (PCC, Wako Pure Chemical Industries, Ltd.). We mixed 40.2 mg PCC with 60 mg silica gel in 3 ml dichloromethane (Wako Pure Chemical Industries, Ltd.), and 10.2 mg 1-octacosanol was dissolved in 1 ml dichloromethane. These two solutions were mixed and stirred in reaction vials for 3 h at 0 °C, and the supernatant liquid was collected. In the same way, 38.0 mg PCC and 10.4 mg 1-triacontanol were mixed and then the supernatant liquid was collected. The supernatant liquids were separately purified on silica gel thin-layer chromatography plates developed with 30% diethyl ester-in-hexane. Single spots with R_f values that were apparently larger than those of the respective alcohols were detected when exposed to iodine vapor. Each spot was scraped off and extracted with 500 μl *n*-hexane for 30 min. The extracted products were confirmed to be the aldehydes, 1-octacosanal or 1-triacontanal, by their mass spectra. We obtained 7.2 mg 1-octacosanal and 2.7 mg 1-triacontanal, and the yields were 70.9% and 26.1%, respectively. The purities of both compounds were estimated to be over 95% through gas chromatography analyses.

Behavioral bioassays

In the two behavioral bioassays described below, we recorded the responses of the three ant species to glass beads (3 mm diam.) treated with the test sample solution. Following this, one of the beads was placed in a glass petri dish (6 cm diam.). An ant worker was then introduced to the dish and its response to the bead was recorded upon its first antennal contact with the bead. Ant responses were categorized as either neutral behavior (ignoring and antennating) or aggressive behavior (mandible-opening, biting, and abdominal-bending). Ants collected from the field were subjected to the bioassays within 3 h following collection. In both the bioassays, the hexane fraction from different colonies of the same ant species was used as positive control. Considering the body sizes of the three ant species, *C.*

japonicus, F. japonica, and *L. japonicus*, 0.05, 0.2 and 0.5 worker equivalent of CHCs were used to treat the beads, respectively.

The first bioassay was designed to investigate if the pupal cuticular lipids stimulate ant aggression. Either the crude lycaenid pupal extract (0.03 pupae equivalent) or the conspecific foreign ant CHCs (positive control) was used as the test sample solution. In this bioassay, all the beads and ants were used just once. From each ant species, 30 ant workers (10 workers from each of the three conspecific colonies) were used, and 15 pupal extract-treated beads and 15 ant CHCs-treated beads were presented to each ant species. The frequencies of the ant aggressive behavior were compared between the treatments by Fisher's exact test.

The second bioassay was designed to evaluate the suppressive effect of the aldehydes which were included in the pupal cuticular lipids (see results). Either the conspecific foreign ant CHCs or the CHC-aldehyde mixture was used as the test sample solution. The synthetic aldehydes, 1-octacosanal and 1-triacontanal, were mixed with the CHCs of each ant at quantities equivalent to those of the respective workers that were used as positive controls. The aldehydes, 1-octacosanal and 1-triacontanal were adjusted to occupy 25% and 35% of the peak areas of total ion chromatograms (TICs) in the CHCs of each ant, respectively, as hydrocarbons, 1-octacosanal, and 1-triacontanal account for about 40%, 25%, and 35% of the peak areas, respectively, in the lycaenid crude pupal extract (see results). In this bioassay, the two kinds of sample, the mixture of aldehydes and CHCs and the positive control were successively presented to a single ant individual at 10 min intervals. Thus, we used each ant twice to record its response to CHC-aldehyde mixture and positive control. The order of presenting the two samples was changed in each test. For each ant species, 45 ant workers (15 workers from each of the three conspecific colonies) were used, and during this bioassay, the duration of physical contact with each bead was also recorded. To evaluate the suppressive effect on ant aggression, we considered the behavioral data of only those ants that displayed aggressive behavior to at least one of the samples for statistical analyses. We used the sign test for statistical analysis of ant behavioral responses and the Wilcoxon signed-rank test for analysis of the duration of physical contact.

Results

Comparison of the larval and pupal cuticular lipids of *L. argyrognomon*

The crude extract of the lycaenid larvae had 13 GC peaks (Fig. 1a). All these peaks, except for peak 15, were identified as hydrocarbons as they were eluted by *n*-hexane from silica gel. Based on their mass spectra and Kováts retention indexes, the compounds of the peaks 1, 2, 3, 4, 6, 8, 9, and 13 were estimated as *n*-alkanes and those of the peaks 7 and 10 were estimated as methyl-branched alkanes (Table 1). The peaks 5, 11, and 15 were too minuscule to estimate the compounds. Peak 15 was present only in the crude extract of larvae.

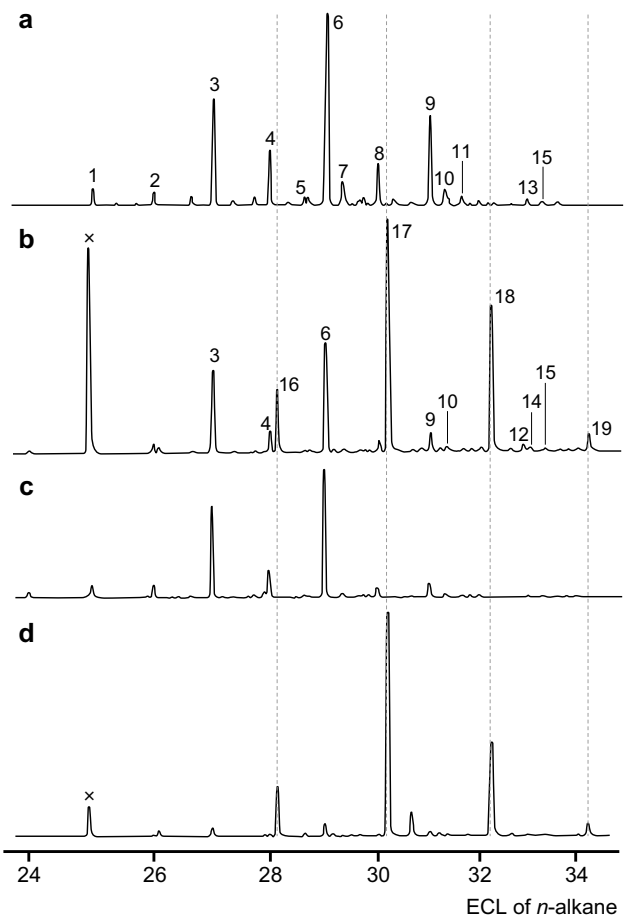

Fig. 1 Total ion chromatograms of the cuticular lipids of a *Lycaeides argyrognomon* larva and pupa. **a** crude hexane extract of one larva. **b** crude hexane extract of one pupa. **c** hexane fraction of pupal cuticular lipids. **d** 5% ether-in-hexane fraction of pupal cuticular lipids. Dotted lines show the positions of the peaks specific to the pupal cuticular lipids. "×" represents dibutylhydroxytoluene impurities which is antioxidant included in diethyl ester

On the other hand, crude extracts of the pupae had 13 GC peaks (Fig. 1b), of which six peaks, 3, 4, 6, 8, 9, and 10, were eluted by *n*-hexane (Fig. 1c). They were also present in the larval hydrocarbons, and their profiles (i.e., relative ratios of the compounds) were quite similar in the larval and pupal hydrocarbons (Fig. 1a, c). Peaks 12 and 14 were too minuscule to infer the chemical identity of the compounds and were detected only in the crude extract of the pupae. The peaks 16, 17, 18, and 19, which were specific to the crude pupal extracts, were eluted with 5%

Table 1 Substances detected in the crude larval and pupal extracts of *L. argyrognomon praeterinsularis*

Peak no.	Kovát's retention index	Tentative identification	Relative peak area of each substance (%)	
			Larva	Pupa
1	2501	*n*-Pentacosane	1.67 ± 0.47	
2	2599	*n*-Hexacosane	1.30 ± 0.32	
3	2701	*n*-Heptacosane	13.67 ± 1.83	9.47 ± 2.71
4	2799	*n*-Octacosane	7.27 ± 0.56	1.71 ± 0.35
5	2865	Trace	1.19 ± 0.09	
6	2902	*n*-Nonacosane	39.76 ± 0.89	15.65 ± 1.60
7	2935	15/13/11-methylnonacosanes	4.50 ± 1.16	
8	2999	*n*-Triacontane	6.88 ± 0.62	1.13 ± 0.15
9	3101	*n*-Hentriacontane	14.70 ± 1.69	3.76 ± 0.44
10	3131	15/13/11-methylhentriacontanes	3.06 ± 0.56	1.19 ± 0.27
11	3165	Trace	3.21 ± 0.85	
12	3287	Trace		1.49 ± 0.22
13	3301	*n*-Tritriacontane	1.57 ± 0.60	
14	3302	Trace		1.43 ± 0.33
15	3330	Trace	1.21 ± 0.19	1.24 ± 0.38
16	2811	1-Hexacosanal		3.43 ± 1.11
17	3016	1-Octacosanal		22.81 ± 3.74
18	3221	1-Triacontanal		31.52 ± 3.41
19	3423	1-Dotriacontanal		5.18 ± 0.83

"Trace" indicates that the substances were too minuscule to be estimated. Relative peak areas are shown as averages and standard errors

diethyl ether-in-hexane when chromatographed on silica gel (Fig. 1d). The fragmentation patterns of those four compounds resembled each other and had diagnostic fragment ions at *m*/*z* 82 and 96 with different Kováts retention indices at about 200 intervals. Hence, they could be a homologous series of compounds. Two peaks, 17 and 18, were identified as 1-octacosanal and 1-triacontanal, respectively, as both their Kováts retention indexes and mass spectra matched those of the respective synthetic compounds, along with their polarity. The peaks, 16 and 19, were tentatively identified as 1-hexacosanal and 1-dotriacontanal based on their Kováts retention indices (Table 1). Hydrocarbons, 1-octacosanal, 1-triactontanal, and a total two other aldehydes of the crude extract of the pupae accounted for 37.06 ± 4.18%, 22.81 ± 3.74%, 31.52 ± 3.41%, and 8.61 ± 0.69% (average ± SE) of the total extracts, respectively.

Ant behavioral responses to the crude pupal extract

All three species of ants often displayed aggressive behavior toward glass beads coated with CHCs from different colonies of the same species. Aggressive behavior was demonstrated by eight of the 15 *C. japonicus* ants (6 mandible-opening, 1 biting, and 1 abdominal-bending), seven of 15 *F. japonica* ants (3 mandible-opening, 3 biting, and 1 abdominal-bending), and 10 of the 15 *L. japonicus* ants (2 mandible-opening, and 8 biting). However, in all three species, the frequencies of aggressive behavior against the crude extract of the lycaenid pupa were significantly lower than those against the positive controls ($p < 0.01$ Fisher's exact test, Table 2). No *C. japonicus* or *F. japonica* ant showed aggressive behavior against the crude pupal extract, while two out of the 15 L. *japonicus* displayed aggressive behavior (1 mandible-opening and 1 biting).

Table 2 Ant behavioral responses to glass beads treated with either crude pupal extracts or conspecific foreign ant CHCs

	C. japonicus			*F. japonica*			*L. japonicus*		
	Aggressive	Neutral	*p*	Aggressive	Neutral	*p*	Aggressive	Neutral	*p*
Foreign ant CHCs	8	7	< 0.01	7	8	< 0.01	10	5	< 0.01
Crude pupal extracts	0	15		0	15		2	13	

Each *p* value is from Fisher's exact test

Suppressive effect of the pupal cuticular aldehydes on ant aggression

Nineteen of the 45 *C. japonicus* ants showed aggressive behavior toward the positive control and/or the CHC-aldehyde mixture. Of the 19 *C. japonicus* workers, 15 showed aggressive behavior (11 mandible-opening and 4 biting) only toward positive controls, whereas two showed aggressive behavior (2 biting) only toward the CHC–aldehyde mixtures. The remaining two showed aggressive behavior toward both samples. The frequency of aggressive behavior toward the CHC-aldehyde mixtures was significantly lower than toward positive controls ($p < 0.01$ by sign test, Fig. 2). Also, the aldehydes significantly shortened the duration of physical contact in *C. japonicus* ($p < 0.01$ by Wilcoxon signed-rank test, Fig. 3).

Sixteen of the 45 *F. japonica* ants showed aggressive behavior toward either or both samples. Of the 16 *F. japonica* workers, 10 showed aggressive behavior (1 mandible-opening and 9 biting) only toward positive controls, and four exhibited aggressive behavior (4 biting) only toward the CHC–aldehyde mixtures. The remaining two exhibited aggressive behavior toward both samples. In *F. japonica*, frequencies of the aggressive behavior did not significantly differ between the two samples ($p = 0.21$ by sign test, Fig. 2). On the other hand, aldehydes significantly shortened the duration of physical contact ($p < 0.01$ by Wilcoxon signed-rank test, Fig. 3).

Thirty-seven of the 45 *L. japonicus* ants showed aggressive behavior toward either or both samples. Of the 37 *L. japonicus* workers, six showed aggressive behavior (4 mandible-opening and 2 biting) only toward positive controls, and five showed aggressive behavior (5 mandible-opening) only toward the CHC-aldehyde mixtures. The remaining 26 showed aggressive behavior toward both samples. In *L. japonicus*, the behavior and the duration of physical contact did not significantly differ between the presentation of positive controls and the CHC-aldehyde mixtures ($p = 0.50$ for the behavior by sign test and $p = 0.83$ for the duration by Wilcoxon signed-rank test, Figs. 2, 3).

Discussion

Our results strongly suggest not CHCs, but aliphatic aldehydes, suppress ant aggression and contribute to maintaining the association with the host ant *C. japonicus* in the pupal stage of *L. argyrognomon*. This is the first study which examined lepidopteran pupal cuticular aldehyde in the context of ant-association. In all three ant species used in our experiments, the aggressive behaviors of the workers were evoked by the glass beads treated with ant CHCs from different colonies of the same species. However, such aggressive behaviors were rarely observed in the case of glass beads treated with the crude pupal extract of *L. argyrognomon*. This suggests that the lycaenid pupae possess certain chemicals capable of effectively suppressing ant aggression. From our GC-MS analyses, it was evident that the pupal extract contained not only hydrocarbons such as *n* -alkanes but also a series of aliphatic aldehydes that were specific to the pupal stage. The two high-content aldehydes, identified as 1-octacosanal and 1-triacontanal, accounted for over half of pupal cuticular lipids. These two aldehydes were confirmed to suppress worker ant aggression effectively upon addition

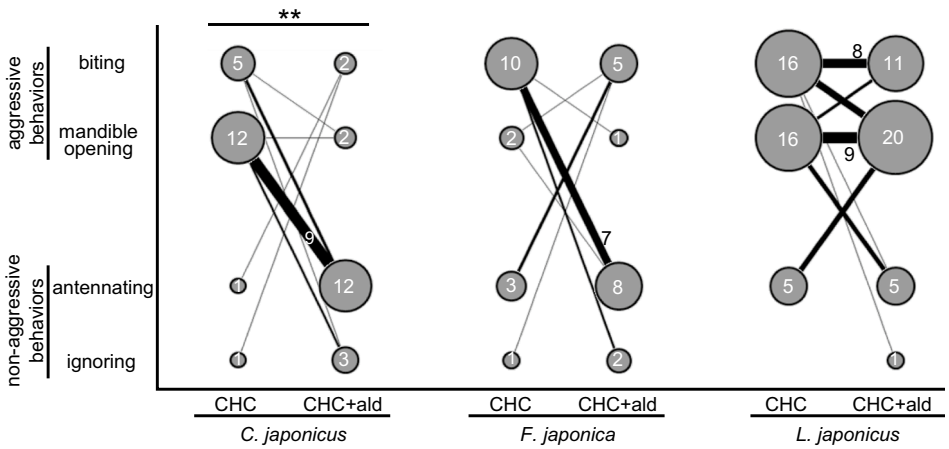

Fig. 2 Behavioral responses of individual ant workers to conspecific foreign ant CHCs and CHC-aldehyde mixtures. The area of each circle and the number inside each circle represent the frequency of each behavior. The lines connecting circles depict combinations of behaviors that an individual ant displayed against the two kinds of beads, positive control (CHC) and CHC-aldehyde mixture (CHC + ald). The width of each line and each number nearby or inside the lines represent the frequencies of each combination. Double asterisk denotes significant difference between ant behaviors to the two kinds of beads ($p < 0.01$ by sign test)

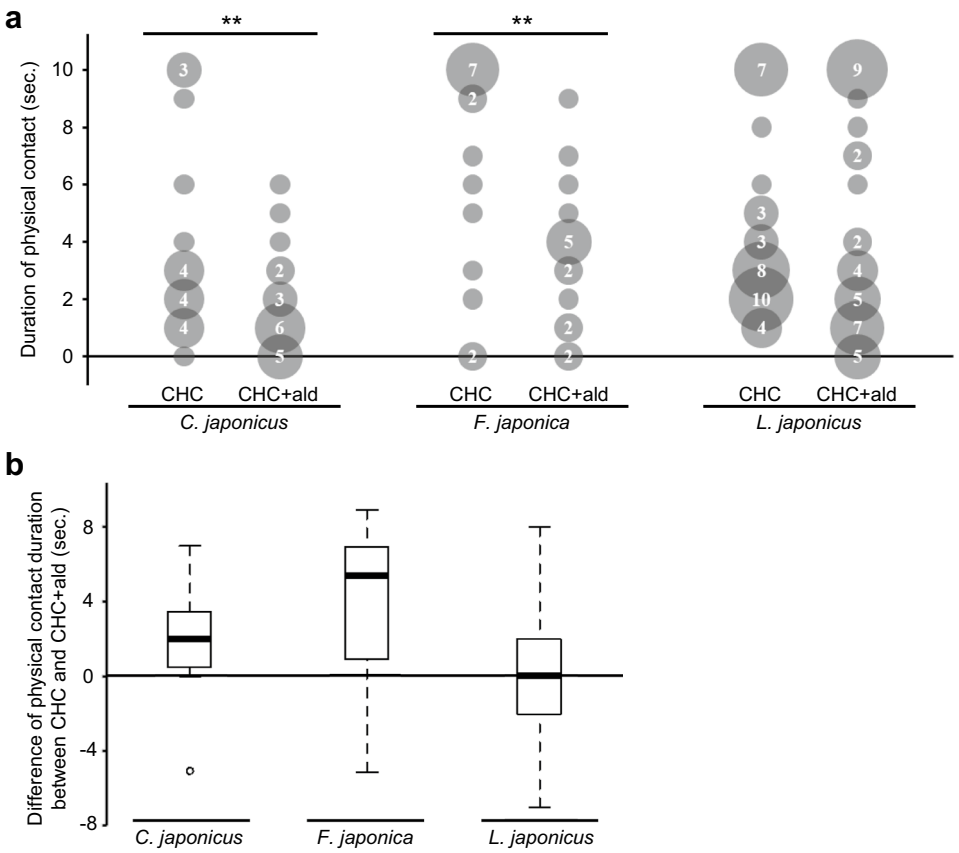

Fig. 3 Duration of physical contact of ants with conspecific foreign ant CHCs and CHC-aldehyde mixtures. **a** Area of each circle and the number inside each circle represent the frequency of each duration of physical contact of the ants with the two kinds of beads, positive control (CHC), and CHC-aldehyde mixture (CHC + ald). Double asterisk denotes significant difference between duration of physical contact with the two kinds of beads ($p < 0.01$ by Wilcoxon signed-rank test). **b** The box plots depict the value obtained by subtracting the physical contact duration with CHC + ald from that of those with CHCs for individual ants

to the conspecific foreign ant CHCs in *C. japonicus*. We also observed that these aldehydes significantly decreased the duration of physical contact of *C. japonicus* and *F. japonica* with the beads, but they hardly affected the behavioral responses of *L. japonicus*. These aliphatic aldehydes, which are peculiar to the pupal stage, could attribute to those chemical tactics employed by *L. argyrognomon* to prevent ants from attacking their pupae. However, the effects are not thoroughgoing but depend on the attending ant species. Alternative explanations are possible: the difference in the concentration of foreign ant CHCs between the positive control and the CHC-aldehyde mixture may affect ant behavior. The concentration of foreign ant CHCs in CHC-aldehyde mixture samples was lower than in positive control as we mixed the aldehydes and the same amount of foreign ant CHCs as a positive control to prepare the samples. However, we assume that the ants we used in the bioassay could detect enough amount of foreign ant CHCs in CHC-aldehyde mixtures to invoke their aggressive behavior, because the absolute amount of foreign ant CHCs (i.e., the amount of CHCs per unit area) in both samples was the same. In many studies, researchers tend to focus on CHCs and choose suitable methods for it, especially in myrmecophiles as CHCs are the nestmate recognition cues for many ant species. This study shows we should focus on polar cuticular lipids as well. It is necessary to carry out bioassays with crude extract and polar fractions not to miss the polar functional substances.

As the predation risk at the pupal stage is quite high for the Lepidoptera (East 1974; Hunter et al. 1997; Grof-Tisza et al. 2015), the choice of pupation site is crucial for their survival. Pupating either inside or nearby an ant nest would be beneficial for lepidopterans by avoiding predators and parasitoids, provided they are not attacked by the worker ants. As Watanabe and Hagiwara (2009) have reported, *L. argyrognomon* occasionally pupate in the nests of *C. japonicus* and never in the nests of *L. japonicus*, while only one case of pupation has been observed in the nest of *F. japonica* (Hagiwara personal observation). This bias in the choice of pupation site could be influenced by the chemical effects of the pupal cuticular aldehydes, which suppress the worker aggression effectively in *C. japonicus* but not so much in *F. japonica* and *L. japonicus*. Additionally, the locations and size of these ant nest entrances may also contribute to the choice. *C. japonicus* is the dominant ant species in the habitat of this lycaenid butterfly in the Yamanashi prefecture. Their nest entrances often open near the base of host plants (Hagiwara personal observation), and the size of the entrances was found to be large enough for the prepupae of the lycaenid. A similar choice of pupation site was also reported in the facultative myrmecophilous lycaenid,

Hemiargus isola (Wagner 1995). It pupates often in the nests of *Formica perpilosa*, which have nest entrances located at the base of their host plants, presumably to avoid risks of predation of the pupae. Several facultative species of lycaenids have been known to enter ant nests to pupate (Wagner 1995). It is presumed that such lycaenid pupae have some chemical tactics to suppress ant aggression, as in the case of the *L. argyrognomon* pupae.

The cuticular lipids of the lycaenid larvae associated with ants have been relatively well investigated compared to those of the lycaenid pupae and most of such studies focus on obligate lycaenid species which have a parasitic association with host ants. Such parasitic lycaenid larvae prey on host ant blood, so they need to mimic their nestmate recognition signal, CHCs, to disguise their identity (Akino et al. 1999; Hojo et al. 2009). Only one study analyzed both pupal and larval CHCs in Lycaenidae. Omura et al. (2009) reported that in *L. argyrognomon* the composition and compositional ratio of pupal CHCs were almost the same as that of the larval CHCs but a few methyl-branched alkanes were lacking in the pupae, which correspond to our result. The predominance of *n*-alkanes in pupae of the lycaenid might contribute to lack of ant aggression as some studies suggest that *n*-alkanes rarely provoke eusocial insect aggression (Dani et al. 2001; Kleeberg et al. 2017). Omura et al. (2009) also reported their CHCs are not similar to CHCs of any species of host ants including *C. japonicus* and *F. japonica*. Unlike parasitic lycaenid larvae, the lycaenid which has a mutualistic association with ant hosts probably do not need to disguise their identity by mimicking ant CHCs, or rather cannot mimic CHCs of a specific ant host to associate with several ant species because ants attack CHCs of other ant species. Omura et al. (2009) did not find pupal aliphatic aldehydes that we found in this study, which might be because of the differences in the storage period of pupae or pupal extracts. If they kept the pupae or the extracts for longer durations even in the freezer the aldehydes might oxidize to carboxylic acids which are not detected by GC-MS with a non-polar column. Although our knowledge on lycaenid pupal cuticular lipids is limited, they can provide important insights into the pupal defensive strategies, especially in those species that maintain associations with ants during their pupal stage.

Some lepidopteran pupae except lycaenid are known to possess polar lipids in their cuticular surface. Pupal cuticular lipids of *Manduca sexta, Heliothis virescens*, and *Helicoverpa zea* include large amounts of aldehydes and other polar lipids (Buckner et al. 1984a, b, 1996) and the major larval cuticular lipids among them are hydrocarbons (Nelson and Buckner 1995; Buckner et al. 1996). Our results of pupal and larval cuticular lipids in *L. argyrognomon* correspond to these previous studies. The differences in cuticular lipids between pupae and larvae we found in this lycaenid might represent an ancestral trait for many Lepidoptera although there are only a limited number of studies available suggesting that it is generally pupae rather than larvae that possess cuticular polar lipids including aldehydes. The function of such aldehydes remains unclear, but it has been suggested that they act as prophylactics for entomopathogenic fungal infections (Ortiz-Urquiza and Keyhani 2013). An aldehyde of the stink bug *Nezara viridula* is suggested to have a fungistatic effect (Sosa-Gomez et al. 1997). Protecting themselves from entomopathogenic fungi is important especially for insects pupating in contact with soil like *L. argyrognomon*.

Host specificity of *L. argyrognomon* is considered to be low, as various ant species have been observed to attend to their larvae (Omura et al. 2009). Our data, however, suggest that *L. argyrognomon* preferably associates with *C. japonicus* rather than with the other two ant species, at least during the pupal stage, because their pupal cuticular aldehydes are capable of influencing *C. japonicus* worker ant behavior. Facultative lycaenid larvae generally benefit from ant-association. In general, the reproductive success and the number of eggs increase with the weight of the female butterfly (Elgar and Pierce 1988; Hill and Pierce 1989; Trager and Daniels 2011). Some studies on facultative lycaenid species show that the larvae-attending ant species influence the body weight of the pupae and adult lycaenids (Fiedler and Hölldobler 1992; Fiedler and Saam 1994; Wagner 1993; Fraser et al. 2001; Kaminski and Rodrigues 2011). For instance, the Reakirt's blue *Hemiargus isola* larvae develop into heavier pupae and adults when they are tended by *Formica perpilosa* (Wagner 1993). The larvae often pupate in the nest of the ant species more than that of the others, and such a choice of pupation site probably increases their survival rate at the pupal stage (Wagner 1995). These previous studies suggest that the fitness of facultative lycaenids can vary depending on the attending ant species. Thus, it would be imperative for the conservation of this endangered species in their habitats to investigate and understand how *L. argyrognomon* are affected by their association with each attending ant species.

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