ORIGINAL ARTICLE

Mutualistic ants and parasitoid communities associated with a facultative myrmecophilous lycaenid, *Arhopala japonica*, and the effects of ant attendance on the avoidance of parasitism

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Abstract

Herbivorous insects have evolved various defensive strategies to avoid their primary enemies, parasitoids. Many species of Lycaenidae (Lepidoptera) have food-for-protection mutualism with ants in their larval stages, where larvae produce nectar for ants and in return ants exclude parasitoids as well as predators. Myrmecophilous relationships are divided into two categories, obligate and facultative, by degrees of myrmecophily. Although parasitoids attacking obligate lycaenids always encounter lycaenid-specific ant species, parasitoids that use facultative lycaenids are likely to encounter diverse ant species showing various defense systems. However, we know little about the parasitoid community of facultative lycaenid larvae. In this study, we investigated the mutualistic ant and parasitoid communities of a facultative myrmecophilous species, *Arhopala japonica*, in seven localities in Japan. The present field observation newly recorded four ant species attending *A. japonica* larvae, and combined with the previous data, the number of attending ant species reached 16, which is nearly the maximum number of reported attending ant species among myrmecophilous lycaenids. However, the present study revealed that almost all parasitized *A. japonica* larvae were attacked by a single braconid species, *Cotesia* sp. near *inducta*. We also assessed the efficiency of facultative ant defense against the parasitoid in the laboratory and revealed that oviposition by *Cotesia* sp. near *inducta* females was almost completely hindered when *A. japonica* larvae were attended by ants. This suggests that the dominant parasitoid does not have effective traits to overcome defensive behavior of ants and that the female wasps oviposit mainly in *A. japonica* larvae without intensive attendance.

Key words: *Charops*, *Cotesia*, Formicinae, geographic variation, Myrmicinae, Theclinae.

INTRODUCTION

Population dynamics and host–plant associations of herbivorous insects are often affected by interactions with natural enemies (Price *et al*. 1980; Faeth 1987; Murphy 2004) as well as with other biotic and abiotic factors (Jeffries & Lawton 1984; Axmacher *et al*. 2009). Parasitoids are one of the influential natural enemies determining a substantial component of the fitness of herbivorous insects (Kato 1994; Teder *et al*. 2000; Murphy 2004). Thus, understanding the

structure of associated parasitoid communities and the defensive mechanism of herbivorous insects is crucial for elucidating the evolution of life history traits in herbivorous insects.

Herbivorous insects have evolved various defensive strategies to avoid their parasitoids by using chemical compounds (Singer *et al*. 2004) and physiological (Carton *et al*. 2008), morphological (Gauld & Bolton 1988) and behavioral characters (Stamp 1982; for a review, Gross 1993; Greeney *et al*. 2012). A unique defensive system in herbivorous insects is the employment of other insect species as bodyguards (Stadler & Dixon 2005). In Lepidoptera (e.g. Pierce *et al*. 2002) and Hemiptera (e.g. Katayama & Suzuki 2002; Amiri-Jami *et al*. 2017), some species have established food-for-protection mutualisms with ants. Attended insects

produce honeydew or nectar for ants, and in return for these rewards, the ants exclude parasitoids and predators of the attended herbivorous insects (Way 1963; Pierce & Mead 1981; Buckley 1987; Pierce *et al.* 1987).

The butterfly family Lycaenidae often show close relationships with ants. Among the species whose life histories have been reported, approximately 75% have relationships with ants in their larval stages (Pierce *et al.* 2002). Lycaenid larvae have evolved unique organs for specialized relationships with ant partners. The larvae secrete nectar from a dorsal nectary organ on the seventh abdominal segment for ants as a reward for protection (Pierce *et al.* 2002). The larvae also secrete volatiles to alert ants from tentacle organs on the eighth abdominal segment and other substances to appease and attract ants from pore cupula organs scattered along the larval bodies (Pierce *et al.* 2002). This means that lycaenid larvae pay a high cost for the maintenance of the protection mutualism (Pierce *et al.* 1987; Fiedler 2006).

For attended insects, relationships with ants are divided into two categories, obligate and facultative, depending on the degree of myrmecophily, and the latter relationship is dominant in Lycaenidae (Pierce *et al.* 2002). In the obligate relationship, lycaenid larvae are attended only by a single species or a single genus of ants, and the larvae never complete their development without ant partners (Pierce *et al.* 2002). In the facultative relationship, lycaenid larvae are associated with various ant species, and they can grow to adults even in the absence of attending ants (Pierce *et al.* 2002). The efficiency of ant defense against parasitoids has been confirmed in several lycaenid species showing both obligate and facultative relationships (Pierce & Mead 1981; Pierce *et al.* 1987; Weeks 2003). However, some parasitoid wasps attacking obligate myrmecophilous lycaenid species have overcome the ant defense of their host larvae. For example, the *Apanteles* (Braconidae) wasp eavesdrops volatiles of partner ants *Iridomyrmex mayri* Forel to detect their host *Jalmenus evagoras* Pfitzner (Elgar *et al.* 2016), and the parasitoid wasp *Ichneumon eumerus* Wesmale (Ichneumonidae) secretes substances that disrupt ant defense of their host *Maculinea rebeli* Hirschke (Thomas *et al.* 2002). These studies imply that obligate myrmecophily is, in turn, likely to lead to the specialization and adaptation of parasitoids.

Although parasitoids associated with lycaenids showing facultative relationships also require overcoming attending ants to achieve successful parasitism, few studies have revealed their parasitization strategies. Parasitoid communities could vary according to ant species if parasitoids are specialized to respective ant species rather than host lycaenid species. In contrast, lycaenid larvae could be parasitized by the same parasitoid species, but in this case, parasitoids are likely to encounter various ant species showing various offense characteristics. However, we know little about the parasitoid communities of lycaenid larvae showing facultative relationships with ants (Pierce & Mead 1981; Weeks 2003; Okubo *et al.* 2009; Kaminski *et al.* 2010).

The lycaenid butterfly *Arhopala japonica* (Murray) (Theclinae), which was also treated as *Narathura japonica*, is a common species in Japan exhibiting a facultative relationship with ants (Fukuda *et al.* 1984; Hojo *et al.* 2014). *Arhopala japonica* is distributed widely from the subtropical Ryukyu Islands to the temperate Honshu Island in Japan and has two or three generations per year (Fukuda *et al.* 1984). The larvae use at least 15 *Quercus* species, but they mainly feed on *Q. glauca* Thunb. at lowland (Fukuda *et al.* 1984). According to Fukuda *et al.* (1984) and Hojo *et al.* (2014), larvae of *A. japonica* are attended by at least 11 ant species, although these surveys were undertaken in only two localities in Japan. Hojo *et al.* (2015) revealed that the secretions from *A. japonica* larvae reduce the dopamine level and locomotive activity of an attendant ant, *Pristomyrmex punctatus* Mayr, which makes the attending ants hardly leave the host larvae. Therefore, *A. japonica* larvae secrete nectar not only for rewards but also for manipulating the behavior of their ant partners (Hojo *et al.* 2015). This approach strengthens the possibilities for *A. japonica* larvae to attract various species of ants, establishing food-for-protection mutualism, and thus the possibilities for larval parasitoids to encounter various ants. To date, the parasitoid community of *A. japonica* has been reported only briefly in an illustrated book of the larval life history of Japanese butterflies (Fukuda *et al.* 1984), and there is no detailed information about the community structure, parasitism rates, seasonality or geographic variations.

In this study, we investigated the attending ant species and the parasitoid community of *A. japonica* larvae and assessed the efficiency of ant attendance for parasitoid avoidance. First, we surveyed the attending ant species by field observation in seven different localities in Japan. Second, we collected *A. japonica* larvae from the seven localities and reared them in the laboratory to confirm how many parasitoid species use the facultative myrmecophilous *A. japonica* larvae as their host. In particular, we focused on the phenological dynamics of *A. japonica* larvae and their associated parasitoids in two of the seven localities. As these field surveys revealed that a single species of parasitoid wasp is dominant on *A. japonica* larvae, we further tested the efficiency of the defense by attending ants against oviposition by the parasitoid.

MATERIALS AND METHODS

Observation of attending ant species

To assess geographic variations of ant species attending *A. japonica* larvae, field investigations were carried out in seven localities ranging from the Ryukyu Archipelago to Honshu Island in Japan as follows: Amami-Oshima Island (28°28–32′N, 129°30–49′E), Kagoshima City (31°55–60′N, 130°49–55′E), Saga City (in Kono Park [33°15′N, 130°16′E] and Shinrin Park [33°14′N, 130°14′E]), Niimi City (34°99′–35°00′N, 133°43′E), Kyoto City (mainly in the Kyoto Botanical Garden [35°04′N, 135°76′E] and additionally in several other sites [35°02–08′N, 135°74–80′E]), Kiso Town (35°73′N, 137°71′E), and Sendai City (38°26′N, 140°83–85′E) (Fig. 1A).

The surveys were undertaken in mid-May in Amami-Oshima Island, in late August in Kagoshima City, in early June, late July and late August in Niimi City, in late July in Kiso Town, and in mid-July and late August in Sendai City, in 2019. In the other two localities (Saga and Kyoto), a periodical route census was undertaken from early summer to autumn, the larval season of *A. japonica*. In Saga, ten and 20 *Q. glauca* trees were selected for monitoring in 2016 and 2017, respectively, and ten shoots were selected as observation shoots from each census tree. Thus, our observations in Saga were based in total on 100 and 200 shoots in 2016 and 2017, respectively. Then the numbers of *A. japonica* larvae and attending ants were recorded weekly for each shoot from early June to early October. In Kyoto, the field census was undertaken weekly in the Kyoto Botanical Garden from early May to mid-October 2019 and in several other sites in Kyoto City irregularly from early May to mid-October 2019.

In the investigations, census shoots were located below 1.6 m, which allowed us to inspect all leaves on the shoots. Identification of ant species followed that of Imai *et al.* (2003). However, as the distinction between *Crematogaster matsumurai* Frel and *Cr. teranishii* Santschi in the field without stereoscopic microscopes was sometimes ambiguous, we recorded the total number of individuals for the two species.

Parasitoid communities and their phenological dynamics

To understand the structure of the parasitoid communities, *A. japonica* larvae were collected from the abovementioned seven localities at the same time as the attending ant observations were undertaken. However,
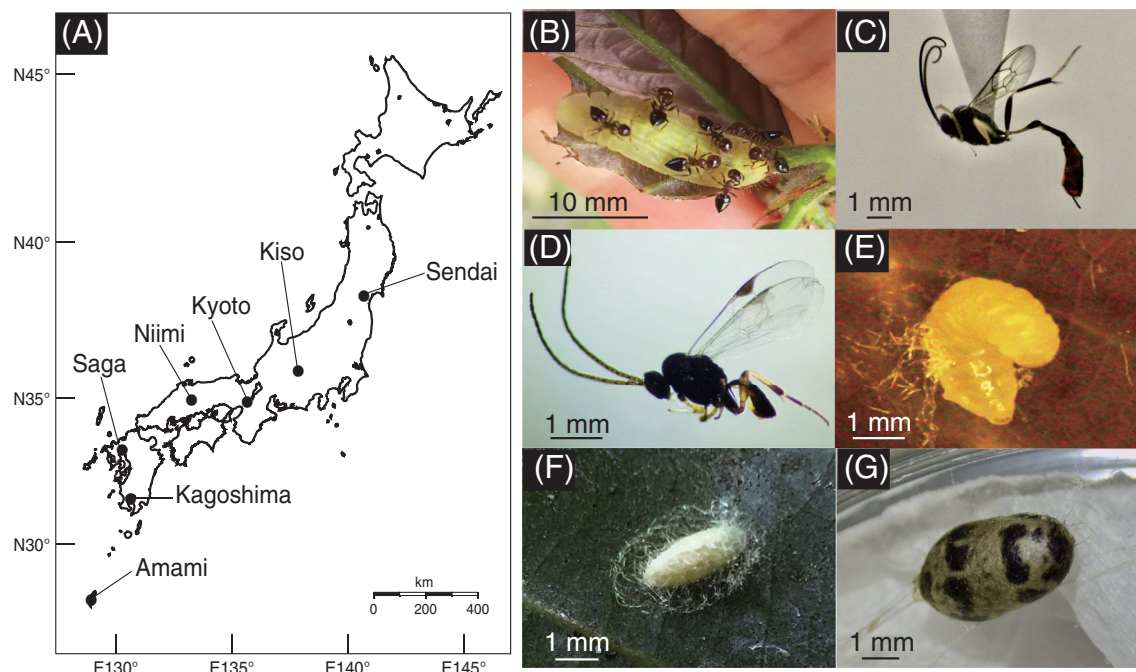

Figure 1 Sampling localities, larvae of *Arhopala japonica* and its parasitoid wasps *Cotesia* sp. near *inducta* and *Charops striata*. (A) Map of sampling localities in Japan. (B) *A. japonica* larva attended by *Crematogaster* sp. (C) Adult of *Ch. striata*. (D) Adult of *Cotesia* sp. near *inducta*. (E) Final instar of *Cotesia* sp. near *inducta*. (F) Cocoon of *Cotesia* sp. near *inducta*. (G) Cocoon of *Ch. striata*.

in Saga, we collected larvae only in 2016 at the same sites from trees that were not used for the ant observation. The samplings were undertaken two or three times per week from early June to early October 2016.

Collected larvae of *A. japonica* were maintained in the laboratory individually in a Petri dish (50 mm diameter and 11 mm high) with wet paper at $25 \pm 1°C$ in light : dark 14:10 or 16:8 photocycles at Saga University for the Saga samples and at Kyoto Prefectural University for the samples from the six other localities, and fresh leaves of *Q. glauca* were supplied daily as food for them.

Some emerged parasitoids were mounted for morphological observation, and the right hind leg was removed from each mounted specimen before drying for DNA extractions. The removed hind legs, other unmounted emerged adults and egressed parasitoid larvae were preserved in 99.5% ethanol and stored at −80°C until DNA extraction.

Total DNA was extracted from the right hind leg of an adult or the whole body of a parasitoid larva (Table S1) using a DNeasy Tissue Kit (Qiagen, Tokyo, Japan) according to the manufacturer's protocol. A primer set LCO1490 + HCO2198 (Folmer *et al.* 1994) was used to amplify the 658 bp fragment of the DNA barcoding region of mitochondrial cytochrome oxidase subunit I (COI). The reaction cycle was 94°C for 3 min followed by 40 cycles of 94°C for 30 s, 50 or 55°C for 30 s and 72°C for 45 s, followed by 72°C for 5 min. Amplified polymerase chain reaction (PCR) products were purified using EconoSpin columns (BIO-BIK, Osaka, Japan) and then subjected to a single-stranded PCR for sequencing. All single-stranded PCRs and sequencing were carried out in the Genewiz Sequencing Center (Saitama, Japan), following the manufacturer's protocol. The obtained sequences were aligned using Mesquite (Maddison & Maddison 2018). We used the "identification tool" in BOLD software (version 3.0; Ratnasingham & Hebert 2007) to search for similar sequences in the database.

In Saga and Kyoto, the phenological dynamics of parasitoids were also surveyed. In Saga, the phenological census was carried out at the same time as investigating the parasitoid community. In Kyoto, field observations were undertaken in the Kyoto Botanical Garden once a week from early May to mid-October in 2019. Larvae were collected after recording attending ant species at the time of the abovementioned route censuses.

Life history of parasitoids

For all *A. japonica* larvae surveyed regarding the parasitoid community in Saga and Kyoto, we recorded the instars when they were collected and when parasitoid larvae had egressed from their bodies. Egressed parasitoid larvae were maintained until adult emergence under the same conditions as those of the *A. japonica* larvae, and the duration (days) required for the emergence as well as their sex was recorded.

Effects of ant attendance on parasitoid avoidance

To test the efficiency of ant attendance for parasitoid avoidance, oviposition trials with or without ant attendance were undertaken in the laboratory. Eggs of *A. japonica* were collected from *Q. glauca* shoots in the Kyoto Botanical Garden to obtain unparasitized host larvae, and hatched larvae were maintained individually until eclosion to the third instar as mentioned earlier. *Cotesia* sp. near *inducta* (Hymenoptera: Braconidae) was used as a parasitoid species for the oviposition experiment because *A. japonica* larvae were mainly parasitized by this braconid in Kagoshima, Saga and Kyoto (see Results). The wasps were obtained by rearing *A. japonica* larvae collected in Kyoto Botanical Garden. Emerged adult wasps were maintained individually in a centrifuge tube (118 mm length, 28 mm diameter) covered with mesh, and a 10% honey solution was provided as food. *Lasius japonicus* Santschi (Hymenoptera: Formicidae) was used as the experimental attending ant species, because this species was frequently observed with *A. japonica* larvae in the mainland of Japan throughout our field surveys. Workers of *L. japonicus* were collected from two colonies on the Kyoto Prefectural University campus (35°04′N, 135°76′E) before experiments or from two laboratory-reared colonies originating from Matsugasaki (35°05′N, 135°77′E), Kyoto City. A 1-day-old third instar of *A. japonica* was introduced in a Petri dish (90 mm diameter and 30 mm high) with two *L. japonicus* workers because third instars of *A. japonica* are attended in the field by two ant workers on average (Y. Nakabayashi, pers. obs., 2019). Thirty minutes after the beginning of ant attendance, a female of *Cotesia* sp. near *inducta* was released in the Petri dish. After the wasp release, we observed the attended larva to determine whether the wasp oviposited successfully for 30 min. As a control, a female wasp was released into a Petri dish containing only a third instar of *A. japonica*.

Statistical analyses

The sex ratio of the emerged adult parasitoids was analyzed with the exact binomial test. The percentage of ant attendance, parasitism rates and successful oviposition rates by *Cotesia* sp. near *inducta* in the laboratory

experiments were analyzed with Fisher's exact test. The parasitism rates by *Cotesia* sp. near *inducta* in the field for each *A. japonica* instar were analyzed with Fisher's exact test and the *P*-value was adjusted by the Benjamini–Hochberg (BH) method with a false discovery rate of 0.05 (Benjamini & Hochberg 1995). All statistical tests were carried out using the R package version 3.3.1 (R Core Team, 2016).

RESULTS

Observation of attending ant species

Ant species attending *A. japonica* larvae in the field are summarized in Table 1. In total, 12 species belonging to two subfamilies (Myrmicinae and Formicinae) were recorded in the field observations. The most widely observed species were *Pr. puncutatus*, *Cr. matsumurai*, *Cr. teranishii* and *L. japonicus*, which were recorded in four localities. Seven species were observed in only one or two localities. The present study was the first to record *Pheidole noda* Smith, *Tetramorium bicarinatum* Bolton, *Formica japonica* Forell and *Polyrhachis moesta* (Emery) as attending ant species of *A. japonica* larvae.

The number and percentage of *A. japonica* larvae attended by each ant species in the respective localities are shown in Table 1. In Saga, 64 (37.9%) of 169 observed *A. japonica* larvae were attended by ants, and the majority (87.5%, *N* = 56) of them were attended by *Cr. matsumurai* and *Cr. teranishii*. In Kyoto, 118 (67.8%) of 174 observed *A. japonica* larvae were attended by ants. Among them, the most frequently observed ant species was *Pr. punctatus* (29.7%, *N* = 35). Although the frequencies of field

observations were almost identical between Saga and Kyoto, the percentage of ant attendance in Kyoto was significantly higher than that in Saga (Fisher's exact test, *P* < 0.001). In Kagoshima, Niimi, Kiso and Sendai, more than 50% of the larvae were attended by any ant species, but only one larva was attended by ants among 16 *A. japonica* larvae in Amami.

Parasitoid communities and their phenological dynamics

The number and percentage of *A. japonica* larvae parasitized by each parasitoid species in the respective localities are shown in Table 2. Parasitoid adults emerged from the host larvae that were collected from three localities (Kagoshima, Saga and Kyoto), and only two species of solitary koinobionts were confirmed in the survey. *Charops striata* Uchida (Hymenoptera: Ichneumonidae) (Fig. 1C), which was identified by morphological observation and sequencing of the DNA barcoding region (Table S1), was only obtained from Kyoto, and its parasitism rate was very low. In contrast, the other parasitoid species emerged from more than 50% of the collected host larvae in Kyoto. In Kagoshima and Saga, this braconid wasp was only the parasitoid that emerged from *A. japonica* larvae. This species was identified as a member of the genus *Cotesia* by morphological observation (Fig. 1D). Although the morphological characteristics of the *Cotesia* species were similar to those of *C. inducta* Papp, there were distinct morphological differences between them (J.L. Fernández-Triana, pers. comm., 2019). The sequence data of all adult and larval samples were identical

Table 1 List of attendant ant species and number (percentage) of *Arhopala japonica* larvae attended by each ant species

Ant species	Locality						
	Amami	Kagoshima	Saga	Niimi	Kyoto	Kiso	Sendai
Myrmicinae							
Pheidole noda	0 (0.0)	6 (11.1)	1 (0.6)	0 (0.0)	13 (7.5)	0 (0.0)	0 (0.0)
Temnothorax congruus	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (0.6)	0 (0.0)	0 (0.0)
Tetramorium tsushimae	0 (0.0)	0 (0.0)	2 (1.2)	0 (0.0)	2 (1.1)	0 (0.0)	0 (0.0)
Tetramorium bicarinatum	0 (0.0)	3 (5.56)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Pristomyrmex punctatus	0 (0.0)	12 (22.2)	1 (0.6)	4 (26.7)	35 (20.1)	0 (0.0)	0 (0.0)
Crematogaster osakensis	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	19 (10.9)	0 (0.0)	2 (33.3)
Cr. matsumurai and *teranishii*	0 (0.0)	12 (22.2)	56 (33.1)	6 (40)	22 (12.6)	0 (0.0)	0 (0.0)
Formicinae							
Lasius japonicus	0 (0.0)	0 (0.0)	4 (2.4)	0 (0.0)	10 (5.7)	1 (50)	1 (16.7)
Formica japonica	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (0.6)	0 (0.0)	0 (0.0)
Camponotus japonicus	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	4 (2.3)	0 (0.0)	0 (0.0)
Camponotus vitiosus	0 (0.0)	4 (7.4)	0 (0.0)	0 (0.0)	9 (5.2)	0 (0.0)	0 (0.0)
Polyrhachis moesta	1 (6.7)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Without attendance	14 (93.3)	13 (24.1)	105 (62.1)	5 (33.3)	56 (32.2)	1 (50)	3 (50)
Total number of larvae	15	54	169	15	174	2	6

Table 2 Number (percentage) of *Arhopala japonica* larvae parasitized by each parasitoid species

	Locality						
Parasitoid species	Amami	Kagoshima	Saga	Niimi	Kyoto	Kiso	Sendai
Cotesia sp. near *inducta*	0 (0.0)	1 (1.9)	44 (21.6)	0 (0.0)	116 (56.6)	0 (0.0)	0 (0.0)
Charops striata	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	4 (2.0)	0 (0.0)	0 (0.0)
Not parasitized[†]	15 (100)	53 (98.1)	160 (78.4)	15 (100)	85 (41.5)	2 (100)	6 (100)
Total number of larvae	15	54	204	15	205	2	6

[†]Number of *A. japonica* larvae pupated.

(Table S1), indicating that all sequenced samples belong to the same species. A BOLD search using the obtained sequences also detected *C. inducta* as the most closely related species, and their sequence divergence was 3.2%, suggesting that the *Cotesia* species obtained from *A. japonica* is different from *C. inducta*. Thus, we treated the species as *Cotesia* sp. near *inducta* in the present study. Although we obtained almost identical numbers of *A. japonica* larvae in Saga and Kyoto (Table 1), the parasitism rates were significantly higher in Kyoto than in Saga (Fisher's exact test, $P < 0.001$).

The phenological dynamics of *A. japonica* larvae and *Cotesia* sp. near *inducta* are shown in Fig. 2. In Saga, *A. japonica* larvae were continuously observed from early June to early October, but the number of individuals decreased to nearly zero in August (Fig. 2A). The parasitism rate by *Cotesia* sp. near *inducta* decreased to zero in August, and that in early June and late September was also almost zero, although a substantial number of host larvae were collected. In Kyoto, *A. japonica* larvae were observed from late May, but they were not found from early June to mid-June (Fig. 2B). After late June, *A. japonica* larvae were continuously observed until mid-October (Fig. 2B). The parasitism rate by *Cotesia* sp. near *inducta* decreased to zero in early to mid-June, early July, mid-August and mid-September, but each peak of the parasitism rate was almost coincident or slightly delayed with the peak of the number of observed host larvae except for the peak at 13 August (Fig. 2B).

Life history of parasitoids

Among the 44 observed *Cotesia* sp. near *inducta* larvae in Saga, 40 (90.9%) egressed from the fourth instar of *A. japonica* and the remaining four (9.1%) from the fifth instar. Among 116 observed parasitoid larvae in Kyoto, one (0.9%) egressed from the third instar, 108 (93.1%) from the fourth instar, and seven (6.0%) from the fifth instar. The egressed larvae of the parasitoid immediately spun cocoons near the host larvae (Fig. 1E, F).

In Saga, no *Cotesia* sp. near *inducta* larvae egressed from the host larvae that were collected at the first instar (Fig. 3A). The rates of egression drastically increased in the host larvae that were collected at the second instar and almost constant in the larvae that were collected at the third and fourth instars. The egression rate decreased to zero in the fifth instar, although there were no significant differences (Table S2A, Fisher's exact test with BH method, P > corrected P-value with a false discovery rate of 0.05) among the host instars collected. In Kyoto, the larvae of *Cotesia* sp. near *inducta* egressed from the host larvae that were collected at the first instar (Fig. 3B). The egression rates were almost constant to those of the host larvae that were collected at the fourth instar and rapidly decreased to zero in the fifth instar. There were significant differences in the egression rates between the fifth and the other instars (Table S2B, Fisher's exact test with BH method, P < corrected P-value with a false discovery rate of 0.05).

Adults of *Cotesia* sp. near *inducta* emerged on average 5.22 ± 0.90 (SD) days ($N = 23$) after egression. Of the 23 emerged adults in Saga, 17 were females and six were males. There was a significant deviation from the 1:1 ratio to a female-biased sex ratio (exact binomial test, $P = 0.035$). Of the 68 adults in Kyoto, 55 were females and 13 were males, and there was also a significant deviation from the 1:1 ratio (exact binomial test, $P < 0.001$).

In the case of *Ch. striata*, two observed larvae egressed from the fourth instar of the host larvae and the remaining two from the fifth instar. The egressed larvae of *Ch. striata* immediately spun a cocoon, and the cocoon was hung on to a thread (Fig. 1G). Of the four *Ch. striata* larvae, one egressed from the host that was collected at the second instar, and the other three egressed from the hosts that were collected at the fourth instar. Two *Ch. striata* adults emerged; one was female, and the other was male (Fig. 1C).

Effects of ant attendance on parasitoid avoidance

When *A. japonica* larvae were attended by ants, only one female (12.5%, $N = 8$) of *Cotesia* sp. near *inducta*
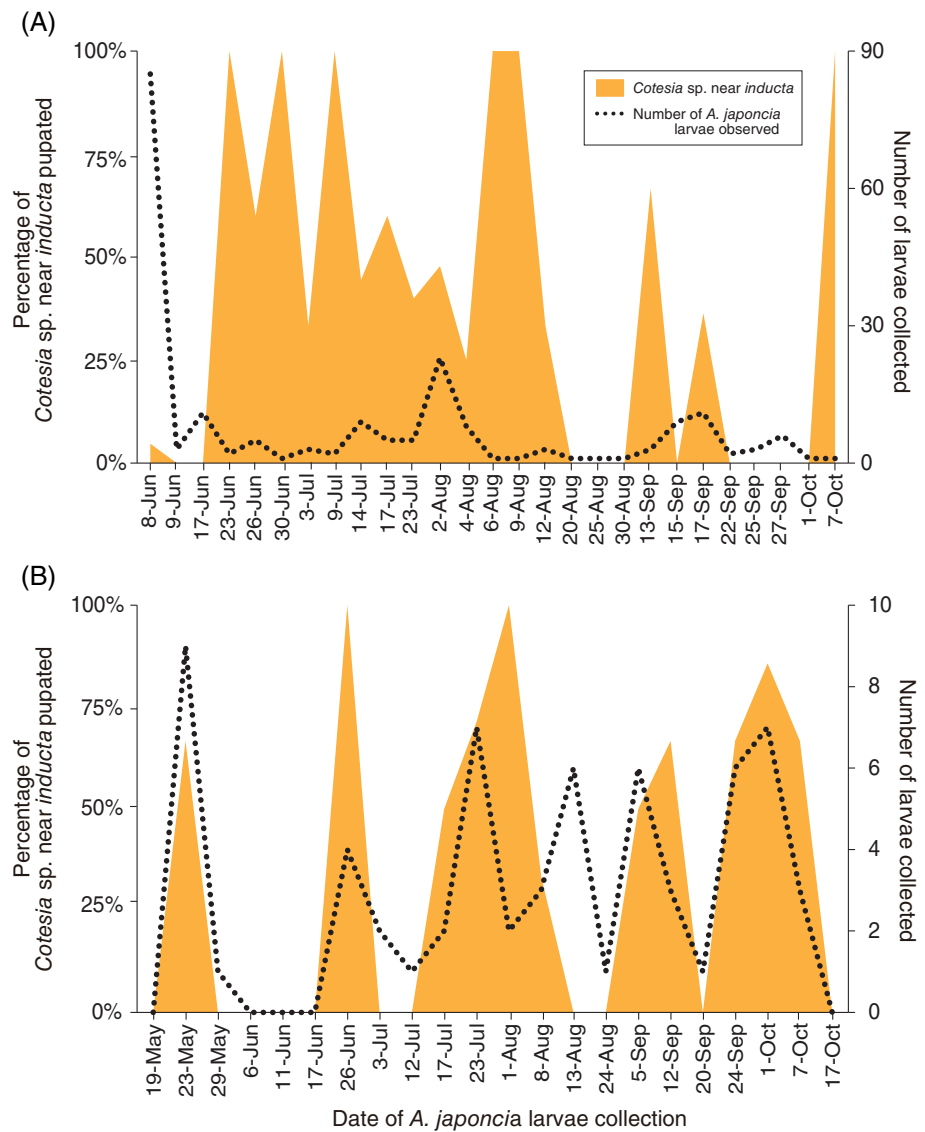
Figure 2 Phenological dynamics of *Cotesia* sp. near *inducta*, in (A) Saga (in 2016) and (B) Kyoto (in 2019). The left axis shows percentages of pupated *Cotesia* sp. near *inducta*, and the right axis shows the numbers of *Arhopala japonica* larvae collected on each sampling day (dashed line).
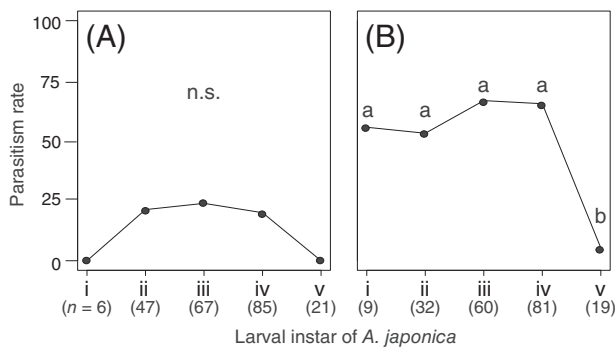

Figure 3 Parasitism rates by *Cotesia* sp. near *inducta* in the wild for each *Arhopala japonica* instar in (A) Saga and (B) Kyoto. Numbers in parentheses show the collected number of *A. japonica* larvae for each instar. Different letters on each plot indicate significant differences in parasitism rates among larval instars. n.s., not significant.

could attack the *A. japonica* larvae, but the other females could not oviposit in the host larvae due to the defense by *L. japonicus* workers. When the host larvae were not attended by ants, all tested wasps succeeded with oviposition into the host larvae ($N = 8$). There was a significant difference in the successful oviposition rates between the attended and unattended larvae (Fisher's exact test, $P = 0.0014$). When the ant workers detected *Cotesia* sp. near *inducta*, they approached the attending host larva and immediately attacked the parasitoid wasp. In this case, the oviposition of the wasp was hindered every time, even though some wasps repeatedly tried to oviposit into the attended larvae. Other wasps gave up on ovipositing and left the attended larvae alone until the end of the trials.

DISCUSSION

The number of ant species attending each facultative myrmecophilous lycaenid is generally approximately five (Peterson 1993; Weeks 2003; Okubo *et al.* 2009) and rarely more than ten (Kaminski *et al.* 2012). In the present study, we newly recorded four ant species attending *A. japonica* larvae and, combined with data shown by Fukuda *et al.* (1984) and Hojo *et al.* (2014), the number of attending ant species reached 16. Therefore, *A. japonica* was revealed to have associations with nearly the maximum number of attending ant species among myrmecophilous lycaenids (Kaminski *et al.* 2012), and, indeed, the attending ant species of *A. japonica* varied among larval individuals. However, the present study revealed that almost all parasitized *A. japonica* larvae were attacked by a single species of braconid wasp, *Cotesia* sp. near *inducta*. These results indicate that *Cotesia* sp. near *inducta* is likely to encounter various ant species when the wasps try to oviposit in *A. japonica* larvae.

The genus *Cotesia* is a large group of Microgasterinae (Hymenoptera: Braconidae) that mainly parasitizes lepidopteran larvae, and the estimated number of species is 1,500–2,000 worldwide (Mason 1981; Shaw & Huddleston 1991). *Cotesia inducta* is a koinobiont larval parasitoid and was first described as *Apanteles inductus* from Hungary (Papp 2003). Since then, this species has been recorded widely in Europe (Slovakia, Bulgaria, British Isles and Spain) (Shaw 2007; Obregón *et al.* 2015) and Asia (Turkey and Korea) (Papp 2003). This species attacks several lycaenid species, and four and three species belonging to Polyommatini and Theclini, respectively, have been recorded as hosts so far (Shaw 2007; Obregón *et al.* 2015). This fact implies that *Cotesia* sp. near *inducta* also parasitizes other lycaenid species. Although Fukuda *et al.* (1984) reported another braconid species, *Apanteles* sp., as a parasitoid of *A. japonica*, the authors provided neither literature nor specimen information on the parasitoid records. Therefore, we could not examine the specimens determined as *Apanteles* sp. However, as the genus *Cotesia* was treated as *Apanteles* in previous works (Mason 1981), the *Apanteles* sp. reported in Fukuda *et al.* (1984) was likely to be the same species as *Cotesia* sp. near *inducta*.

Four *A. japonica* larvae collected from Kyoto were parasitized by the ichneumonid wasp *Ch. striata*, and this is the first record of *Ch. striata* parasitizing butterfly species. *Charops striata* has been recorded as a larval parasitoid of zygaenid moths, *Artona martini* Efetov in Japan (Momoi 1969) and *Elcysma westwoodii* Vollenhoven in South Korea (Cho *et al.*

2010). These studies, coupled with the present results, imply that *Ch. striata* has a wide host range and that *Ch. striata* is not a specialist of *A. japonica*. Therefore, the parasitism rate of *Ch. striata* in *A. japonica* larvae could be much lower than that of *Cotesia* sp. near *inducta*. However, there is a possibility that females of *Ch. striata* oviposit in the same instar of host larvae as *Cotesia* sp. near *inducta*, suggesting that multiparasitism by the two parasitoid species could occur in *A. japonica* larvae. Braconid larvae often possess massive fighting mandibles that are used for physical attack against coexisting parasitoids (Mayhew & van Alphen 1999; Vinson & Mourad 2000; Shi *et al.* 2004; Chau & Maeto 2008; Aoyama & Ohshima 2019). This observation implies that *Ch. striata* larvae in superparasitized *A. japonica* larvae are likely to be killed by the larvae of *Cotesia* sp. near *inducta*, resulting in the decreased parasitism rate of *Ch. striata* in such a coexisting population.

According to Fukuda *et al.* (1984), *A. japonica* larvae were also parasitized by *Paradrino laevicula* Mesnil (Diptera: Tachinidae). *Paradrino laevicula* is distributed in the Oriental and Australian regions, and they use a broad range of lepidopteran species as hosts, for example, *Amyna punctum* Fabricius (Noctuidae) and *Ostrinia furnacalis* (Guenée) (Pyralidae) in Japan and *Euploea core corinna* MacLeay (Nymphalidae) in Australia (Shima 1984; McAuslane & Bennett 1995). As *Pa. laevicula* has a very wide host range, this species is probably not a specialist of *A. japonica* larvae, and thus, it might not be found in the present study.

In parasitoids of myrmecophilous butterflies, female wasps are often reported to oviposit mainly into the early instars of their hosts (e.g. Pierce *et al.* 1987). This observation suggests that there could be advantages for choosing early instars as ovipositing targets. The reason could be that earlier instars are more suitable hosts for koinobiont parasitoids because later instar hosts have stronger physiological and behavioral defenses (Gunasena *et al.* 1989; King 1989; Mackauer 1996; Gols & Harvey 2009). Therefore, future experiments comparing the oviposition preference of female wasps between early and later instar hosts as well as those assessing the strength of physiological and behavioral defenses in different host instars will contribute to uncovering the factors promoting oviposition in early instars. Otherwise, early instar hosts are less attractive for ants and thus might have lower ant defenses (Pierce *et al.* 1987). This means that parasitoids might be expected to encounter attending ants at lower probabilities when they try to oviposit in earlier instars. Furthermore, the establishment of ant attendance in late instars is likely to provide defense not only for the

hosts but also for parasitizing koinobionts in the host body because host survival is essential for the survival of koinobiont parasitoids, and the ant defense could be effective for excluding their hyperparasitoids and competitors. In fact, parasitized *A. japonica* larvae are as continuously attended by ants as unparasitized larvae (Y. Nakabayashi, pers. obs., 2019); thus, the ant attendance of *A. japonica* probably also protects their parasitoid larvae.

Although *Cotesia* sp. near *inducta* predominantly parasitized *A. japonica* larvae in our census localities, oviposition by *Cotesia* sp. near *inducta* females was almost completely hindered when *A. japonica* larvae were attended by *L. japonicus* in the laboratory experiment. These results suggest that *Cotesia* sp. near *inducta* does not bear any effective traits to overcome the defensive behavior of attending ants and that parasitoid females oviposit mainly in *A. japonica* larvae without intensive ant attendance. Several studies have also shown that the attendance of *L. japonicus* is effective for the exclusion of natural enemies in other insect species (Kaneko 2002; Katayama & Suzuki 2003; Takizawa & Yasuda 2006). Thus, our present results strengthen the possibility that *L. japonicus* is a universally efficient partner for various myrmecophilous insects. Other major attending ants, *Cr. matsumurai*, *Cr. teranishii* and *Pr. punctatus*, are also aggressive ant species and efficiently exclude herbivores and parasitoids (e.g. Kaneko 2003; Yamawo *et al*. 2014, 2017). Therefore, these dominant attending ants can also be effective partners for *A. japonica* larvae. The efficiency of facultative ant defense is quite different among lycaenid species (e.g. Pierce & Mead 1981; Pierce & Easteal 1986; Peterson 1993; Savignano 1994; Weeks 2003), and the strength of defense is also different among ant species (Fraser *et al*. 2001; Kaneko 2003, 2007). However, the ant attendance of *A. japonica* larvae appears to be very effective for avoiding their dominant parasitoid wasps.

Attending ant communities of *A. japonica* larvae were partially different among the seven census localities, but no clear relationships were observed between localities and attending ant species. Comparative approaches also detected no relationships between the lycaenid phylogeny and attending ant taxa (Ward *et al*. 2015, 2016). Myrmicine species are divided into six tribes (Ward *et al*. 2015), and among seven species of Myrmicine found with *A. japonica* larvae in our study (Table 1), only *Ph. noda* belongs to Attini, and the other six belong to Crematogastrini. Formicine species are also divided into six tribes (Ward *et al*. 2016), and *L. japonicus* is a member of Lassini, *F. japonica* Motschoulsky belongs to Formicini and two

Camponotus species and *Po. moesta* are Camponotini (Table 1). Therefore, *A. japonica* larvae are attended not by specific ant species but by various ant taxa inhabiting host plants of *A. japonica*. This implies that host plants of *A. japonica* influence the establishment of ant defense because association with host plants where partner ants frequently visit can increase the likelihood of ant attendance.

The host choice of herbivorous insects is important for escaping from parasitoids (Murphy 2004). Some facultative myrmecophilous lycaenids prefer to oviposit on the shoots on which other myrmecophilous insects are present or on the plants producing extrafloral nectaries, and such oviposition behaviors facilitate ant defense for their offspring (Kaminski *et al*. 2010; Mota & Oliveira 2016; Alves-Silva *et al*. 2018). *Arhopala japonica* larvae are associated with various *Quercus* (Fagaceae) species, and these *Quercus* species are often visited by various ant species because their leaves are often infested by other myrmecophilous insects such as *Greenidea nipponica* Suenaga and *Lachnus tropicalis* Van der Goot (Hemiptera: Aphididae) (Moritsu 1983). Some fagaceous trees secrete nectars from leaf wounds created by herbivores, and the nectar secreted from the wounds attracts ants as extrafloral nectaries (Staab *et al*. 2017). Thus, the association with *Quercus* might make it easier for myrmecophilous herbivores to encounter ants.

Arhopala japonica is expanding its distribution range toward the northern area in Japan (Takahashi 1998). Range expansion in myrmecophilous insects is often restricted by the lack of suitable ant species as partners (Fiedler 2001). However, the fact that *A. japonica* larvae can be attended by the maximum number of ant species recorded so far suggests that *A. japonica* could establish mutualism with novel ant species in newly invaded areas. Thus, future studies comparing the parasitoids, attending ants and ant attractiveness of *A. japonica* larvae between invaded and originally distributed areas could provide a unique opportunity to assess the effects of range expansion of myrmecophilous herbivores on the modification of trophic interactions.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the Supporting Information section at the end of the article.

Table S1. Sampling information and GenBank accession numbers of parasitoid wasps of *Arhopala japonica* larvae.

Table S2. *P*-values of pairwise comparisons of parasitism rates among different instars of *Arhopala japonica* larvae shown in Fig. 3. *P*-values in bold letters are lower than the corrected *P*-values (in parentheses). (A) *P*-values of pairwise comparisons of the parasitism rate in Saga. (B) *P*-values of pairwise comparisons of the parasitism rate in Kyoto.