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Title: Ant attendance reduces flight muscle and wing size in the aphid *Tuberculatus quercicola*

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Short running title: Reduction in flight apparatus of aphids

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In otherwise mutualistic relationships between aphids and ants, attendance by ants often has negative impacts on aphids. For example, in a previous study using traps in the field, the aphid *Tuberculatus quercicola*, which exhibits mutualistic interactions with ants, showed extremely low dispersal rates, despite having long wings. This study investigates whether components of the flight apparatus (mesonotum length, flight muscle, and wings) differ between aphids attended by ants and not attended by ants. Randomized block analysis of variance, using body length as a covariate, showed that ant attendance has a negative influence on aphid flight apparatus. This result indicates that aphids produce honeydew at the expense of resource investment in flight apparatus. Since the dispersal of *T. quercicola* is limited under ant attendance, the reduction in flight apparatus could precede a decrease in body size. The present study also showed that flight apparatus was more developed in aphids under ant-exclusion conditions. This may imply that *T. quercicola* fly when ants are not available. The maintenance of flight apparatus in *T. quercicola* might therefore be partly explained by gene flow on the rare occasions that this aphid species disperses.

Keywords: mutualism; flight apparatus; *Quercus dentata*

1. INTRODUCTION

In otherwise mutualistic relationships between aphids and ants, attendance by ants often has negative impacts on aphids. For instance, aphid body size or embryo number may decline due to the increased cost of honeydew production [1, 2]. Alternatively, colony

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development may be suppressed [3]. Moreover, attending ants act as inhibitory agents of the dispersal of aphids [4]. Ant mandibular secretions may inhibit alate development [5], while ant semiochemicals may reduce the walking activity of apterous aphids [6]. To date, studies of how ants influence aphid dispersal have been based on observational studies, where the number of dispersing aphids was compared against the presence and absence of ants. However, it remains unknown whether ant attendance directly influences the development of flight apparatus (e.g. wings and flight muscle) in aphids.

The aphid *Tuberculatus quercicola* feeds on *Quercus dentata*, and exhibits mutualistic interactions with ants. During the summer, regardless of colony density or ant attendance, all nymphs develop into alate viviparous females that produce parthenogenetic offspring. The lack of wing dimorphism in *T. quercicola* provides a simple system to evaluate the extent of dipersal ability. In a previous study using traps and weekly observations, *T. quercicola* exhibited low dispersal rates, in which the total numbers of winged individuals trapped and observed in trees across all seasons were 8/1342 (trapped/observed) [4]. This suggests that, despite possessing of wings, the dispersal of *T. quercicola* is limited by attendance by ants, leading to the hypothesis that attending ants have negative impacts on the flight apparatus of *T. quercicola*.

This study investigates whether the flight apparatus of aphids differs between those attended by ants and not attended by ants. The following parameters were evaluated for each condition: (1) differences in mesonotum length and wing areas, and (2) differences in flight muscle development and whole thorax section. All parameters were corrected against body length. Based on the findings, the implications of ant-attendance on resource investment to flight apparatus by aphids are discussed.

2. MATERIAL AND METHODS

(a) *Rearing aphids*

Individuals of *T. quercicola* were reared on the host plant *Q. dentata* at the Ishikari Coast of Hokkaido, North Japan (43 ºN, 141 ºE). At the field site, colonies of *T. quercicola* were attended by the red wood ant *Formica yessensis*. All experiments were conducted in a predator-free environment using four *Q. dentata* trees that were, on average, 3 m in height. In early June 2011, five to eight branches with a pair of shoots were randomly selected from each tree for the experiments. All of the leaves were removed from each shoot except for one, so that only two leaves remained on each branch. Each pair of leaves was located at the apex of the branch, forming a Y-shaped twig. On one leaf, two plastic tubes (diameter 4 mm, length 6 cm) were attached with plastic tape along the petiole to allow the approach of ants. To remove the effects of genetic differences among the aphids on the results, prior to the experiment one aphid clone was reared on each of the study trees. One fourth-instar nymph was collected from each study tree, and transferred to the leaf connected the tubes by using a small brush. After transfer, the leaf was bagged with a nylon net (33×22 cm) to propagate the clone with ant attendance (ant-attended treatment) for about two weeks. In late June, seven to 10 second- or third-instar individuals of ant-attended colonies were transferred onto the other leaf, which was then bagged with a nylon net (ant-excluded treatment). To maintain a low density of eight to 15 individuals per leaf, and prevent overcrowding, several individuals were removed during the course of the experiment. Two to three weeks after transfer, the collection of winged aphids from both treatments was started. Because winged aphids of more than 2 d old start to produce the first-instar, analyses are required to quantify resource allocation for both winged adults and the first-instar nymphs. Therefore, to

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calculate the entire resource investment of an aphid, the aphids used to quantify flight muscle were collected within two days after the emergence of winged adults. To avoid potential variation in aphid nutritional status, which might be influenced by the nutritional quality of *Q. dentata*, the collection interval for a pair of ant-attended and ant-excluded treatments was arranged to be less than one week. A total of 21 branches were established from the four trees between 7 July and 21 September 2011. Four to 14 aphids were collected per bagged leaf and fixed in 99.5% ethanol. The average number of aphids for each treatment per branch and per tree (mean ± SD) was as follows: 8.5 ± 2.2 for the ant-attended treatment and 8.8 ± 2.2 for the ant-excluded treatment.

(b) *Measurements of aphids*

Mesonotum length (Fig. 1a) was measured as indices of the development of flight apparatus because fore-wing is connected to flight muscle in mesonotum. Body length was measured as a covariate in statistical analyses. The body part dimensions were measured by using an eyepiece micrometre installed in a binocular microscope (1000× magnification). The measurements of aphids were orderd for each colony (branch), and the medians were used for statistical analyses.

(c) *Image analysis of wings and flight muscle*

Only aphids within the 2 d period after emergence as adults were used in the present study, to exclude the effects of autolysis on flight muscle development. Either the left or the right fore-wing and hind-wing were cut from a body, and the areas were captured using the eyepiece of a digital microscope AM-423X (Bigc.com, Torrance, CA). The areas were measured using Image J [7]. After measuring body size and wing area, the aphids

were dehydrated in xylene and embedded in paraffin wax. The embedded paraffin blocks were cut to a series of sections of flight muscle (6-8 μm) using a microtome, and the sections were stained by using a haematoxyline-eosin method. For each aphid, one to six pieces of the sections were captured with the eyepiece of a digital microscope. The whole thorax section and flight muscle area (Fig. 1d) were measured using Image J, and the sections that had the maximum whole thorax section and flight muscle area were selected as representative values. The measurements of wings, the whole thorax section, and flight muscle area were ordered for each colony (branch), and the medians were used for statistical analyses.

(d) *Statistical analysis*

To control for individual variation in the host plant, randomized block analysis of variance (ANOVA) was used for all measurement scores. This strategy should minimize any effects peculiar to single leaves. In this randomized block design, the study trees and branches were assigned to blocks. Body length was included as a covariate in the ANOVA model. The ANOVA model contained 'tree', 'branch nested within tree', 'ant', 'body length', and an interaction term 'ant×body length'. ANOVA was performed twice. It was first performed to include an interaction term. Then, if no significant difference was found in the first ANOVA, it was performed without an interaction term. *P*-values were adjusted with Bonferroni multiple corrections. Besides ANOVA, the regression between body length and each parameter were analysed. The same analyses were also applied to the data of all individuals collected from both treatments.

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3. RESULTS

No significant difference was found in any of the interaction terms, but it was found for the whole thorax section ($F_{1,18} = 10.98$, $p = 0.004$) in the first ANOVAs. In the second ANOVA without interaction terms, all traits, except for the whole thorax section, were significantly larger in individuals from the ant-excluded treatment compared to the ant-attended treatment (table 1). The means and SDs for the traits were as follows: the mean mesonotum length was 0.38 ± 0.03 mm in the ant-attended treatment and 0.41 ± 0.04 mm in the ant-excluded treatment (fig. 1b). The mean total wing area was 1.47 ± 0.24 mm^2 in the ant-attended treatment and 1.66 ± 0.27 mm^2 in the ant-excluded treatment (fig. 1c). The mean whole thorax section was 0.21 ± 0.03 mm^2 in the ant-attended treatment and 0.25 ± 0.05 mm^2 in the ant-excluded treatment (fig. 1e). The mean flight muscle area was 0.07 ± 0.03 mm^2 in the ant-attended treatment and 0.09 ± 0.04 mm^2 in ant-excluded treatment (fig. 1f). In ANOVAs for all individuals, no significant difference was found in any of the interaction terms in the first ANOVAs. In the second ANOVA without interaction terms, all traits were significantly larger in individuals from the ant-excluded treatment compared to the ant-attended treatment (supplementary table 1 and figure 1).

4. DISCUSSION

The present study demonstrated that ant attendance has a direct negative influence on the development of flight apparatus in aphids. Existing research has shown that when *T. quercicola* is under ant attendance, honeydew excretions are increased by up to two fold

compared to aphids under ant-exclusion conditions, causing the amino acids to increase in the honeydew [2]. Therefore, it is possible that a shortage of amino acids causes lower protein synthesis, and hence a reduction in the flight muscle of *T. quercicola*. Since *T. quercicola* dispersal is limited under ant attendance conditions, it is possible that a reduction in flight apparatus precedes a decrease in body size.

The results also showed that the flight apparatus of aphids under ant-exclusion conditions was significantly more developed. This may then imply that *T. quercicola* fly when the ants are not available. Indeed, almost all *T. quercicola* caught in the field traps were observed from early July to mid-July, when lowest number of ants were attending aphid colonies, perhaps because honeydew-foraging ants were being recruited into nest budding (unpublished data). Hence, aphids may have an opportunity to fly and colonise new host plants of high nutritional quality. The maintenance of flight apparatus in *T. quercicola*, which exhibits low dispersal, may therefore be partly explained by continued gene flow on the rare occasions that winged aphids disperse.

The flexibility in the development of flight apparatus in aphids suggests that mutualistic interactions with ants might be subjected to fluctuations in host plant quality or the availability of ants. Unstable mutualisms have been reported by molecular phylogenetic studies, where gains or losses of ant attendance have occurred at least five times in the course of the evolution of *Chaitophorus* aphids [8] and *Tuberculatus* aphids [9]. Further studies on other aphid species are needed to clarify whether flexibility in the development of flight apparatus is associated with labile aphid-ant mutualisms.

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Figure legends

Table 1. ANOVA for (a) Mesonotum length, (b) Total wing area, (c) Whole thorax

selection, and (d) Flight muscle area

Figure 1. Measurement parts are indicated in (a) a picture of a typical *Tuberculatus*

species after [10] and (d) a photograph of flightmuscle. Comparison of ant-attended and

ant-excluded aphids for (b) mesonotum length, (c) wing area, (d) whole section, and (e)

flight muscle. The linear regression lines shown as solid lines fitted to the closed circles

and the broken lines fitted to the open circles indicate the relationships between body

length and each parameter for ant-attended and ant-excluded aphids, respectively.

Regression equation, regression coefficient, and statistical significance for the regression

coefficient of solid and broken lines are as follows: (b) $y = 0.167x + 0.098$, $R^2 = 0.637$, p

< 0.0001 and $y = 0.231x - 0.002$, $R^2 = 0.705$, $p < 0.0001$, (c) $y = 1.427x - 0.93$, $R^2 = 0.795$,

$p < 0.0001$ and $y = 1.546x - 1.072$, $R^2 = 0.738$, $p < 0.0001$, (e) $y = 0.177x - 0.083$, $R^2 =$

0.784, $p < 0.0001$ and $y = 0.279x - 0.243$, $R^2 = 0.827$, $p < 0.0001$, and (f) $y = 0.04x - 0.001$, $R^2 = 0.036$, $p = 0.407$ and $y = 0.116x - 0.12$, $R^2 = 0.212$, $p = 0.036$.

Table 1. ANOVA for (a) Mesonotum length, (b) Total wing area, (c)

sorce of variation	d.f.	ss	F	p
(a) Mesonotum length				
Tree	3	####	4.5687	0.0143
Branch (Tree)	17	####	2.5515	0.0257
Ant	1	####	9.5221	0.0061
Body length	1	####	#####	0.0014
Error	19	####		
(b) Total wing area				
Tree	3	####	2.9058	0.0614
Branch (Tree)	17	####	4.7048	0.0008
Ant	1	####	#####	0.0042
Body length	1	####	##### <	0.0001
Error	19	####		
(c) Whole thorax section				
Tree	3	####	5.4298	0.0077
Branch (Tree)	17	####	2.5318	0.0291
Ant	1	####	#####	0.0028
Body length	1	####	##### <	0.0001
Ant*Body length	1	####	#####	0.0039
Error	18	####		
(d) Flight muscle area				
Tree	3	####	9.6288	0.0004
Branch (Tree)	17	####	9.6586 <	0.0001
Ant	1	####	5.2005	0.0343
Body length	1	####	#####	0.0047
Error	19	####		

Figure 1.