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Title: Seasonal changes in the flight apparatus of winged females and sexual males of the

aphid *Tuberculatus quercicola* (Hemiptera: Aphididae)

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Abstract   *Tuberculatus quercicola* (Matsumura) feeds on *Quercus dentata* Thunberg, and exhibits mutualistic interactions with ants. *Tuberculatus quercicola* has two winged morphs in its life cycle, winged females appear in summer and sexual males appear in autumn. Previous studies have shown that winged females exhibit higher wing loading (ratio of body volume to wing area) due to ant attendance, resulting in extremely low dispersal rates. It is known that the nutritional quality of host plants is high in spring and autumn, when leaves are growing or senescent, and low in summer when leaves are mature. The present study examined the effects of seasonal plant deterioration on the development of flight apparatus (wing size and flight muscle) of winged females and males. Moreover, field intercept traps were used to examine the extent of dispersal by males. The results showed that seasonal plant deterioration affected flight apparatus development in winged females, particularly flight muscle. Flight muscle development was significantly higher in winged males in autumn than in winged females. However, winged males were not caught in any of the traps. The difference in resource allocation to the flight apparatus between winged females and males is discussed.

Introduction

Tuberculatus quercicola (Matsumura) feeds on *Quercus dentata* Thunberg, and exhibits mutualistic interactions with ants. *Tubercualuts quercicola* does not alternate host plants during its life history. In late spring, stem mothers of *T. quercicola* hatch on the trunk, from eggs that developed overwinter and move to the underside of growing leaves. During the summer, all nymphs of the aphid develop into alate viviparous females (hereafter termed winged females), which produce offspring parthenogenetically. In autumn, alate males (hereafter termed males) and apterous oviparous females appear. After mating, oviparous females move from the leaves to the branches to deposit eggs (Fig. 1).

A number of studies have documented that morphology and behavior of *T. quercicola* are affected by ants and nutritional quality of host plants. For example, winged females exhibited higher wing loading (ratio of body volume to wing area) due to ant attendance, resulting in extremely low dispersal rates (Yao 2010, 2011, 2012; Yao and Katagiri 2011). It is known that the concentration of nitrogen and carbohydrate in phloem sap are high in spring and autumn, when leaves are growing or senescent, and low in summer when leaves are mature (Dixon 1970). Shibata et al. (2001) reported that water content and nitrogen concentration in *Q. dentata* decline with progressive of seasons. In accordance with the decline of amino acid concentration of *Q. dentata*, body width, embryo number, and honeydew excretion behavior of the aphids decrease from spring to autumn (Yao 2004; Yao and Akimoto 2002; Yao et al. 2000). These studies suggest that *T. quercicola* is subject to seasonal plant deterioration; hence, the development of flight apparatus might be directly influenced by the nutritional status of host plants.

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Population dynamics, including the extent of dispersal in *T. quercicola*, has been studied in winged females, yet little is known about winged males. This may be because male abundance is low, with short life spans (approximately one month). Knowledge about the dispersal of males would contribute information about the evolution of flight apparatus in aphids. To date, studies on the development of flight apparatus in aphids has been conducted on individuals reared in laboratory (Kobayashi and Ishikawa 1993; Ishikawa and Miura 2009). However, how seasonal changes in host plant in the wild affect the development of flight muscle has given scant attention.

The present study examined (1) the effects of seasonal plant deterioration on the development of flight apparatus in winged females and males, (2) the difference in resource allocation between winged females and males, and (3) the extent of male dispersal using flight traps. The difference in resource allocation to the flight apparatus between winged females and males is discussed.

Materials and methods

Rearing aphids and the sampling of males in the wild

Individuals of *T. quercicola* were reared on the host plant *Q. dentata* at the Ishikari Coast, Hokkaido, North Japan (43 ºN, 141 ºE). At the field site, colonies of *T. quercicola* were attended by the red wood ant *Formica yessensis* Forel. All experiments were conducted in a predator-free environment, using four *Q. dentata* trees that were, on average, 3 m in height. In this experiment, clonal aphids were used to compare the body size and flight apparatus of aphids under ant-attended conditions. For this purpose, one aphid clone was

propagated prior to the experiment. In early June 2011, five to eight branches were randomly selected from each tree for the experiments. All of the leaves were removed from each shoot except one, so that only a single leaf remained on each branch. 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. One fourth-instar nymph was collected from each study tree, and transferred to the leaf connected 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 for about two weeks. 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. In total, 21 branches were established from the four trees between 7 July and 21 September 2011. Because winged females of more than two days old start to produce the first-instar, analyses would be required to quantify resource allocation for both winged adults and the first-instar nymphs. Therefore, to calculate the entire resource investment of an aphid, the aphids used to quantify body size and flight muscle were collected within two days after the emergence of winged adults. Four to 14 aphids were collected per bagged leaf and fixed in 99.5% ethanol. The number of replicates and the average number of aphids used in the treatment per month, per tree, and per branch (mean $\pm$ SD) were as follows: 14 replicates with 5.2 ± 2.6 aphids in July, 13 replicates with 3.9 ± 2.5 aphids in August, and nine replicates with 6 ± 3 aphids in September.

Because the nutritional quality of *Q. dentata* declined in October, only five branches on a tree lasted until apperance of sexual males. The average number of aphids per branch (mean $\pm$ SD) in October was 1.6 ± 1.3 for males and one for winged females. To reinforce the measurement scores of body size or flight apparatus for males, 18 males were collected in the field.

Measurements of aphids

Body length was measured as an index of the development of body size. The dimension was measured by using an eyepiece micrometer installed in a binocular microscope (×1000 magnification). Measurements of aphids were ordered for each colony (branch), and the medians were used for statistical analyses.

Image analysis of wings and flight muscle

Either the left or the right fore-wing and hind-wing were cut from each body, and the wing area was captured using the eyepiece of a digital microscope AM-423X (Bigc.com, Torrance, California). The wing areas were measured using Image J (U.S. National Institutes of Health, Bethesda, Maryland) (Abramoff et al. 2004). After measuring body size and wing area, the aphids were dehydrated in xylene and embedded in paraffin wax (Wako, Osaka, Japan). 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 area of flight muscle and the percentage of flight muscle area in the whole section area were measured using Image J, and the sections with the maximum percentage of flight muscle area were selected as a representative values. The measurements of wings and flight muscles were ordered for each colony (branch), and the medians were used for statistical analyses.

Statistical analysis

To control for individual variation of host plants, the study trees and branches were assigned to blocks. This strategy should minimize any effects peculiar to single leaves. To determine how different months affect each response variable, randomized block ANOVAs were conducted with Bonferroni corrections. In this randomized block design, the interaction terms, including those relating to branches and trees, were part of the error term (Sokal and Rohlf 1995). Randomized block ANOVA was applied for body length. The ANOVA model contained 'tree' and 'branch nested within tree' as blocks and 'month' as main effects. When wing size and the percentage of flight muscle area in the whole section area were analyzed, body length was included as a covariate in ANCOVA model. The ANCOVA model contained 'tree' and 'branch nested within tree' as blocks and 'month' as main effects, 'body length', and an interaction term 'month × body length'. ANCOVA was performed twice. It was first performed to include an interaction term. Then, if no significant difference was found in the first ANCOVA, it was performed without an interaction term. Because the number of replicates was small, ANCOVA failed to perform for comarisons of body size and flight apparatus between winged females and males. Instead of that, the comparisons of body size, wing size, and flight muscle development between winged females and males were performed using a Wilcoxon's signed-rank test. The percentages of flight muscle area in the whole section area were transformed to arcsine square-root in order to satisfy the requirement of normality.

Trap survey

To examine the extent of dispersal by males, flight intercept traps were set up in the field. A flight intercept trap was constructed of a cylindrical tomato cage (120 cm tall and 30 cm in diameter) and transparent packaging tape. Six tape clippings (6 cm width and 80 cm long) were vertically attached to the cages. Ten traps were located at an average distance of 1 m from trees adjacent to the study site. To investigate the demographic patterns of males that colonized host trees, eight *Q. dentata* host trees located adjacent to the traps were chosen, with 20 shoots being randomly selected from each tree. The numbers of males caught in the traps and observed in the trees were counted at weekly intervals from 28 September to 22 October.

Results

Seasonal changes in the development of flight apparatus of winged females

ANOVA for body length showed that no significant difference was found in month effect ($F_{2, 13} = 2.62$, $p = 0.11$). No significant difference was found in any of the interaction terms (wing size, $F_{2, 10} = 2.4$, $p = 0.14$; flight muscle, $F_{2, 10} = 1.13$, $p = 0.36$) in the first ANCOVAs. In the second ANCOVA without interaction terms, a significant month effect was found in the percentage of flight muscle area in the whole area, but not in wing size (wing size, $F_{2, 12} = 2.02$, $p = 0.18$; flight muscle, $F_{2, 12} = 11.3$, $p = 0.002$; Table 1 and Fig. 2). The percentage of flight muscle area in the whole area in September decreased by approximately 68% in September compared to July.

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Comparison of body size, wing size, and flight muscle in winged females and males

The mean body size was significantly larger for winged females compared males ($df = 10$, $z = -2.02$, $p = 0.043$). No significant difference was found in wing size between winged females and males ($df = 10$, $z = -0.944$, $p = 0.345$). The percentage of flight muscle area in the whole area was significantly higher for males compared winged females ($df = 10$, $z = -2.02$, $p = 0.043$) (Fig. 3). The means and SDs of body size, wing size, and the percentage of flight muscle area in the whole area of males collected in the field were 1.502 ± 0.133 mm, 1.96 ± 0.244 mm^2, and 48.341 ± 5.572 %, respectively.

Trap survey

A total of 64 males were observed in the eight trees. However, males were not caught in any of the traps.

Discussion

The present study showed that the development of flight apparatus of winged females decreased with the progression of seasons from spring to autumn. Flight muscle exhibited considerably lower development than wings, suggesting that the formation and maintenance of flight muscle is more costly than that of wings. In a comparison of long-winged and wingless individuals from three aphid families, the development of flight apparatus prolongs the time required for maturation into adults, and results in a

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20% reduction in gonad size (Dixon et al. 1993). This study suggests that selection should favor the loss of the wings in *T. quercicola* during September. It remains unclear why *T. quercicola* developed functionless wings in autumn. This may be attributed to gene expression in distinct regions involving flight apparatus. While flight muscle is derived from mesoderm (Hummon and Costello 1992), wings are derived from ectoderm (Jockusch and Ober 2004). Since wings are developed from body, which is ectoderm-derived organs, cooridinated development might occur in the body and wings. The recovery of host plant nutritional quality in autumn leads *T. quercicola* to switch from resource allocation for both flight muscle and reproduction to focus on reproductive investment, which corresponds to oviparous females and males. This results in increases in body length and wing size during September.

Recent studies on the effects of photoperiod on perthenogenesis lifecycle have been developed in the field of research for gene expression (Ramos et al. 2003; Cortes et al. 2008; Le Trionnaire et al. 2009). Therefore, the hypothesis that short photoperiod and/or temperature in autumn affects the development of flight apparatus of *T. quercicola* could not be ruled out.

Compared to winged females, the body size of males was smaller but flight muscle development was larger. The wing loading of males calculated from the body size and wings was smaller compared to winged females (data not shown), implying that males have dispersal capability. The number of aphid colonies in a host tree decreases in number in autumn, so that competition for oviparous females may lead to increased male dispersal. Therefore, the development of flight muscle might be adaptive for males. However, males were not caught in the traps. This may be due to the low abundance of males and/or inbreeding on the natal trees. Even though males have the ability to fly,

dispersal does not seem to contribute much to outcrossing, because there were a few *Q. dentata* trees that maintained aphid colonies until September. Indeed, the *T. quercicola* population on Hokkaido exhibits a genetic structure with low allele numbers and high genetic differentiation between subpopulations (Yao 2010). Moreover, isolation by distance is not found in *T. quercicola* populations at microgeographical scales, where the mean distance between host trees is 240 m (Yao and Akimoto 2009). These studies indicate that gene flow is limited to within a small range. To clarify whether inbreeding occurs, genotyping with microsatellits on the individuals in matings are needed.

The present study exhibited discordance between the development of wings and flight muscle of winged females, and the opportunity for male dispersal. However, the reason for the maintenance of flight muscle in males remains unclear. Further studies on other *Tuberculatus* males and their phylogenetic relationships are required to elucidate the evolution of flight apparatus in aphids.

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Figure Captions

Fig. 1 Life cycle of *T. quercicola*

Fig. 2 Means ± SDs for seasonal changes in (a) body length, (b) wing size, and (c) the percentage of flight muscle area in the whole area of *T. quercicola* winged females. The photographs of wings and the cross sections of flight muscle correspond to the means for each month. Bold lines indicate scale bars. Statistically significant difference: $**P < 0.01$

Fig. 3 Means ± SDs for the comparison of (a) body length, (b) wing size, and (c) the percentage of flight muscle area in the whole area of *T. quercicola* winged females and sexual males. Males collected in the wild are shown in comparison to the males reared in bags. The photographs of wings and the cross sections of flight muscle correspond to the means for winged females and males. Bold lines indicate scale bars. Statistically significant difference: $*P < 0.05$

All figures were made by Adobe Illustrator.

Table 1 ANCOVA for (a) wing size and (b) the percentage of flight

Source	df	nean square	F	p
(a) Wing size				
Month	2	0.046777	2.0214	0.175
Tree	3	0.046999	1.354	0.304
Branch (Tree)	17	0.176877	0.8992	0.59
Body length	1	0.168069	14.526	0.003
Error	12	0.138845		
(b) Flight muscle				
Month	2	1701.493	11.223	0.002
Tree	3	203.6857	0.8957	0.472
Branch (Tree)	17	971.7322	0.7541	0.71
Body length	1	234.6378	3.0954	0.104
Error	12	909.6113		

Stem mother

Winter

egg

Spring

Oviparous female   male   Viviparous female

Autumn

Summer

Winged female

(a)

Body length (mm)

(b)

Wing size (mm2)

1mm

0.1mm

(c)

Flight muscle (%)

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July   August   September