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**Comparing wing loading, flight muscle, and lipid content in ant-attended and non-attended
Tuberculatus aphid species**

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Short title. Flight apparatus in aphids

Abstract. While all *Tuberculatus* aphids possess wings, some species associated with ants exhibit extremely low levels of dispersal compared to those not associated with ants. Furthermore, phylogenetic interspecific comparisons have found significantly higher wing loading (higher ratio of body volume to wing area) in ant-attended species. This observation indicates that ant-attended species may allocate more of their body resources to reproductive traits (i.e. embryos) rather than flight apparatus (i.e. wings, flight muscle, and lipid). This study focuses on two sympatric aphid species to investigate the hypothesised trade-off in resource investment between fecundity and the flight apparatus; specifically, the ant-attended *Tuberculatus quercicola* (Matsumura) and non-attended *Tuberculatus paiki* Hille Ris Lambers. Species differences are compared in (1) morphology, (2) embryo production, (3) triacylglycerol levels, and (4) wing loading and flight muscle. The results show that *T. quercicola* has a larger body volume, higher fecundity, and higher wing loading compared to *T. paiki*, which has a smaller, slender-shaped body, lower fecundity, and lower wing loading. No significant difference is found between the species with respect to the percentage of triacylglycerol content in dry body weight. The flight muscle development is significantly lower in *T. quercicola* than in *T. paiki*. These results indicate that the additive effect of higher wing loading and the lower amount of flight muscle development in *T. quercicola* may increase the physical difficulty of flight, and hence be responsible for its lower dispersal ability. The trade-off between fecundity and dispersal documented in wing-dimorphic insects may therefore be applicable to *T. quercicola*, which has fully developed wings.

Key words. mutualisms, ant attendance, wings, triacylglycerol, body shape, wing loading

Introduction

The aphid genus *Tuberculatus* feeds primarily on Fagaceae, does not alternate host plants during its life history, and exhibits various interactions with ants, ranging from non-attendance to strong associations. During the summer, the nymphs of *Tuberculatus* develop into long-winged (alate) viviparous females, regardless of the nutritional quality of host plants, colony density, or ant attendance. In a previous field study that used flight intercept traps and weekly observations, it was found that two strongly ant-attended species (*T. quercicola* and undescribed *Tuberculatus* sp. A) exhibited extremely low dispersal levels compared to two non-attended species (*Tuberculatus japonicus* Higuchi and *T. paiki*). The total number of winged individuals trapped and observed in trees across all seasons was 8/1342 (trapped/observed) for *T. quercicola* and 2/194 for *T. sp. A*, compared to 52/200 for *T. japonicus* and 137/1315 for *T. paiki*. In addition, the two ant-attended species also showed low genetic diversity and high genetic differentiation between subpopulations compared to the two non-attended species (Yao, 2010). Moreover, isolation by distance (IBD) was not found in *T. quercicola* populations at micro-geographical scales, where the average distance between host trees was 240m (Yao & Akimoto, 2009). These studies indicate that gene flow in ant-attended species is limited to within a small range. Dispersal behaviour benefits non-attended aphid species by facilitating predator escape or movement to alternative host plants that have higher nutrition levels. Wings function as flight apparatus in these mobile species, while the function of wings in ant-attended species remains less clear.

Wing dimorphism has been documented in many insect species, whereby wingless or short-winged morphs exist in the same species, providing evidence for the trade-off between flight and fecundity (Roff & Fairbairn, 1991; Zera & Denno, 1997, Zera & Brink, 2000; Zera, 2004, but see Guerra & Pollack, 2009). In a comparison of long-winged and wingless (apterous) individuals from three aphid families, the development of flight apparatus prolonged the time

required for maturation into adults, and resulted in a 20% reduction in gonad size (Dixon *et al.*, 1993). While species in the genus *Tuberculatus* have neither wingless nor short-winged morphs, phylogenetic comparison of 20 *Tuberculatus* species revealed that ant-attended species have proportionally smaller wings and larger bodies compared to non-attended species (Yao, 2011). This finding indicates that ant-attended species may allocate more of their body resources to reproductive parameters rather than the wings. Hence, the body size and the number of embryos would be expected to be relatively greater in *T. quercicola* compared to *T. paiki*. By contrast, the relative content of lipid and flight muscle, which are components of flight apparatus, would be expected to be relatively lower in *T. quercicola* compared to *T. paiki*.

Lipid exists mainly as triacylglycerol in the fat body of insects, and is used as fuel in flight muscle (Chino & Downer, 1982). The synthesis of triacylglycerol is costly (Zera *et al.*, 1994); hence it is subject to selection pressure that decreases investment in flight apparatus. Higher triacylglycerol content is more commonly found in the long-winged morphs than in flightless morphs of aphid species (Dixon *et al.*, 1993; Itoyama *et al.*, 2000; Xu *et al.*, 2011), in addition to planthoppers (Itoyama *et al.*, 1999) and crickets (Zera *et al.*, 1994; Zera & Larsen, 2001). In parallel, well developed flight muscle has been reported for the long-winged morphs of aphid species (Ishikawa & Miura, 2009), crickets (Mole & Zera, 1993; Tanaka, 1993; Zera *et al.*, 1997), and the firebugs (Socha & Sula, 2006).

This study evaluates whether the formation and maintenance of aphid wings have evolved in the framework of aphid-ant interactions. This study investigates whether morphology, flight muscle, and triacylglycerol content development differ between *T. quercicola*, which is a low migratory aphid species attended by ants, and *T. paiki*, which is a high migratory species that is not attended by ants. To accomplish this objective, the following parameters were evaluated for each species: (1) differences in morphology, focusing on specialised body structures specific to

dispersal ability, (2) embryo production, (3) triacylglycerol levels, and (4) wing loading and flight muscle.

Materials and methods

Rearing aphids

Tuberculatus quercicola and *T. paiki* were reared on the host plant *Quercus dentata* Thunberg (on which they occur sympatrically in the wild) at the Ishikari Coast, Hokkaido, North Japan (43 °N, 141 °E). 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 early June of 2008 and 2010, six to seven branches were randomly selected from each tree containing a pair of shoots for the experiments. From each shoot, all of the leaves were removed except one, so that only two leaves remained on each branch. Each pair of leaves was situated at the apex of the branch, forming a Y-shaped twig. On the opposite leaves of each branch, a *T. quercicola* and *T. paiki* colony were established. In this experiment, clonal aphids were used to compare the two aphid species. For this purpose, one aphid clone was propagated prior to the experiment on each pair of leaves, which was bagged with a nylon net (33 × 22 cm). On the other leaf, two plastic tubes (diameter 4 mm, length 6 cm) were attached with plastic tape along the petiole, and a net was bound over the tubes. To maintain a constant population of 10-50 individuals per leaf and prevent overcrowding, we removed individuals at each census. In general, fourth-instar nymphs of aphids are sedentary, feeding exclusively on the phloem sap of host plants. Thus, conspicuous differences in the body shape of the two species were expected to be apparent following the emergence of the fourth-instar nymphs into alate adults.

To compare changes in body shape between the consecutive stages of the two species,

fourth-instar and winged adults were collected from each bagged leaf between 4 July and 10 August 2008. The total number of embryos contained by the winged female adults of both species was compared. For the comparison of triacylglycerol levels, winged adults of both species were collected between 2 and 27 July 2010. For the comparison of the flight muscle and wing loading, adults of both species were collected from 7 to 28 July 2008 and 13 to 27 July 2010. Because winged aphids that are more than two days old start to produce the first-instar, resource allocation would be required to be quantified for both winged adults and the first-instar nymphs. Therefore, to calculate the entire resource investment of an aphid, the aphids used in this study were collected within two days after the emergence of winged adults.

Measurements of aphids

The following parameters were measured as indices of growth and reproduction: (1) change in the ratio of body length to body width from fourth-instar to winged adults, and (2) total number of embryos including mature and immature embryos. The body part dimensions were measured using an eyepiece micrometre installed in a binocular microscope ($\times 1000$ magnification). The number of embryos present in each female was counted after dissection. Measurements of aphids were averaged for each colony (branch), and the averages were used for statistical analyses. In total, there were 23 and 19 fourth-instar colonies of *T. quercicola* and *T. paiki*, and 23 and 23 winged colonies for *T. quercicola* and *T. paiki*, respectively. A normal distribution for the ratio of body length to body width, both for fourth-instar and winged adults, was confirmed before statistical testing (Shapiro-Wilk test; $P = 0.65$ and $P = 0.3$ for fourth-instar and winged *T. quercicola*, respectively; $P = 0.1$ and $P = 0.07$ for fourth-instar and winged *T. paiki*, respectively). The average number of dissected aphids for each species per branch and per tree (mean $\pm$ SD) was as follows: for fourth-instar, 9.3 ± 5.5 for *T. quercicola* and 9.1 ± 4.3 for *T. paiki*; for winged adults,

10.8 ± 5.1 for *T. quercicola* and 11.7 ± 6.7 for *T. paiki*.

Lipid assay

Aphids from each bagged leaf were collected in 0.6 mL tubes, then placed in a cool bag to stop all activity, and subsequently dried at 55 °C for 3 h. Each aphid was weighed with an Ultramicro Balance SE-2 (Sartorius, Tokyo, Japan), and homogenised in a 0.6 mL cylindrical vial with 100 µL extraction solvent (a chloroform/methanol 2:1 (v/v) mixture). 20 µL (0.5 µg µL⁻¹) of cholesteryl acetate (Nacalai tesque, Kyoto, Japan) was added to each vial as an internal standard. After nitrogen gas was used to dry the solvent, 10 µL of *n*-hexane was added to each vial. The mixture was subsequently was applied to a thin layer quartz rod CHROMAROD (Mitsubishi Chemical Medience Co., Tokyo, Japan). Samples were developed in diethyl ether:hexane (at a ratio of 1:6) and analysed with IATROSCAN TH-10 (Mitsubishi Chemical Medience Co.) (Katagiri & Kort, 1991). For dry body weight (µg), lipid weight (µg), and the percentage of lipid weight in dry body weight, the data were averaged for each colony (branch), and the values were used for statistical testing, resulting in 22 pairs of colonies each for *T. quercicola* and *T. paiki*. The average number of aphids analysed for each species per branch and per tree (mean ± SD) was as follows: 10.7 ± 3.2 for *T. quercicola* and 11.7 ± 2.9 for *T. paiki*.

Image analysis of wing loading and flight muscle

Autolysis of flight muscle has been reported in some studies (Dixon *et al.*, 1993; Tanaka, 1993; Tanaka *et al.*, 2001; Socha & Sula, 2006). However, only aphids within the two days period after an emergence as adults were used in the present study. This criterion was used to exclude the effects of autolysis on flight muscle development. Five to six aphids were collected per bagged leaf and fixed in 99.5% ethanol. The body volume (V) of winged adults was

calculated by using the formula of an ellipsoid, $V = 4\pi ab^2/3$, where body length is (a) and body width is (b). 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 the Image J (Abramoff, 2004). Wing loading was calculated as the ratio of body volume (mm^3) to total wing area (mm^2). After completing the wing loading measurements, 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. One to six pieces of the sections per aphid were captured with the eyepiece of a digital microscope. The area of flight muscle and the percentage of flight muscle area in whole section area were measured using Image J, and the sections that had the maximum percentage of flight muscle area were selected as a representative value. The flight apparatus measurements were averaged for each colony (branch), and the averages were used for statistical analyses. In total, 16 pairs (six pairs from 2008 and 10 from 2010) of colonies of *T. quercicola* and *T. paiki* were measured. The average number of aphids for each species per branch and per tree (mean $\pm$ SD) was as follows: 4.3 ± 1.7 for *T. quercicola* and 4.2 ± 1.9 for *T. paiki*.

Statistical analysis

To control for individual variation in the host plant, the study trees and branches were assigned to blocks. This strategy should minimize any effects peculiar to single leaves. Randomized block analysis of variance (ANOVA) was applied for (1) the comparisons of changes in the ratio of body length to body width from fourth-instar to winged adults, and (2) the total number of embryos. A null hypothesis was rejected at a level of 0.05. In this randomized block design, the interaction terms, including those relating to branches and trees, were part of the error term

(Sokal & Rohlf, 1995). To determine how the two species and stadium affect each response variable, randomized block ANOVAs were conducted with Bonferroni corrections. The ANOVA model contained 'tree' and 'branch nested within tree' as blocks and 'species', 'stadiums', and 'species*stadium' as main effects. A Wilcoxon's signed-rank test was used for species comparisons of the lipid assay, flight muscle development, and wing loading.

Results

Body volume and number of embryos

For the ratio of body length to body width, a significant difference was found in the effects of 'species', 'stadium', and 'species*stadium' interaction (Randomized block ANOVA; species*stadium: $F_{1,57} = 39$; $P < 0.0001$) (Table 1a). There was a two-fold increase in the difference of the index between the two species from fourth-instar (0.26) to winged adult (0.52) (Fig. 1a). This result indicated that *T. paiki* was more slender than *T. quercicola*, which was more conspicuous in winged adults. The total number of embryos found in winged females was significantly greater in *T. quercicola* than in *T. paiki* (Randomized block ANOVA; species: $F_{1,22} = 84.2$; $P < 0.0001$) (Table 1b and Fig. 1b).

Lipid assay

Mean dry body weight and mean triacylglycerol weight were significantly higher in *T. quercicola* than in *T. paiki* (Wilcoxon's signed-rank test; mean dry body weight: $n = 22$ pairs; $P < 0.0001$; mean triacylglycerol weight: $n = 22$ pairs; $P = 0.0001$) (Figs. 2a and b). No significant difference was found for the percentage of triacylglycerol content in dry body weight between the two species (Wilcoxon's signed-rank test; $n = 22$ pairs, $P = 0.41$) (Fig. 2c).

Image analysis of wing loading and flight muscle

Body volume and the total wing area were significantly greater in *T. quercicola* than in *T. paiki* (Wilcoxon's signed-rank test; body volume: $n = 16$ pairs; $P = 0.0005$; the total wing area: $n = 16$ pairs; $P = 0.0023$) (Figs. 3a and b). Wing loading was higher in *T. quercicola* than in *T. paiki* (Wilcoxon's signed-rank test; $n = 16$ pairs, $P = 0.0005$) (Fig. 3c). The area of flight muscle was higher in *T. quercicola* than in *T. paiki* (Wilcoxon's signed-rank test; $n = 16$ pairs; $P = 0.0113$) (Fig. 4a). The coefficient of variation in the flight muscle area was larger in *T. quercicola* than in *T. paiki* (Wilcoxon's signed-rank test; $n = 16$ pairs; $P = 0.0386$) (Fig. 4b). The percentage of wing muscle area of the whole area was significantly lower in *T. quercicola* than in *T. paiki* (Wilcoxon's signed-rank test; $n = 16$ pairs; $P = 0.0005$) (Fig. 4c).

Discussion

A noticeable increase in the ratio of body length to body width at emergence indicates that the body shape of the alate of *T. paiki* is specific for flight. In addition, lower wing loading may also play an important role in the dispersal behaviour of *T. paiki*. These results suggest that the ability to fly may have evolved in non-attended aphids as a strategy to evade/escape natural enemies or to seek alternative nutritional host plants. By contrast, the larger body volume of *T. quercicola* was associated with higher numbers of embryos. However, the larger body volume in this species resulted in larger wing loading, preventing frequent flight. These results support existing knowledge about the dispersal abilities of these two species, whereby dispersal have been recorded in *T. paiki*, while a comparatively low level of dispersal has been recorded in *T. quercicola*. In addition, the recorded differences in body size between the two species were consistent with a study of 68 aphid species, in which the body size of long-winged morphs was found to be significantly smaller than that of flightless morphs (Dixon & Kindlmann, 1999). Our

results suggest that the trade-off between flight and fecundity (i.e. oogenesis-flight syndrome) (Johnson, 1969), may also be applicable to *T. quercicola* and *T. paiki*. For example, in previous experiments with *T. quercicola*, the removal of ants always resulted in the extinction of the aphid colonies (Yao *et al.*, 2000), which was caused by predation. Because *T. quercicola* benefitted from ant attendance, reduced levels of dispersal, in parallel to larger body mass and increased wing loading, may be adaptive.

A significant difference in the flight apparatus between the two species was found in the development of flight muscle, but not in relative triacylglycerol content. The similarity in triacylglycerol content may have been due to the small amounts of triacylglycerol that accumulate after moulting. Some studies of the cricket *Modicogryllus confirmatus* Walker and planthopper *Nilaparvata lugens* (Stal) have demonstrated that the difference in triacylglycerol content between winged-morphs was highest in adults at three days after emergence because of differences in longevity, copulation, and migration (Itoyama *et al.*, 1999; Tanaka *et al.*, 1999). The aphids used in the present study were new adults within two days of emergence; hence more time may have been required for *T. paiki* to produce large enough amounts of triacylglycerol for detection. Another explanation for the lack of difference in triacylglycerol content is that triacylglycerol is also used as fuel by tissues other than flight muscle, such as insect eggs (Ziegler & Antwerpen, 2006). Hence, it is possible that mixed triacylglycerol content was detected in the present study. For example, triacylglycerol in *T. quercicola* may be mainly derived from a large numbers of embryos. Hence, lipid analysis conducted separately on the flight muscle and other tissues might provide more exact quantification of the triacylglycerol levels used in flight.

In addition to lipid, carbohydrate is used as fuel for the flight muscles via the haemolymph (Beenakkers *et al.*, 1984; Gäde & Marco, 2009). In the blowfly *Phormia regina* Meigen, the migratory locust *Locusta migratoria* (L.), the spittle bug *Locris arithmetica* Walker, and the stink

bug *Encosternum delegorguei* Spinola, carbohydrate is utilised mainly in the form of trehalose as a fuel for flight during the first 0.5-30 minutes of flight. Aphids do not exhibit continuous flight, but primarily fly short distances with short intervals (Dixon, 1998). This behaviour indicates that the fat body glycogen, a source of trehalose, may be a more critical substrate for flight in *T. paiki*. Further analyses are needed to study the role of carbohydrates in relation to flight.

The percentage of flight muscle area of the whole area in *T. quercicola* (40.3%) was lower than in *T. paiki* (51.4%), which may primarily account for the low dispersal behaviour in *T. quercicola*. However, an additive effect of the lower flight muscle development and higher wing loading in *T. quercicola* would increase the physical difficulty of flight, and therefore provide a more plausible explanation for the known differences in dispersal ability between the two species. In *T. quercicola*, resource allocation appears to be directed towards increasing body size rather than flight muscle, resulting in either an increased level of fecundity or the production of larger volumes of honeydew. Aside from the lower average of flight muscle development, a large coefficient variation in the flight muscle area of *T. quercicola* was detected. It is known that the stabilising of natural selection reduces phenotypic variance (Falconer, 1989), suggesting that the flight muscle of *T. quercicola* appears to be free from natural selection. A number of studies on wing dimorphic insects have demonstrated that the development of flight muscle is negatively associated with fecundity or longevity (Mole & Zera, 1993; Tanaka, 1993). Although viviparous *T. quercicola* has no apterous morph, selection acting on increased fecundity, rather than flight muscle development, seems to follow a similar direction to that of flightless morphs in wing dimorphic insects.

It is unclear, however, why full or partial wing loss has not evolved in *T. quercicola*. Flightlessness in long-winged insects has been reported in three Coleoptera, *Heptophylla picea* Motschulsky (Scarabaeidae) (Tada *et al.*, 1991), *Anomala schonfeldti* Ohaus (Scarabaeidae) (Tada

et al., 1993), *Geotrupes laevistriatus* Motschulsky (Geotrupidae) (Suzuki *et al.*, 2001), and two Heteroptera, *Gerris remigis* Say (Gerridae) (Fairbairn & Desranleau, 1987) and *Pyrrhocoris apterus* (L.) (Pyrrhocoridae) (Socha & Zemek, 2000). Flightlessness in the above species has been reported in the context of benefits, such as a pre-oviposition season and an arrest of ovariole maturation. However, the adaptive significance of wing retention in flightless species remains unknown. Ontogenetic constraints or hidden adaptive significance may be possible explanations. If morphological constraints render wing-loss difficult, changes in allometric relationship between body size and wing area could precede loss of wings. Alternatively, wing loss may not have evolved because a degree of dispersal may still be required to maintain some level of outcrossing, or because the insects require a certain amount of low level of dispersal to colonize new plants. To determine whether the retention of wings arises from morphological or phylogenetic constraints, histological studies and phylogenetic approaches for a larger number of species in the *Tuberculatus* genus are required.

Recent interspecific comparative studies have been developed in the framework of phylogenetic relationships (Felsenstein, 1985), indicating that the results of the current study, except that of wing loading, should be treated with caution. Because *T. quercicola* and *T. paiki* are not sister species (Yao, 2011), it is possible that the differences in the traits between the two species may be attributed to phylogenetic similarity rather than mutualistic interactions with ants. However, Yao (2011) used phylogenetic comparative methods to demonstrate the parallel evolution of higher wing loading with ant attendance. Ultimately, extensive studies on the morphological, physiological, reproductive and phylogenetic relationships of other *Tuberculatus* species would contribute towards elucidating the evolutionary processes in aphid-ant interactions.

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Table 1. Randomized block ANOVAs for (a) the ratio of body length to body width and (b) the total number of embryos. *P* values were adjusted using Bonferroni multiple corrections.

	d.f.	SS	<i>F</i>	<i>P</i>
(a) Body length/body width				
Tree	3	0.01	0.56	0.6407
Branch (Tree)	20	0.24	1.35	0.1857
Species	1	2.79	316.64	< 0.0001
Stadium	1	4.91	558.01	< 0.0001
Species*Stadium	1	0.34	38.96	< 0.0001
Error	57	0.50		
(b) Total number of embryos				
Tree	3	19.74	2.95	0.0550
Branch (Tree)	20	40.88	0.92	0.5751
Species	1	187.65	84.16	< 0.0001
Error	22	49.05		

Figure legends

Fig. 1. Comparing *Tuberculatus quercicola* and *Tuberculatus paiki* for (a) changes in the ratio of body length to body width from the fourth-instar to winged adult (mean $\pm$ SE) and (b) the total number of embryos (mean $\pm$ SD). The grey and open bar represent *T. quercicola* and *T. paiki*, respectively. *** denotes significant difference at $P < 0.001$.

Fig. 2. Comparing *Tuberculatus quercicola* and *Tuberculatus paiki* for (a) dry body weight (μg), (b) triacylglycerol weight (μg), and (c) triacylglycerol in dry body weight (%). The grey and open bar represent *T. quercicola* and *T. paiki*, respectively. Mean $\pm$ SD. *** denotes significant difference at $P < 0.001$.

Fig. 3. Comparing *Tuberculatus quercicola* and *Tuberculatus paiki* for (a) body volume (mm^3), (b) wing area (mm^2), and (c) wing loading ($\text{mm}^3 / \text{mm}^2$). The grey and open bar represent *T. quercicola* and *T. paiki*, respectively. Mean $\pm$ SD. ** and *** denote significant difference at $P < 0.01$ and $P < 0.001$, respectively.

Fig. 4. Comparing *Tuberculatus quercicola* and *Tuberculatus paiki* for box plots of the flight muscle for (a) the area (mm^2), (b) the coefficient variation, and (c) the ratio of flight muscle to the whole section area. Open circles indicate outliers. (d) Images of cross-sections showing the flight muscle of *T. quercicola* and *T. paiki*. The upper, middle, and lower images of the sections in each species correspond to the individuals that exhibit the near the maximum (60.2% for *T. quercicola* and 64.7% for *T. paiki*), average (40.5% for *T. quercicola* and 57.2% for *T. paiki*), and minimum (15.3% for *T. quercicola* and 38.3% for *T. paiki*) percentage of flight muscle, respectively. * and *** denote significant difference at $P < 0.05$ and $P < 0.001$,

respectively.







