Published in final edited form as:

Evolution. 2005 April ; 59(4): 921–926.

THE ORIGIN OF A MUTUALISM: A MORPHOLOGICAL TRAIT PROMOTING THE EVOLUTION OF ANT-APHID MUTUALISMS

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Abstract

Mutualisms are mutually beneficial interactions between species and are fundamentally important at all levels of biological organization. It is not clear, however, why one species participates in a particular mutualism whereas another does not. Here we show that pre-existing traits can dispose particular species to evolve a mutualistic interaction. Combining morphological, ecological, and behavioral data in a comparative analysis, we show that resource use in *Chaitophorus* aphids (Hemiptera: Aphididae) modulates the origin of their mutualism with ants. We demonstrate that aphid species that feed on deeper phloem elements have longer mouthparts, that this inhibits their ability to withdraw their mouthparts and escape predators and that, consequently, this increases their need for protection by mutualist ants.

Keywords

Ants; aphids; comparative analysis; mutualism; myrmecophily; preadaptation

Mutualisms are mutually beneficial interactions between species and are ubiquitous, often ecologically dominant and fundamentally important at all levels of biological organization (Boucher et al. 1982; Herre et al. 1999; Hoeksema and Bruna 2000). However, their evolution, and in particular their origin, has challenged biologists for decades. Several studies have shown that the traits involved in maintaining a mutualistic interaction are generally modifications of pre-existing traits, rather than evolutionary novelties (Ward 1991; Pellmyr et al. 1996; Anstett et al. 1997). This suggests that preadaptation is central to the origin of mutualisms, with certain traits predisposing a species to forming a mutualistic relationship. Nevertheless, there are no clear examples of how interspecific variability in a particular trait influences the pattern of mutualisms among closely related species. A promising approach is to study mutualisms that are particularly evolutionarily labile and have multiple evolutionarily independent origins. One example of such a mutualism is between ants and aphids.

Aphids often, but not always, form a mutualistic association with ants. The aphid provides the ant with carbohydrates in the form of “honeydew,” the waste product of its sugar-rich diet of plant sap. In return the ant may defend the aphid from predators (Nixon 1951; Banks 1962; Way 1963). Although almost all aphids produce honeydew, are susceptible to predation, and co-occur in the same habitats as ants (Bristow 1991), only 60% of aphid species have a

relationship with ants (Stadler 1997). The mutualism is extremely evolutionarily labile and may be lost or gained multiple times within a single genus (Shingleton and Stern 2003). Ant-aphid interactions are therefore an ideal model for the study of the evolution of mutualisms, providing multiple independent examples of its gain and loss. It has been hypothesized that the pattern of ant mutualism among aphids may be explained by differences in feeding position on their host plant (Dixon 1998). Aphids feed by extending their elongated mouthparts into their host plant's phloem elements. Those feeding on the branches and trunks of trees need to have particularly long mouthparts to reach the large, deeply located vascular bundles of the phloem. Their long mouthparts may, however, impede their ability to withdraw from their feeding position and escape from predators. Such aphids may therefore benefit from protection by ants more than those feeding on smaller and shallower vascular bundles, and this may be why some aphids form ant mutualisms whereas others do not. There is some evidence that this is the case. In a phylogenetically uncontrolled comparative analysis of 112 European aphid species there was a strong association between feeding on woody parts of the host plant and ant tending (Stadler et al. 2003).

Here we test whether (1) feeding position on the host plant is related to interspecific variation in mouthpart length in aphids; (2) whether this explains interspecific variation in ability to withdraw from feeding position; (3) whether this explains interspecific variation in susceptibility to predators; and (4) whether this, consequently, explains why some aphid species are ant-tended whereas others are not. Such analysis requires a known phylogeny in order to generate multiple evolutionarily independent contrasts. In this study we used a molecular phylogeny of the *Chaitophorus* aphids (Shingleton and Stern 2003). The genus constitutes approximately 70 species worldwide, all of which feed on either willow (*Salix*) or poplar (*Populus*) (Family: Salicaceae) (Blackman and Eastop 1994). Ant tending evolved or was lost at least five times during *Chaitophorus* evolution (Shingleton and Stern 2003). We concentrated our study on a subset of 13 species. These species show a range of interactions with ants, from obligate mutualism through facultative mutualism to being untended. Nevertheless, all 13 co-occur in the same habitat as ants and many of the untended species share the same host plant as tended species (Hille Ris Lambers 1960; Pintera 1987). Therefore, lack of a mutualistic relationship is not due to lack of a potential mutualistic partner. The 13 species also show a range of feeding positions, with some feeding on the young shoots and leaf petioles, others on the midvein of the leaf, and others on the lateral leaf veins. Vascular bundles are more deeply located in the petioles and shoots than in the leaves (Elliott and Hodgson 1996). Using the phylogeny, and combining morphological, ecological, and behavioral data, we demonstrate that mouthpart length is a primary factor determining the benefits, and hence evolution, of the aphid's mutualistic relationship with ants.

Materials and Methods

Aphid Species

The study included all the known species of British *Chaitophorus* (*C. capreae*, *C. horii beuthani*, *C. leucomelas*, *C. niger*, *C. populeti*, *C. populialbae*, *C. salicti*, *C. tremulae*, *C. truncatus*, and *C. vitellinae*), as well as one species from Austria (*C. nassanowi*) and two from the United States (*C. populicola* and *C. neglectus*). Of these species, six (*C. leucomelas*, *C. nassanowi*, *C. populeti*, *C. populicola*, *C. salicti*, *C. vitellinae*) are obligate mutualists, one (*C. populialbae*) is a facultative mutualist, and six are never or almost never visited by ants (*C. capreae*, *C. horii beuthani*, *C. neglectus*, *C. niger*, *C. tremulae*, *C. truncatus*) (Pintera 1987; Blackman and Eastop 1994; Shingleton 2001). These relationships are, to our knowledge, constant across each species' range. In all analyses, the obligatorily and facultatively tended species were considered tended, and all other species were considered untended.

Interspecific Variation in Mouthpart Length

We collected between 11 and 47 adults of each species, each from a different colony. Aphids were stored in 80% alcohol and subsequently cleared, stained, and mounted as described by Blackman and Eastop (1994). We measured the mouthpart length (labium sheath length) and body length and width from digital images (measurement error was estimated as 2.5% of the mean value) using ImageJ (vers. 1.3; available free at <http://rsb.info.nih.gov/ij/>).

Interspecific Variation in Ability to Withdraw from Feeding Position

All the British *Chaitophorus* were tested for their ability to withdraw from their feeding position in response to an aggressive stimulus. The stimulus was exposure to aphid alarm pheromone (trans- β -farnesene) released by a crushed conspecific aphid placed adjacent (< 5 mm) to the tail of the test aphid (we assumed that an aphid releases sufficient alarm pheromone to alert its conspecifics). The escape response was the time taken for an adult individual to withdraw its mouthparts and begin to walk away from its feeding position, after initiation of the stimulus. Individuals who began to escape before stimulation (evident from lateral movement of the head as the mouthparts are withdrawn) were excluded from analysis. All individuals were untended at the time of testing, and each came from a different host plant. We tested 50 individuals of each species. The data were collected over five days, during which climatic conditions remained approximately constant. We randomized the order of aphid species tested across all days.

We also examined whether there was an intraspecific relationship between mouthpart length and an individual's ability to withdraw from its feeding position. We tested a further 50 individuals of *C. populeti* for their escape ability and subsequently collected each individual and measured their mouthpart length and body length and width, as described above.

Interspecific Variation in Susceptibility to Predators

A subset of the British *Chaitophorus* was tested for susceptibility to predation and was selected because of their availability and because, collectively, they show a wide interspecific range of mouthpart lengths. We established 25 laboratory populations of 15–25 fourth-instar *C. populialbae*, *C. populeti*, *C. tremulae*, *C. leucomelas*, and *C. vitellinae* on 10-cm-long leaf-bearing twigs of *Populus alba*, *P. tremula*, *P. nigra*, and *Salix alba*, respectively. Each twig was held in a plastic cylinder (height 115 mm, diameter 50 mm) sealed with muslin. On each twig, we introduced a single adult two-spotted ladybird (*Adalia bipuncta*) that had been starved for 24 h. After 12 h, we counted the number of remaining aphids in each population, including aphids that had walked off the twig.

Statistical Analysis

For all interspecific tests, we used only phylogenetically independent contrasts. We used the program CAIC (Purvis and Rambaut 1995) to generate suitable contrasts, which were then analyzed using one-sample *t*-tests or linear regressions. We used the topology of the maximum likelihood (ML) tree of the *Chaitophorus* (see inset in Fig. 1), assuming a punctuational mode of evolution by setting all branch lengths as equal. We also repeated the analysis assuming a gradualistic mode of evolution by incorporating branch lengths from the ML tree. In all comparisons, both methods produced the qualitatively same results, and only the results of the former are reported. Where appropriate, branch lengths were transformed to ensure that the residual variance of the contrasts was homogeneous (Garland et al. 1992). Prior to analysis, mean mouthpart lengths were adjusted for mean body size (body length $\times$ width) by analyzing the relationship between mouthpart dimension and body size using CAIC and regressing the former against the latter through the origin. The slope of this line was then fitted to the raw data, and the residuals used for subsequent analysis (Purvis and Rambaut 1995). Other statistics

were performed using the statistical package R (available via <http://www.r-project.org>). All measurements were log-transformed prior to analysis. Significance was set at $P < 0.05$.

Results

Aphid mouthpart length varied with feeding position. Of the 13 species examined, four fed on the petioles of their host plant and nine fed on the leaves. Petiole-feeding species had longer mouthparts than leaf-feeding species (Fig. 1).

Differences in mouthpart length influenced an aphid's ability to escape from predators. There was a significant positive relationship between the mean length of a species' mouthparts, as measured above, and the mean time for that species to escape (Fig. 2A,B). This relationship was also evident intraspecifically. Individual *C. populeti* with longer mouthparts escaped more slowly than individuals with shorter mouthparts (generalized linear model: escape time = size + mouthpart length, $F_{\text{mouthpart length (1,49)}} = 4.91$, $P = 0.0315$).

Differences in escape ability influence an aphid's susceptibility to predation. There was a significant positive relationship between the mean escape time of a species, as measured above, and the level of predation it suffered by the two-spotted ladybird (Fig. 2C,D). There was also a positive relationship between mean mouthpart length for a species, as measured above, and the level of predation it suffered (contrasts at nodes v, vii, viii, and x in Fig. 1; $r^2 = 0.80$, $df = 3$, $P = 0.0389$).

Given these findings, we then used the data to test whether there was a relationship between mouthpart dimension and ant tending. Tended aphids (obligate and facultative) had longer mouthparts than untended aphids amongst the *Chaitophorus* aphids as a whole (contrasts at nodes ii, vi, vii, and x in Fig. 1; $t_4 = 3.312$, $df = 3$, $P = 0.0452$) and within leaf-feeding aphids alone (contrasts at nodes ii and vi in Fig. 1; $t_2 = 32.127$, $df = 1$, $P = 0.0198$).

Discussion

These comparisons demonstrate an evolutionary relationship between feeding position, dimensions of the mouthparts, ability to escape, and the risk of predation suffered by an aphid species. This suggests that some species may consequently benefit more from ant tending than others. Feeding site seems to be one factor that may explain why some aphids form a mutualistic interaction with ants whereas others do not.

It is possible that feeding position evolved as an adaptation to ant tending and not vice versa: aphids may have evolved to feed on deeper phloem elements to attract ants, if such elements provided sap in greater quantity and with a higher sugar concentration. However, this hypothesis is incompatible with the general understanding of phloem function in higher plants. Under the widely accepted mass flow model (Münch 1930), sap moves down a pressure gradient from the leaf phloem to the stem phloem and on to the rest of the plant. Aphids are largely passive feeders, relying on sap pressure to force liquid through their mouthparts (although they can actively suck up food if necessary; Auclair 1963). Since sap pressure is highest in the leaves, it seems unlikely that the quantity of sap available to an aphid will be greater in the petioles than in the leaves. Further, the sugar concentration of sap is unlikely to be higher in the petioles than in the leaves, since the pressure gradient is established by the difference in sugar concentration between the source (leaves) and sink (nonphotosynthetic growing tissue; Münch 1930). Consequently, the deeper phloem elements of the petioles should not provide sap in greater quantity or with a higher sugar concentration than the shallower phloem elements of the leaves. Although this has yet to be investigated specifically in tree-feeding aphids, experimental data show this to be true for barley-feeding *Sitobion yakini* (Matsiliza and Botha 2002). Therefore, based on sap sugar concentration and quantity alone,

Chaitophorus aphids do not appear to be selecting their feeding site in order to better attract ants.

The selection of feeding site is likely to result from interspecific competition. *Chaitophorus* species that share the same host plant always have significantly different mouthpart lengths (e.g., *C. capreae* and *C. salicti* on goat willow, *C. niger* and *C. vitellinae* on white willow; Fig. 1) even when both species are tended (e.g., *C. leucomelas* and *C. nassanowi* on black poplar, *C. populialbae* and *C. populeti* on aspen; Fig. 1). This relates to observable differences in their feeding site, even between species sharing the same host-plant leaves. For example, *C. salicti* feeds on the deeper midveins of goat willow leaves, whereas *C. capreae* feeds on the shallower lateral veins. Ant tending may therefore be one method of escaping interspecific competition by allowing an aphid to feed at a site unavailable to untended species.

These arguments do not, in themselves, explain why aphids that feed on shallower elements are not tended. Such aphids still produce honeydew and, for the ant, the stylet length of its partner is probably unimportant. It follows that aphids feeding on deeper phloem elements have evolved additional traits to better attract ants, as an evolutionary response to tending. Several of these traits have been identified. For example, tended aphids appear to adjust the quality and quantity of their honeydew to successfully attract ants (Takeda et al. 1982; Fischer and Shingleton 2001; Yao and Akimoto 2001). In particular, tended aphids increase the honeydew concentration of melezitose, a trisaccharide particularly attractive to ants (Fischer and Shingleton 2001). There is evidence that such adjustments incur costs to the aphids (Stadler and Dixon 1998; Yao et al. 2000), which are likely to increase if there is competition between aphid species for ant partners. We suggest that only in species feeding on deeper phloem elements will the costs of these traits be outweighed by the benefits of protection from predators.

We used only a small number of the approximately 70 *Chaitophorus* species worldwide in our analyses. Nevertheless, the 13 species examined, which included all the British *Chaitophorus*, were representative of the genus as a whole. Approximately 41% of *Chaitophorus* species are untended, with 28% living on the petioles or twigs of their host plant (Hille Ris Lambers 1960; Pintera 1987). Among the *Chaitophorus* species we examined, 46% are untended with 31% living on the petioles of their host plant. We were unable to include any of the few *Chaitophorus* species that feed on the bark of their host plant, although all of these species are reported as ant tended (Hille Ris Lambers 1960; Pintera 1987), as would be expected under the hypothesis.

A potential problem with comparative analyses using independent contrasts arises from uncertainty in both the topology and branch lengths of the phylogeny used (Huelsenbeck et al. 2000; Huelsenbeck and Rannala 2003). In the case of the *Chaitophorus* phylogeny, there is poor resolution at some of the nodes of the maximum likelihood (ML) tree, and the ML tree and the trees constructed using neighbor-joining (NJ) or maximum parsimony (MP) all differ slightly in their topology (Shingleton and Stern 2003). To check the robustness of our conclusions we reanalyzed the data using both the NJ and MP trees. Under both punctuated and gradualistic models of evolution, the analyses yielded qualitatively the same results as for the analyses based on the ML tree. The only exception was the analysis testing the relationship between feeding position and mouthpart dimension using the MP tree with equal branch lengths, which was close to significance at $P = 0.051$. Therefore, the data appear to be sufficiently robust to uphold our interpretations.

In conclusion, the data suggest that the origin of ant-aphid mutualisms may depend on traits that have evolved under unrelated selective pressures, but that influence the benefits of the mutualism. Evidence from other organisms also suggests that preadaptations are important in

the evolution of mutualisms. For example, the mutualism between yuccas and yucca moths (Pellmyr et al. 1996; Pellmyr 1997) and between various plants and ants (Janzen 1966; Ward 1991; Yu and Davidson 1997) appear to depend largely on pre-existing traits rather than coevolved ones, suggesting that preadaptations are central to the origin of these mutualisms. This study is one of the first, however, to demonstrate that variation in a pre-existing trait may account for variation in a mutualism among closely related species.

Acknowledgments

We thank C. Cartwright-Finch for her help measuring specimens, M. Akam for the use of his equipment, and J. Brisson for her advice and assistance with preparing the manuscript. We thank two anonymous reviewers for their helpful comments. AWS was supported by a Biotechnology and Biological Sciences Research Council (U.K.) Research Studentship.

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Fig. 1.

Mean mouthpart lengths of 13 *Chaitophorus* species, their feeding position, and their relationship with ants. Inset shows phylogenetic relationships of species. Petiole-feeding aphids have significantly longer mouthparts than leaf-feeding aphids (contrasts at nodes vii, ix, and x on phylogeny; one-sample t -test: $t_3 = 15.018$, $df = 2$, $P = 0.004$).

Fig. 2.

(A) Relationship between mouthpart dimension and escape time, uncorrected for overall body size and phylogenetic position. (B) The relationship is significant for independent contrasts adjusted for body size (labels refer to nodes on phylogeny in Fig. 1; $r^2 = 0.71$, $df = 8$, $P = 0.0024$). (C) Relationship between escape time and predation, uncorrected for phylogenetic position. (D) The relationship is significant for independent contrasts (labels refer to nodes on phylogeny in Fig. 1; $r^2 = 0.852$, $df = 3$, $P = 0.0253$).