

Symbiont modifies host life-history traits that affect gene flow

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The evolution of herbivore–host plant specialization requires low levels of gene flow between populations on alternate plant species. Accordingly, selection for host plant specialization is most effective when genotypes have minimal exposure to, and few mating opportunities with individuals from, alternate habitats. Maternally transmitted bacterial symbionts are common in insect herbivores and can influence host fecundity under a variety of conditions. Symbiont-mediated effects on host life-history strategies, however, are largely unknown. Here, we show that the facultative bacterial symbiont *Candidatus Regiella insecticola* strikingly alters both dispersal and mating in the pea aphid, *Acyrtosiphon pisum*. Pea aphids containing *Regiella* produced only half the number of winged offspring in response to crowding and, for two out of three aphid lineages, altered the timing of sexual reproduction in response to conditions mimicking seasonal changes, than did aphids lacking *Regiella*. These symbiont-associated changes in dispersal and mating are likely to have played a key role in the initiation of genetic differentiation and in the evolution of pea aphid–host plant specialization. As symbionts are widespread in insects, symbiont-induced life history changes may have promoted specialization, and potentially speciation, in many organisms.

Keywords: *Acyrtosiphon pisum*; *Candidatus Regiella insecticola*; symbiosis; wing induction; sexual induction; host plant specialization

1. INTRODUCTION

Limited gene flow between herbivore populations on alternate plant species leads to genetic differentiation, which can include divergence in genes affecting host plant use. If host plant specialization is adaptive, natural selection further restricts gene flow between populations. Thus, because of the broad evolutionary consequences, it is of paramount importance to understand the determination of herbivore life-history traits that affect gene flow.

Many insects, including most Homoptera, harbour vertically transmitted bacterial symbionts (Buchner 1965; Douglas 1989), yet the degree to which symbionts may alter gene flow between herbivore populations is unknown. Pea aphid, *Acyrtosiphon pisum*, houses an obligate bacterial symbiont, *Buchnera*, which synthesizes amino acids. Additionally, any of a suite of facultative bacterial symbionts can also be present. These facultative symbionts are not present in all individuals, and are not necessary for survival and reproduction. Beneficial effects of some of the facultative symbionts include heat shock resistance (Montllor *et al.* 2002), parasitoid resistance (Oliver *et al.* 2003) and host plant adaptation (Tsuchida *et al.* 2004).

In at least some geographical areas, aphids containing the facultative bacterial symbiont *Candidatus Regiella insecticola* (also known as U-type or PAUS, and here, henceforth referred to as *Regiella*; Moran *et al.* 2005) are genetically differentiated from other pea aphids (France, Simon *et al.* 2003; California, T. E. Leonardo, unpublished work), indicating at least partial reproductive isolation. Pea aphids containing *Regiella* are commonly

observed to be more abundant on clover than on alternate plants (Japan, Tsuchida *et al.* 2002; California, USA, Leonardo & Muir 2003; France, Simon *et al.* 2003; England, Ferrari *et al.* 2004). Both symbiont and host genes affect this host plant specialization; while *Regiella* increases clover-specific fecundity in some genotypes (Tsuchida *et al.* 2004) host genes determine specialization for other genotypes (Via *et al.* 2000; Hawthorne & Via 2001).

In this study, we investigate whether *Regiella* might contribute to the evolution of host plant specialization by altering life-history traits that reduce gene flow; specifically, the production of winged and sexual offspring. Although aphids are wingless and asexual throughout much of the year (Dixon 1998), maternal exposure to particular environmental cues alters gene expression in developing offspring. Production of winged offspring can be rapidly induced under crowded conditions. The proximate cues resulting in wing induction are not fully understood, but include direct contact with other aphids, decreased plant nutritional quality, and exposure to natural enemies (Muller *et al.* 1999; Weisser 2001; Mondor *et al.* 2004, 2005; Podjasek *et al.* 2005). Sexual morphs are produced in response to conditions that precede the onset of winter in temperate climates, specifically, increasing dark phase of each day and decreasing temperature (Lees 1989; Via 1992).

Here, we show that *Regiella* reduces winged offspring production and changes the timing of sexual morph production. These altered life-history traits have the potential to reduce gene flow between host populations on alternate plant species, thereby promoting the evolution of host plant specialization.

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2. MATERIAL AND METHODS

(a) Genetic materials

Pea aphid lineages were collected from white clover plants, *Trifolium repens*, in the Central Valley of California and are among those described in Leonardo & Muir (2003). Removal of *Regiella* by ampicillin injection is described in Leonardo (2004). Ampicillin treatment was performed more than 2 years (*ca* 50 generations) before conducting the described experiments.

(b) DNA extraction

DNA was extracted from single aphids using a high salt extraction procedure. Aphids were homogenized in 100 μ l of lysis solution (0.1 M Tris, 0.1 M EDTA, 1% SDS), and incubated at 65 °C for 30 min. Subsequently, 33 μ l of protein precipitation solution (3 M K, 5 M Ac) was added, and the mixture was briefly vortexed and incubated on ice for 20 min. Tubes were spun for 5 min at 14 000 r.p.m., the supernatant removed, and spun again to remove residual proteins. One hundred microlitres of isopropanol was added to the second supernatant to precipitate the DNA, and spun at 14 000 r.p.m. to pellet the DNA. The pellet was washed in ethanol and re-suspended in 20 μ l TE.

(c) Identification of symbiont

To confirm that the symbiont was *R. insecticola*, the 16S gene was amplified and sequenced from each lineage, i.e. T1, T4 and GC15. The 16S gene was amplified with the *Regiella* specific primer U99: ATCGGGGAGTAGCTTGCTAC (Sandström *et al.* 2001) and a general bacterial reverse primer CTACGGTTACCTTGTTACGAC. Amplification cycle was 3 min at 94 °C, followed by 33 cycles of 20 s at 94 °C, 1 min at 54 °C, 1.5 min at 72 °C and then a final 3 min extension at 72 °C. Sequencing was carried out by Genewiz sequencing service.

(d) Confirmation of symbiotype across treatments

Presence/absence of *R. insecticola* within sublineages was verified by performing symbiont-specific PCR to the *Regiella* 16S gene (using the primer set described above). Verification was performed on two adult asexual or sexual female offspring per lineage, collected shortly before or during the crowding and sexual induction experiments.

(e) Wing induction

(i) Experimental design

Less than 24 h of maternal exposure to crowded conditions can result in the production of offspring that will develop into winged adults (Sutherland 1969). We evaluated the wing induction response for two aphid lineages that naturally contained *Regiella* (T1_{Reg}, T4_{Reg}) and for representatives of these lineages from which the symbiont had been experimentally removed by ampicillin treatment (T1_{amp}, T4_{amp}) (Leonardo 2004).

For each lineage and treatment, two to six independent sublineages (depending on the experimental block) were established at least two generations prior to the experiment. Twelve-day-old aphids from these colonies were transferred to fresh plants for 24 h, generating a large number of same-age offspring. When the offspring were 10-days-old they were pooled and randomly assigned to either the crowding or single aphid treatment.

For the crowding treatment, ten, 10-day-old aphids were placed together in Petri dishes containing a single white clover

leaf. Leaf tissue was trimmed to enhance the treatment and to standardize leaf area to *ca* 0.5 cm². Cloverleaf stems were embedded in agar to slow desiccation. There were three experimental blocks, each run on a separate date, and each of which contained both host lineages (T1 and T4) and treatments (control- and ampicillin-treated). The number of replicates per treatment per host lineage was 2, 8 or 12, depending on the block. In all cases, a similar number of control colonies (i.e. single aphids on intact leaves) were evaluated to determine whether there were baseline differences in wing production among lineages. For both treatments, offspring produced over the first 48 h were reared to maturity and scored for wing presence/absence.

(ii) Statistical analysis

To determine if *Regiella* influenced the production of winged offspring under crowded conditions, we conducted a two factor analysis of covariance using JMP IN 5.1 (SAS Institute 2005). Main factors in the analysis were: lineage (T1 versus T4) and *Regiella* (present versus absent). Covariates in our model were: block (1–3) and total offspring born during the experiment (0–44). These covariates controlled for differences in wing-induction responses over different trials and density induced wing-induction responses, respectively. Our dependent variable was the proportion of winged offspring produced. To normalize the data, proportions were transformed ($x' = \arcsin\sqrt{x}$) prior to analysis.

(f) Sexual induction

(i) Experimental design

We evaluated the sexual-induction responses for three aphid lineages that naturally contained *Regiella* (T1_{Reg}, T4_{Reg}, GC15_{Reg}), and for representatives of these lineages from which the symbiont had been experimentally removed by ampicillin treatment (T1_{amp}, T4_{amp}, GC15_{amp}; Leonardo 2004). In contrast to wing induction, aphid embryos require sexual induction cues very early in development. Maternal exposure to suitable conditions produces either grand-daughter or great-granddaughter sexual offspring depending upon the developmental stage of the induced aphid (Lees 1989; Via 1992).

Aphids were reared on white clover plants, covered with cellophane bags, in an incubator at 16 °C and a 12 h light : 12 h dark (12L : 12D) photoperiod, which corresponds to the photoperiod in mid-October for the region where these lineages were collected. To control for grand-maternal and maternal effects three sublineages per lineage per treatment (six for GC15_{amp}) were independently maintained for at least two generations prior to the start of the experiment.

To initiate sexual induction for each sublineage, two 9-day-old aphids (fourth instars or newly moulted adults) were transferred to a single plant and placed in the 12L : 12D induction incubator. Two, 12-day-old adult offspring per sublineage were transferred to fresh plants and allowed to reproduce for 2–4 days. Two of these offspring per sublineage (thus, six replicates per lineage per treatment) were transferred when in their fourth instar to individual Petri dishes containing a clover leaf.

We evaluated the sexual phenotype of all offspring produced by these 'experimental' aphids. Every 3–6 days experimental aphids were transferred to a fresh dish. Offspring were transferred every 4–5 days until the aphids reached adulthood and could be scored as males, female

sexuals or asexuals. In a small number of cases (less than 5% of offspring collections across all replicates), the leaf wilted prematurely resulting in substantial mortality. These collections were not included in the statistical analyses. Additionally, in five of 42 cases the experimental aphid was a sexual female, indicating that induction had happened in the preceding generation. These replicates were also eliminated from the analyses.

(ii) Statistical analysis

Numbers of male, female sexual and asexual offspring produced over the course of the experiment were analysed by Repeated Measures MANOVA, using JMP IN 5.1 (SAS Institute 2005). Independent variables in the analysis were: lineage (T1 versus T4 versus GC15), *Regiella* (present versus absent) and sublineage (nested within lineage). The dependent variables were the numbers of each phenotype produced over successive time periods. To simplify the analysis, numbers of male, female sexual and asexual offspring were analysed separately. It is noted, however, that numbers of morphs are not truly independent due to constraints on aphid reproduction.

3. RESULTS

(a) Confirmation of symbiont presence

The *Regiella* 16S DNA sequences obtained from all three host lineages were identical (Gen Bank accession number DQ228285) and 99.4% similar to *Regiella* 16S reference sequence AY296734 (1336/1344 identical nucleotides). PCR confirmed that all ampicillin-treated sublineages remained negative for the presence of *Regiella*, and that all control sublineages retained *Regiella*.

(b) Wing induction

Symbionts dramatically altered the production of dispersal morphs. *Regiella*-infected aphids produced only half the number of winged offspring, as did aphids from which *Regiella* had been removed ($p=0.015$; figure 1). Though lineage T1 produced over twice as many winged offspring as T4 ($p=0.0033$; figure 1), there was no significant interaction between host lineage and symbiont presence/absence ($p=0.80$). This result indicates that *Regiella* has a similar effect on phenotypic expression across these lineages.

We also examined whether aphid density (the number of adults surviving till the end of the crowding trial) affected the wing induction response and found a slight, but non-significant trend towards larger numbers of winged offspring with higher aphid densities ($p=0.28$). Finally, aphids did not produce any winged offspring when reared singly, suggesting that differences in winged offspring production are the result of symbiont-mediation of an environmentally induced transgenerational effect. It remains possible, however, that *Regiella* may alter winged offspring production even in aphids reared singly, particularly for genotypes that produce higher numbers of winged offspring than the lineages used in our study.

(c) Sexual induction

Symbiont presence affected the production of sexual morphs, but not in all lineages (lineage \times *Regiella*: males, $p=0.044$; female sexuals, $p=0.0009$; asexuals, $p=0.0057$). Symbiont presence had a strong effect on

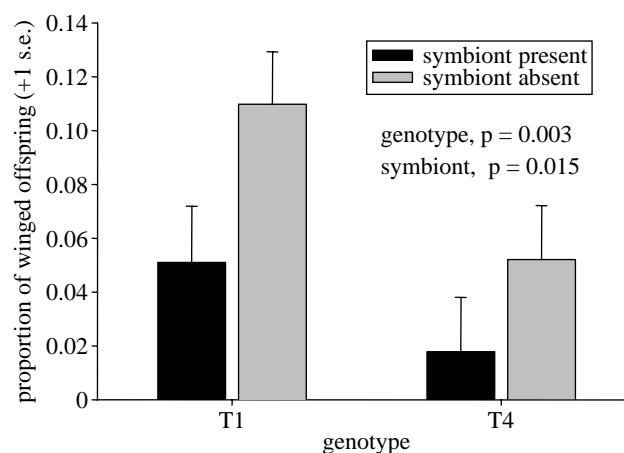


Figure 1. Symbiont alters response to crowding. Production of winged progeny in response to a crowding stimulus for two aphid lineages, T1 and T4, when the symbiont was present (black bars) versus absent (grey bars). Data are pooled from three experimental blocks ($n=2, 8$ and 12 replicates per sublineage per treatment combination; $n=88$ total).

the sexual induction profiles of GC15 and T1, but no effect on T4 (figure 2). Sublineage was only significant in the analysis of lineage T1, influencing the production of female sexuals ($p=0.0016$) and female asexuals ($p=0.0064$). For GC15, *Regiella* removal decreased sexual offspring production (males, $p<0.0008$; female sexuals, $p<0.0006$), while simultaneously increasing asexual offspring production ($p=0.0028$). *Regiella* removal had a similar effect on lineage T1, with a trend towards smaller numbers of males ($p=0.15$), a decreased number of female sexuals ($p<0.0010$) and an increased number of asexual offspring ($p=0.0010$). *Regiella*, however, did not influence the sexual induction profile of lineage T4 (males, $p=0.80$; female sexuals, $p=0.22$; asexuals, $p=0.59$).

4. DISCUSSION

The facultative bacterial symbiont *Candidatus* R. insecticola substantially influenced both wing and sexual induction in the pea aphid. These symbiont-mediated effects are unique from those previously identified in aphids as they are transgenerational (the parental symbiont affects the phenotype of aphid offspring), they involve extreme changes in morphology, and they have the potential to reduce gene flow between host populations.

(a) Understanding sexual induction

A genotype's sexual induction profile stereotypically changes as the light regime changes (MacKay 1987). At short dark phase, reproduction is exclusively asexual. As photoperiod decreases, males are produced. A further reduction in photoperiod causes male production to cease and sexual females to be produced. Some asexual offspring are generally produced at the beginning or end of the reproductive sequence (Lees 1989). Numerous studies have indicated that the critical photoperiods that trigger the production of males and sexual females may differ between genotypes (MacKay 1989; Via 1992). For lineages T1 and GC15, aphids with and without *Regiella* have different critical dark phases for the initiation of male and sexual female production. For these lineages,

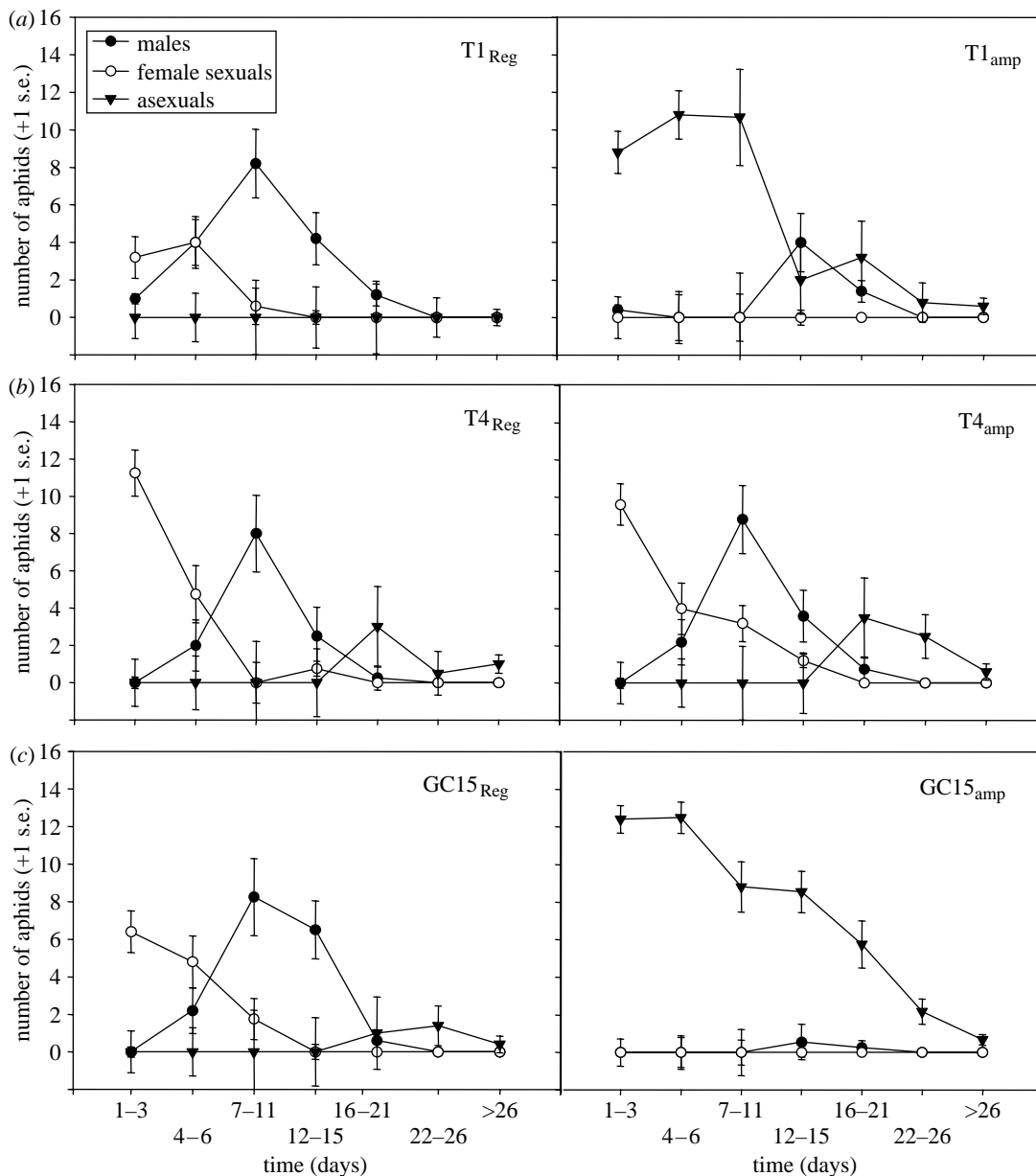


Figure 2. Symbiont alters production of male, sexual female and asexual female morphs. Sexual induction profiles for lineages (a) T1, (b) T4 and (c) GC15 when the symbiont was present (reg) versus absent (amp). Points indicate the mean numbers of male, female sexual, and asexual offspring produced during each time period. *Regiella* presence had a significant effect on the sexual induction profiles of lineages T1 and GC15. Number of replicates: T1_{Reg}, 5; T1_{amp}, 5; T4_{Reg}, 4; T4_{amp}, 5; GC15_{Reg}, 5; GC15_{amp}, 12; $n=36$ total.

Regiella-containing aphids would produce sexual offspring earlier in the season than would aphids from which *Regiella* is absent.

(b) Interactions between host and symbiont genomes

Regiella removal did not affect the sexual induction profiles of all lineages, indicating that the consequences of infection depend upon host and (or) symbiont genotype. While little is known about the role of genetic variation in shaping symbiotic interactions, this is likely to be a general phenomenon. For example, different isolates of the bacterial symbiont, *Halmitonella defensa* conferred different levels of pea aphid resistance to parasitoid attack when in a common host genetic background (Oliver *et al.* 2005). Furthermore, in *Drosophila melanogaster*, *Wolbachia* infection can result in increased, decreased, or unchanged

survival and fecundity depending upon host genetic background (Fry *et al.* 2004). Additional experiments, using a larger number of host and symbiont genotypes, are required to determine the generality of the effects documented here.

(c) Are these traits adaptive?

Direct selection favouring the evolution of modified wing and sexual induction responses is highly plausible. Symbionts with high rates of vertical transmission, such as this one, will spread rapidly if they increase host fitness. In pea aphid, as well as many other aphid species, there has clearly been selection for the evolution of host plant specialization. If specialization is a favourable lifestyle, wing and sexual induction modification would be selected for if they increase the frequency of mating with other individuals using the same resource. Offspring of such

crosses would be more highly specialized, thus with greater fitness.

Symbiont-mediated induction responses could also have evolved as the byproduct of selection on another trait, such as the ability to perform well on the host plant. Wing induction in pea aphid is influenced by host plant quality (Muller *et al.* 1999) and the same is possible for sexual induction. In some genetic backgrounds, *Regiella* has been shown to increase aphid fecundity on clover (Tsuchida *et al.* 2004), suggesting that dispersal or sexual induction might result from *Regiella* increasing aphid nutrient uptake. We believe that nutrient uptake is not likely to be the sole factor resulting in these induction responses, for several reasons. First, for the genotypes used in this study there was no detectable effect of *Regiella* on clover-specific fecundity (Leonardo 2004). Additionally, *Regiella* affected wing induction but not sexual induction of lineage T4, indicating that different mechanisms underlie the production of these two phenotypes.

(d) A role in the evolution of host plant specialization?

The symbiont-mediated changes in dispersal and the timing of mating described here may contribute to the evolution of host plant specialization or reinforce it in cases of secondary contact. *Regiella* may limit gene flow by: (i) reducing movement of *Regiella*-containing aphids between environments, thus increasing selection for performance on particular host plants, and (ii) altering the timing of mating of aphids with and without *Regiella*, thus reducing gene flow between these groups. However, factors controlled for in our experiments may affect the efficacy of these mechanisms under natural conditions. For instance, if sexual morph production is also influenced by host plant species and (or) host plant quality, the role of *Regiella* in limiting gene flow could be largely reduced. Field studies and modelling will be necessary to determine the degree to which symbiont-modified induction responses affect gene flow under field conditions.

As insect symbionts are extremely common (Buchner 1965; Douglas 1989), we suggest that symbiont-induced changes in herbivore life-history patterns may have played a key role in the evolution of herbivore–host plant specialization in many species. Symbionts may promote specialization, and perhaps speciation, by changing countless aspects of a host organism's life-history, including altering host phenotypic expression.

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