Symbiont modifies host life-history traits that affect gene flow

Teresa E. Leonardo\textsuperscript{1,*} and Edward B. Mondor\textsuperscript{2}

\textsuperscript{1}Department of Ecology & Evolutionary Biology, Princeton University, Princeton, NJ 08544, USA
\textsuperscript{2}Kaua‘i Agricultural Research Center, University of Hawai‘i at Manoa, 7370 Kuamo‘o Road, Kapa‘a, HI 96746, USA

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 \textit{Candidatus Regiella insecticola} strikingly alters both dispersal and mating in the pea aphid, \textit{Acyrthosiphon pisum}. Pea aphids containing \textit{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 \textit{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.

\textbf{Keywords:} \textit{Acyrthosiphon pisum}; \textit{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, \textit{Acyrthosiphon pisum}, houses an obligate bacterial symbiont, \textit{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 \textit{Candidatus Regiella insecticola} (also known as U-type or PAUS, and here, henceforth referred to as \textit{Regiella}; Moran et al. 2005) are genetically differentiated from other pea aphids (France, Simon et al. 2003; California, T. E. Leonard, unpublished work), indicating at least partial reproductive isolation. Pea aphids containing \textit{Regiella} are commonly observed to be more abundant on clover than on alternate plants (Japan, Tsuchida et al. 2002; California, USA, Leonardo & Muiru 2003; France, Simon et al. 2003; England, Ferrari et al. 2004). Both symbiont and host genes affect this host plant specialization; while \textit{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 \textit{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 \textit{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.

\* Author for correspondence (teresal@princeton.edu).
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 & Muiru (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 μl of lysis solution (0.1 M Tris, 0.1 M EDTA, 1% SDS), and incubated at 65 °C for 30 min. Subsequently, 33 μl 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 μl 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: ATCGGGGAAGTAGCTTGCTAC (Sandström et al. 2001) and a general bacterial reverse primer CTACGGTTACCTGTAGGAC. 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 symbiont type 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* (*T1Reg, T4Reg, GC15Reg*), and for representatives of these lineages from which the symbiont had been experimentally removed by ampicillin treatment (*T1amp, T4amp, GC15amp*; 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 GC15amp) 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 (four 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.

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 ($\chi^2 = \arcsin(\sqrt{x})$) prior to analysis.

(f) Sexual induction
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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
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Numbers of male, female sexual and asexual offspring were analysed 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.

(ii) Statistical analysis

The 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 lineages 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 × Regiella: males, p = 0.044; female sexuals, p = 0.0009; asexuals, p = 0.0057). Symbiont presence had a strong effect on 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,
Regiella-containing aphids would produce sexual offspring earlier in the season than would aphids from which Regiella is absent.

**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, *Hamiltonella 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.

**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 (Mueller 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 (Tsuihida 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. Symbions 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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