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Facultative endosymbionts mediate dietary breadth in a polyphagous herbivore

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Summary

1. Intraspecific variation in dietary breadth can influence important ecological and evolutionary processes, yet the mechanisms generating this variation are usually unknown. Maternally transmitted bacterial symbionts frequently infect insect herbivores, and many have been shown to mediate key ecological interactions. For polyphagous herbivores, infection with particular symbionts is often strongly correlated with feeding on particular plant species, suggesting that facultative symbionts might directly determine herbivore food plant specificity. However, previous tests of this hypothesis have returned inconsistent results, providing little empirical support for a causal relationship between facultative symbiont infection and dietary breadth.

2. Here, we investigate whether heritable facultative symbionts mediate dietary breadth in the polyphagous aphid, *Aphis craccivora*. We first determined that asexual clones of the aphid differ dramatically in performance across two leguminous food plants, locust and alfalfa, and could be considered biotypes with distinct ecological characteristics. The heritable symbiont *Arsenophonus* is strongly associated with locust-origin aphids.

3. We created experimental lines that share aphid genotypes but differed with respect to *Arsenophonus* infection status, and compared performance across three food plant species. Naturally *Arsenophonus*-infected clones performed 2–4× better on locust and up to 75% worse on two alternate plant species than uninfected controls, clearly demonstrating that *Arsenophonus* promotes specialization on locust. In both laboratory and field experiments, uninfected locust- and alfalfa-origin clones exhibited similar and modest performance on locust, indicating that the 'locust-associated biotype' would not exist without *Arsenophonus*.

4. We also hypothesized that moving *Arsenophonus*, via transinfection, to an alfalfa-origin lineage would improve performance on locust and serve to expand dietary breadth. Indeed, transinfection doubled aphid performance on locust and halved aphid performance on alfalfa. However, because this aphid lineage naturally performs better on alfalfa, the transinfected symbiont functionally equalized aphid performance between locust and alfalfa, making the alfalfa biotype more generalized. Thus, the same symbiont can either reduce or expand dietary breadth, depending on host genotype.

5. Our results unequivocally demonstrate that symbiont gain or loss can instantaneously and substantially change the topology of food plant use in a polyphagous insect, modifying diet in ways that potentially influence the insect's ecological niche, evolutionary trajectory and pest status.

Key-words: *Arsenophonus*, biotype, generalist, *Hamiltonella*, host races, host-associated differentiation, intraspecific variation, microbial symbiosis

Introduction

Dietary breadth is a key descriptive characteristic for herbivorous insects, often defining our expectations for phenomena such as ecological connectivity, co-evolution and speciation (Agosta 2006; Nyman 2010; Janz 2011; Singer *et al.* 2014). Diet is often not homogeneous within a species; polyphagous species are frequently composed of individuals that differ from one another in both identity

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and breadth of resources used (Drès & Mallet 2002; Araújo, Bolnick & Layman 2011). Such intraspecific variation is increasingly recognized as an important driver for community ecology and eco-evolutionary dynamics (Bolnick *et al*. 2011; Hersch-Green, Turley & Johnson 2011; Schoener 2011), yet the breadth of mechanisms underlying intraspecific variation in diet is only beginning to be explored (Araújo, Bolnick & Layman 2011; Janz 2011).

One intriguing hypothesis is that differential infection by facultative bacterial symbionts may play a role in determining food plant usage by herbivorous insects (Leonardo & Muiru 2003). Insects are often infected by maternally inherited symbionts that confer ecologically relevant traits to their host (Feldhaar 2011; White 2011; Oliver & Martinez 2014). Heritable symbionts can improve the environmental tolerance of their hosts and/or provide protection against a range of natural enemies (Oliver *et al*. 2010; Oliver, Smith & Russell 2014). They can also influence the interactions of herbivorous insects with plants (Hosokawa *et al*. 2007; Frago, Dicke & Godfray 2012; Hansen & Moran 2014). Indeed, ancient obligate nutritional endosymbionts allowed the exploitation of nutrient-poor ecological niches, including plant sap, which facilitated the subsequent adaptive radiation of numerous species-rich insect clades (Moran & Telang 1998). Facultative symbionts, which are not strictly required for host survival, have been hypothesized to influence diet breadth either by allowing the use of additional plant species or promoting dietary specialization by increasing performance on one plant species while decreasing performance on others (Frago, Dicke & Godfray 2012). Theoretically, acquisition of microbes that mediate dietary breadth could facilitate use of novel ecological niches, host-associated differentiation and potentially even sympatric speciation (Ferrari & Vavre 2011; Medina, Nachappa & Tamborindeguy 2011; Henry *et al*. 2013). Additionally, symbiont-mediated use of food plants would have important implications for pest management, as symbiont gain or loss may allow previously innocuous species to invade economically important crops, resulting in the creation of novel pests (Hosokawa *et al*. 2007; Brown *et al*. 2014).

To date, however, there is little empirical evidence that facultative symbionts directly affect herbivore dietary breadth (Hansen & Moran 2014). Most studies of the role of symbionts in host plant specificity involve *Acyrthosiphon pisum* (pea aphid), which has diversified into numerous genetically distinct host races that specialize on cultivated legumes (Hawthorne & Via 2001; Ferrari, Via & Godfray 2008; Peccoud *et al*. 2009). While aphid genes generally form the basis for host races in this cyclically parthenogenetic aphid (Hansen & Moran 2014), some host races show significant positive associations with particular symbionts. The most notable association is between clover-feeding *Ac. pisum* and the bacterial symbiont *Regiella insecticola* (Tsuchida *et al*. 2002; Leonardo & Muiru 2003; Ferrari *et al*. 2012; Russell *et al*. 2013). While phylogenetic evidence suggests *Regiella* facilitated host plant shifts to clover (Henry *et al*. 2013), empirical studies on the causative association between *R. insecticola* and clover use have produced inconsistent results. In Japan, Tsuchida, Koga & Fukatsu (2004) found that one strain of *Regiella* infecting one aphid genotype expanded host plant range by dramatically improving performance on clover while having minimal effects on aphid performance on vetch. However, additional studies from North America and Europe, using a larger array of *Ac. pisum* clones and *Regiella* strains, have not found a consistent effect of *Regiella* on food plant specificity (Leonardo 2004; Ferrari, Scarborough & Godfray 2007; McLean *et al*. 2011). Thus, symbiont mediation of herbivore dietary breadth has not been generally established even within *Ac. pisum*, much less other herbivore species.

Here, we investigate the role of facultative symbionts in the dietary breath in *Aphis craccivora* Koch (cowpea aphid). We previously reported a strong correlation between particular facultative symbionts and food plant use in *A. craccivora* (Brady & White 2013; Brady *et al*. 2014), a polyphagous aphid with a cosmopolitan distribution (Blackman & Eastop 2006). In particular, the facultative symbiont *Arsenophonus* was prevalent in *A. craccivora* feeding on black locust (*Robinia pseudoacacia* L.), whereas the facultative symbiont *Hamiltonella defensa* was associated with aphids feeding on alfalfa (*Medicago sativa* L.). Several aphid species, including *A. craccivora*, receive partial to complete protection from hymenopteran parasitoids when infected with *Hamiltonella* (Oliver *et al*. 2003; Schmid *et al*. 2012; Asplen *et al*. 2014). *Arsenophonus* is also widespread in aphids (Jousselin *et al*. 2013), but no functional roles have been established for this symbiont within this host group (Wulff *et al*. 2013).

In the present paper, we test whether *Arsenophonus* influences dietary breadth in *A. craccivora*. First, we established that *A. craccivora* has 'host-associated biotypes' that differ in their performance across locust and alfalfa plants. We are using this term as the parthenogenetic equivalent of host races, as *A. craccivora* is believed to be strictly parthenogenetic over the vast majority of its range (Blackman & Eastop 2006). Secondly, we tested whether *Arsenophonus* promotes specialization on locust in naturally infected clones by experimentally removing the symbiont and comparing performance of differentially infected isolines (which have the same aphid genetic background) across three food plants. Thirdly, we tested whether *Arsenophonus* would have similar effects in a novel genetic background, by transinfecting the symbiont into an alfalfa-origin clone that naturally performs well on alfalfa and poorly on locust, and again comparing performance of isolines across food plant species. Fourthly, we determined the relative importance of facultative symbionts in generating the observed locust-associated biotype by comparing performance of multiple differentially infected locust- and alfalfa-origin aphid clones on locust. Finally, we replicated our experiments in the field, to establish whether our results are robust and relevant to the ecology of the aphid under natural conditions.

Materials and methods

ORIGIN AND MAINTENANCE OF LINES

Aphis craccivora clones were collected in central Kentucky, USA, from one of two plants: black locust or alfalfa. Clonal lines were initiated from single, parthenogenetic females and maintained separately on fava (*Vicia faba* L.) in 3·78-L plastic cages with mesh panels for ventilation (White *et al.* 2009), at 16 L:8 D and 22 °C. Fava is an acceptable host for all tested clones of *A. craccivora*, as it is for *Ac. pisum* (Ferrari, Via & Godfray 2008). Clonal progeny were transferred to fresh fava plants at approximately 2-week intervals. Experimental lines are listed in Table S1 (Supporting information), along with their microsatellite profiles. Primers and reaction conditions for microsatellite loci can be found in Dykstra *et al.* (2014).

EXPERIMENTS 1A AND 1B: DOES *APHIS CRACCIVORA* HAVE HOST-ASSOCIATED BIOTYPES?

We preliminarily tested whether *A. craccivora* clones show differential performance across two food plants in the field. We conducted two experiments to compare performance among four locust-origin aphid clones (LW, LE, LJ and LS) and four alfalfa-origin clones (AC, AO, AL and AV). Experiment 1a compared clone performance in alfalfa, in a managed field at the University of Kentucky's Spindletop Research Farm (Fayette Co., KY; 38°07′N 84°31′W). Eighty plants, separated from each other by a minimum of 1 m, were randomly assigned to one of the eight aphid clones, and we enclosed a single stem of each plant in a polyester organza cage (30 × 18 cm) with 10 fourth instar aphids. Experiment 1b compared clone performance on locust saplings at the University of Kentucky Ecological Research and Education Center (Fayette Co., KY; 38°05′N 84°29′W). Sixty-four actively growing branch tips from sapling trees (1–3 m tall) were each randomly assigned to an aphid clone, and each tip was caged with 10 fourth instar aphids. For both experiments, aphids were recollected and counted after 10 days. Cages containing predators were excluded from final data analysis, resulting in a final sample size of 5–10 experimental units per aphid clone per experiment. For each experiment, we ln-transformed final aphid population size per plant and compared clone performance in SPSS (v 21, IBM Corp., Armonk, NY, USA) using a one-way ANOVA followed by a planned contrast to compare the locust-origin clones with the alfalfa-origin clones.

ENDOSYMBIONT IDENTIFICATION AND ESTABLISHMENT OF EXPERIMENTAL LINES

We screened the aphid clones for common aphid endosymbionts using (i) symbiont-specific diagnostic PCR for all known *A. craccivora* symbionts and (ii) denaturing gradient gel electrophoresis (DGGE) following amplification of a variable region of 16S rRNA to detect any unexpected symbionts. Diagnostic PCR was conducted using previously published primers and protocols (Brady & White 2013; Brady *et al.* 2014) following a 10% w/v Chelex DNA extraction (Wulff *et al.* 2013), and DGGE primers and protocols were conducted as in Dykstra *et al.* (2014). Most locust-origin clones were infected with *Arsenophonus*, whereas most alfalfa-origin clones were infected with *Hamiltonella* (Table S1); no other facultative symbionts were detected in our experimental lines. *Hamiltonella* is readily lost in laboratory colonies through imperfect vertical transmission and fitness differentials between uninfected and *Hamiltonella*-infected individuals (Dykstra *et al.* 2014). Therefore, at time of experimental use, multiple clones were available that originated from alfalfa but were negative for *Hamiltonella*.

To create experimental lines that shared the same aphid genotype (i.e. isoline) but differed with respect to symbiont infection status, we first used an antibiotic curing protocol, modified from Douglas, Francois & Minto (2006), to selectively cure two *Arsenophonus*-infected clones (from locust) and one *Hamiltonella*-infected clone (from alfalfa). In brief, infected fourth instar aphids were fed on artificial diet infused with 50 μg mL^{-1} gentamicin, cefotaxime and/or ampicillin for 3 days, then individually transferred to and reared on fava. Offspring were tested for symbiont infection using diagnostic PCR (Brady & White 2013); clonal siblings from individuals testing negative were used to initiate cured lineages of each initial aphid clone. Aphids used in experiments were at least 10 generations removed from antibiotic treatment. Infection status of all clones was validated periodically using diagnostic PCR for *Arsenophonus* and/or *Hamiltonella*, as appropriate. All experimentally manipulated clones exhibited distinct genotypes as indicated by microsatellite profiles (Table S1).

We subsequently used haemolymph microinjection to transinfect *Arsenophonus* from a locust-origin clone (LE) to an alfalfa-origin clone (AL). Individual donor aphids and recipient aphids were restrained with a vacuum apparatus and microinjected as described in Wulff *et al.* (2013). Surviving recipient aphids were individually reared on fava leaves imbedded in 1% agar in 35-mm Petri dishes until offspring were produced. Following 5 days of reproduction, aphids were screened for *Arsenophonus* using diagnostic PCR as described above; offspring of aphids testing positive for this symbiont were retained for continued rearing. This procedure was repeated for three generations, after which a single stably infected aphid was used to initiate the AL *Ars*+ clonal line. Subsequent generations were screened to verify stable *Arsenophonus* infection; experimental aphids were approximately 10 generations removed from injection.

EXPERIMENTS 2A, 2B AND 2C: DOES *ARSENOPHONUS* AFFECT LOCUST-ORIGIN APHID PERFORMANCE ACROSS HOST PLANTS?

Because locust-origin clones of *A. craccivora* are predominantly associated with the symbiont *Arsenophonus* (Brady & White 2013; Brady *et al.* 2014), we tested whether this symbiont differentially affects aphid fitness on locust vs. other plants. We evaluated population growth of *Arsenophonus*-infected vs. cured aphid isolines across three plants: black locust, alfalfa and fava. We replicated this greenhouse experiment three times: Experiments 2a and 2b each evaluated a single *Ars*+/− isoline pair (Total $N = 36$) and Experiment 2c evaluated both locust-origin isoline pairs simultaneously (Total $N = 60$). In Experiments 2a and 2b, we infested each of 12 pots per plant species with 10 fourth instar aphids from either the *Arsenophonus*-infected or the cured isoline. Black locust was seed propagated at a commercial nursery and *c.* 1 year old at the time of purchase. Each seedling was planted in a 28-cm nursery pot filled with ProMix potting media and was maintained in the greenhouse at 16 L:8 D, 22 ± 3 °C for at least 2 months prior to experimental use. Fava (var. Windsor) was propagated from seed, two seeds per 10-cm pot, and was a minimum of 10 days old before experimental use. Alfalfa (var. Summer) was seeded at 10 seeds per 10-cm pot and was at least 17 days old before experimental use. For all pots, the surface of the soil was covered with plaster of Paris around the growing plants, to provide visual contrast for aphid counting at the conclusion of the experiment. Ten aphids were enclosed on each plant type in an organza cage. Each locust cage enclosed a single branch tip, whereas fava and alfalfa cages enclosed all plant material in the pot. Enclosed plant material from all three species was approximately equal and did not limit aphid population growth within the time span of the experiment. After 10 days in the greenhouse, final aphid numbers were recorded per pot. Experiment 2c was identical, except (i) black

locust was also grown from seed and at least 7 weeks old (*c*. 15 cm high) at the time of experimental use and (ii) the sample size per treatment was reduced to five pots per plant species (3 factor levels) per infection status (2 factor levels) per isoline (2 factor levels), to accommodate inclusion of both isoline pairs simultaneously ($N = 60$ pots total). Final aphid population size per pot was ln-transformed, and data were analysed using PROC GLM with type III SS in SAS (v. 9.3, SAS Institute, Cary, NC, USA), with plant and infection status incorporated as fixed factors and aphid clone incorporated as a random factor in Experiment 2c.

EXPERIMENT 3: DOES *ARSENOPHONUS* SIMILARLY AFFECT THE PERFORMANCE OF ALFALFA-ORIGIN APHIDS ACROSS HOST PLANTS?

To determine whether *Arsenophonus* had similar effects in a novel genetic background, we evaluated the effects of the symbiont in our transinfected alfalfa-origin clone (AL). This experiment was conducted 8 months after the previous experiments, once the artificially infected line became available. We compared aphid population growth of AL *Ars*+ and AL *Ars*−, as well as the previously tested LW isoline pair, across the three plant species as in the previous experiment ($N = 60$ pots total), except that this experiment was conducted in the laboratory rather than greenhouse to maximize containment of the transinfected clone. Caged plants were maintained under our standard colony maintenance conditions at 22 ± 1 °C under fluorescent shop lights at 16 L:8 D for the 10-day duration of the experiment. Ln-transformed data were analysed as described previously; one datapoint was excluded from final analysis due to the presence of a predatory hemipteran within the cage.

EXPERIMENT 4: IS THE LOCUST-ASSOCIATED BIOTYPE ATTRIBUTABLE TO SYMBIONTS?

Experiment 4 was similar to Experiment 1b, in which we compared the performance of locust-origin and alfalfa-origin clones on locust. However, Experiment 4 focused on the degree to which endosymbionts might contribute to host-associated biotypes in *A. craccivora* by comparing clones of known infection status that originated from both plant species. We tested the performance of 11 clones on locust in the greenhouse: six clones that originated from locust and five that originated from alfalfa. Some of these clones were naturally infected with their characteristic facultative endosymbiont and some lacked facultative endosymbionts, either though antibiotic curing or natural loss (Table S1). We conducted this experiment in the same manner as the previous greenhouse experiments: we used 6-week-old locust seedlings, with five locust pots per aphid clone (Total $N = 55$), each infested with 10 fourth instar aphids that were allowed to reproduce for 10 days. We analysed ln-transformed data using one-way ANOVA in SPSS followed by two planned contrasts. First, we contrasted the performance of the three *Arsenophonus*-infected locust-origin vs. two *Hamiltonella*-infected alfalfa-origin clones, to verify that host-associated clones showed the expected differential performance pattern when their characteristic endosymbionts were present. Secondly, we contrasted performance of the three uninfected locust-origin vs. three uninfected alfalfa-origin clones, to determine whether the clones retained a signature of host plant specialization when their facultative endosymbionts were absent.

EXPERIMENTS 5A AND 5B: FIELD VALIDATION

To validate the patterns we observed under controlled greenhouse settings, we compared the performance of aphid clones on locust in the field in two replicate experiments. Each experiment evaluated both locust-origin isoline pairs (LW *Ars*+ and LW *Ars*−, LE *Ars*+ and LE *Ars*−); Experiment 5a also included unrelated *Hamiltonella*-infected (AC *Ham*+) vs. uninfected (AV *Ham*−) alfalfa-origin clones, whereas Experiment 5b included the alfalfa-origin isoline pair (AC *Ham*+ and AC *Ham*−). We conducted these experiments at the University of Kentucky's Ecological Research and Education Center using the same field protocol as described previously with eight branch tips per aphid clone (Total $N = 48$) per experiment. We again used one-way ANOVA on square-root- or ln-transformed data in SPSS to compare final population size among treatments for each experiment. We used planned contrasts to compare (i) the performance of each *Arsenophonus*-infected clone to its corresponding uninfected isoline, (ii) the performance of naturally *Arsenophonus*-infected locust-origin clones to the naturally *Hamiltonella*-infected alfalfa-origin clone and (iii) the performance of the uninfected locust-origin clones to the uninfected alfalfa-origin clone.

Results

APHIS CRACCIVORA HAS HOST-ASSOCIATED BIOTYPES (EXPERIMENTS 1A AND 1B)

When we compared performance between four locust-origin and four alfalfa-origin aphid clones on the two plant species in the field, we found that clones performed significantly better on their host plant of origin than clones originating from the alternative host plant (Fig. 1). After 10 days on alfalfa, the mean population of alfalfa-origin clones was >10× higher than locust-origin clones (ANOVA $F_{7,64} = 9{\cdot}74$, $P < 0{\cdot}001$; planned contrast $t_{64} = 7{\cdot}2$, $P < 0{\cdot}001$; Fig. 1a). Similarly, on locust, mean population size of locust-origin clones was *c*. 5× higher than alfalfa-origin clones (ANOVA $F_{7,44} = 4{\cdot}52$, $P < 0{\cdot}001$; planned contrast $t_{44} = -4{\cdot}34$, $P < 0{\cdot}001$; Fig. 1b). However, one locust-origin clone, LS, performed relatively poorly on locust and was approximately equivalent to the best performing alfalfa-origin clone (AL). Upon investigation for facultative symbionts, clone LS proved to be the only locust-origin clone naturally lacking infection with *Arsenophonus* (Table S1), suggesting that *Arsenophonus* may influence aphid performance on locust. This hypothesis was experimentally tested with the next series of experiments.

NATURAL *ARSENOPHONUS* INFECTION PROMOTES APHID SPECIALIZATION ON LOCUST (EXPERIMENTS 2A, 2B AND 2C)

When we compared population growth of naturally *Arsenophonus*-infected clones to cured experimental isolines sharing the same aphid genotype, we found that *Arsenophonus* promoted dietary specialization, strongly improving aphid performance on locust, but decreasing performance on two other plants, alfalfa and fava. In three replicate greenhouse experiments, we found a significant interaction between *Arsenophonus* infection and performance on specific food plants (Fig. 2, Table S2). On locust, *Arsenophonus*-infected populations were 2–4× larger than uninfected populations

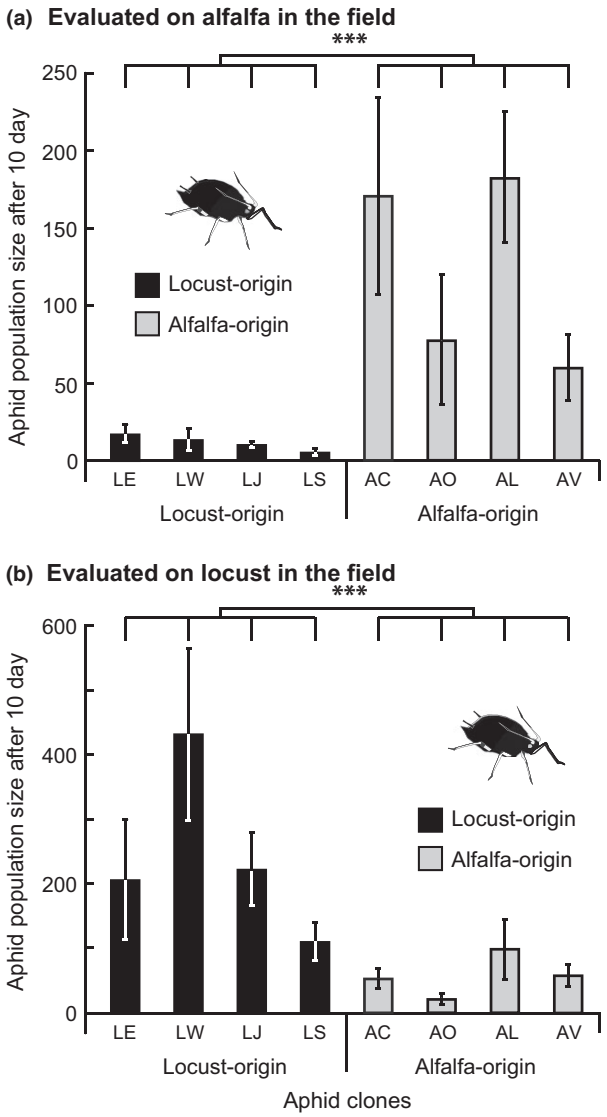

Fig. 1. *Aphis craccivora* clones perform best on their plant of origin. Mean ± SE population size of locust-origin and alfalfa-origin *A. craccivora* clones after 10 days in the field (a) on alfalfa (Experiment 1a) and (b) on locust (Experiment 1b). Aphid clones (*x*-axes) fully described in Table S1. Brackets indicate treatments that were contrasted statistically: *** indicates $P < 0{\cdot}001$.

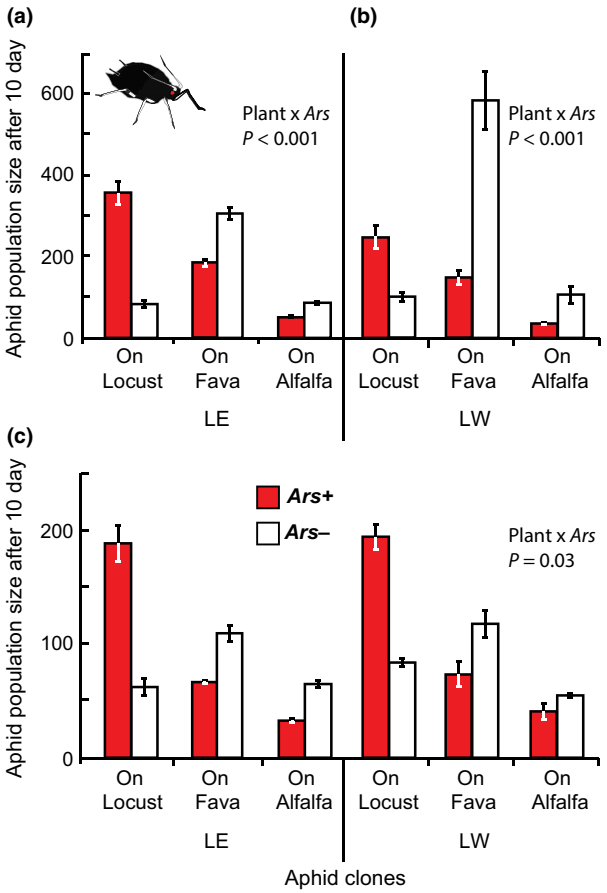

Fig. 2. Three replicate experiments demonstrate that natural *Arsenophonus* infection promotes aphid specialization on locust. Mean ± SE population size of *Arsenophonus*-infected (*Ars*+) locust-origin clones vs. experimentally cured (*Ars*−) isolines after 10 days on one of three plant species in the greenhouse. (a) Experiment 2a tested performance of the LE clone, (b) Experiment 2b tested performance of the LW clone, and (c) Experiment 2c included both the LE and the LW clones of *Aphis craccivora*. In all experiments, *Ars*+ clones performed best on locust and worst on alfalfa, while *Ars*− performed best on the universal host fava, but poorly on both locust and alfalfa. In contrasts between infected and uninfected isolines, *Ars*+ clones performed 2–4× better than *Ars*− on locust, but 25–75% worse than *Ars*− on fava and alfalfa.

after 10 days. In contrast, on fava and alfalfa, *Arsenophonus* infection reduced overall aphid population growth relative to uninfected aphids, by 30–60% on fava and 25–70% on alfalfa. In Experiment 2c, we evaluated performance of two different *Arsenophonus*+/− isoline pairs simultaneously and found no significant main or interactive effects of 'clone' (Table S2), indicating that *Arsenophonus* improved aphid performance on locust and decreased aphid performance on other plants in both of these aphid genotypes. We next tested whether *Arsenophonus* would have a similar effect when transferred into a completely novel genetic background, that of an alfalfa-origin aphid clone that naturally performs poorly on locust and well on alfalfa.

TRANSINFECTED *ARSENOPHONUS* MAKES ALFALFA-ORIGIN APHIDS MORE GENERALIZED (EXPERIMENT 3)

In an alfalfa-origin line transinfected with *Arsenophonus* (AL *Ars*+), infection doubled aphid performance on locust, slightly reduced performance on fava and halved performance on alfalfa relative to the uninfected isoline (AL *Ars*−; Fig. 3), a comparable effect as seen in the locust-origin clones (Fig. 2). However, because this uninfected alfalfa-origin clone naturally performs *c.* 4× better on alfalfa than locust (Fig. 3), *Arsenophonus*-mediated benefits on locust, and costs on alfalfa, effectively acted to equalize aphid performance across food plants, rendering this alfalfa-specialist clone into a more generalized feeder.

We also included one of the previously tested locust-origin isoline pairs (LW) in this experiment and found a

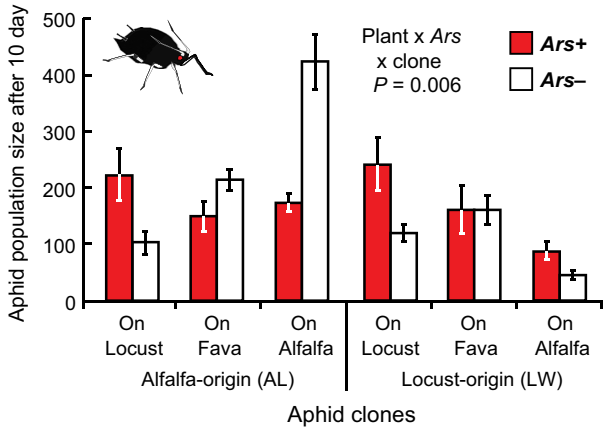

Fig. 3. Transinfection of *Arsenophonus* into an alfalfa-origin clone equalizes aphid performance across plants. Experiment 3: mean ± SE population size of *Arsenophonus*-infected (*Ars*+) and uninfected (*Ars*−) aphid clones after 10 days feeding on one of three plant species in the laboratory. The naturally uninfected alfalfa-origin clone (AL *Ars*−) performed 4× better on alfalfa than locust, whereas the transfected AL *Ars*+ clone performed moderately and equivalently across all three plant species. In contrasts between infected and uninfected AL isolines, the *Ars*+ clone performed 2× better than *Ars*− on locust, but 50% worse on alfalfa. A locust-origin clone (LW) was tested simultaneously as a control; consistent with previous experiments (Fig. 2), the *Ars*+ clone performed best on locust and *Ars*− clone best on fava.

strong three-way interaction between plant species, *Arsenophonus* infection and clone (Table S2), primarily driven by differences in performance among the clones on alfalfa. In the locust-origin LW clone, *Arsenophonus* also doubled performance on locust, but in contrast with previous experiments (Fig. 2) had no effect on performance on fava, and somewhat improved performance on alfalfa relative to the uninfected isoline (Fig. 3). Nevertheless, both the LW *Ars*+ and LW *Ars*− clones showed similar patterns of performance across host plants as previous experiments, with the *Arsenophonus*-infected clone performing best on locust (more locust specialized) and the uninfected clone performing best on fava (not locust specialized). The newly transinfected AL clone performed just as well on locust as the locust-origin LW clone when both had *Arsenophonus*, and when *Arsenophonus* was lacking, the locust-origin LW clone performed as poorly on locust as the alfalfa-origin AL clone. This pattern suggested that the locust-associated biotype might be primarily a function of *Arsenophonus* infection, rather than a historical association between an aphid clone and host plant. The inference that *Arsenophonus* underlies the locust-associated biotype was further supported in the next experiment.

THE LOCUST-ASSOCIATED BIOTYPE OF *APHIS CRACCIVORA* IS ATTRIBUTABLE TO SYMBIONTS (EXPERIMENT 4)

When we tested the performance on locust of multiple aphid clones originating from each host plant, we found that symbionts were a more important predictor of performance than plant of origin. Overall population size differed significantly among aphid clones after 10 days ($F_{10,44} = 43{\cdot}56$, $P < 0{\cdot}001$; Fig. 4). When infected with their natural symbionts, locust-origin clones performed 5× better on locust than alfalfa-origin clones ($t_{44} = 17{\cdot}94$, $P < 0{\cdot}001$), but when the symbionts were absent, there was no difference in performance between locust- and alfalfa-origin clones ($t_{44} = -0{\cdot}26$, $P = 0{\cdot}80$). Thus, following *Arsenophonus* removal, the locust-associated biotype performed no better on locust than the alfalfa-associated biotype.

FIELD RESULTS RECAPITULATE GREENHOUSE FINDINGS (EXPERIMENTS 5A AND 5B)

Field experiments corroborated greenhouse results (Fig. 5). *Arsenophonus*-positive clones performed 2–6× better on locust than their cured isolines (Exp. 5a: $t_{42} = 9{\cdot}9$, $P < 0{\cdot}001$; Exp. 5b: $t_{40} = 7{\cdot}6$, $P < 0{\cdot}001$), which was very comparable to the greenhouse results (Fig. 2). The locust-origin clones also performed 8–31× better than alfalfa-origin clones when all were infected with their natural symbionts (Exp. 5a: $t_{42} = 12{\cdot}3$, $P < 0{\cdot}001$; Exp. 5b: $t_{40} = 11{\cdot}4$, $P < 0{\cdot}001$), again comparable to differences observed in previous experiments. However, this high

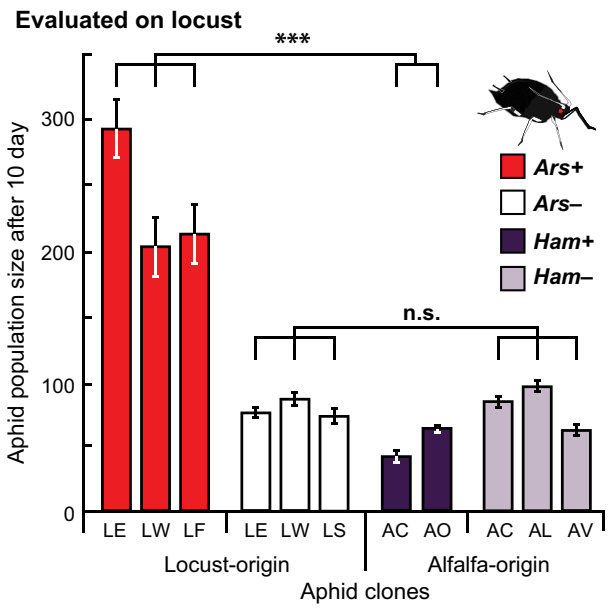

Fig. 4. Without *Arsenophonus,* locust-origin aphid clones perform no better on locust than alfalfa-origin clones. Experiment 4: mean ± SE population size of *Arsenophonus* and *Hamiltonella*-infected and uninfected clones on locust after 10 days in the greenhouse. Brackets indicate treatments that were contrasted statistically: *** indicates $P < 0{\cdot}001$ and n.s. indicates nonsignificant. When infected with their respective natural symbionts, locust-origin clones substantially outperformed alfalfa-origin clones, but when the symbionts were absent, performance of locust-origin and alfalfa-origin clones was indistinguishable.

performance of locust-origin aphids on locust was erased when *Arsenophonus* was removed, and we observed no statistical difference between locust- and alfalfa-origin aphids when their symbionts were absent (Exp. 5a: $t_{42} = 0{\cdot}7$, $P = 0{\cdot}52$; Exp. 5b: $t_{40} = 1{\cdot}4$, $P = 0{\cdot}18$).

Discussion

We provide the first unambiguous demonstration that diet breadth in a polyphagous herbivore is mediated by heritable facultative symbionts. We first established that *A. craccivora* is composed of host-associated biotypes that differ dramatically in their performance across two common food plants, locust and alfalfa. We then showed that dietary specialization on locust in this aphid is mediated by the heritable bacterial symbiont *Arsenophonus*; clonal lines infected with *Arsenophonus* exhibited greatly improved performance on locust relative to uninfected controls sharing the same aphid genotype, while *Arsenophonus* infection reduced aphid performance on two alternate host plants. This effect was consistent across naturally infected and artificially transinfected aphids, but the outcome differed with respect to resultant dietary breadth: in the transinfected clone that originated from alfalfa, *Arsenophonus* equalized performance across alfalfa and locust, expanding dietary breadth and making the aphid more generalized, whereas in naturally infected aphid clones, *Arsenophonus* increased dietary specialization on locust. In fact, locust-origin clones with their *Arsenophonus* removed perform no better on locust than uninfected clones originating from alfalfa, indicating that the locust-associated biotype is largely attributable to bacterial symbionts. Previous diagnostic surveys corroborate this finding, showing that *Arsenophonus* infections are highly prevalent in *A. craccivora* collected from locust, but less common in aphids from other food plants (Brady & White 2013; Brady *et al.* 2014).

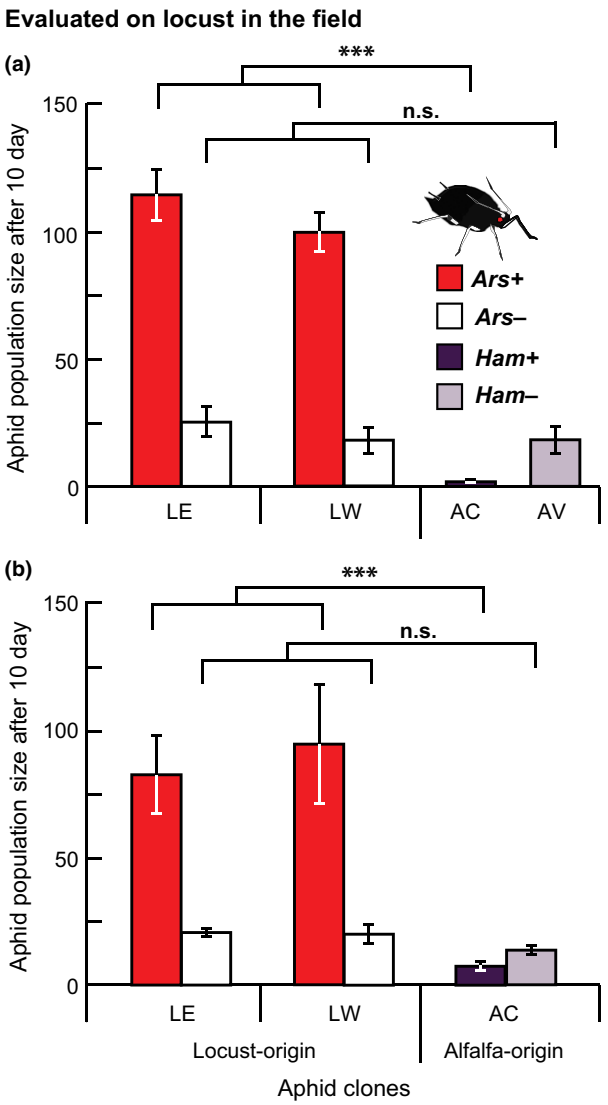

Fig. 5. Field results validate that locust-origin *Aphis craccivora* do not perform well on locust without *Arsenophonus*. Mean ± SE population size of *Arsenophonus*-infected (*Ars*+), *Arsenophonus*-uninfected (*Ars*−), *Hamiltonella*-infected (*Ham*+) and *Hamiltonella*-uninfected (*Ham*−) aphid clones on locust after 10 days in the field. (a) Experiment 5a and (b) Experiment 5b were performed similarly, but differed in the identity of the *Ham*− clone. Brackets indicate treatments that were contrasted statistically: *** indicates $P < 0{\cdot}001$ and n.s. indicates nonsignificant.

The effect of *Arsenophonus* in determining *A. craccivora* diet breadth appears to be more general than that of *Regiella* in *Ac. pisum*, which has had inconsistent effects on aphid performance on clover among studies (Leonardo 2004; Tsuchida, Koga & Fukatsu 2004; Ferrari, Scarborough & Godfray 2007; McLean *et al.* 2011; Tsuchida *et al.* 2011). *Arsenophonus*, in contrast, improved *A. craccivora* performance on locust in every instance across multiple laboratory and field experiments that comprised multiple aphid genotypes. *Arsenophonus* also consistently decreased performance on other food plants, excepting one instance. Very few studies have evaluated symbiont effects on natural populations of insects (Harmon, Moran & Ives 2009). That we were able to confirm laboratory findings under field conditions provides confidence of their importance in nature. Notably, the genetic diversity of *A. craccivora* and *Arsenophonus* used in the present study was limited. Of four *A. craccivora* accessions originating from locust, only one (the LW clone) could be identified as genetically distinct from the others using seven microsatellite loci (Table S1). This level of genetic homogeneity is consistent with that exhibited by other invasive aphid species that lack sexual reproduction (Harrison & Mondor 2011; Caron, Ede & Sunnucks 2014), and indicates that our results are likely ecologically relevant over a widespread geographical range. In asexual species, such as *A. craccivora*, the presence or absence of particular maternally transmitted symbionts may comprise the bulk of heritable genetic variation, and shifts to new ecological niches may require such microbial facilitators.

Many herbivorous insects, including *A. craccivora*, are composed of host races or host-associated biotypes (Drès

& Mallet 2002). In our study, we show that the locust-associated biotype of *A. craccivora* is largely driven by the symbiont *Arsenophonus*. In both the greenhouse and the field, cured locust-origin and alfalfa-origin clones of *A. craccivora* performed modestly and equivalently on locust. It was only in the presence of *Arsenophonus* that a locust-associated biotype was evident. The additional presence of *Hamiltonella* in some alfalfa-origin clones likely further reinforces divergent host plant usage (Brady & White 2013; Brady *et al*. 2014). In contrast with the food plant-specific costs and benefits demonstrated by *Arsenophonus*, *Hamiltonella* exerts a general fitness cost on infected aphids in exchange for providing parasitism protection (Oliver *et al*. 2008; Vorburger & Gouskov 2011; Dykstra *et al*. 2014). Consequently, it is not surprising that alfalfa-origin aphids infected with *Hamiltonella* tended to perform even more poorly on locust than their uninfected counterparts (Fig. 4). Ongoing studies will assess whether differentially infected *A. craccivora* biotypes also exhibit distinct food plant preferences, which would bolster distinct evolutionary trajectories among lineages.

Symbiont mediation of diet breadth can have important ecological and evolutionary consequences. Niche expansion or restriction can affect the biotic networks and environmental conditions experienced by herbivores, generating disruptive selection among biotypes with different plant associations and potentially driving speciation (Stireman, Nason & Heard 2005; Matsubayashi, Ohshima & Nosil 2010). As mediated by facultative symbionts, these processes of change may be particularly abrupt: facultative symbionts can be gained via horizontal transfer events (Russell *et al*. 2003; Oliver *et al*. 2008; Caspi-Fluger *et al*. 2012; Gehrer & Vorburger 2012) or lost through imperfect vertical transmission (Dykstra *et al*. 2014). Our data show that either symbiont gain or loss can rapidly and dramatically alter the topology of food plant use for an herbivore, abruptly changing the ecological specifications of the lineage in potentially unexpected ways. Where these changes improve performance on economically important food plant species, a previously innocuous herbivore may become a pest of consequence. We therefore suggest that emergent pests can potentially arise as a result of symbiont transmission dynamics.

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Data accessibility

Data deposited in the Dryad Digital Repository: http://doi.org/10.5061/dryad.f7301 (Wagner *et al*. 2015).

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Supporting Information

Additional Supporting information may be found in the online version of this article:

Table S1. Aphid clone origins and characterization.
Table S2. ANOVA tables for Experiments 2a, 2b, 2c, and 3.