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Differential aphid toxicity to ladybeetles is not a function of host plant or facultative bacterial symbionts

Jennifer A. White*, Joshua S. McCord, Kelly A. Jackson, Allison C. Dehnel and Paul A. Lenhart

Department of Entomology, University of Kentucky, Lexington, KY 40515, USA

Summary

1. Herbivores often defend themselves from predation by transmitting toxic plant-produced chemicals to their enemies. Polyphagous herbivores sometimes exhibit differential toxicity when found on various host plant species, which is generally assumed to reflect variation in plant chemistry.

2. Here, however, we provide evidence that host-associated herbivore lineages can intrinsically differ in their toxic properties. Lineages of *Aphis craccivora* originating from black locust (*Robinia pseudoacacia*) are unsuitable food for the ladybeetle *Harmonia axyridis*, resulting in death of both larvae and adults, whereas aphid lineages originating from alfalfa (*Medicago sativa*) support larval development and adult reproduction. We show that locust-origin aphids remain toxic and alfalfa-origin aphids remain non-toxic when reared on any of three legume plants (fava, alfalfa or locust).

3. Furthermore, toxicity is not a function of the facultative bacterial symbiont *Arsenophonus*, which is naturally present in locust-origin aphid lineages and facilitates aphid use of locust. Experimentally cured locust-origin lineages remain toxic, and an experimentally transinfected alfalfa-origin lineage remains non-toxic to *H. axyridis*.

4. Instead, *Arsenophonus* plays an indirect role in the distribution of toxic aphid lineages by facilitating aphid use of locust. It is the parthenogenetic coinheritance of *Arsenophonus* and the toxic trait that observationally correlates locust-feeding with toxicity in *A. craccivora*, rather than host plant chemistry *per se*.

5. Our results clearly demonstrate that aphid lineages intrinsically vary in their toxic properties in a way that neither plant chemistry nor bacterial symbionts can explain. A more inclusive paradigm is needed for understanding variation in herbivore defence against predators.

Key-words: defence, facultative bacterial symbionts, polyphagy, sequestration

Introduction

Protection against natural enemies is critical for survival, and different species have evolved a myriad of defences against potential consumers (Edmunds 1974). Despite this pivotal importance, defensive properties can vary substantially among individuals and populations within a species (Miyatake *et al.* 2004; Gols *et al.* 2008), in ways that can differentially affect associated consumers and communities (Bolnick *et al.* 2011). Mechanistically, intraspecific variation in anti-predator defence can be due to factors such as phenotypic plasticity (Agrawal 2001), symbiont infection (Haine 2008) or genotype (Brodie, Ridenhour & Brodie 2002), and elucidating the source of variation can often be difficult. Chemical defences, which are widespread among insect herbivores, are often thought to be derived from plant metabolites (Schmidt 1990). These products may be passed directly to the herbivore's enemies in unmodified form, or first sequestered and/or altered via a variety of biosynthetic pathways (Nishida 2002; Opitz & Müller 2009). When populations of the same insect on different host plants vary in their defensive properties, the standing paradigm is that these differences reflect underlying variation in plant chemistry (Michaud 2005; Ode 2006; Hodek & Evans 2012). For example, the classic sequestration defence of monarch butterflies varies among milkweed host plant species, depending on plant production of cardiac glycosides (Brower *et al.* 1968).

The cowpea aphid, *Aphis craccivora* Koch, would appear to fit within this paradigm, showing host plant-associated variation in defence against predatory ladybeetles (Hodek

*Correspondence author. E-mail: jenawhite@uky.edu

& Evans 2012). This aphid is entirely parthenogenetic and broadly polyphagous, occurring on over 19 plant families, with particularly high prevalence on legumes (Fabaceae) (Blackman & Eastop 2007). When *A. craccivora* was tested as prey for the multicoloured Asian ladybeetle, *Harmonia axyridis* (Pallas), aphid populations drawn from black locust (*Robinia pseudoacacia* L.) were found to be lethal, whereas populations from other host plants were entirely suitable for ladybeetle development (Hukusima & Kamei 1970; Kamo, Tokuoka & Miyazaki 2010). Subsequent bioassays of various chemicals isolated from the foliage of black locust identified three compounds, canavanine, ethanolamine and cyanamide, which caused mortality of the ladybeetle (Obatake & Suzuki 1985; Kamo, Tokuoka & Miyazaki 2012). Cyanamide was not present in common vetch (Kamo, Tokuoka & Miyazaki 2012), a plant from which *A. craccivora* populations had previously been shown to be suitable for *H. axyridis* (Hukusima & Kamei 1970), further supporting the idea that the toxicity of locust-origin *A. craccivora* is a by-product of plant chemistry.

An alternative hypothesis, however, is that the aphids vary intrinsically in their toxicity, independent of host plant. The aforementioned studies all used wild-collected *A. craccivora* populations (Takeda, Hukusima & Yamada 1964; Hukusima & Kamei 1970; Kamo, Tokuoka & Miyazaki 2010, 2012), which may have differed from one another in more respects than their current host plant. *Aphis craccivora* populations collected from different host plants show evidence of genetic structuring (Brady *et al.* 2014) and are often infected with different heritable bacterial endosymbionts (Brady & White 2013; Brady *et al.* 2014). In fact, most locust-origin *A. craccivora* are infected with a strain of the maternally inherited bacteria *Arsenophonus*, which improves aphid fitness on locust, but decreases aphid fitness on other plants (Wagner *et al.* 2015). Many facultative symbionts of aphids have been shown to provide defence against a diversity of natural enemies (Oliver *et al.* 2003; Scarborough, Ferrari & Godfray 2005; Łukasik *et al.* 2013), including ladybeetles (Costopoulos *et al.* 2014), but a defensive role has not yet been ascribed to *Arsenophonus* (Wulff *et al.* 2013).

In this study, we tested the hypotheses: (i) that differential suitability of *A. craccivora* to *H. axyridis* is attributable to aphid lineage rather than host plant and (ii) that the toxicity of some *A. craccivora* lineages is caused by the heritable facultative symbiont *Arsenophonus*. Our study establishes that different host plants and symbiont infections correlate with, but do not cause, variable toxicity of this widespread aphid pest, re-emphasizing the perils of inferring causality from observed associational patterns.

Materials and methods

ORIGIN AND MAINTENANCE OF INSECTS

Aphis craccivora clones were collected from either black locust or alfalfa (*Medicago sativa* L.) in central Kentucky, USA. Individual clonal lines were initiated from parthenogenetic females and maintained on fava (*Vicia faba* L.) as described in Wagner *et al.* (2015). For the present experiments, we primarily used two locust-origin clones (clones LE and LW) and three alfalfa-origin clones (clones AC, AL and AV) that all had distinct genotypes (Wagner *et al.* 2015; Table S1, Supporting Information). All clones were molecularly screened for facultative bacterial symbionts (Brady *et al.* 2014); none were present except for *Arsenophonus* in the locust-origin clones. Within each host plant of origin, these aphid clones were used interchangeably in feeding trials, based on aphid availability. In a separate experiment, we individually assessed the suitability of eight locust-origin and three alfalfa-origin clonal aphid accessions and corroborated that the results presented herein are representative of locust-origin and alfalfa-origin aphid lineages collected on a broad geographical and temporal scale (Fig. S1). All clones were maintained on fava for a minimum of 10 months (~30 aphid generations) before experimental use, to ensure the absence of residual effects from the original host plants. For experiments conducted with aphids feeding on locust or alfalfa, aphid lineages were moved from stock laboratory colonies on fava to the new host plant at least 2 weeks prior to experimental use. *Harmonia axyridis* adults were also collected in Kentucky, USA. Paired male and female *H. axyridis* were maintained in individual Petri dishes (100 × 25 mm) on pea aphid, *Acyrthosiphon pisum* (Harris), in an incubator at 25 °C, 16-h : 8-h light : dark cycle, 65% humidity. All experimental beetles were no more than two generations removed from field-collected individuals.

INTRINSIC SUITABILITY OF *APHIS CRACCIVORA* LINEAGES FOR *HARMONIA AXYRIDIS*

To evaluate whether intrinsic suitability differed between locust-origin and alfalfa-origin *A. craccivora*, we compared *H. axyridis* larval development and survivorship on these aphid lineages when both aphid types had been maintained on fava. Fava is a permissive host plant for all lineages of *A. craccivora*, whereas locust and alfalfa are relatively poor hosts for aphid lineages that originated from the other plant (Wagner *et al.* 2015), limiting aphid number and quality. Rearing the aphid lineages on a mutually permissive host plant species allowed us to compare intrinsic suitability, while excluding potentially confounding maternal host plant or aphid quality effects. As a control, we included a third treatment of locust-origin aphids reared on locust, which we expected to be toxic to *H. axyridis* (Kamo, Tokuoka & Miyazaki 2010).

Neonate *H. axyridis* were removed from their egg clutches before natural dispersal, to prevent sibling cannibalism. Each larva was randomly assigned to one of the three aphid treatments: alfalfa-origin aphids that had been maintained on fava, locust-origin aphids that had been maintained on fava or locust-origin aphids that had been maintained on locust. Each *H. axyridis* larva was placed in a separate 35 × 10 mm Petri dish and given *ad libitum* access to their assigned aphid type over the course of the experiment. Beetle larvae were monitored for survival and larval instar daily and transferred to larger (100 × 25 mm) Petri dishes when they moulted to the third instar. The experiment was concluded when all *H. axyridis* had either reached adulthood or died. A total of 66 larvae were evaluated, of which one escaped and was eliminated from the data set. We constructed Kaplan–Meier survivorship curves in SIGMAPLOT 13 (Systat Software, San Jose, CA, USA) and compared treatments using the log-rank test (Bland & Altman 2004) followed by Holm–Šídák multiple comparisons.

Because not all treatments in the previous experiment produced *H. axyridis* adults, we compared adult survivorship and fecundity between locust-origin and alfalfa-origin *A. craccivora* in a separate experiment. In this case, we only used aphids that had been maintained on fava. Adult *H. axyridis* were field-collected and separated by sex. Field-collected wild beetles undoubtedly varied

widely in age and condition, but were randomized across treatments, ensuring that any observed treatment effects were robust. Thirty-nine females were placed individually in Petri dishes, satiated with *Ac. pisum* for 24 h and then deprived of food for 24 h. Each female then received her treatment of locust-origin or alfalfa-origin *A. craccivora* aphids *ad libitum* for 14 days. We replenished aphids daily, monitored survivorship and counted eggs laid. We evaluated differences in survivorship between treatments using the log-rank test on Kaplan–Meier survivorship curves. We compared egg production using *t*-tests on ln-transformed data, to account for heteroscedasticity. In addition to comparing total egg production over the 14-day assay, we also calculated and compared average daily egg production, dividing each beetle's total egg production by the number of days it survived. In this way, we were able to evaluate the treatment effect on egg production independently of any effect on adult survival.

Finally, to investigate whether differences in suitability of locust-origin vs. alfalfa-origin aphids remained consistent across host plants, we compared *H. axyridis* larval development when (i) both aphid lineages had been reared on locust, and (ii) both aphid lineages had been reared on alfalfa. The experimental protocol for these two experiments was identical to previous larval development assays on fava-reared aphids, except that aphid numbers were limited as a consequence of the relatively poor development of locust-origin aphids on alfalfa, and of alfalfa-origin aphids on locust (Wagner *et al.* 2015). To accommodate this limitation, each experiment was concluded after 6 days, before surviving *H. axyridis* larvae reached the voracious later instars. For each experiment, survivorship to day 6 was compared between treatments using the log-rank test on Kaplan–Meier survivorship curves.

EFFECT OF THE SYMBIONT *ARSENOPHONUS* ON APHID SUITABILITY

To test whether the bacterial symbiont *Arsenophonus* affects the suitability of *A. craccivora* as prey for *H. axyridis*, we compared larval development and survivorship on aphid lineages in which symbiont infection had been experimentally manipulated. Natural locust-origin *A. craccivora* clones are almost always infected with *Arsenophonus*, whereas alfalfa-origin clones are not (Brady & White 2013; Brady *et al.* 2014). As described in Wagner *et al.* (2015), we fed aphids a diet containing antibiotics (gentamicin, cefotaxime and/or ampicillin) and monitored offspring for loss of *Arsenophonus* using diagnostic PCR, resulting in two locust-origin clones that were cured of *Arsenophonus*. Similarly, we transinfected *Arsenophonus* into one alfalfa-origin line of *A. craccivora* by microinjecting hemolymph from an *Arsenophonus*-infected locust-origin aphid, and monitoring offspring for the presence of *Arsenophonus* using diagnostic PCR (Brady & White 2013). All symbiont-manipulated aphid lines were maintained on fava for more than a year (>35 generations) before the testing of *H. axyridis* larvae, with appropriate infection status verified by diagnostic PCR (Brady & White 2013). Larval assays of *H. axyridis* survival and development were conducted as described previously, comparing the *Arsenophonus*-infected aphid lines (naturally infected locust origin and transinfected alfalfa origin) to their uninfected counterparts (cured locust origin and naturally uninfected alfalfa origin). The trial lasted until all *H. axyridis* reached adulthood or died. We again calculated Kaplan–Meier survival curves and compared treatments using the log-rank test followed by pairwise multiple comparisons using the Holm–Šídák method.

Results

When we reared locust-origin and alfalfa-origin lineages of *A. craccivora* on the same host plant and compared their suitability as food for *H. axyridis*, we found that toxicity was primarily a function of aphid lineage, not the aphid's current host plant. When fed locust-origin *A. craccivora*, 100% (= 43/43) of *H. axyridis* larvae died within 7 days, regardless of whether the aphids had been maintained on locust or fava (Fig. 1). None of these beetle larvae moulted out of the first instar before death. In contrast, when fed alfalfa-origin aphids maintained on fava, only 14% (= 3/22) of *H. axyridis* larvae died during the 17-day trial, and all the survivors achieved adulthood. The survivorship curves of all three treatments differed significantly from one another at $\alpha = 0{\cdot}05$ (overall $\chi^2 = 80{\cdot}8$, d.f. = 2, $P < 0{\cdot}001$). All larvae fed locust-origin aphids died, but larvae fed on locust-maintained aphids survived twice as long (Mean ± 1 SE = 5·3 ± 0·3 days) as fava-maintained aphids (2·7 ± 0·2 days). However, this effect is minor compared with the vast difference in beetle survivorship between locust-origin and alfalfa-origin aphid treatments (Fig. 1).

Adult *H. axyridis* also suffered when fed locust-origin *A. craccivora*. About 73% (= 16/22) of the beetles died during the 14-day assay period when fed locust-origin aphids (reared on fava), whereas only one (1/17 = 6%) died when fed alfalfa-origin aphids reared on fava ($\chi^2 = 15{\cdot}5$, d.f. = 1, $P < 0{\cdot}001$; Fig. 2a). Overall egg production was greatly diminished for beetles fed locust-origin aphids (22·3 ± 3·8 eggs) relative to those fed alfalfa-origin aphids (166·9 ± 23·7 eggs; $t_{37} = 6{\cdot}8$, $P < 0{\cdot}001$; Fig. 2b). This difference is not entirely explained by differential beetle survival: when we calculated eggs produced per day survival, beetles fed locust-origin aphids still produced 78% fewer eggs than beetles fed alfalfa-origin aphids (locust

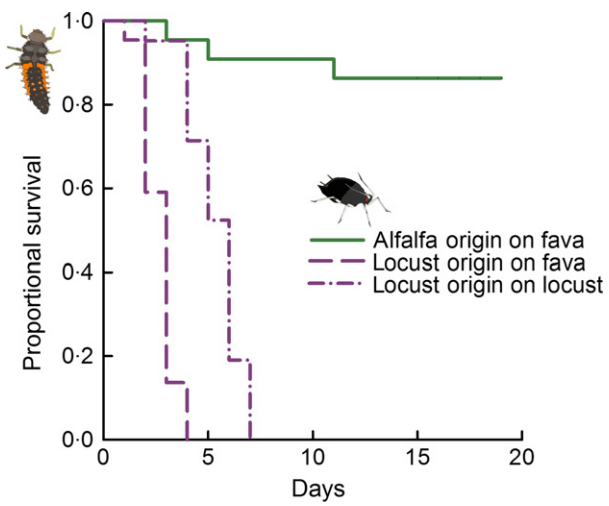

Fig. 1. Larval survival of *Harmonia axyridis* is primarily affected by aphid lineage rather than the aphid's current host plant. Larval *H. axyridis* were fed either alfalfa-origin or locust-origin *Aphis craccivora* that had been reared on fava for at least 10 months. Locust-origin *A. craccivora* that had been reared on locust were included as a control treatment. $N = 22$ beetle larvae per treatment.

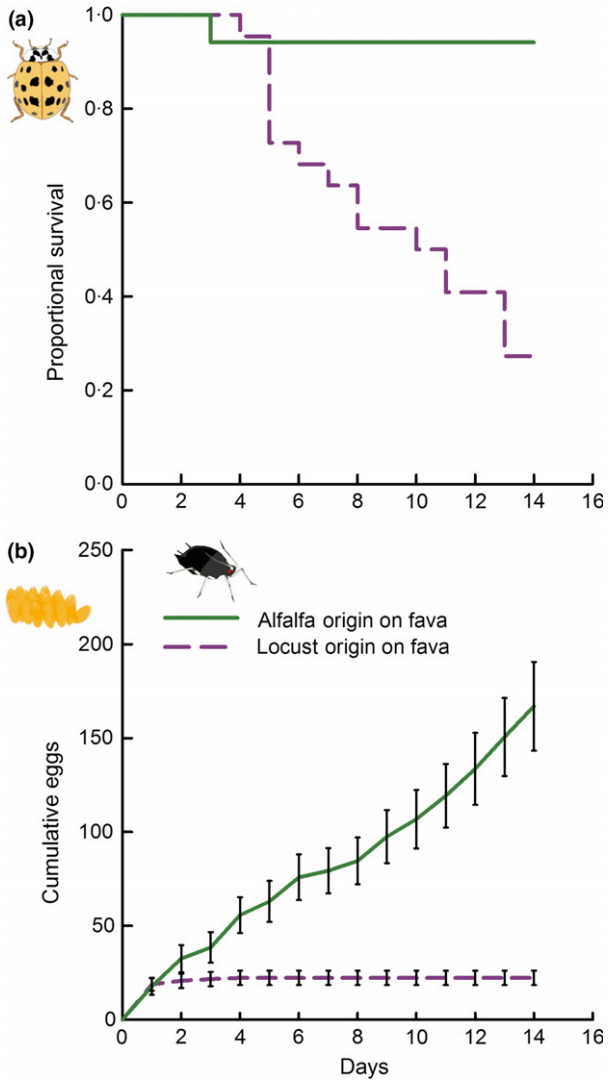

Fig. 2. Adult *Harmonia axyridis* have reduced (a) survival and (b) mean ± SE cumulative egg production over 14 days when fed locust-origin *Aphis craccivora* relative to alfalfa-origin aphids. All aphids were reared on fava, from lineages that had been reared on fava for over a year. $N = 22$ beetles on locust-origin and 17 beetles on alfalfa-origin aphids.

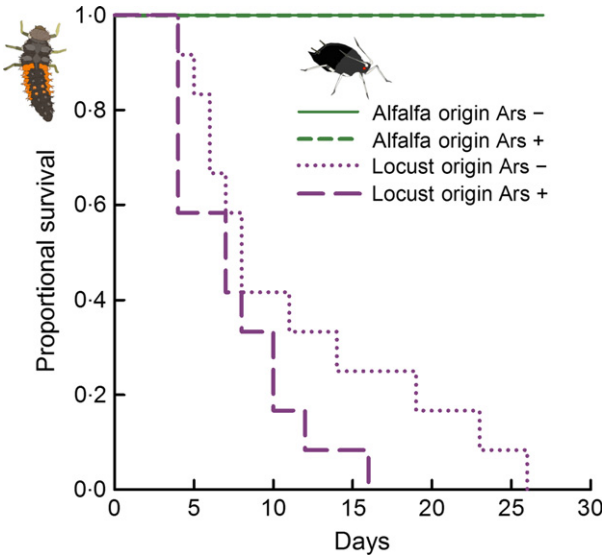

Fig. 3. The facultative symbiont *Arsenophonus* in *Aphis craccivora* does not affect survival of larval *Harmonia axyridis*. Locust-origin aphids were either naturally infected with the symbiont *Arsenophonus* or were from experimentally cured lineages. Alfalfa-origin aphids were either naturally uninfected, or were from a lineage artificially transinfected with *Arsenophonus*. All aphids were reared on fava, from lineages that had been reared on fava for over a year. $N = 12$ beetle larvae per treatment.

origin = 2·7 ± 0·5 eggs/day alive, alfalfa origin = 12·0 ± 1·7 eggs/day alive; $t_{37} = 5{\cdot}0$, $P < 0{\cdot}001$). For the beetles fed locust-origin aphids, almost all egg production was on the first day of the experiment, and for that day alone, egg production did not differ between the two treatments (locust origin = 18·7 ± 3·3 eggs, alfalfa origin = 17·8 ± 4·5 eggs; $t_{37} = 0{\cdot}2$, $P = 0{\cdot}86$).

Furthermore, when we reciprocally transferred the two aphid lineages to one another's host plants, they retained their respective toxicity properties. When both aphid lineages were reared on locust, 77% (= 10/13) of *H. axyridis* larvae fed locust-origin aphids died within the 6-day assay period, whereas only 7% (= 1/15) of beetle larvae fed alfalfa-origin aphids died ($\chi^2 = 12{\cdot}3$, d.f. = 1, $P < 0{\cdot}001$). Beetles fed alfalfa-origin aphids reared on locust were uniformly in the third instar at the end of the assay, whereas the few surviving beetles fed locust-origin aphids were still in the first instar. We observed the same result when both lineages of aphids were reared on alfalfa: 100% (= 15/15) of *H. axyridis* larvae fed locust-origin aphids died within 6 days without ever moulting, whereas only 7% (= 1/15) of beetle larvae fed alfalfa-origin aphids died ($\chi^2 = 25{\cdot}1$, d.f. = 1, $P < 0{\cdot}001$). Beetle larvae fed alfalfa-origin aphids were uniformly in the fourth instar at the end of the assay.

The toxic properties of locust-origin *A. craccivora* were not related to *Arsenophonus* infection. All (= 24/24) *H. axyridis* larvae fed locust-origin aphids (reared on fava) died, regardless of whether they were fed aphids that bore a natural *Arsenophonus* infection, or aphids from lineages that had been cured of *Arsenophonus* (Fig. 3). In contrast, none (= 0/24) of the *H. axyridis* larvae fed alfalfa-origin aphids died and all survived to adulthood, even if the aphids were from a lineage that had been transinfected with *Arsenophonus*. Overall, there were significant differences in survivorship among treatments in this experiment ($\chi^2 = 58{\cdot}9$, d.f. = 3, $P < 0{\cdot}001$), but these differences were entirely driven by the contrast between larvae fed locust-origin vs. alfalfa-origin aphids. For the locust-origin aphid treatments, mean *H. axyridis* survival was longer than in previous experiments (9·5 ± 1·3 days), and some larvae managed to moult to the second (two larvae), third (two larvae) or even fourth instar (one larva) before perishing. However, survival did not differ statistically between beetle larvae fed the *Arsenophonus*-infected vs. uninfected aphids ($\chi^2 = 2{\cdot}3$, d.f. = 1, $P = 0{\cdot}23$).

Discussion

The wide variation in suitability of *A. craccivora* as a food source for *H. axyridis* is a property of aphid lineage, not host plant. When reared on any of three legume plants (fava, alfalfa or locust) aphid lineages that had originated from alfalfa supported *H. axyridis* larval development to adulthood, whereas aphid lineages originating from locust did not support *H. axyridis* development, with larvae generally dying within a week while still in the first instar. Likewise, adult *H. axyridis* fed alfalfa-origin aphids thrived and had high reproductive output, whereas adult beetles fed locust-origin aphids produced very few eggs, and most died within the 2-week assay. Such intrinsic variation in aphid defensive properties, independent of host plant yet correlated with host plant of collection, is surprising, and runs counter to the expectations derived from decades of experimentation on tritrophic chemical interactions (reviewed in Nishida 2002; Ode 2006; Hodek & Evans 2012).

Once host plant was eliminated as an explanation for differential toxicity in *A. craccivora*, the most logical alternative hypothesis was differential infection by a facultative defensive symbiont, as is the case in pea aphid, *Ac. pisum*. *Acyrthosiphon pisum* is composed of genetically distinct host races that are specialized onto different host plants (Via 1991; Ferrari, Via & Godfray 2008), some of which were found to be intrinsically more susceptible to parasitism than others, even when the aphids were reared on the same host plant species (Hufbauer & Via 1999). It was ultimately shown that the inherited facultative symbiont *Hamiltonella defensa*, which provides parasitism protection, is more prevalent in some *Ac. pisum* host races than others (Oliver, Moran & Hunter 2005; Ferrari *et al.* 2012). This same facultative symbiont in *Ac. pisum* has also been shown to have a moderate effect on survival of the ladybeetle *Hippodamia convergens* (Costopoulos *et al.* 2014), leading to a clear hypothesis that facultative symbionts might also provide the explanation for differential toxicity among *A. craccivora* lineages. This hypothesis was initially bolstered by the observation that toxic locust-origin aphids are predominantly infected with the bacterial symbiont *Arsenophonus* and non-toxic alfalfa-origin aphids lack *Arsenophonus* (Brady & White 2013; Brady *et al.* 2014; Wagner *et al.* 2015).

However, symbiotically mediated defence in *A. craccivora* was not supported in the present study. Toxic locust-origin aphid lineages remained toxic after their *Arsenophonus* had been removed, and a non-toxic alfalfa-origin aphid lineage remained non-toxic even after it had been stably transinfected with *Arsenophonus*. While it is possible that alternate facultative symbionts might be present in locust-origin aphids that cause toxicity, we currently have no evidence of such hypothetical symbionts. Previous microbiome characterization of locust-origin *A. craccivora* did not reveal the presence of any other facultative bacterial associates (Brady & White 2013), nor do there appear to be any endosymbiotic fungi associated with toxic lineages (J. White, unpublished fungal next-gen sequencing data).

At this stage, alternative explanations for toxicity that do not invoke symbiotic partners are equally plausible. *Aphis craccivora* is almost exclusively parthenogenetic, and locust- and alfalfa-origin lineages show slight but consistent sequence divergence from one another (Brady *et al.* 2014). The lineages may have diverged from one another on some ecologically relevant axis, such as *de novo* defensive chemistry or by-products of enzymatic nutritional cascades, that results in differential toxicity to *H. axyridis*. Ongoing transcriptomic and functional investigations will help elucidate the mechanism of toxicity within this system, and perhaps also shed light on the source of plasticity in toxicity observed among experiments.

Even though *Arsenophonus* does not cause toxicity, it is noteworthy that this symbiont still plays a role in the distribution of toxic aphids across the landscape. Previous research has shown that *Arsenophonus* improves aphid performance on locust but decreases performance on other plants (Wagner *et al.* 2015), which likely explains the global propensity for locust-associated *A. craccivora* to be infected with *Arsenophonus* (Brady *et al.* 2014). Given that *Arsenophonus* is maternally inherited and *A. craccivora* is parthenogenetic, co-occurrence of *Arsenophonus* and the toxic trait (whatever its cause) is persistent over time; it is this linkage that secondarily correlates locust-feeding aphids with toxicity towards the ladybeetle, rather than chemicals derived from locust *per se* (Obatake & Suzuki 1985; Kamo, Tokuoka & Miyazaki 2012). Thus, locust is shown to be a correlative rather than causative factor in the decades-old observation that *A. craccivora* from locust are toxic to *H. axyridis* (Hukusima & Kamei 1970; Kamo, Tokuoka & Miyazaki 2010). We expect that other lineages of *A. craccivora*, which are uninfected with *Arsenophonus* and not associated with locust, may also be toxic to *H. axyridis*; ongoing investigations will determine how widespread this toxicity trait may be.

While there are certainly well-documented instances where differential toxicity of polyphagous herbivores across host plants are direct functions of host plant chemistry (e.g. Brower *et al.* 1968; Snook *et al.* 1993; Singer *et al.* 2004), our results emphasize that other paradigms for differential defence should be considered, particularly in systems where herbivores exhibit genetic structuring across host plants and may have diverged evolutionarily from one another (Drès & Mallet 2002; Stireman, Nason & Heard 2005). When decoupled from host plant, toxicity polymorphisms can provide an elegant and tractable framework for generally assessing the efficacy of defensive traits and their consequences for predator–prey population dynamics and community structure.

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Data accessibility

Data deposited in the Dryad Digital Repository http://dx.doi.org/10.5061/dryad.37dv8 (White *et al*. 2016).

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Supporting Information

Additional Supporting Information may be found online in the supporting information tab for this article:

Fig. S1. (a) Survival and (b) mean ± SE final developmental stage of *Harmonia axyridis* larvae fed individual clones of *Aphis craccivora*.

Table S1. *Aphis craccivora* clonal origins.