MOLECULAR ECOLOGY

Molecular Ecology (2015) **24**, 1135–1149 doi: 10.1111/mec.13095

Patterns, causes and consequences of defensive microbiome dynamics across multiple scales

ANDREW H. SMITH,* PIOTR ŁUKASIK,* MICHAEL P. O'CONNOR,* AMANDA LEE,* GARRETT MAYO,* MILTON T. DROTT,* STEVEN DOLL,* ROBERT TUTTLE,* RACHAEL A. DISCIULLO,* ANDREA MESSINA,* KERRY M. OLIVER† and JACOB A. RUSSELL*

**Department of Biology, Drexel University, Philadelphia PA 19104, USA, †Department of Entomology, University of Georgia, Athens GA 30602, USA*

Abstract

The microbiome can significantly impact host phenotypes and serve as an additional source of heritable genetic variation. While patterns across eukaryotes are consistent with a role for symbiotic microbes in host macroevolution, few studies have examined symbiont-driven host evolution or the ecological implications of a dynamic microbiome across temporal, spatial or ecological scales. The pea aphid, *Acyrthosiphon pisum*, and its eight heritable bacterial endosymbionts have served as a model for studies on symbiosis and its potential contributions to host ecology and evolution. But we know little about the natural dynamics or ecological impacts of the heritable microbiome of this cosmopolitan insect pest. Here we report seasonal shifts in the frequencies of heritable defensive bacteria from natural pea aphid populations across two host races and geographic regions. Microbiome dynamics were consistent with symbiont responses to host-level selection and findings from one population suggested symbiont-driven adaptation to seasonally changing parasitoid pressures. Conversely, symbiont levels were negatively correlated with enemy-driven mortality when measured across host races, suggesting important ecological impacts of host race microbiome divergence. Rapid drops in symbiont frequencies following seasonal peaks suggest microbiome instability in several populations, with potentially large costs of 'superinfection' under certain environmental conditions. In summary, the realization of several laboratory-derived, a priori expectations suggests important natural impacts of defensive symbionts in host-enemy eco-evolutionary feedbacks. Yet negative findings and unanticipated correlations suggest complexities within this system may limit or obscure symbiont-driven contemporary evolution, a finding of broad significance given the widespread nature of defensive microbes across plants and animals.

Keywords: *Acyrthosiphon pisum*, *Aphidius ervi*, contemporary evolution, *Pandora neoaphidis*, selection, symbiosis

Received 19 September 2014; revision received 18 December 2014; accepted 19 December 2014

Introduction

Rapid evolution has been widely documented in nature and has often been linked to adaptive processes. While major drivers of short-term adaptive evolution can be hard to pinpoint, climatic conditions and natural enemies are thought to be of key importance. Both show seasonal variation, and thus, while most contemporary evolution has been documented across multiple years, important adaptation may occur over shorter timescales (Reznick *et al*. 1997; Reimchen & Nosil 2002). The cyclical nature of selective pressures may favour the maintenance of functionally significant genetic diversity, while the ecological feedbacks of short-term evolution may alter the dynamics of terrestrial or aquatic communities (for review see Thompson 1998; Carroll *et al*. 2007; Fussmann *et al*. 2007).

Correspondence: Andrew H. Smith, Fax: 215 895 1273;
E-mail: ahs55@drexel.edu

The genetic diversity encoded in the microbiomes of plants and animals is becoming increasingly evident, and thus, studies of phenotypic evolutionary change must now consider the combination of host and symbiont genomes—the holobiont—as the component acted upon by natural selection (Zilber-Rosenberg & Rosenberg 2008). This is especially true for systems in which symbiotic microbes are passed on from parent to offspring with high fidelity. Such faithful transfer couples host and microbe fitness, enabling host-level natural selection to govern the trajectories of symbionts that shape host phenotypes. Insects are renowned for such faithful associations, with most species possessing transovarially transmitted bacteria (Hilgenboecker *et al.* 2008; Moran *et al.* 2008). Intriguingly, many of these heritable symbionts are defensive, protecting hosts against enemies such as RNA viruses (Teixeira *et al.* 2008), fungal pathogens (Łukasik *et al.* 2013c), parasitic nematodes (Jaenike *et al.* 2010), parasitoid wasps (Oliver *et al.* 2003) or predators (Piel *et al.* 2004).

Variation in heritable symbiont frequencies has been documented across broad spatial scales (Skaljac *et al.* 2010; Toju & Fukatsu 2011) and over the span of several years in natural populations (Jaenike *et al.* 2010; Himler *et al.* 2011). Yet few studies have examined fine-scale seasonal dynamics in nature (but see Hoffmann *et al.* 1998) and thus the potential contributions of symbionts to rapid adaptation. Our lack of knowledge on the feedbacks between hosts, symbionts and the environment over shorter timescales is notable given symbionts' contributions towards insect pest status (Chu *et al.* 2013; Chung *et al.* 2013; Brown *et al.* 2014), their impacts on insect-vectored disease (Hedges *et al.* 2008; Hoffmann *et al.* 2011) and their potential to disrupt or enhance biological control strategies (Oliver *et al.* 2010).

The pea aphid (*Acyrthosiphon pisum*) provides a tractable system for the study of microbiome-driven, contemporary evolution. This species is a specialized feeder on herbaceous legumes and forms races differentiated by host plant (Peccoud *et al.* 2009; Ferrari *et al.* 2012). In temperate regions, pea aphids are cyclically parthenogenetic, undergoing more than eight clonal generations annually prior to sexual reproduction and overwintering as eggs (Markkula 1963). Pea aphid populations typically exhibit polymorphism for associations with seven heritable, facultative bacteria not necessary for growth and reproduction. Each of these has been implicated in variable levels of defence against either the parasitoid wasp *Aphidius ervi* (Oliver *et al.* 2003; Guay *et al.* 2009) or fungal pathogens (*Pandora neoaphidis* and *Zoophthora occidentalis*) (Scarborough *et al.* 2005; Łukasik *et al.* 2013c; Parker *et al.* 2013). Best-established are the protective properties of the symbionts *Hamiltonella defensa* (vs. *A. ervi*) and *Regiella insecticola* (vs. fungal pathogens) (Ferrari *et al.* 2004; Moran *et al.* 2005; Scarborough *et al.* 2005; Oliver *et al.* 2008, 2009; Łukasik *et al.* 2013c; Parker *et al.* 2013). Confirmation of the protective properties of these two symbionts has been extensive, including studies performed on aphids and enemies from two continents, multiple symbiont strains tested in multiple aphid genetic backgrounds against multiple enemy species and genotypes. Further, yet limited, support for resistance to *A. ervi* has been obtained for the X-type symbiont (Guay *et al.* 2009) and *Serratia symbiotica* (Oliver *et al.* 2003), with additional evidence for protective roles of *Rickettsiella viridis*, *Rickettsia* and *Spiroplasma* against *P. neoaphidis* (Łukasik *et al.* 2013c). In addition, some symbiont species may play multiple roles, such as *S. symbiotica*, which provides tolerance to heat stress (Montllor *et al.* 2002; Russell & Moran 2006; Burke *et al.* 2009).

To date, the majority of studies within this system have focused on single infections under controlled laboratory conditions. While laboratory-based studies have developed a solid foundation to our understanding of aphid–symbiont–environment interactions, few studies have examined symbiont frequencies in relation to environmental factors under natural conditions. While field surveys have reported defensive microbiome divergence across pea aphid host races (Ferrari *et al.* 2012) and symbiont–climate correlations across geographic scales (Tsuchida *et al.* 2002; Henry *et al.* 2013; Russell *et al.* 2013), comprehensive surveys across temporal scales in relation to environmental factors have been comparatively rare (Montllor *et al.* 2002; Oliver *et al.* 2014). At least one study observed temporal shifts in *S. symbiotica* frequencies, suggesting symbiont contributions towards thermal adaptation (Montllor *et al.* 2002). Completely lacking have been efforts to assess the relationships between symbionts and their hosts' natural enemies. Considering *A. ervi* and *P. neoaphidis* inflict high levels of mortality that vary seasonally and between host races (Hufbauer 2002a), and costs have been detected for several of the aforementioned symbionts in the absence of enemies or under different temperature treatments (Bensadia *et al.* 2006; Russell & Moran 2006; Oliver *et al.* 2008; Guay *et al.* 2009; Simon *et al.* 2011), we would predict that symbiont frequencies will vary over spatial, temporal and ecological scales in conjunction with fluctuating enemy pressures (Oliver *et al.* 2014).

To assess the predictions of rapid, seasonal adaptation of the defensive microbiome, we repeatedly sampled pea aphids across a single season within four populations, relating symbiont frequencies to pressures from both natural enemies and temperature. Our study spanned nine dates across 6 months, encompassing two host races from distinct crops from each of two regions

in the northeastern United States. Relationships between aphids' facultative symbiont frequencies and natural enemies across these scales provide detailed insights into contemporary evolution at the microbiome level and the role of symbionts in contemporary host adaptation. Our findings also suggest the potential for symbiont-mediated defensive phenotypes to impact enemy populations, suggesting important consequences of defensive symbioses in the real world.

Methods

Sampling

Aphids were collected within three replicate alfalfa (*Medicago sativa*) and red clover (*Trifolium pratense*) fields from each of two locations: the Finger Lakes region of New York state (NY) and Berks County in the state of Pennsylvania (PA) (Table S1, Supporting information), where these crops are typically grown in monoculture. The same fields were sampled every 3 weeks starting 9th May and ending 24th October 2011, resulting in nine separate sampling dates with occasional collection gaps due to low aphid densities. Individual aphids were collected using beat sampling from plants separated by approximately 20 m to minimize resampling of the same clones. Aphids were either preserved in 95% ethanol and stored at −20 °C prior to symbiont screening or maintained alive (2nd to 4th instars only) at 20 °C and 16D:8N within an environmental chamber prior to assessment of mortality factors. Eight days post-collection, the numbers of aphids succumbing to *A. ervi* or *Praon* spp. parasitoids, or to fungal infection were counted. Enemies causing death were identified based on stereotypical characteristics of parasitoid 'mummies' (hardened outer shells of aphids that contain wasp pupae) and fungal cadavers. All fungal cadavers were checked under an 80× dissecting microscope to verify the presence of fungal spores. And while a vast majority of cadavers fit the phenotype of a fatal *P. neoaphidis* infection, some (~10%) had degraded substantially, making the pathogen difficult to identify. For this reason, we refer generically to fungal-induced mortality for our measures of pathogen pressures. We should note that a study conducted in a temperate region of the United States found *P. neoaphidis* drove 81.5% of fungal mortality in alfalfa populations, while *Zoophthora occidentalis* (previously *Erynia occidentalis*) caused 14.8% (Hutchinson & Hogg 1984). Combined with this and our own observations, we fully expect that the vast majority of fungus-induced mortality was caused by enemies that are overcome by *R. insecticola* (Scarborough *et al.* 2005; Parker *et al.* 2013). In addition, most fungal pathogen-triggered death was very likely caused by the fungus (*P. neoaphidis*) that is overcome by the remaining symbionts from the fungal defender guild (*Rickettsia*, *Rickettsiella*, and *Spiroplasma*) (Łukasik *et al.* 2013c).

Sweep net sampling was performed in each field to measure densities of pea aphids, *A. ervi*, other parasitoid wasps and generalist aphid predators. Six replicates of 30 sweeps each were taken per field, and each replicate sample was separated by ≥20 m. The contents of each replicate were stored in a kill jar containing ethyl acetate, brought back to the laboratory, preserved in 95% ethanol, then stored at −20 °C prior to counting the contents under a dissection microscope (see Supporting Information and Appendix S1: Sweep Sampling). To measure within-canopy temperature, one temperature probe (Watchdog B100 2K temperature logger, Spectrum Technologies, Aurora, IL, USA) was placed in each field approximately one inch above ground level. Temperatures were recorded every 30 min throughout the sampling period.

DNA extraction, PCR, microsatellite genotyping and sequencing

DNA from preserved aphids was extracted following prior protocols (Russell *et al.* 2003) (see Supporting Information). Template quality was verified by performing a polymerase chain reaction (PCR) to detect *Buchnera aphidicola*, a symbiont possessed by all pea aphids. To test individual aphids for the seven species of facultative symbionts that exist in United States populations (see Russell *et al.* 2013), DNA samples were subjected to diagnostic PCRs for each symbiont to amplify a fragment of 16S rRNA or a portion of this gene along with 23S rRNA and the spacer region in between (Table S2, Supporting information). A subset of aphids was subject to Sanger sequencing (GenBank Accession nos KP710314–KP710505) or real-time qPCR to verify the accuracy of diagnostic PCRs (see Supporting Information and Appendix S1: Symbiont Master). All PCRs included a positive and negative control, and presence/absence of bacteria was determined via gel electrophoresis. Results from reaction batches yielding amplification of the negative control or failed positive controls were discarded, and PCR screens rerun.

Microsatellite genotyping was performed on a subset of aphids collected for symbiont screening over various time points and fields to determine the capacity for symbiont hitchhiking on proliferating clones. Genotyping was performed on 164 aphids collected over three consecutive time points in the same three alfalfa fields in PA. The time points include the sampling period with the highest level of superinfection (i.e. the average number of facultative symbionts per aphid) and the

preceding and anteceding sampling periods. Multiplex PCRs were run and products submitted for genotyping on an Applied Biosystems 3130XL at the University of Pennsylvania Sequencing Center (see Supporting Information and Appendix S1).

Statistical analyses

All analyses were carried out using the lme4 package in R version 2.14.2 (R Development Core Team 2011). Variation in the proportions of aphids possessing specific symbionts and in the numbers of symbionts per aphid was measured across states, crops and sampling dates using repeated measures generalized linear models. This analysis treats each aphid as a separate replicate within a field. To determine differences between states and crops, models included the state, crop and dates sampled as fixed effects and the collection field as a random or block effect. Models to determine differences between sampling dates were analysed separately for each crop and location; these included sampling date as the fixed effect and collection field as a random or block effect. The binomial family function was included in all models when the response variable (symbiont presence/absence) was binary.

A repeated measures Linear Model was used to analyse differences in insect counts, parasitoid- and fungal-induced mortality, and numbers of symbionts per aphid between states, crops and sampling dates. To improve normality, insect counts and numbers of symbionts per aphid were square root transformed, while proportions of aphids dying due to parasitoids or fungal pathogens were arcsin transformed.

Correlations between environmental factors (predator density, parasitoid density, parasitoid-induced mortality, fungal-induced mortality) and symbionts were analysed using repeated measures generalized linear models. Models included mortality and enemy density as fixed effects and collection field as a random or block effect. For analyses of symbiont frequencies vs. enemy densities, both predator and parasitoid density were included in the initial model; predators were subsequently removed from the model after goodness of fit was assessed using the Akaike information criterion (AIC). To standardize enemy densities, the number of wasps or predators was divided by the number of pea aphids from the same collection. These values were log transformed before the aforementioned analyses to improve normality. While many correlations were detected between symbionts and environmental variables (see Supporting Information), we limit our discussion to those with the clearest origins and ecological relevance.

Results

Host plant, spatial and temporal differences in facultative symbiont frequencies

Across the 1753 pea aphids screened for the seven known facultative symbionts (NY alfalfa: 806, NY Clover: 351, PA alfalfa: 580, PA clover: 226), we found several symbionts that varied in prevalence between regions and crops (Fig. 1, Table 1). Regional differences were detected for the X-type ($F = 11.2$, $P < 0.001$), which was enriched in NY ($F = 11.20$, $P < 0.001$), and both *S. symbiotica* ($F = 17.47$, $P < 0.0001$) and *R. viridis* ($F = 16.16$, $P < 0.0001$), which were more frequent overall in PA. The prevalence of *H. defensa* (NY: $F = 12.62$, $P < 0.001$, PA: $F = 7.78$, $P < 0.01$), X-type (NY: $F = 8.93$, $P < 0.01$, PA: $F = 17.6$, $P < 0.0001$) and *Rickettsiella* (NY: $F = 6.21$, $P < 0.05$, PA: $F = 5.61$, $P < 0.05$) was higher in alfalfa populations in both locations, while *R. insecticola* was more prevalent in clover populations in both states

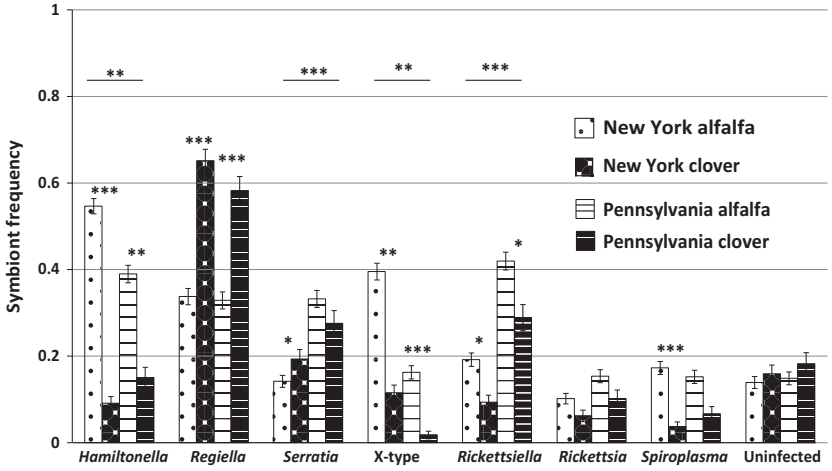

Fig. 1 Overall symbiont frequencies in four pea aphid populations. Columns are the mean (±SEM) proportion of aphids harbouring specific facultative symbionts or no symbionts from three alfalfa and three clover fields in New York and Pennsylvania collected over 9 dates in 2011. Asterisks above lines indicate significant differences between states and asterisks above columns indicate differences between crops. (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).

Table 1 Comparison of symbiont frequencies between states, crops and dates

Source of variation	d.f.	*Hamiltonella*		*Regiella*		*Serratia*		*Rickettsia*		X-type		*Rickettsiella*		*Spiroplasma*	
		Chi-square	*P*	Chi-square	*P*	Chi-square	*P*	Chi-square	*P*	Chi-square	*P*	Chi-square	*P*	Chi-square	*P*
State	1	6.83	0.009	0.01	0.7614	17.47	3.0E-5	3.22	0.07268	11.20	<0.001	16.16	<0.0001	1.87	0.1712
Crop															
New York	1	12.62	<0.0010	13.79	<0.0010	4.06	0.0439	1.59	0.2080	8.93	0.0028	6.21	0.0127	11.90	0.0010
Pennsylvania	1	7.77	0.0050	14.81	<0.0001	1.24	0.2664	2.14	0.1438	17.60	<0.0001	5.61	0.0178	2.70	0.1003
State × Crop Interaction	1	5.12	0.0240	1.08	0.2976	5.42	0.0199	0.03	0.8674	1.58	0.2093	0.68	0.4090	0.05	0.1595
Date															
NY Alfalfa	8	7.48	0.4860	23.76	0.0025	46.26	<0.0001	9.86	0.2748	13.20	0.1051	46.52	<0.0001	16.47	0.0361
NY Clover	6	2.77	0.9050	5.33	0.5028	40.27	<0.0001	8.29	0.2175	8.61	0.1966	11.78	0.0671	14.24	0.0271
PA Alfalfa	8	57.62	<0.0001	134.98	<0.0001	121.40	<0.0001	57.81	<0.0001	129.70	<0.0001	110.70	<0.0001	20.72	0.0079
PA Clover	6	63.96	<0.0001	9.91	0.1285	95.47	<0.0001	11.01	0.2011	10.92	0.2061	50.03	<0.0001	8.84	0.3563

Chi-square and *P*-values for comparisons of symbiont frequencies in three alfalfa and three clover fields repeatedly sampled in New York and Pennsylvania in 2011.

(NY: $F = 0.13.8$, $P < 0.001$, PA: $F = 14.81$, $P < 0.001$). Significant state × crop interactions existed for *Hamiltonella* ($F = 5.12$, $P < 0.05$) and *Serratia* ($F = 5.42$, $P < 0.05$) frequencies. The mean number of facultative symbionts per aphid (superinfection level) was higher in alfalfa than clover in both states (Fig. 2, Table S3, Supporting information; NY Alfalfa = 1.87 vs. NY Clover = 1.23, $F = 12.69$, $P < 0.001$; PA Alfalfa = 1.93 vs. PA Clover = 1.48; $F = 3.77$, $P = 0.052$). Superinfection levels reached a notable, data set-wide high at the second sampling date in PA alfalfa (3.73, Fig. 2).

When considering symbionts together as defensive guilds, the proportion of aphids with ≥1 parasitoid defender (*Hamiltonella*, *Serratia* and X-type) and the numbers of parasitoid defending symbionts per aphid were higher in alfalfa than in clover ($F = 17.5$, 15.5, respectively, $P < 0.0001$, Fig. 3, Table S3, Supporting information). Similarly, the proportion of aphids with ≥1 fungal defender (*Regiella*, *Rickettsia*, *Rickettsiella*, *Spiroplasma*) was higher in clover vs. alfalfa in NY ($F = 7.68$, $P < 0.01$), but not PA ($F = 0.35$, $P < 0.56$) (Fig. 3, Table S3, Supporting information). Also, the proportion of fungal defenders and the average number of fungal defending symbionts per aphid were higher in PA than NY ($F = 4.88$, $P < 0.05$; $F = 10.14$, $P < 0.01$, respectively) (Fig. 3, Table S3, Supporting information).

Analyses of temporal dynamics for individual symbionts, superinfection levels and proportions of uninfected aphids (i.e. no facultative symbionts) within the four populations yielded 36 separate analyses, of which 23 showed significant change overtime (~64%, $P < 0.05$, Fig. 2, Table 1, Table S3, Supporting information). All symbionts exhibited significant frequency shifts in at least one population, with only *Serratia* showing significant changes across all four. Frequencies of uninfected aphids also differed overtime in all but the NY clover population ($F = 12.47$, $P = 0.052$, Table S3, Supporting information). *Hamiltonella* showed a regional signature, fluctuating in both PA populations ($P < 0.0001$), but not significantly in either NY population (Fig. 2, Table 1). *Regiella*, in contrast, showed a host race signature, with frequencies changing overtime in alfalfa populations ($P < 0.01$), but not on clover in either locale (Fig. 2, Table 1).

Temporal dynamics were the most extensive in the PA alfalfa population, with significant shifts in the frequencies of each facultative symbiont, the proportion of symbiont-free aphids, and the level of superinfection ($P < 0.01$, Table 1, Table S3, Supporting information). These dynamics stemmed partially from high prevalence of many symbionts on the second sampling date (superinfection level = 3.73 symbionts per aphid, uninfected frequency = 1%), followed by a rapid drop in frequencies 3 weeks later (superinfection level = 1.19

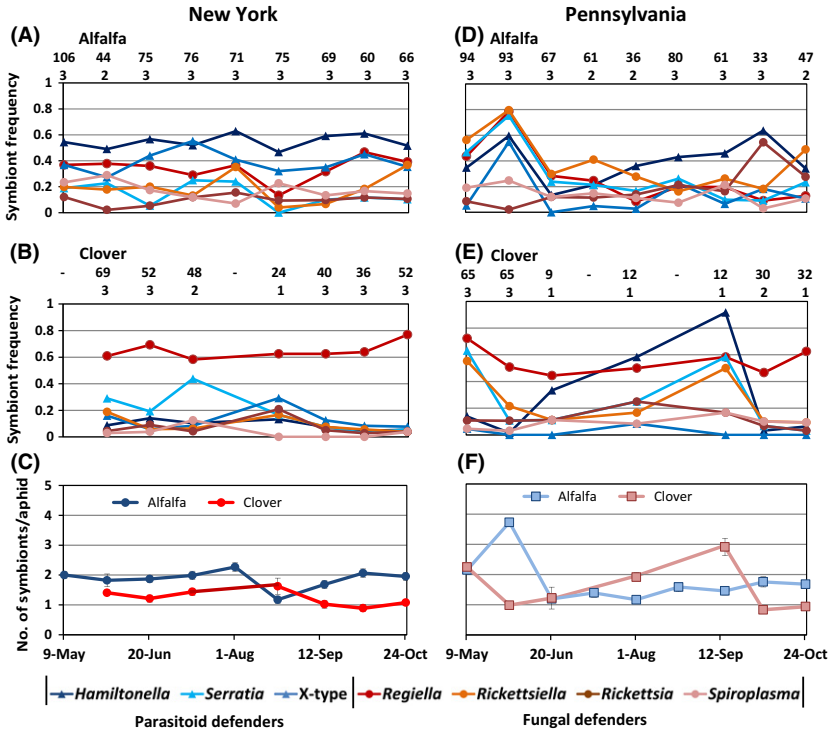

Fig. 2 Symbiont frequencies and superinfection level overtime. The average proportion of pea aphids possessing facultative symbionts (A, B, D, E) and average number of symbionts per aphid (superinfection level) (C, F) in 3 alfalfa and 3 clover fields repeatedly sampled in New York (A, B, C) and Pennsylvania (D, E, F) in 2011. Numbers above graphs indicate the total number of aphids sampled on a particular date (top row) and the number of fields sampled (bottom row). A dash (–) indicates no aphids were found in any fields on those dates sampled.

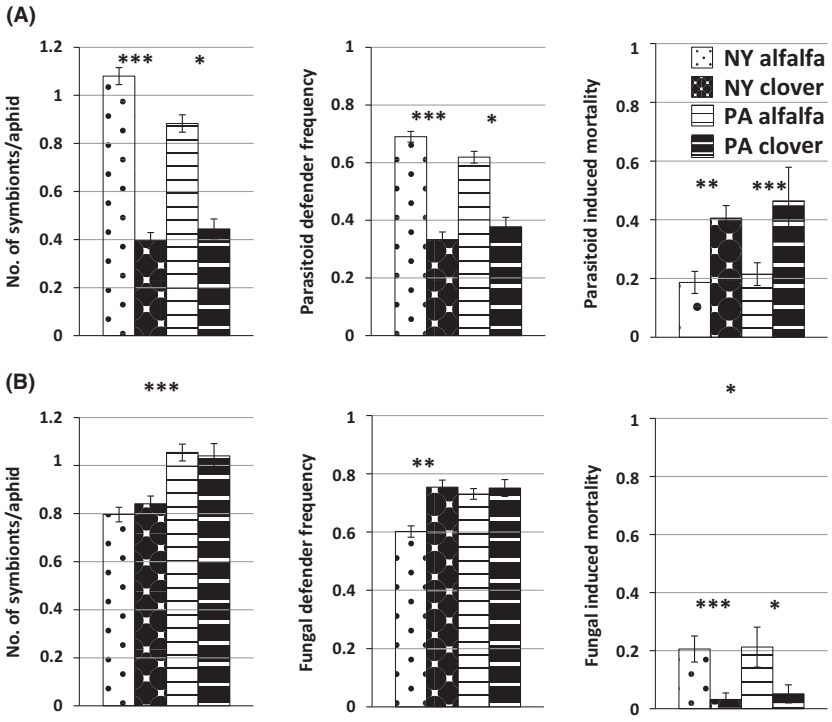

Fig. 3 Defensive symbiont superinfection level, frequency and enemy-induced aphid mortality. Columns are the mean (±SEM) number of symbionts per aphid (superinfection level), proportion of aphids possessing at least one defensive symbiont (symbiont frequency) and proportion of aphids dying due to *Aphidius ervi* or fungal pathogens in three alfalfa and three clover fields repeatedly sampled in New York and Pennsylvania in 2011. (A) Parasitoid defenders: *Hamiltonella*, *Serratia*, X-type. (B) Fungal defenders: *Regiella*, *Rickettsiella*, *Rickettsia*, *Spiroplasma*. Asterisks above columns indicate significant differences between crops within a state, and asterisk above bars indicate differences among states. (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).

symbionts per aphid, uninfected frequency = 23.9%) (Fig. 2). A similar parallel drop was observed in PA clover between the first and second collection dates (Fig. 2). In both populations, these symbiont shifts showed strong consistency between replicate fields (Fig. S1, Supporting information). Microsatellite genotyping of aphids from these time points indicated that the rapid shift in alfalfa populations was not the result of hitchhiking on successful/unsuccessful clonal backgrounds (Fig. S2; Table S4, Supporting information), as

we found high superinfection levels across many different clonal genotypes (Appendix S1, Supporting information: Microsatellite Data).

To further explore the unexpectedly high levels of superinfection across multiple clonal backgrounds, we ran PCRs on DNA from surface sterilized aphids from these same time points, while also performing sequence confirmation to rule out false positives. Details of this work are described in Supporting Information, but in short, our findings did not suggest methodological error or batch contamination effects. Instead, we conclude that superinfections can reach high levels at particular times of the year.

In all but the NY clover population, parallel drops in symbiont frequencies were seen across replicate fields 3 weeks after times of peak or near-peak superinfection (Fig. 2; Fig. S1, Supporting information). The timing of two events across PA host races occurred within a 3-week span (May 9–30 vs. May 30–June 20 for clover and alfalfa, respectively). The other parallel drops in infection frequencies occurred in mid-season in NY alfalfa (August 1–22) and late season in PA clover (September 12–October 3). These trends were unusual in terms of their magnitude, replication across fields and the numbers of affected symbionts with similar simultaneous trajectories. All coincided with a rise in the proportion of uninfected aphids, which was driven by symbiont absence and not reduced symbiont titre, based on real-time PCR results (Table S5, Supporting information).

Insect densities and aphid mortality

Pea aphid mortality assays and sweep net sampling were performed in conjunction with aphid sampling to determine the proportion of aphids dying due to parasitoids or fungal infection, along with aphid, parasitoid and predator densities. In depth analyses of regional, temporal and host race impacts on aphid and natural enemy densities can be found in the supplemental materials (Fig. S3; Table S5, Supporting information). Most notably, standardized *A. ervi* densities (# *A. ervi* wasps: pea aphid) did not differ between crops, although they were higher in PA than NY ($F = 5.52$, $P < 0.05$, Table S5, Supporting information). Furthermore, the densities of aphids, *A. ervi* (standardized and unstandardized), and all predators fluctuated significantly overtime in all populations but NY clover ($P < 0.05$, Table S5, Supporting information)—the population with the most subtle symbiont dynamics—where only *A. ervi* and *Orius insidiosus* (minute pirate bug) densities showed no significant shifts.

The proportions of aphids dying due to parasitoids and pathogens also varied overtime, space and/or host race. Of the 448 aphids that mummified after succumbing to parasitism, 387 were killed by *A. ervi*, while 61 were killed by *Praon* spp. We found no state or state × crop interaction effects on *A. ervi*-, total parasitoid- or fungal-induced mortality; however, all three measures fluctuated temporally in all populations except NY clover ($P < 0.05$, Fig. S4, Table S6, Supporting information). Additionally, mortality resulting from parasitism by *A. ervi*, and parasitoids overall, was 20–27% higher in clover than alfalfa ($F = 14.39$, $P < 0.001$, $F = 13.63$, $P < 0.001$, respectively, Fig. 3, Fig. S4; Table S6, Supporting information), while that caused by fungal pathogens, was 16–17% higher in alfalfa ($F = 9.97$, $P < 0.01$, Fig. 3, Fig. S4, Table S6, Supporting information). Our findings of higher parasitoid resistance among alfalfa host races and higher fungal resistance among clover host races match laboratory and field assays conducted, in some cases, from similar regions (Hufbauer & Via 1999; Hufbauer 2001, 2002a,b; Ferrari & Godfray 2003) as this study.

Correlations between facultative symbionts, natural enemies and temperature

The most notable positive correlations between facultative symbionts and natural enemy pressures existed in PA alfalfa populations between *A. ervi* mortality and all known or suspected parasitoid defenders, *Hamiltonella* ($F = 24.75$, $P < 0.0001$), *Serratia* ($F = 46.48$, $P < 0.0001$) and X-type ($F = 80.02$, $P < 0.0001$) (Fig. 4, Table S7, Supporting information). These were characterized by early spikes in all three symbionts at times of high parasitoid-induced mortality, followed by a smaller rebound in symbiont frequencies coinciding with a slight rise in successful parasitism in late August.

Regiella frequencies surprisingly showed a similar positive correlation with *A. ervi*-induced mortality in PA alfalfa populations ($F = 64.32$, $P < 0.0001$) (Table S7, Supporting information). Yet *Hamiltonella-Regiella* coinfections also showed a similar trend for this population ($F = 58.63$, $P < 0.0001$) as did *Hamiltonella*-X-type coinfections ($F = 63.51$, $P < 0.0001$), and overall superinfection level ($F = 133.73$, $P < 0.0001$, Table S7, Supporting information), suggesting the potential for symbiont hitchhiking or unforeseen synergistic impact of coinfection symbionts.

In this same population, only *Hamiltonella* frequencies (among the three parasitoid defenders) correlated with *A. ervi*:aphid ratio, this time exhibiting a negative relationship ($F = 7.72$, $P < 0.01$, Table S8, Supporting information). However, the three highest values for *A. ervi* density (standardized and unstandardized) were reached on the dates of highest *Hamiltonella* frequencies (May 30, October 3) or 3 weeks after (June 20),

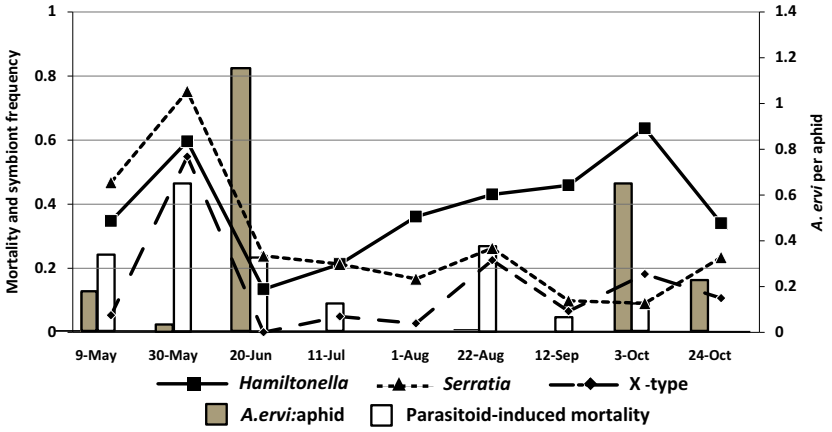

Fig. 4 *Aphidius ervi*-induced mortality, *A. ervi* per aphid density and symbiont frequencies in Pennsylvania alfalfa. Points or columns on the graphs are mean proportions of aphids successfully parasitized by *A. ervi* or infected with symbionts and average *A. ervi*:aphid ratio in three alfalfa fields repeatedly sampled in Pennsylvania in 2011. Missing columns for *A. ervi*:Aphid indicate values of zero. Missing columns for parasitoid mortality on August 1 and October 24 indicate aphid numbers were too low in all three fields to perform mortality assays.

consistent with an association between *Hamiltonella* frequency shifts and parasitoid selection pressures (Figs 4 and S3, Supporting information).

In PA clover populations, there was a negative relationship between *A. ervi*-induced mortality and parasitoid defender frequency ($F = 44.73$, $P < 0.0001$, Fig. 5, Table S7, Supporting information), with a notable rise in mummification coinciding with two separate drops in defender frequencies (May 9–May 30, September 12–October 3), followed by a rise in *A. ervi* density and *A. ervi*:aphid ratio 3 weeks later (Figs S3 and S4, Supporting information). In NY clover populations, we found a marginally significant negative correlation between the frequency of aphids with a fungal defender and fungal-induced mortality ($F = 3.32$, $P = 0.068$, Table S9, Supporting information), with the only instances of fungal-induced mortality coming in two of the three time points in which defender frequencies fell below 70%. Such negative correlations between symbionts and their targeted enemies were also seen in pooled analysis across populations ($P < 0.05$, Fig. 5, Tables S7 and S9, Supporting information). These trends were especially notable for the most established defenders, *Hamiltonella* (parasitoids) and *Regiella* (pathogens), and were largely driven by differences between host races in mortality and symbiont prevalence (Fig. 5, Tables S7 and S9, Supporting information), suggesting suppressive impacts of symbionts on the success of targeted enemies.

While temperature correlated with some seasonal symbiont dynamics, our findings were not consistent

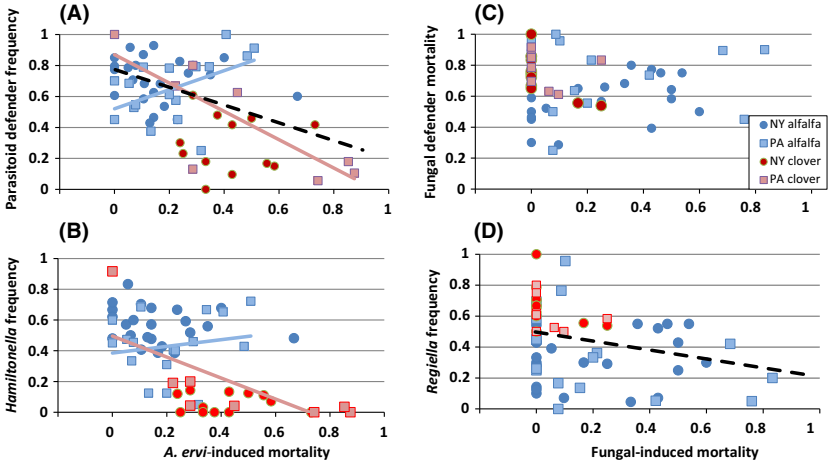

Fig. 5 Symbiont by mortality factor linear regression. Linear regression of (A) the proportion of aphids possessing a parasitoid defender (*Hamiltonella*, *Serratia*, X-type) or (B) *Hamiltonella*, vs. the proportion dying from *Aphidius ervi* parasitism and (C) the proportion of aphids possessing a fungal defender (*Regiella*, *Rickettsiella*, *Rickettsia*, *Spiroplasma*) or (D) *Regiella*, vs. those dying from fungal infection. Each point represents an individual field and time point sampled in 2011. The linear trendline for all data points is represented by the black dashed line and is shown only when the relationship significantly deviates from zero. Trendlines for regressions that differed significantly ($P < 0.05$) at the population level (for a given host race within the represented state) are shown on each graph in coloured lines.

across host races and locations (Table S10, Supporting information), arguing against a clear symbiont response. In fact, thermotolerance-conferring *Serratia* showed a negative overall correlation with temperature across the four populations. However, higher frequencies of *Serratia* were consistently found in warmer PA regions ($F = 17.47$, $P < 0.0001$, Fig. 1, Table 1), revealing a split between temporal and geographic temperature patterns.

Discussion

We found that the heritable microbiome of pea aphids is highly dynamic over short timescales. While symbiont dynamics varied across populations, we generally found all symbionts to change in frequency overtime in at least one population. These fluctuations were greatest in PA alfalfa, and lowest in NY clover where enemy dynamics were intriguingly also muted. While genetic drift and migration may have driven some microbiome dynamics, many symbiont shifts were large in magnitude and occurred in parallel across replicate fields. As aphid genotyping ruled out hitchhiking on favoured aphid clones, the magnitude and consistency of symbiont dynamics across replicate fields suggest nonrandom symbiont-mediated aphid evolution (see Table S11, Supporting Information). Gradual-to-rapid rises in symbiont prevalence suggest roles for symbionts in seasonal aphid adaptation. Yet the most extreme parallel changes involved rapid drops in symbiont frequencies, arguing for symbiont costs or instability under some contexts. Considering the host race and regional symbiont variation found in our study, these seasonal shifts further underscore the impressive dynamics of facultative symbionts in the pea aphid species.

Correlates of symbiont frequencies

All seven pea aphid facultative symbionts have been implicated in defence against parasitoids or fungal pathogens. While analyses on defensive guilds (e.g. parasitoid defenders) uncovered intuitive trends (discussed further below), those focused on the two most established defenders, *Hamiltonella* (vs. parasitoids) and *Regiella* (vs. fungal pathogens), revealed some of the clearest symbiont–enemy correlations. Notably, *Hamiltonella* frequencies (like those of *Serratia* and X-type) showed positive correlations with parasitoid-induced mortality in PA alfalfa, adding to a growing body of literature documenting temporal correlations between defensive traits and shifting enemy pressures. As prior discoveries have emphasized host-encoded traits (Reznick *et al.* 1997; Reimchen & Nosil 2002), our findings provide, to our knowledge, the first natural signal of symbiont-driven adaptation in response to natural enemies. That such a response unfolded across just weeks or months suggests that symbionts may enable seasonal adaptation in multivoltine organisms.

In addition to this temporal correlation, trends seen mostly across host races suggest important ecological impacts of symbiont variability. First, higher frequencies of *Hamiltonella* in alfalfa host races correlated with lower levels of *A. ervi*-induced mortality; and second, higher *Regiella* frequencies in clover host races were associated with lower rates of fungal-induced mortality. Although similar findings on symbionts and pea aphid mortality have been reported separately (Hufbauer 2002a; Henry *et al.* 2013) (see Oliver *et al.* 2014 for review), our study is the first to explicitly link variable mortality with variable symbiont frequencies in the pea aphid system. Discoveries from insects (Jaenike *et al.* 2010) and plants (Clay *et al.* 2005) have also demonstrated symbiont-driven alterations in host-enemy outcomes, with the potential for community-wide ecological impacts.

The last of the clear, novel patterns identified in this study involved the propensity for multiple symbionts to super-infect the same hosts, a property that varied significantly overtime. Specifically, for three of our four populations, nearly ubiquitous and often drastic reductions in symbiont frequencies occurred within 3 weeks of a seasonal spike in superinfection, levels not observed in previous field surveys (Ferrari *et al.* 2012; Russell *et al.* 2013). In PA clover populations, two such events occurred at different times of the year; and like the singular events in PA and NY alfalfa, the early season PA clover event was consistent across symbionts and replicate fields.

This population-level 'instability' is intriguing and largely unforeseen considering our general knowledge on transmission rates, transmission routes and costs of infection from laboratory studies (see Oliver *et al.* 2014). First, in the laboratory, single symbiont infections are stable with near 100% transmission efficiency from mother to offspring (Chen & Purcell 1997; Darby & Douglas 2003; Weldon *et al.* 2013). While some instances of symbiont loss have been observed under exposure to high temperatures (Burke *et al.* 2009), or in individuals infected with multiple symbionts (Sandstrom *et al.* 2001; Moran & Dunbar 2006), no studies have observed transmission failure to the extent required to explain our findings of drastic symbiont declines. Second, while horizontal symbiont transfer must occur over evolutionary timescales (Sandstrom *et al.* 2001; Russell *et al.* 2003), there is little evidence that it is frequent enough to drive the observed superinfection spikes. For instance, laboratory studies have found little, if any, transfer between aphids feeding on the same plants (Chen & Purcell 1997; Darby & Douglas 2003; Oliver

et al. 2008). However, parasitoids can vector symbionts between black bean aphids under laboratory conditions (Gehrer & Vorburger 2012), but rates under field conditions are presumably lower and it is unclear if transmission rates in this manner can approach that required to explain the symbiont frequency and superinfection spikes observed in our study. Third, and finally, high costs of single symbiont infections have not been observed in the absence of natural enemies or heat shock in the laboratory (Russell & Moran 2006; Oliver *et al.* 2008) (see Supplementary Information: *Hamiltonella defensa* dynamics), at least not under the temperate spring and summer conditions that are characteristic of our field locations. However, strong fitness detriments have been reported for an artificially created superinfection in pea aphids (Oliver *et al.* 2006). Considering the high superinfection levels observed in this study, infection costs clearly warrant future consideration as a potential driver of symbiont instability and frequency shifts in natural populations.

Emerging complexity in the pea aphid-defensive symbiont system

While the above findings suggest the reciprocal impacts of symbionts and natural enemies, several other analyses on enemies and symbionts lacked consistent or intuitive correlations (see correlations: Tables S7 and S9; time lag correlations: Tables S12 and S13, Supporting information) revealing complexities inherent in multipartite interactions operating under natural conditions (Fig. 6). While this may partially expose room for improvement in our measures of enemy pressures, (e.g. the timing and frequency of our sampling), it also raises important biological questions. Most pertinent are those relating to the strength and direction of selection under temporal and spatial environmental variability and how these govern symbiont-mediated eco-evolutionary feedbacks between aphids and enemies (Fig. 6). In the sections below, we discuss four candidate factors with the potential to alter these feedbacks and their implications for the design of future field studies, including (i) strong impacts of alternative environmental forces; (ii) unforeseen sources of defensive variability; (iii) symbiont-symbiont hitchhiking; and (iv) rapid enemy counter-adaptation.

Emerging complexity: alternative environmental forces

Laboratory-based findings indicate that pathogens and parasitoids are not the only factors influencing the pea aphid microbiome; thus, the strength of selection imposed by alternative forces may outweigh enemy impacts in the field, as seen in other systems (Parker 1991; Burdon & Thompson 1995). Climate is among the strongest candidates, as climate-mediated selection

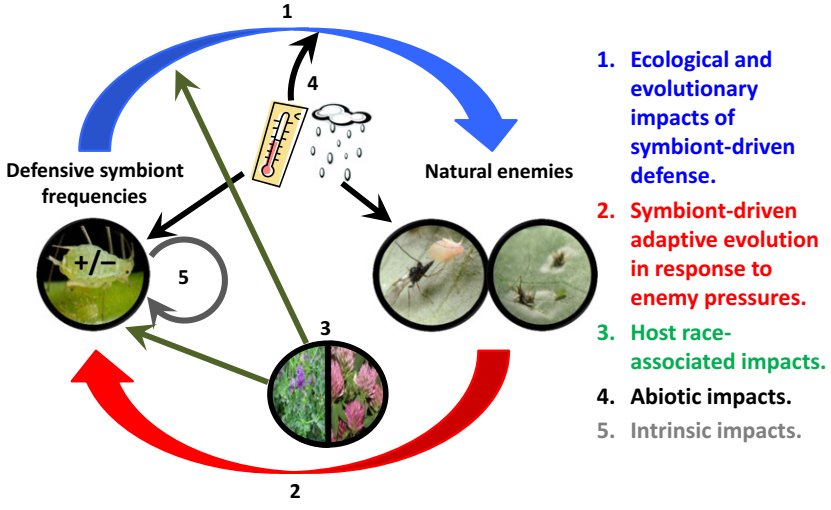

Fig. 6 Eco-evolutionary feedback model for interactions between aphids, their symbionts and their natural enemies. Natural enemy populations and evolution should be shaped by the prevalence of defensive symbionts in the field (1). Enemy pressures should play a role in governing symbiont prevalence (2). Symbiont-mediated feedbacks between enemies and aphids may also involve co-evolution, with negative frequency dependence between enemy genotypes and the specific strains that they can or cannot overcome (1) & (2). However, countervailing selective factors or the efficacy of defence in the field may vary across host races (3) or climates (4), potentially limiting symbiont-enemy feedbacks. Climate will also directly impact enemy performance (4) independently of aphid defence, helping to shape the seasonality of enemy populations seen for this system. Finally, superinfections may be unstable due to intrinsic fitness costs or transmission failure (5), ultimately governing symbiont dynamics at certain times of year.

plays a major role in shaping natural phenotypic variation (Siepielski *et al.* 2009), and as symbiont stability and benefits are impacted by temperature in other systems (Berkelmans & Van Oppen 2006). Pea aphids and their symbionts serve as a prime example, as laboratory-based heat shock can favour aphids with *Serratia* symbionts (Montllor *et al.* 2002), while apparently reducing the rates of *Serratia* transfer from mother to offspring (Burke *et al.* 2010). Unlike Montllor *et al.* (2002), we did not detect temporal patterns of *Serratia*-driven adaptation to warming temperatures. However, enrichment of *Serratia* in warmer PA vs. NY populations is consistent with temperature-mediated selection favouring aphids with this symbiont, adding to a small list of correlations between symbiont frequencies and climactic variables (Tsuchida *et al.* 2002; Berkelmans & Van Oppen 2006; Henry *et al.* 2013).

The costs and benefits of other symbionts are not firmly known to vary in consistent ways across temperature (but see Russell & Moran 2006), yet *Hamiltonella*-mediated defence against parasitoids has been proposed to falter under hot conditions (Bensadia *et al.* 2006; Guay *et al.* 2009). Thus, the expected seasonality of defensive penetrance, the observed seasonality of parasitoid and pathogen dynamics (prevalent in spring and fall), and the known impacts of climate on *Pandora* pathogens (Shah *et al.* 2002) (favoured under cool, humid conditions), may have driven the mostly negative correlations between temperature and symbionts. Similarly, positive correlations between uninfected aphids and temperature may stem from symbiont-imposed costs in the absence of natural enemies and not temperature alone.

Direct and indirect impacts of climate are just one possible contributor to our unexplained symbiont dynamics, with possibilities including competition, predators and host plant (Tsuchida *et al.* 2004; Ferrari *et al.* 2007; Oliver *et al.* 2008; Costopoulos *et al.* 2014). This latter factor is intriguing in the light of differing constraints to symbiont spread seen across pea aphid host races. Indeed, persistently low levels of antiparasitoid symbionts were detected in clover host races in spite of similar numbers of *A. ervi* wasps (per aphid) in alfalfa and clover fields. In contrast, limited spread of anti-pathogen *Regiella* symbionts was seen among alfalfa host races, in spite of higher pathogen mortality. While the host plant could directly shape these differences, aphid genetic background, the identities of other predominant symbionts and surrounding community composition serve to further differentiate pea aphid host races. Distinguishing among these factors will, thus, be crucial to understanding the drivers of microbiome divergence.

Emerging complexity: unforeseen sources of defensive variability and hitchhiking

While pea aphids have outsourced some defence to facultative symbionts, more recent discoveries suggest variability in host-encoded factors that govern the outcomes of aphid-parasitoid and aphid–fungal pathogen interactions (Martinez *et al.* 2014; Parker *et al.* 2014). The genetic basis for these outcomes is not yet known but the general influence of aphid genotype on symbiont costs and benefits (Ferrari *et al.* 2007; Łukasik *et al.* 2013b) suggests a need for systematic exploration on the relative contributions of hosts vs. symbionts towards defensive variability.

Underexplored defensive variability may also arise at the microbiome level due to strain diversity and superinfection. For instance, different *Hamiltonella* and *Spiroplasma* strains confer varying levels of defence against pea aphid parasitoids and pathogens, respectively (Oliver *et al.* 2005; Łukasik *et al.* 2013c). Given the potential for interspecific symbiont transfer (Russell *et al.* 2003) and entirely different defensive roles across related symbiont strains from various aphid species (Vorburger *et al.* 2010; Hansen *et al.* 2012; Łukasik *et al.* 2013a), confident assignment of a protective property to a symbiont species requires extensive experimentation. In addition to strain differences, variation in the protective abilities or general costs and benefits of symbiont species may arise due to variable superinfections. To date, few studies have explicitly explored the impacts of single vs. multiple infection, but superinfection has been argued to alter host-symbiont outcomes in important ways (Oliver *et al.* 2006; Guay *et al.* 2009). As aphid superinfection varies overtime and across host races, and as multiple strains of the same symbiont species exist in the same populations (Russell *et al.* 2013), it is evident that a tremendous amount of cytoplasmically inherited genetic diversity exists within this system.

An additional complication stemming from common superinfection involves hitchhiking, where a symbiont *not* under selection increases or decreases in the population due to common cohabitation with a symbiont that *is* under selection. The early season PA dynamics in both clover and alfalfa provide an example, as both pathogen and parasitoid defenders (known and suspected), showed similar trajectories. Parasitoids appeared to be the dominant selective force at this time, among the enemies studied. So it would be reasonable to propose that pathogen defenders were hitchhiking in favoured aphids due to the benefits of parasitoid defenders. In general, hitchhiking by coinfecting symbionts would certainly help to explain some of our nonintuitive correlations. However, benefits may be realized

specifically because of superinfection, suggesting a tantalizing avenue for future investigation.

Emerging complexity: enemy counter-adaptation

Our results, plus those from a prior laboratory-based experimental evolution study, have shown aphids can evolve at the microbiome level, likely in response to their natural enemies (Oliver *et al.* 2008). However, *P. neoaphidis* and *A. ervi* are not static targets and possess sufficient variation in their ability to overcome host resistance (Milner 1985; Henter 1995), indicating the potential for natural counter-adaptation. Importantly, recent studies have implicated symbionts as targets of enemy virulence, as parasitoids can employ behavioural responses (Oliver *et al.* 2012) and rapidly evolve to overcome *Hamiltonella*-mediated defence (Dion *et al.* 2011). The specificity of wasp virulence towards particular *Hamiltonella* variants (Rouchet & Vorburger 2012, 2014) suggests that field studies in this system should shift their emphasis from the symbiont species to the strain level.

Combined, these findings suggest the microbiome's potential as a linchpin in the antagonistic co-evolution between pea aphids and their enemies. The negative frequency-dependence extending from such interactions could help to maintain the diverse suite of heritable, defensive elements found in pea aphid populations and explain the subtleties of our results, should aphids switch between modes of defence not easily discriminated through our methodologies. This possibility clearly warrants further investigation in the pea aphid system and beyond given the widespread nature of defensive symbiosis, the importance of antagonistic co-evolution in plant and animal systems, and the resulting implications for the microbiome in global biodiversity.

Conclusions

Our findings of symbiont frequency shifts across just a few generations suggest that symbionts are part of the adaptive arsenal of their pea aphid hosts, meeting challenges unfolding across short time spans, but also imposing large costs under some contexts. Although symbiont response to enemy-mediated pressures over seasonal timescales is not always clear, perhaps due to stronger counter-acting selective pressures or other complexities within the system (e.g. superinfection, hitchhiking, frequency-dependent selection, methodology), our data suggest that symbionts impact aphid-enemy feedbacks, shaping divergence in ecologically important defensive properties across pea aphid populations. Our understanding of the real world impacts of a dynamic microbiome is still in its infancy, with examples in the field undoubtedly more common than documented thus far. Considering the near ubiquity of microbial symbionts across eukaryotic hosts, future field studies should prove highly informative in our understanding of the eco-evolutionary dynamics shaped by nature's diverse microbiome.

Acknowledgements

We would like to thank the Cornell University (Ithaca, NY) Dairy Research Center and Potato Breeding Program and the following farmers for access to fields for sampling: Troy Sherman; John Flemming; William Wisleff; Vernon Burkholder; Norman Burkholder; Edward Burkholder; Lawrence Burkholder and Steven Brubacher. This study was funded by the National Science Foundation award No. 1050098. We dedicate this study to the memory of our co-author, Andrea Messina, and her inspiring enthusiasm. Her warmth and effervescent spirit will not be forgotten.

References

Bensadia F, Boudreault S, Guay J-F, Michaud D, Cloutier C (2006) Aphid clonal resistance to a parasitoid fails under heat stress. *Journal of Insect Physiology*, **52**, 146–157.

Berkelmans R, Van Oppen MJ (2006) The role of zooxanthellae in the thermal tolerance of corals: a 'nugget of hope'for coral reefs in an era of climate change. *Proceedings of the Royal Society B: Biological Sciences*, **273**, 2305–2312.

Brown AMV, Huynh LY, Bolender CM, Nelson KG, McCutcheon JP (2014) Population genomics of a symbiont in the early stages of a pest invasion. *Molecular Ecology*, **23**, 1516–1530.

Burdon JJ, Thompson JN (1995) Changed patterns of resistance in a population of *Linum marginale* attacked by the rust pathogen *Melampsora lini*. *Journal of Ecology*, **83**, 199–206.

Burke G, Fiehn O, Moran N (2009) Effects of facultative symbionts and heat stress on the metabolome of pea aphids. *ISME Journal*, **4**, 242–252.

Burke G, Fiehn O, Moran N (2010) Effects of facultative symbionts and heat stress on the metabolome of pea aphids. *The ISME Journal*, **4**, 242–252.

Carroll SP, Hendry AP, Reznick DN, Fox CW (2007) Evolution on ecological time-scales. *Functional Ecology*, **21**, 387–393.

Chen D-Q, Purcell AH (1997) Occurrence and transmission of facultative endosymbionts in aphids. *Current Microbiology*, **34**, 220–225.

Chu C-C, Spencer JL, Curzi MJ, Zavala JA, Seufferheld MJ (2013) Gut bacteria facilitate adaptation to crop rotation in the western corn rootworm. *Proceedings of the National Academy of Sciences*, **110**, 11917–11922.

Chung SH, Rosa C, Scully ED *et al.* (2013) Herbivore exploits orally secreted bacteria to suppress plant defenses. *Proceedings of the National Academy of Sciences*, **110**, 15728–15733.

Clay K, Holah J, Rudgers JA (2005) Herbivores cause a rapid increase in hereditary symbiosis and alter plant community composition. *Proceedings of the National Academy of Sciences of the United States of America*, **102**, 12465–12470.

Costopoulos K, Kovacs JL, Kamins A, Gerardo NM (2014) Aphid facultative symbionts reduce survival of the predatory lady beetle *Hippodamia convergens*. *BMC Ecology*, **14**, 5.

Darby A, Douglas A (2003) Elucidation of the transmission patterns of an insect-borne bacterium. *Applied and Environmental Microbiology*, **69**, 4403–4407.

Dion E, Zélé F, Simon JC, Outreman Y (2011) Rapid evolution of parasitoids when faced with the symbiont-mediated resistance of their hosts. *Journal of Evolutionary Biology* **24**, 741–750.

Ferrari J, Godfray HCJ (2003) Resistance to a fungal pathogen and host plant specialization in the pea aphid. *Ecology Letters*, **6**, 111–118.

Ferrari J, Darby AC, Daniell TJ, Godfray HCJ, Douglas AE (2004) Linking the bacterial community in pea aphids with host-plant use and natural enemy resistance. *Ecological Entomology*, **29**, 60–65.

Ferrari J, Scarborough CL, Godfray HCJ (2007) Genetic variation in the effect of a facultative symbiont on host-plant use by pea aphids. *Oecologia*, **153**, 323–329.

Ferrari J, West JA, Via S, Godfray HC (2012) Population genetic structure and secondary symbionts in host-associated populations of the pea aphid complex. *Evolution*, **66**, 375–390.

Fussmann GF, Loreau M, Abrams PA (2007) Eco-evolutionary dynamics of communities and ecosystems. *Functional Ecology*, **21**, 465–477.

Gehrer L, Vorburger C (2012) Parasitoids as vectors of facultative bacterial endosymbionts in aphids. *Biology Letters*, rsbl20120144.

Guay J-F, Boudreault S, Michaud D, Cloutier C (2009) Impact of environmental stress on aphid clonal resistance to parasitoids: role of *Hamiltonella defensa* bacterial symbiosis in association with a new facultative symbiont of the pea aphid. *Journal of Insect Physiology*, **55**, 919–926.

Hansen AK, Vorburger C, Moran NA (2012) Genomic basis of endosymbiont-conferred protection against an insect parasitoid. *Genome Research*, **22**, 106–114.

Hedges LM, Brownlie JC, O'Neill SL, Johnson KN (2008) Wolbachia and virus protection in insects. *Science*, **322**, 702.

Henry LM, Peccoud J, Simon J-C *et al.* (2013) Horizontally transmitted symbionts and host colonization of ecological niches. *Current Biology*, **23**, 1713–1717.

Henter HJ (1995) The potential for coevolution in a host-parasitoid system. II. Genetic variation within a population of wasps in the ability to parasitize an aphid host. *Evolution*, **49**, 439–445.

Hilgenboecker K, Hammerstein P, Schlattmann P, Telschow A, Werren JH (2008) How many species are infected with Wolbachia?–a statistical analysis of current data. *FEMS Microbiology Letters*, **281**, 215–220.

Himler AG, Adachi-Hagimori T, Bergen JE *et al.* (2011) Rapid spread of a bacterial symbiont in an invasive whitefly is driven by fitness benefits and female bias. *Science*, **332**, 254–256.

Hoffmann AA, Hercus M, Dagher H (1998) Population dynamics of the Wolbachia infection causing cytoplasmic incompatibility in *Drosophila melanogaster*. *Genetics*, **148**, 221–231.

Hoffmann AA, Montgomery BL, Popovici J *et al.* (2011) Successful establishment of Wolbachia in Aedes populations to suppress dengue transmission. *Nature*, **476**, 454–457.

Hufbauer RA (2001) Pea aphid-parasitoid interactions: have parasitoids adapted to differential resistance? *Ecology*, **82**, 717–725.

Hufbauer RA (2002a) Aphid population dynamics: does resistance to parasitism influence population size? *Ecological Entomology*, **27**, 25–32.

Hufbauer RA (2002b) Evidence for nonadaptive evolution in parasitoid virulence following a biological control introduction. *Ecological Applications*, **12**, 66–78.

Hufbauer RA, Via S (1999) Evolution of an aphid-parasitoid interaction: variation in resistance to parasitism among aphid populations specialized on different plants. *Evolution*, **53**, 1435–1445.

Hutchinson WD, Hogg DB (1984) Demographic statistics for the pea aphid (Homoptera: Aphididae) in Wisconsin and a comparison with other populations. *Environmental Entomology*, **13**, 1173–1181.

Jaenike J, Unckless R, Cockburn SN, Boelio LM, Perlman SJ (2010) Adaptation via symbiosis: recent spread of a *Drosophila* defensive symbiont. *Science*, **329**, 212–215.

Łukasik P, Dawid MA, Ferrari J, Godfray HCJ (2013a) The diversity and fitness effects of infection with facultative endosymbionts in the grain aphid, *Sitobion avenae*. *Oecologia*, **173**, 985–996.

Łukasik P, Guo H, van Asch M, Ferrari J, Godfray HCJ (2013b) Protection against a fungal pathogen conferred by the aphid facultative endosymbionts *Rickettsia* and *Spiroplasma* is expressed in multiple host genotypes and species and is not influenced by co-infection with another symbiont. *Journal of Evolutionary Biology*, **26**, 2654–2661.

Łukasik P, van Asch M, Guo H, Ferrari J, Godfray HCJ (2013c) Unrelated facultative endosymbionts protect aphids against a fungal pathogen. *Ecology Letters*, **16**, 214–218.

Markkula M (1963) Studies on the pea aphid, *Acyrthosiphon pisum* harris (hom., aphididae), with special refference to the differences in the biology of the green and red forms.

Martinez AJ, Weldon SR, Oliver KM (2014) Effects of parasitism on aphid nutritional and protective symbioses. *Molecular Ecology*, **23**, 1594–1607.

Milner RJ (1985) Distribution in time and space of resistance to the pathogenic fungus *Erynia neoaphidis* in the pea aphid *Acyrthosiphon pisum*. *Entomologia Experimentalis et Applicata*, **37**, 235–240.

Montllor CB, Maxmen A, Purcell AH (2002) Facultative bacterial endosymbionts benefit pea aphids *Acyrthosiphon pisum* under heat stress. *Ecological Entomology*, **27**, 189–195.

Moran NA, Dunbar HE (2006) Sexual acquisition of beneficial symbionts in aphids. *Proceedings of the National Academy of Sciences of the United States of America*, **103**, 12803–12806.

Moran NA, Russell JA, Koga R, Fukatsu T (2005) Evolutionary relationships of three new species of Enterobacteriaceae living as symbionts of aphids and other insects. *Applied and Environmental Microbiology*, **71**, 3302–3310.

Moran NA, McCutcheon JP, Nakabachi A (2008) Genomics and evolution of heritable bacterial symbionts. *Annual Review of Genetics*, **42**, 165–190.

Oliver KM, Russell JA, Moran NA, Hunter MS (2003) Facultative bacterial symbionts in aphids confer resistance to parasitic wasps. *Proceedings of the National Academy of Sciences of the United States of America*, **100**, 1803–1807.

Oliver KM, Moran NA, Hunter MS (2005) Variation in resistance to parasitism in aphids is due to symbionts not host genotype. *Proceedings of the National Academy of Sciences of the United States of America*, **102**, 12795–12800.

Oliver KM, Moran NA, Hunter MS (2006) Costs and benefits of a superinfection of facultative symbionts in aphids. *Proceedings of the Royal Society B-Biological Sciences*, **273**, 1273–1280.

Oliver KM, Campos J, Moran NA, Hunter MS (2008) Population dynamics of defensive symbionts in aphids. *Proceedings of the Royal Society B-Biological Sciences*, **275**, 293–299.

Oliver KM, Degnan PH, Hunter MS, Moran NA (2009) Bacteriophages encode factors required for protection in a symbiotic mutualism. *Science*, **325**, 992–994.

Oliver KM, Degnan PH, Burke GR, Moran NA (2010) Facultative symbionts in aphids and the horizontal transfer of ecologically important traits. *Annual Review of Entomology*, **55**, 247–266.

Oliver KM, Noge K, Huang EM *et al.* (2012) Parasitic wasp responses to symbiont-based defense in aphids. *BMC Biology*, **10**, 11.

Oliver KM, Smith AH, Russell JA (2014) Defensive symbiosis in the real world – advancing ecological studies of heritable, protective bacteria in aphids and beyond. *Functional Ecology*, **28**, 341–355.

Parker MA (1991) Nonadaptive evolution of disease resistance in an annual legume. *Evolution*, **45**, 1209–1217.

Parker BJ, Spragg CJ, Altincicek B, Gerardo NM (2013) Symbiont-mediated protection against fungal pathogens in pea aphids: a role for pathogen specificity? *Applied and Environmental Microbiology*, **79**, 2455–2458.

Parker BJ, Garcia JR, Gerardo NM (2014) Genetic variation in resistance and fecundity tolerance in a natural host-pathogen interaction. *Evolution*, **68**, 2421–2429.

Peccoud J, Ollivier A, Plantegenest M, Simon J-C (2009) A continuum of genetic divergence from sympatric host races to species in the pea aphid complex. *Proceedings of the National Academy of Sciences*, **106**, 7495–7500.

Piel J, Höfer I, Hui D (2004) Evidence for a symbiosis island involved in horizontal acquisition of pederin biosynthetic capabilities by the bacterial symbiont of Paederus fuscipes beetles. *Journal of Bacteriology*, **186**, 1280–1286.

R Development Core Team (2011) *R: A Language and Environment for Statistical Computing*. R Foundation for Statistical Computing, Vienna, Austria.

Reimchen TE, Nosil P (2002) Temporal variation in divergent selection on spine number in three spine stickleback. *Evolution*, **56**, 2472–2483.

Reznick DN, Shaw FH, Rodd FH, Shaw RG (1997) Evaluation of the rate of evolution in natural populations of Guppies (*Poecilia reticulata*). *Science*, **275**, 1934–1937.

Rouchet R, Vorburger C (2012) Strong specificity in the interaction between parasitoids and symbiont-protected hosts. *Journal of Evolutionary Biology*, **25**, 2369–2375.

Rouchet R, Vorburger C (2014) Experimental evolution of parasitoid infectivity on symbiont-protected hosts leads to the emergence of genotype specificity. *Evolution*, **68**, 1607–1616.

Russell JA, Moran NA (2006) Costs and benefits of symbiont infection in aphids: variation among symbionts and across temperatures. *Proceedings of the Royal Society B-Biological Sciences*, **273**, 603–610.

Russell JA, Latorre A, Sabater-Muńoz B, Moya A, Moran NA (2003) Side-stepping secondary symbionts: widespread horizontal transfer across and beyond the Aphidoidea. *Molecular Ecology*, **12**, 1061–1075.

Russell JA, Weldon S, Smith AH *et al.* (2013) Uncovering symbiont-driven genetic diversity across North American pea aphids. *Molecular Ecology*, **22**, 2045–2059.

Sandstrom JP, Russell JA, White JP, Moran NA (2001) Independent origins and horizontal transfer of bacterial symbionts of aphids. *Molecular Ecology*, **10**, 217–228.

Scarborough CL, Ferrari J, Godfray HCJ (2005) Aphid protected from pathogen by endosymbiont. *Science*, **310**, 1781.

Shah PA, Aebi M, Tuor U (2002) Effects of constant and fluctuating temperatures on sporulation and infection by the aphid-pathogenic fungus *Pandora neoaphidis*. *Entomologia Experimentalis et Applicata*, **103**, 257–266.

Siepielski AM, DiBattista JD, Carlson SM (2009) It's about time: the temporal dynamics of phenotypic selection in the wild. *Ecology Letters*, **12**, 1261–1276.

Simon JC, Boutin S, Tsuchida T *et al.* (2011) Facultative symbiont infections affect aphid reproduction. *PLoS One*, **6**, e21831.

Skaljac M, Zanic K, Ban SG, Kontsedalov S, Ghanim M (2010) Co-infection and localization of secondary symbionts in two whitefly species. *BMC Microbiology*, **10**, 142.

Teixeira L, Ferreira Á, Ashburner M (2008) The bacterial symbiont Wolbachia induces resistance to RNA viral infections in *Drosophila melanogaster*. *PLoS Biology*, **6**, e1000002.

Thompson JN (1998) Rapid evolution as an ecological process. *Trends in Ecology & Evolution (Personal edition)*, **13**, 329–332.

Toju H, Fukatsu T (2011) Diversity and infection prevalence of endosymbionts in natural populations of the chestnut weevil: relevance of local climate and host plants. *Molecular Ecology*, **20**, 853–868.

Tsuchida T, Koga R, Shibao H, Matsumoto T, Fukatsu T (2002) Diversity and geographic distribution of secondary endosymbiotic bacteria in natural populations of the pea aphid, *Acyrthosiphon pisum*. *Molecular Ecology*, **11**, 2123–2135.

Tsuchida T, Koga R, Fukatsu T (2004) Host plant specialization governed by facultative symbiont. *Science*, **303**, 1989.

Vorburger C, Gehrer L, Rodriguez P (2010) A strain of the bacterial symbiont *Regiella insecticola* protects aphids against parasitoids. *Biology Letters*, **6**, 109–111.

Weldon SR, Strand MR, Oliver KM (2013) Phage loss and the breakdown of a defensive symbiosis in aphids. *Proceedings of the Royal Society B: Biological Sciences*, **280**, 20122103. doi: 10.1098/rspb.2012.2103.

Zilber-Rosenberg I, Rosenberg E (2008) Role of microorganisms in the evolution of animals and plants: the hologenome theory of evolution. *FEMS Microbiology Reviews*, **32**, 723–735.

A.H.S., J.A.R. and K.M.O. designed the experiments. A.H.S. directly implemented and oversaw all field and laboratory components of this research. A.H.S., M.O. and J.A.R. designed the approach for data analysis and presentation. A.H.S., J.A.R., P.L. and K.M.O. wrote the manuscript. A.L., G.M., M.T.D., S.A.D. and A.M. performed molecular work. P.L. helped in the development of molecular protocols. M.T.D., S.D., N.T. and R.A.D. assisted in field surveys and collections, along with mortality assays. M.T.D. and R.A.D. identified and counted arthropods from sweep net samples.

Data accessibility

Sanger sequences are deposited in GenBank under the Accession nos KP710314–KP710505. Additional data that include sequence alignments, microsatellite reads, collection information, diagnostic PCR results, aphid samples sequenced and subject to qPCR, field cut and spray records, temperature readings, sweep net counts and mortality assay results are downloaded in dryad as Appendix S1 (Supporting information) (doi: 10.5061/dryad.mk159).

Supporting information

Additional supporting information may be found in the online version of this article.

Fig. S1 Symbiont dynamics in four pea aphid populations repeatedly sampled in 2011.

Fig. S2 Average multiple infection in microsatellite genotyped aphids over three dates in Pennsylvania alfalfa, 2011.

Fig. S3 Arthropod dynamics in four populations repeatedly sampled in 2011.

Fig. S4 Pea aphid parasitoid and fungal mortality dynamics in four populations repeatedly sampled in 2011.

Appendix S1 Microsatellite Data, Symbiont Master, Sweep Sampling, Mortality Assay, Cut and Spray Records, Temperature Measures (doi: 10.5061/dryad.8kf98).

Table S1 Collection locations.

Table S2 Primers and thermocycling conditions.

Table S3 Comparison of uninfected frequency and superinfection levels between states, crops and dates.

Table S4 Average superinfection levels of aphids undergoing microsatellite genotyping.

Table S5 Comparisons of arthropod natural enemy densities between states, crops and over time.

Table S6 Comparisons of parasitoid- and fungal-induced mortality between states, crops and over time.

Table S7 Correlations between facultative symbionts and *Aphidius ervi*-induced mortality.

Table S8 Correlations between facultative symbionts and *A. ervi* density.

Table S9 Correlations between facultative symbionts and fungal-induced mortality.

Table S10 Correlations between facultative symbionts and temperature.

Table S11 Selection coefficients and probability of drift for *H. defensa* frequencies in Pennsylvania alfalfa pea aphid populations.

Table S12 Time lag correlations between facultative symbionts and *Aphidius ervi*-induced mortality.

Table S13 Time lag correlations between facultative symbionts and fungal-induced mortality.

Table S14 Target genes and primer sequences for qPCR assays.