Host-parasitoid coevolution
The role of parasitoid adaptation to endosymbiont-mediated defence in aphids

Author(s):
Rouchet, Romain

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Host-parasitoid coevolution: The role of parasitoid adaptation to endosymbiont-mediated defence in aphids

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Presented by

ROMAIN ROUCHET

Master of Science in Biology, University of Rennes 1
Born April 13th, 1984
Citizen of France

Accepted on the recommendation of
Prof. Dr. Christoph Vorburger
Prof. Dr. Jukka Jokela
Prof. Dr. Wolf Blanckenhorn
Dr. Julia Ferrari

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Host-parasite interactions may result in open-ended cycles of adaptations and counter-adaptations for the traits involved in the outcome of the interactions, such as host resistance and parasite virulence. Reciprocal selection in host-parasitoid interactions is particularly intense, because the outcome of the interaction is always lethal for one of the antagonists. In aphids, a large part of the variation in resistance to parasitoids is due to protective endosymbionts that some individuals harbour. Although the effects of these protective symbionts on aphid interactions with parasitoids have been under intense investigation over the last decade, little is known about the role of parasitoids in this three-level interaction. In my doctoral research, I studied parasitoid adaptation to endosymbiont-mediated defences in aphids. I used the black bean aphid, *Aphis fabae*, which may be infected by the highly protective symbiont *Hamiltonella defensa*, and its Hymenopteran parasitoid *Lysiphlebus fabarum*.

In Chapter 1, I tested for specificity in the interaction between *L. fabarum* and *A. fabae* clones harbouring *H. defensa*. Specificity in the interaction between hosts and parasites is important, because it can lead to negative-frequency-dependence, which is known to promote the maintenance of genetic variation in both antagonists. Previous studies reported a lack of genotype-specificity between unprotected aphids and parasitoids. I observed large variation in host resistance and parasitoid infectivity, as well as a significant aphid clone-by-parasitoid line interaction, the hallmark of a genotype-specific host-parasitoid interaction. This genotype-specificity appears to be mediated by *H. defensa*, highlighting the important role that symbionts can play in host-parasite coevolution.

In Chapter 2, I assessed the diversity and frequency of facultative endosymbionts in five aphid taxa of the genus *Aphis* that are all important hosts of *L. fabarum*. I conducted a PCR screen of a large number of aphids originating from seventeen sites in Switzerland and Southern France for the presence of six facultative symbionts. The most common facultative symbionts were *H. defensa*, *Serratia symbiotica*, *Rickettsia*, and *Regiella insecticola*. These symbionts were present in all five aphid taxa, and their frequencies differed significantly among taxa. There was significant geographic variation in the frequency of these symbionts, but no parallels among aphid species, indicating that the forces structuring symbiont community act independently in different hosts.

Following-up on the high frequency of *H. defensa* in natural populations of aphids and the observed variations among locations (Chapter 2), I predicted that parasitoids may become locally adapted to the prevalence of this symbiont in their host populations. In Chapter 3, I estimated the ability of *L. fabarum* wasps from various populations to overcome *H. defensa*-mediated defences, and related this ability to the frequency of infection by *H. defensa* in their local host populations. For this purpose, parasitoids were exposed to four sublines of a single clone of *A. fabae*,
three of which harboured artificial infections with different isolates of *H. defensa*. Parasitoids from sites with a high prevalence of *H. defensa* in the aphid hosts indeed tended to be more infective on protected hosts, but this relationship was not significant. I further observed a significant *H. defensa* isolate-by-parasitoid genotype interaction, supporting the results of Chapter 1, and I found that parasitoid infectivity depended on which aphid species parasitoids were collected from.

In **Chapter 4** I tested for the ability of parasitoids to adapt to symbiont-mediated-defences in their hosts by using an experimental evolution approach. Parasitoids were exposed for eleven generations to the exact same clone of *A. fabae* artificially harbouring different isolates of *H. defensa*. Parasitoids experienced a fast adaptation to *H. defensa*, and this adaptation was in part specific to the symbiont isolate harbouring by the host line they evolved on. These results confirm that symbionts mediate the genotype-specificity between hosts and parasitoids in this system.

In summary, the studies I conducted during my doctoral thesis show that protective symbionts play a major role in mediating the interaction between aphids and their parasitoids. Protective symbionts such as *H. defensa* are found at relatively high frequencies in natural aphid populations. These symbionts strongly increase the genotype-specificity of the interaction between aphids and parasitoids. Hence, parasitoids have to adapt not only to the prevalence of *H. defensa* in their hosts, but also to the very isolates of the symbiont they are facing. These outcomes raise appealing prospects for future research on host-parasite interactions.
Résumé

Les interactions hôtes-parasites peuvent amener à une sélection fréquence-dépendante négative sur les traits impliqués dans le résultat de l’interaction, comme la résistance des hôtes et l’infectivité des parasites. La sélection réciproque dans les interactions hôtes-parasitoides est particulièrement intense, car le résultat de l’interaction est toujours fatal pour un des antagonistes. Chez les pucerons, une large partie de la variabilité dans la résistance des hôtes aux parasitoides est due à la présence d’endosymbiontes que certains clones possèdent. Alors que les effets de ces endosymbiontes sur les interactions des pucerons avec les parasitoides a été l’objet d’études approfondies au cours de la dernière décennie, le rôle des parasitoides dans cette interaction à trois niveaux demeurent inconnu. Durant ma thèse, j’ai étudié l’adaptation des parasitoides à la résistance des pucerons qui leur est conférée par leur endosymbiontes. J’ai utilisé le parasitoïde Lysiphlebus fabarum et son hôte, le puceron Aphis fabae, pour lequel la présence du symbionte Hamiltonella defensa, conférant un haut niveau de résistance contre les parasitoides, a été rapportée pour une partie des clones.

Dans le Chapitre 1, j’ai testé la spécificité dans l’interaction entre L. fabarum et des clones d’A. fabae infectés par H. defensa. Une telle spécificité est importante, car elle peut mener à une sélection à fréquence dépendance négative, connue pour promouvoir la maintenance de la variabilité génétique chez les deux antagonistes. Des études précédentes ont montré une absence de spécificité génotypique entre pucerons n’arborant pas le symbionte et les parasitoides. J’ai observé une grande variabilité dans la résistance des hôtes et dans l’infectivité des parasitoides, ainsi que interaction significative en entre clone du puceron et ligne du parasitoïde. Cette spécificité entre génotypes apparaît comme étant arbitrée par H. defensa, démontrant le rôle essentiel que peuvent jouer les symbiontes dans la coévolution entre hôtes et parasites.


Dans la continuation des résultats sur la fréquence de H. defensa dans les populations naturelles de pucerons observés en fonction de la localisation géographique (Chapitre 2), j’ai prédit que les parasitoides pouvaient être adapté localement à la prévalence de ce symbionte dans leurs populations d’hôtes. Dans le Chapitre 3, j’ai estimé l’habilité de guêpes de L. fabarum provenant de différentes
populations à contourner la résistance provoquée par *H. defensa*, et relié cette habilité à la fréquence de *H. defensa* dans les populations naturelles de pucerons. Pour cela, les parasitoides ont été exposés à quatre sous-lignées d’un unique et même clone d’*A. fabae*, parmi lesquelles trois étaient artificiellement infectées par différentes souches d’*H. defensa*. Les parasitoides provenant de sites avec une forte prévalence de la bactérie dans leur hôtes avaient tendance à avoir un plus niveau d’infectivité, mais cette relation n’étaient pas significative. J’ai de plus observé une interaction significative entre souches d’*H. defensa* et génotypes des parasitoides, confirmant les résultats obtenus dans le Chapitre 1, et j’ai pu montré que l’infectivité des parasitoides dépend de l’espèces hôtes dont ils proviennent.

Dans le **Chapitre 4**, j’ai testé l’habilité de parasitoides à s’adapter à la résistance provoquée par les symbiontes dans leurs hôtes en utilisant une expérimentation par évolution expérimentale. Les parasitoides étaient exposés pendant onze générations au même clone d’*A. fabae* infecté par différentes souches d’*H. defensa*. Les parasitoides ont connu une adaptation rapide à *H. defensa*, et cette adaptation était en partie spécifique à la souche du symbionte contenu par leur hôte sur lequel ils ont évolué. Ces résultats confirment que les symbiontes arbitrent la spécificité génotypique entre hôtes et parasitoides dans ce système.

En résumé, les études que j’ai réalisées durant la thèse de doctorat montrent que les symbiontes jouent un rôle majeur en arbitrant les interactions entre les pucerons et leurs parasitoides. Les symbiontes conférant une résistance comme *H. defensa* sont retrouvés à des fréquences importantes dans les populations naturelles de pucerons. Ces symbiontes accroissent fortement la spécificité génotypique dans l’interaction entre pucerons et parasitoides. Les parasitoides doivent donc s’adapter non-seulement à la prévalence de *H. defensa* dans leurs hôtes, mais aussi à la souche du symbionte auquelle ils font face. Ces résultats ouvrent de nouvelles perspectives pour le futur de la recherche sur les interactions hôte-parasite.
Introduction

Host-parasite interactions

Parasites are ubiquitous and affect all living organisms (Price, 1980; Windsor, 1998). Host-parasite interactions often provide rare genotypes with a selective advantage and can lead to intense and continuous processes of reciprocal adaptation for the traits involved in the outcome of the interaction such as host resistance and parasite infectivity (Thompson 1994). This continuous arm race between coevolving antagonists is referred to as the Red Queen hypothesis (van Valen, 1977; Bell, 1982). Negative frequency-dependent reciprocal selection is a main force in the maintenance of genetic variation in natural populations (Judson, 1995), and possibly even in the maintenance of sexual reproduction (Jaenike, 1978; Hamilton, 1980). Negative frequency-dependence requires genetic variation for resistance in hosts and infectivity in parasites, as well as costs for resistance and infectivity and/or a high genetic specificity of the host-parasite interaction (Hamilton et al., 1990; Agrawal & Lively, 2002). Due to its importance in the dynamics of coevolving systems, evidences for genotype-specificity have been sought in various host-parasite interactions, from which many studies reported significant host genotype-by-parasite genotype interactions (Carius et al., 2001; Lambrechts et al., 2006; Salvaudon et al., 2007).

Insect host-parasitoid interactions

Parasitoids are parasites that, in order to successfully develop, have to kill their hosts. Parasitoids have been suggested to be the most important cause of mortality in herbivorous insects (Hawkins et al., 1997), and the practice of introducing parasitoids for the biological control of insect pests provides evidence that they can significantly reduce host densities (Beddington et al., 1978). Since the outcome of the interaction is always fatal to one of the antagonists, host-parasitoid systems presumably exhibit very intense reciprocal selection, and thus represent a great scope for the study of coevolution (Godfray, 1993; Kraaijeveld et al., 1998). There is overwhelming evidence for genetic variation in host resistance and parasitoid infectivity in these systems (Fellowes & Godfray, 2000; Ferrari et al., 2001; Kraaijeveld & Godfray,
The relevance of genotype-specificity in host-parasitoid interactions, however, has received little attention and remains a matter of contention. While Dubuffet et al. (2007) found a significant genotype-by-genotype interaction between *Drosophila melanogaster* and its parasitoid *Leptopilina boulardi*, other host-parasitoid systems showed no clear evidence for such specificity (Kraaijeveld & Godfray, 2001; Sandrock *et al.*, 2010).

**Endosymbionts in aphids**

In insects, associations with symbiotic microorganisms are pervasive (Ferrari & Vavre, 2011). Most of them are bacteria that are predominantly vertically transmitted, and can profoundly influence the ecology and evolution of their hosts (Klepzig *et al.* 2009). Among insects, aphid endosymbionts in particular have received considerable attention (Oliver *et al.* 2010). Almost all aphids require the primary or obligate symbiont *Buchnera aphidicola*, housed in specialized cells called bacteriocytes, which provides them with essential nutrients missing from their plant sap-sucking diet (Buchner, 1965; Douglas, 1998). The long association between aphids and *B. aphidicola* has led to pure vertical transmission of the symbiont. Additionally to *B. aphidicola*, aphids may harbour a large variety of facultative or secondary endosymbionts that are generally not essential to their survival and reproduction (reviewed by Oliver *et al.* 2010). Facultative symbionts may inhabit a large variety of tissues and usually undergo some horizontal transmission (Oliver *et al.*, 2010). In aphids, secondary symbionts can influence diverse ecological interactions and confer strong fitness benefits in particular environments. The endosymbiotic bacterium *Hamiltonella defensa* is probably the most-studied secondary symbiont, and has been shown to provide different aphid species with potentially strong protection against their parasitoid wasps (Oliver *et al.*, 2003; Ferrari *et al.*, 2004; Oliver *et al.*, 2005; Vorburger *et al.*, 2009). Other well-studied symbionts of aphids are *Regiella insecticola*, which has been proposed to increase the resistance to a fungal pathogen and influence host plant specialization in the pea aphid *Acyrthosiphon pisum* (Ferrari *et al.*, 2004; Tsuchida *et al.*, 2004; Scarborough *et al.*, 2005), and *Serratia symbiotica*, which can increase resistance to parasitoids and increase thermal tolerance in the pea aphid (Montllor *et al.*, 2002; Oliver *et al.*, 2003).
Hence, endosymbionts constitute an important part of the heritable variation and may contribute significantly to aphid adaptation.

The role of *Hamiltonella defensa*

Due to the strong protection it can provide to aphids against their parasitoids, *H. defensa* has been subject to considerable interest since its discovery. The protection conferred by *H. defensa* to aphids against parasitoids can be linked to the production of different eukaryote-targeted toxins encoded by lysogenic bacteriophages called APSE found within *H. defensa* genome (Moran et al., 2005; Degnan & Moran, 2008; Oliver et al., 2009). Different strains of *H. defensa* seem to be associated with different bacteriophages variants, and there is evidence that different strains of *H. defensa* provide different levels of protection against parasitoids depending by which bacteriophage they are infected (Oliver et al., 2005). Recent studies suggest that the resistance conferred by *H. defensa* against parasitoids acts more specifically than the hosts’ own defences (Vorburger et al., 2009; Schmid et al., 2012). Moreover, in an extensive screening of natural populations of the pea aphid for the presence of secondary symbionts, Ferrari et al. (2012) found that 26% of *A. pisum* clones harbour *H. defensa*. These findings suggest that *H. defensa* may impose strong selection on parasitoids to adapt to symbiont-mediated defences.

Coevolution in the *Aphis fabae*/Lysiphlebus fabarum* system*

The black bean aphid, *Aphis fabae*, is an important pest of many agricultural crops (Blackman & Eastop, 2000). One of its main host plants is the broad bean, *Vicia faba*, which can easily be cultivated in the laboratory. *Aphis fabae* reproduces by cyclical parthenogenesis, with many asexual generations in spring and summer followed by a sexual generation in autumn, or by obligate parthenogenesis in areas with mild winter (Sandrock et al., 2011a). Hence, this aphid can indefinitely be maintained clonally in the laboratory under long day-length conditions. *Aphis fabae*’s most important parasitoid is the Hymenopteran *Lysiphlebus fabarum* (Starý, 2006). *Lysiphlebus fabarum* is a parasitoid of many *Aphis* species, and, exceptionally among aphid parasitoids, reproduces by thelytokous parthenogenesis in most European
populations (Belshaw et al., 1999; Sandrock et al., 2011b). However, L. fabarum also comprises sexual (arrhenotokous) lineages, limited to some aphid host species in most European locations (Sandrock et al. 2011). By offering the possibility to establish genetically uniform lines for both antagonists with the asexual wasp lineages, and the possibility to realise artificial selection or experimental evolution experiments with the sexual lineages of the parasitoid, the A. fabae - L. fabarum system allows for powerful and simple tests of genetic interaction and antagonistic coevolution.

Sandrock et al. (2010) found genetic variation in both host resistance and parasitoid infectivity, but no genotype-specificity in the interactions between A. fabae and L. fabarum. However, their experiment used aphids without any secondary endosymbions. There is growing evidence that the presence of H. defensa in A. fabae may dramatically increase the genotype specificity in the interaction between L. fabarum and its host (Vorburger et al., 2009; Schmid et al., 2012). Finally, the prevalence of H. defensa in natural populations of A. fabae remains poorly known, but it has been suggested to be significant (Vorburger et al., 2009). Hence, this system represents a great scope for the study of host-parasitoid coevolution mediated by protective endosymbionts.

**Thesis outline**

Whilst the interaction between aphids and their facultative endosymbionts has received much attention over the last decade, little is known about the effects of these symbionts on the natural enemies of aphids. This thesis aims to study parasitoid adaptation to the protective symbiont H. defensa in aphids. For this purpose, I focussed on the interaction between A. fabae and its parasitoid wasp L. fabarum.

Specifically, in **Chapter 1** I attempted to measure the genetic variation in host resistance and parasitoid infectivity in the interaction between L. fabarum and A. fabae hosts infected by H. defensa. I also tested for specificity in this three-level' interaction. This is essential to assess the dynamics of coevolution in this system, as genetic variation for the traits involved in the outcome of the interaction and genotype specificity are required for coadaptation between antagonists and can lead to negative-frequency dependence. Consecutively, in **Chapter 2** I sampled a large number of
aphids from five taxa of the genus *Aphis* that are important hosts of *L. fabarum* across seventeen sites in Switzerland and Southern France and screened them for the presence of six known facultative endosymbionts. I developed a multiplex PCR assay to simultaneously test for the presence of these symbionts by amplifying a part of their 16S rDNA gene. The first aim of this survey was to measure the prevalence of *H. defensa* in the aphid hosts of *L. fabarum*. This study was also aiming to characterise the full symbiont communities in different aphid species. This is important to test for geographic variation, potential horizontal transfer of symbionts between aphid species, and interactions between symbiont species. I also sampled *L. fabarum* wasps from the seventeen natural populations of aphids analysed in Chapter 2, and used them in **Chapter 3** to test for local adaptation of the parasitoid to the prevalence of *H. defensa* in its aphid hosts. The frequencies of *H. defensa* were obtained from the aphid populations screened in Chapter 2, and parasitoids were sampled at the exact same locations. Parasitoid ability to overcome *H. defensa*-mediated defences was quantified in the laboratory on a single clone of *A. fabae* that I artificially infected with different isolates of *H. defensa*. Local adaptation of *L. fabarum* to symbiont-mediated defences would show the importance of selection imposed by the aphids' defensive symbionts on their hymenopteran parasitoids. In **Chapter 4**, I explored the potential of parasitoids to adapt to symbiont-mediated defences using an experimental evolution approach. I used sexual populations of *L. fabarum* and exposed them to different treatments consisting of the same *A. fabae* clone harbouring artificial infections with different isolates of *H. defensa*. In addition to observing the evolution of parasitoid counterdefences in real-time, the design of this experiment allowed me to test for genotype-specificity in the potential adaptation of parasitoids to *H. defensa*, as well as for potential costs related to this adaptation.


Chapter 1

Strong specificity in the interaction between parasitoids and symbiont-protected hosts

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Romain Rouchet & Christoph Vorburger

**Abstract:** Coevolution between hosts and parasites may promote the maintenance of genetic variation in both antagonists by negative-frequency-dependence if the host-parasite interaction is genotype-specific. Here we tested for specificity in the interaction between parasitoids (*Lysiphlebus fabarum*) and aphid hosts (*Aphis fabae*) that are protected by a heritable defensive endosymbiont, the \(\gamma\)-proteobacterium *Hamiltonella defensa*. Previous studies reported a lack of genotype-specificity between unprotected aphids and parasitoids, but suggested that symbiont-conferred resistance might exhibit a higher degree of specificity. Indeed, in addition to ample variation in host resistance as well as parasitoid infectivity, we found a strong aphid clone-by-parasitoid line interaction on the rates of successful parasitism. This genotype-specificity appears to be mediated by *H. defensa*, highlighting the important role that endosymbionts can play in host-parasite coevolution.

**Keywords:** Genotype-by-genotype interaction, Infectivity, Endosymbiont, *Aphis fabae*, *Hamiltonella defensa*, *Lysiphlebus fabarum*. 
Introduction

Hosts and their parasites are engaged in evolutionary arms-races of adaptation and counter-adaptation (Dawkins & Krebs, 1979). Reciprocal adaptation requires genetic variation for traits involved in the final outcome of the interaction (Thompson, 1994), and it may result in negative frequency-dependent selection (Hamilton, 1980). By providing rare genotypes with a selective advantage, negative frequency-dependence has been suggested to be an important force for the maintenance of genetic variation in natural populations (Judson, 1995). However, negative frequency-dependence may only arise under certain conditions. It either requires substantial costs of resistance and infectivity, respectively, or a high genetic specificity of the host-parasite interaction (Hamilton et al., 1990; Agrawal & Lively, 2002). In the latter case, the relative resistance of host genotypes varies depending on which parasite genotypes they are exposed to, and vice versa. Because of its importance for the dynamics of coevolution, evidence for genotype specificity has been sought in several host-parasite systems, of which many did indeed exhibit significant host genotype-by-parasite genotype interactions (Carius et al., 2001; Lambrechts et al., 2006; Salvaudon et al., 2007), although others did not show such specificity (e.g. Paterson, 2005).

In insect host-parasitoid systems, reciprocal selection is particularly strong because the interaction is always fatal for one of the antagonists (Godfray, 1993). These kinds of interactions are thus good candidates for investigating genetic variation and specificity by exposing multiple host strains to parasitoid strains of variable infectivity. In such experiments, genetic specificity is reflected in a statistical interaction between host and parasitoid strains, i.e. a genotype-by-genotype interaction. However, this approach has rarely been applied to insect host-parasitoid systems. The relevance of genotype-specificity in host-parasitoid interactions is thus still a matter of contention, whereas the evidence for substantial genetic variation for resistance and infectivity in natural populations is overwhelming (Fellowes & Godfray, 2000; Ferrari et al., 2001; Kraaijeveld & Godfray, 2001; von Burg et al., 2008). Best studied is the interaction between *Drosophila melanogaster* and its larval parasitoids *Leptopilina boulardi* and
1. Specificity in a symbiont-mediated interaction

Asobara tabida (Kraaijeveld & Godfray, 1999; Dupas et al., 2003). While the genetic specificity in the *D. melanogaster/A. tabida* system appears to be very low (Kraaijeveld & Godfray, 2001) there is clear evidence for genotype-by-genotype interactions between *D. melanogaster* and *L. boulardi* (Dubuffet et al., 2007). Their interaction is determined by simple underlying genetics that are well described by a gene-for-gene model (Flor, 1956), which entails that despite the significant specificity, universal infectivity is possible in parasitoids that are homozygous for so-called 'virulence alleles' (Dupas et al., 2003).

The interaction between *Aphis fabae*, the black bean aphid, and its main parasitoid *Lysiphlebus fabarum* is another of the few that has been investigated for such specificity between host and parasitoid genotypes (Vorburger et al., 2009; Sandrock et al., 2010). *Aphis fabae* is a cyclical parthenogen and can be maintained clonally in the laboratory. *Lysiphlebus fabarum*, exceptionally among aphid parasitoids, also reproduces by parthenogenesis in most populations (Belshaw & Quicke, 2003; Starý, 1988, 1999). This allows for powerful tests of genotype-specificity by working with genetically uniform lines of both antagonists. Using a fully crossed factorial design with multiple asexual lines of host and parasitoid, Sandrock et al. (2010) detected no evidence for genotype specificity in the direct interaction between *A. fabae* and *L. fabarum*. However, aphids frequently rely on facultative bacterial endosymbionts for defence against parasitoids (Oliver et al., 2003, 2010; Vorburger et al., 2010). In one of these symbionts, the γ-proteobacterium *Hamiltonella defensa*, protection of pea aphids against parasitoids could be linked to the production of eukaryote-targeted toxins encoded by lysogenic bacteriophages called APSE, found within the *H. defensa* genome (Moran et al., 2005; Degnan & Moran, 2008; Oliver et al., 2009). Different strains of *H. defensa* are associated with different bacteriophage variants, and there is evidence that different strains of *H. defensa* provide different levels of protection against parasitoids (Oliver et al., 2005). This raises the possibility that defence conferred by symbionts may act more specifically than the aphids' own defences, which is supported by two studies (Vorburger et al., 2009; Schmid et al., 2012). Thus, we hypothesize that aphids infected with *H. defensa*, possessing an additional layer of heritable variation for resistance, are more likely to exhibit genotype specificity in the interaction with their parasitoids. We tested
1. Specificity in a symbiont-mediated interaction

this hypothesis in a fully crossed factorial experiment that, in contrast to the experiment by Sandrock et al. (2010), used A. fabae clones harbouring the defensive symbiont H. defensa. Our experiment did indeed reveal a strong host clone-by-parasitoid line interaction on the rates of successful parasitism by L. fabarum.

Materials and methods

The host-parasitoid system

The black bean aphid, Aphis fabae, is a widely distributed pest of agricultural crops in the northern hemisphere (Blackman & Eastop, 2000). It uses many species from several plant families as hosts, including broad bean (Vicia faba) and several species of Chenopodiaceae such as sugar beet (Beta vulgaris), where it is particularly damaging. Aphis fabae reproduces by cyclical parthenogenesis (sexual reproduction only before winter) or - in areas with mild winters - by obligate parthenogenesis (Sandrock et al., 2011). Parasitoids are among the most important natural enemies of aphids (Schmidt et al., 2003), and A. fabae's most common parasitoid is Lysiphlebus fabarum (Starý, 2006), which reproduces by thelytokous parthenogenesis in most populations (Belshaw et al., 1999; Starý, 1999; Sandrock & Vorburger, 2011). Females of L. fabarum oviposit a single egg into aphids. The larva then hatches and develops through several instars inside the still active host, which is only killed before parasitoid pupation. Metamorphosis takes place within a cocoon spun inside the host's dried remains, forming a 'mummy' from which the adult wasp emerges.

Aphis fabae frequently harbours the facultative bacterial endosymbiont H. defensa. Based on estimates from Swiss and French populations, the frequency of infection with these vertically transmitted symbionts is approximatively 50% (Rouchet R. & Vorburger C., unpublished data). When infected with H. defensa, aphids enjoy strongly increased resistance to parasitism by L. fabarum (Vorburger et al., 2009; Schmid et al., 2012), but they suffer from a reduced lifespan in the absence of parasitoids (Vorburger & Gouskov, 2011), which may explain why H. defensa does not go to fixation.
Host and parasitoid collection and care

Aphids were collected in June and July 2006 as part of a Europe-wide sampling program (Sandrock et al., 2011a). Single females were brought into the laboratory and used to establish isofemale clonal lines on caged seedlings of *V. faba*. The lines were maintained at 20°C and a 16-h photoperiod. These summer-like conditions allow us to maintain them clonally for any number of generations. All clones were genotyped at eight microsatellite loci (Coeur d'Acier et al., 2004) and screened for the presence of facultative endosymbionts as described in Vorburger et al. (2009). The 15 clones used in this experiment originated from France, Italy, Germany and Switzerland (Supplementary Table 1). We used 13 aphid clones that were naturally infected with *H. defensa*. They represented all *H. defensa*-infected clones we maintained in the laboratory at the time that had not been used in any of our earlier experiments. We only used previously untested clones to avoid potential biases from using infected clones for which we already had preliminary evidence of genotype-by-genotype interactions with parasitoids (Vorburger et al., 2009). In addition, we included two clones without any secondary endosymbiont. These two clones (nrs. A06-405 and A06-407) were chosen as reference points because in an earlier experiment, they were the most resistant and the most susceptible of all *H. defensa*-free clones tested (Vorburger et al., 2009). All clones had unique microsatellite multilocus genotypes (MLGs) except for two of the *H. defensa*-infected clones, A06-69 and A06-82, which shared the same MLG. Both were collected in southern France where mild winters permit parthenogenetic overwintering and are thus likely to represent the same clonal lineage. We used both lines in the experiment because they were collected from different sites separated by 200 km. The collection information and microsatellite genotypes of all *A. fabae* clones are detailed in Supplementary Table 1.

Parasitoids were collected from parasitized aphid colonies in the field that were recognized by the presence of mummies. Such colonies were collected into air-permeable containers where wasps were allowed to emerge. Upon emergence of the wasps, single asexual females of *L. fabarum* were isolated and allowed to attack caged *A. fabae* colonies in the laboratory to establish parthenogenetic isofemale lines of parasitoids. Line 06-297 was collected in 2006 in France, lines 07-59 and 07-64 were both collected in 2007 in Switzerland. These lines were then maintained in a climatized room at 20°C on
our standard, *H. defensa*-free clone of *A. fabae* (A06-256) that was not included in the experiment. All lines were genotyped at 12 microsatellite loci (Fauvergue et al., 2005; Sandrock et al., 2007) to ensure that we collected representatives of genetically different asexual lines. Their microsatellite genotypes together with more detailed collection information are provided in Supplementary Table 2. Note that *L. fabarum* lines 06-297 and 07-59 are the same as those used in Vorburger et al. (2009). Line 07-64 was additionally included because of results that suggested it to be relatively infective also on *H. defensa*-protected aphids (Schmid et al., 2012), which would increase the variation in parasitism success and thus improve the chances of observing interactions if they existed.

**Experiment**

The experiment quantified the susceptibility of the 15 clones of *A. fabae* to the three thelytokous lines of *L. fabarum* in a fully crossed factorial design. As in Henter & Via (1995), the general trial was to expose a group of aphid nymphs to wasps for a fixed period of time and measure the proportion of individuals mummified (i.e. successfully parasitized). This measure does not distinguish between pre-ovipositional defences (e.g. avoidance behavior) and physiological resistance against the parasitoid egg or larva, but previous studies have shown that it largely reflects the latter. Clonal differences in mummification rates do not originate from differences in parasitoid oviposition (Henter & Via, 1995), and parasitoids seem equally likely oviposit in aphids with and without defensive endosymbionts (Oliver et al., 2003). The proportion of individuals mummified is thus a reliable measure of host susceptibility.

Each combination of aphid clone and wasp line (45 combinations) was replicated six times, resulting in 270 colonies of aphid nymphs tested. At the beginning of the experiment, we split each aphid clone into six sublines maintained on *V. faba* seedlings for two generations. From these sublines three mature aphid females from each of the 15 lines were placed for 24 hours on a *V. faba* seedling grown in 0.07-L-plastic pot and covered with a cage. Two days after adult removal, aphid nymphs (48 to 72h old) were counted for each colony and the plants placed randomly in one of the 6 blocks. Two female wasps from one of the parasitoid lines were introduced in the aphid colony for 12
1. Specificity in a symbiont-mediated interaction

hours. Nine days after wasp exposure, all successfully parasitized aphids had turned to mummies and were counted.

**Statistical analysis**

Data were analyzed in R version 2.11.1 (R Development Core Team, 2010). The proportion of aphids mummified was analyzed using a generalized linear model (GLM) with logit link and a quasibinomial error distribution to account for overdispersion (Crawley, 2005). We tested for the effects of aphid clone, parasitoid line, the aphid clone by parasitoid line interaction and experimental block. Because there was some variation in aphid colony size among replicates, we also included the initial number of nymphs exposed to parasitoids as a covariate in the model.

**Results**

The number of nymphs per colony did not affect the proportion of aphids mummified (Table 1), but there was a marginally non-significant block effect. Host clone and parasitoid line both had a highly significant effect on the proportion of mummified aphids. The two *H. defensa*-free aphid lines, A06-405 and A06-407, were susceptible to all three parasitoid lines, albeit to different extents (Fig. 1). The clones harboring *H. defensa* showed complete or near-complete resistance to at least one of the three parasitoid lines, but the relative resistance to the three parasitoids differed widely among clones (Fig. 1). Notably, the two aphid clones with identical MLGs (A06-69 and A06-82) showed almost the same pattern, that is resistance to parasitoid lines 06-297 and 07-59, but susceptibility to line 07-64 (Fig. 1). Parasitoid line 07-64 was generally very infective on hosts harboring *H. defensa*: only two of these clones showed resistance. Overall, the specific patterns of resistance and infectivity were reflected in a highly significant host clone-by-parasitoid line interaction in the analysis (Table 1). This interaction remained significant when only the 13 *H. defensa*-protected clones were considered ($F_{24, 189} = 8.164, P < 0.001$), but it was non-significant when the analysis was restricted to the two unprotected clones ($F_{2, 24} = 1.739, P = 0.197$). The latter result is consistent with two previous studies finding no such interaction between unprotected aphids and parasitoids (Sandrock *et al.*, 2010; Vorburger *et al.*, 2009) but we acknowledge that with only two
unprotected clones in the present experiment, the power to detect an interaction was very limited.
1. Specificity in a symbiont-mediated interaction

**Fig. 1.** Mean susceptibility of fifteen *Aphis fabae* clones to three thelytokous lines of the parasitoid *Lysiphlebus fabarum*. All clones harboured natural infections with the facultative bacterial endosymbiont *Hamiltonella defensa*, except for A06-405 and A06-407.

**Table 1:** Results of the generalized linear model on the proportion of aphid infection by parasitoids. The model was a quasi-likelihood fit with logit link and binomial errors, using a dispersion parameter of 2.72.

<table>
<thead>
<tr>
<th></th>
<th>df</th>
<th>Deviance</th>
<th>F</th>
<th>P</th>
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<tbody>
<tr>
<td>Number of nymphs</td>
<td>1</td>
<td>0.16</td>
<td>0.06</td>
<td>0.806</td>
</tr>
<tr>
<td>Block</td>
<td>5</td>
<td>30.01</td>
<td>2.20</td>
<td>0.054</td>
</tr>
<tr>
<td>Parasitoid line</td>
<td>2</td>
<td>913.78</td>
<td>168.15</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Host clone</td>
<td>14</td>
<td>1010.35</td>
<td>26.56</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Host clone x parasitoid line</td>
<td>28</td>
<td>767.85</td>
<td>10.09</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Residual</td>
<td>223</td>
<td>646.26</td>
<td></td>
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</table>
**Discussion**

Genetic variation for host resistance and parasite infectivity as well as genotype-by-genotype interactions are important assumptions of coevolutionary theory (Hamilton, 1980). The present study demonstrates ample variation for resistance and infectivity in an insect host-parasitoid system, and we found a strong interaction between parasitoid lines and host clones on rates of parasitism when aphids were protected by the bacterial endosymbiont *H. defensa*. This contrasts with an earlier study that investigated the same interaction in the absence of defensive endosymbionts and revealed no evidence for such specificity (Sandrock et al., 2010). An important caveat is that because we worked with different aphid clones harbouring natural infections with *H. defensa*, we could not separate the effects of host and endosymbiont genotypes on the observed variation in host resistance. However, the evidence so far shows that symbiont-conferred effects tend to overwhelm any underlying variation among host genotypes (Oliver et al., 2005; Vorburger et al., 2009). A second caveat is that aphids and parasitoids came from multiple sites that were geographically quite distant. We cannot exclude that some of the effects seen might be influenced by patterns of local adaptation, because the study by Sandrock et al. (2010) reporting a lack of genotype-by-genotype interactions only used syntopic combinations of *H. defensa*-free *A. fabae* and *L. fabarum* genotypes. On the other hand, the study by Vorburger et al. (2009) used aphid clones collected at distances of up to 200 km apart and did not detect any evidence for genotype-specificity in the interaction of parasitoids and *H. defensa*-free aphids either. At least at a small spatial scale, local adaptation may not be very likely anyway in this system, because *A. fabae* is a highly mobile species that exhibited a very shallow genetic population structure and a lack of isolation-by-distance across 18 European populations (Sandrock et al., 2011a). The parasitoid *L. fabarum* is generally less mobile than its host, but due to its reproduction by thelytokous parthenogenesis, many asexual lineages can be found over large geographic areas (Sandrock et al., 2011b).

The above caveats notwithstanding, the clear difference between the patterns seen with symbiont-protected aphids here and unprotected aphids in an otherwise similar experiment (Sandrock et al., 2010) is at least suggestive of the symbionts mediating the
observed specificity. This was supported recently by an experiment in which sublines of a single aphid clone were experimentally infected with different isolates of *H. defensa* and exposed to multiple parasitoid genotypes (Schmid *et al.*, 2012). The sublines' relative resistances changed according to which parasitoid they were exposed to, i.e. there was a significant host line-by-parasitoid line interaction. In that case it was clear that the endosymbiont was responsible for the observed specificity because all aphids were genetically identical (Schmid *et al.*, 2012). For the present experiment, the critical test would require to cure all lines of *H. defensa* with antibiotics (McLean *et al.*, 2011) and test whether the host line-by-parasitoid line interaction disappeared as a result.

One of the parasitoid lines (07-64) was particularly efficient in overcoming symbiont-mediated defenses (Fig. 1). On most of the symbiont-protected hosts it achieved rates of parasitisms that were as high or even higher than those on unprotected hosts, but it was unable to parasitize two clones that were also resistant to the other two parasitoid lines. Note that by simply determining the number of mummies formed, we may overestimate somewhat the fitness of parasitoids on symbiont-protected hosts. Work by Nyabuga *et al.* (2010) and Schmid *et al.* (2012) has shown that even if they are able to overcome symbiont-conferring resistance, aphid parasitoids may suffer from sublethal effects of developing in aphids infected with *H. defensa*, such as slightly delayed development, reduced emergence from mummies and smaller body size. However, this would not have altered our interpretation of a specific interaction, because there was no evidence of genotype-by-genotype interactions on these sublethal effects (Schmid *et al.*, 2012). That parasitoid populations exhibit genetic variation in their ability to overcome symbiont-conferring resistance has also been supported by an experimental evolution experiment using the aphid parasitoid *Aphidius ervi* (Dion *et al.*, 2011). Populations maintained on pea aphids harbouring *H. defensa* increased their infectivity significantly within just a few generations. However, the molecular mechanisms underlying parasitoid counter-adaptation to the presence of defensive endosymbionts in their hosts remain unknown and need further investigation.

In conclusion, our results demonstrate a genotype-by-genotype interaction between symbiont-protected aphids and their parasitoids on the rate of successful parasitism. This
is important because genetic specificity may lead to negative frequency-dependence and thus maintain genetic variation. In comparison with previous studies on the same system (Vorburger et al., 2009, Sandrock et al., 2010) our results further suggest that the endosymbionts may play a role in mediating this specificity. The present experiment does not demonstrate a causal link, but evidence for a causal role was provided in another study on the same system (Schmid et al., 2012). Such effects may be more common than is currently appreciated. Vertically transmitted symbionts are ubiquitous in invertebrates (Haine, 2008), and theory predicts that they are under selection to evolve host protection in the presence of horizontally transmitted parasites that can co-infect the same hosts (Jones et al., 2011). Thus, we predict that additional cases of specific, symbiont-mediated host-parasite interactions may be found by investigating other systems with defensive endosymbionts. This may influence our expectations for the genetic dynamics under antagonistic coevolution between hosts and parasites, especially if defensive endosymbionts retain some ability for horizontal transmission, as is the case for *H. defensa* (Moran & Dunbar, 2006; Gehrer & Vorburger, in press). Simulations of a host-parasite interaction model that incorporated such a symbiont showed that under some conditions, the symbiont can engage in cycles of reciprocal adaptation with the parasite, while host genetic variation can be lost (Kwiatkowski et al., in press). It will also be important to establish whether the observed genotype-by-genotype interactions are sensitive to environmental perturbations. For a number of host-parasite (Bryner & Rigling, 2011; Sadd, 2011) or plant-herbivore systems (Tétard-Jones et al., 2007), it was shown that genotype-by-genotype interactions can be modified by the biotic or abiotic environment in which they take place. This is referred to as a genotype-by-genotype-by-environment interaction and has important implications for the dynamics of coevolution in variable environments (Mostowy & Engelstädter, 2011). For the aphid/symbiont-parasitoid system studied here, we have preliminary evidence that genotype-by-genotype interactions are robust to temperature variation (L. Cayetano & C. Vorburger, unpublished data), but sensitivity to other types of environmental variation has not been investigated so far.
Acknowledgments

We thank C. Sandrock for field collecting and A. Gouskov for insect maintenance. Two anonymous reviewers provided very constructive comments on the manuscript. This study was supported by the Swiss National Science Foundation.
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References


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SUPPLEMENTARY ONLINE MATERIAL

Table S1. Collection information and genotypes at eight microsatellite loci (Coeur d'Acier et al., 2004) for the 15 clones of Aphis fabae used in this study.

<table>
<thead>
<tr>
<th>Sample ID</th>
<th>Collection site</th>
<th>Sampling date</th>
<th>Host plant</th>
<th>Secondary symbiont</th>
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<th>AF-50</th>
<th>AF-82</th>
<th>AF-85</th>
<th>AF-86</th>
<th>AF-181</th>
<th>AF-beta</th>
<th>AF-F</th>
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<tbody>
<tr>
<td>A06-09</td>
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<td>08/05/2006</td>
<td>Vicia faba</td>
<td>Hamiltonella defensa</td>
<td>315 321</td>
<td>257 272</td>
<td>171 177</td>
<td>220 222</td>
<td>219 219</td>
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</tr>
<tr>
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<td>08/05/2006</td>
<td>Vicia faba</td>
<td>Hamiltonella defensa</td>
<td>315 321</td>
<td>257 272</td>
<td>171 177</td>
<td>220 222</td>
<td>217 219</td>
<td>309 309</td>
<td>280 282</td>
<td>127 136</td>
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<td>220 220</td>
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<td>311 311</td>
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<td>127 132</td>
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<td>257 272</td>
<td>171 177</td>
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<td>257 272</td>
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<td>257 272</td>
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<td>280 282</td>
<td>127 127</td>
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</table>
1. Specificity in a symbiont-mediated interaction

**Table S2.** Collection information and genotypes at 12 microsatellite loci (Fauvergue *et al.*, 2005; Sandrock *et al.*, 2007) for the three thelytokous lines of *Lysiphlebus fabarum* used in this study.

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<thead>
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<th>Isofemale line</th>
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<th>07-59</th>
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<td>Collected from:</td>
<td><em>Aphis fabae cirsiicanthoides</em> on <em>Cirsium arvense</em></td>
<td><em>Aphis hederae</em> on <em>Hedera helix</em></td>
<td><em>Aphis fabae fabae</em> on <em>Chenopodium album</em></td>
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**Microsatellite genotypes:**

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**Supplementary References**


Chapter 2

Ample variation but little congruence in the secondary symbiont communities of five congeneric aphid hosts

Romain Rouchet, Jenny Herzog & Christoph Vorburger

Abstract:

In addition to the obligate symbiont Buchnera aphidicola, aphids harbor a large variety of secondary (facultative) bacterial endosymbionts that may play an important role in their hosts' ecology. These heritable symbionts were mostly discovered in the pea aphid, Acyrthosiphon pisum, and knowledge about their occurrence in aphids other than this model species is still scarce. We conducted an extensive PCR screen for the facultative symbionts Hamiltonella defensa, Regiella insecticola, Serratia symbiotica, Rickettsia, Spiroplasma and X-type in five abundant and widespread aphids of the genus Aphis: Aphis fabae cirsiiacanthoidis, A. fabae fabae, A. hederae, A. ruborum and A. urticata. Although these aphids use different host plants, they occur in close proximity within the same habitats, which could potentially lead to an assimilation of their symbiont communities due to shared selection pressures and/or frequent lateral transfer of symbionts. We analyzed 1592 individuals originating from 17 sites in Switzerland and Southern France and detected all of the symbionts we tested for. The most frequent secondary symbionts were H. defensa, S. symbiotica, Rickettsia and R. insecticola, which were present in all aphid species, albeit at very different frequencies. There was also significant geographic variation in the frequencies of the different symbionts, but this variation showed no parallels among the five aphid species, indicating that the forces structuring their symbiont communities act independently in the different hosts. Interestingly, S. symbiotica was found in 95% of A. urticata individuals, suggesting the possibility that this bacteria is in a transition state from a facultative to an obligate symbiont in this aphid. We also found three pairwise combinations of secondary symbionts for which co-infections were observed less frequently than expected in one of the species, pointing at potential incompatibilities or competitive exclusion among different symbionts.

Keywords:
bacterial community, Aphis, facultative endosymbiont, geographic structure, intra- and interspecific variations


**Introduction**

Aphids engage in symbiotic associations with multiple heritable bacteria that affect their ecology and evolution (Oliver *et al.*, 2010). Nearly all aphid species harbour the obligate or primary endosymbiont *Buchnera aphidicola*, which provides their hosts with essential amino acids and other nutrients missing from their phloem diet (Buchner, 1965; Douglas, 1998). Unlike obligatory symbionts, facultative or secondary symbionts are generally not required for aphid survival or reproduction, although they often appear to replicate only within their hosts (Sandström *et al.*, 2001; Tsuchida *et al.*, 2002; Moran *et al.*, 2005) and have been shown to be mutualistic in various ecological interactions (reviewed by Oliver *et al.*, 2010). The best studied secondary symbiont in aphids is *Hamiltonella defensa*, which can provide various aphid species with strong protection against parasitoid wasps (Oliver *et al.*, 2003; Ferrari *et al.*, 2004; Oliver *et al.*, 2005; Vorburger *et al.*, 2009). Other well-studied symbionts of aphids are *Regiella insecticola*, which can increase the resistance of pea aphids (*Acyrthosiphon pisum*) to a fungal pathogen and influences their host specialization (Ferrari *et al.*, 2004; Tsuchida *et al.*, 2004; Scarborough *et al.*, 2005), and *Serratia symbiotica* which increases thermal tolerance and resistance to parasitoids in pea aphids (Montllor *et al.*, 2002; Oliver *et al.*, 2003). Other facultative endosymbionts have been described in aphids, but their possible ecological functions remain mostly unknown. For example a member of the insect symbiont group *Arsenophonus* was found in the soybean aphid *Aphis glycines* (Wille & Hartman, 2009). Several other endosymbionts have been described in *A. pisum*, namely bacteria from the genera *Rickettsia* and *Spiroplasma*, and a bacterium referred to as X-type (Fukatsu *et al.*, 2001; Tsuchida *et al.*, 2002; Simon *et al.*, 2003; Guay *et al.*, 2009; Ferrari *et al.*, 2011). Some of these symbionts have been shown to have ecologically relevant effects in insects other than aphids, for example *Spiroplasma* has been shown to rescue *Drosophila neotestacea* females from the sterilizing effects of nematode parasitism (Jaenike & Brekke, 2011), and both *Spiroplasma* and *Rickettsia* have been reported to be male-killers in various arthropods such as *Drosophila* species (Watts *et al.*, 2009) and beetle species (Werren *et al.*, 1995; Lawson *et al.*, 2001). Due to their predominantly vertical transmission, endosymbionts are part of the heritable variation and thus contribute to adaptation. Many insects harbour entire communities of co-occurring endosymbionts,
which in turn affect their hosts' ecological communities by modifying interactions between the host and other species (Ferrari & Vavre, 2011). In this context, determining the frequencies of facultative symbionts and the composition of symbiont complements in insect populations is of strong ecological and evolutionary interest.

The aphid genus *Aphis* contains more than 400 species and is the largest genus within the family Aphididae (Blackman & Eastop, 2006). Many species of this genus are notorious agricultural pests, including the soybean aphid *A. glycines* (Ragsdale et al. 2004), the cotton or melon aphid *A. gossypii* (Kennedy et al., 1975; Cauquil et al., 1982) and the black bean aphid *A. fabae fabae* (Völkl & Stechmann, 1998). *Aphis* species exploit a large number of host plants, yet the morphologies of congeneric species are highly similar compared to other aphid genera (Blackman & Eastop, 2006). The black bean aphid *A. fabae* represents a complex of several subspecies which use different groups of secondary host plants during the asexual spring and summer generations, but return to the same primary host plants (mainly the spindle tree *Euonymus europaeus*) for sexual reproduction (Stroyan, 1984). For example, in Europe, *A. fabae fabae* occurs on the secondary host plants *Vicia faba* (broad bean) and many species of the family Chenopodiaceae, cultivated plants (e.g. sugar beet) as well as weeds (e.g. *Chenopodium album*), whereas *A. fabae cirsiiacanthoidis* mainly occurs on thistles such as the creeping thistle *Cirsium arvense* or the spear thistle *Cirsium vulgare*. *Aphis fabae fabae* and *A. f. cirsiiacanthoides* are thus typical aphids of agricultural, ruderal and suburban habitats in Europe. Other numerically abundant species of *Aphis* in such habitats are *Aphis hederae* on ivy (*Hedera helix*), *Aphis ruborum* mainly on blackberries of the genus *Rubus* and *Aphis urticae* on stinging nettles (*Urtica dioica*) (Blackman & Eastop, 2006). These aphid species use different host plants, but otherwise they are embedded in similar ecological communities within the same habitat. All are typically tended by ants (Way 1963; personal observations) and they share many natural enemies such as predators and parasitoids. For example, wasps of the *Lysiphlebus fabarum* group are the main parasitoids of all these aphids (Starý, 1988; 2006; C. Vorburger & R. Rouchet, personal observation). In addition to exerting similar selection on different species, aphid parasitoids may also act as vectors for horizontal transfer of facultative endosymbionts (Gehrer & Vorburger, 2012). It is thus conceivable that symbiont frequencies assimilate
between aphid species within communities, especially for symbionts like *H. defensa* that provide protection against parasitoids. On the other hand, a study by Ferrari *et al.* (2012), showed that the frequencies of facultative symbionts in pea aphids depended mainly on the host plant, even for *H. defensa*, suggesting that the host plants have primacy in structuring the symbiont composition of aphids.

In this context we investigated the composition and frequencies of facultative endosymbionts in natural populations of five species/subspecies of the genus *Aphis*: *A. fabae cirsiiacanthoidis*, *A. fabae fabae*, *A. hederae*, *A. ruborum* and *A. urticata*. In total, we sampled 1595 individuals from 17 localities across Switzerland and Southern France and screened them for the presence of six known aphid endosymbionts: *H. defensa*, *R. insecticola*, *S. symbiotica*, *Rickettsia*, *Spiroplasma* and X-type. We analyzed the effect of aphid species and sampling location on symbiont distributions, looked for possible interactions between different symbionts in a given aphid species by analyzing overall co-infections as well as correlations between symbionts at a geographic level, and looked for evidence of horizontal symbiont transfer between the different aphid species.

**Materials and methods**

**Aphid collection**

From May to early July 2009, we collected aphids belonging to five taxa at fourteen sites in Switzerland and three sites in France (Fig. 1). *Aphis fabae fabae* was collected from *Chenopodium album*, *A. fabae cirsiiacanthoidis* mainly from *Cirsium arvense* with a few individuals from *C. vulgare* (29 out of 290), *A. hederae* from the common ivy *Hedera helix*, *A. ruborum* from the blackberry shrub *Rubus fruticosus* and *A. urticata* from the stinging nettle *Urtica dioica*. Although *A. fabae fabae* and *A. fabae cirsiiacanthoidis* are taxonomically treated as two subspecies of the same species, for simplicity we will refer to them as species in this study. Potential host plants were checked for aphid colonies and one individual was collected from each infested plant, with a minimum distance of 5 meters between two colonies of the same species to avoid collecting clonal descendents from the same individual. The goal was to collect at least 20 aphids from each taxon per
site, although this was not quite achieved for all sites (Table S1, supporting information). Aphids were collected into individual tubes and stored at -80°C until DNA extraction.

**DNA extraction**

Aphid DNA was prepared using a Chelex® protocol (Bio-Rad, Hercules, CA, USA). Aphids were crushed in 1.5 ml tubes with 100 µl of 5% Chelex® suspension and 5 µl of 20 mg/ml Proteinase K (Sigma, Saint-Louis, MO, USA). The samples were then incubated at room temperature overnight. The next morning, samples were incubated at 65°C for 15 min, and at 100°C for 6 min. Extracts were briefly vortexed between each stage of the extraction. Samples were finally centrifuged and the supernatant stored at -20°C until use in diagnostic PCRs.

**Symbiont detection**

We developed a multiplex PCR assay to simultaneously test for the presence of six facultative endosymbionts by amplifying a part of their 16S rDNA gene. We used the universal bacterial forward primer 16SA1 for all the tested symbionts (Fukatsu & Nikoh 1998), and a specific backward primer labeled with a fluorescent dye for each endosymbiont species, including the obligate endosymbiont *B. aphidicola* as an internal positive control for the success of the DNA extraction (Table 1). The PCR was conducted in a final volume of 10 µl with 5 µl of 2x Qiagen® Multiplex PCR Mastermix, the primers, ddH₂O and 1 ul of DNA extract. The PCR program used a first step of denaturation at 95°C for 3 minutes followed by 10 cycles at 95°C for 1 min, 65-56°C (touchdown, -1°C per cycle) for 1 min, 72°C for 2 min, followed by an additional 25 cycles at 95°C for 1 min, 55°C for 1 min, 72°C for 2 min and a final extension step at 72°C for 6 min. Fragment analysis was done in GeneMapper®. Due to overlapping peaks between *B. aphidicola* and *S. symbiotica*, when unclear we verified the presence of *S. symbiotica* by doing additional single-locus PCRs using the same forward primer and the
2. Facultative symbionts in natural populations of aphids

same reverse undyed primer specific to *S. symbiotica* used in the multiplex PCR. PCR reactions were run on 2% agarose gels and checked for positive bands.

**Statistical analysis**

The effects of aphid host species and sampling site on the distribution of facultative endosymbionts were analyzed using a generalized linear model (GLM) with a logit link function and a binomial error distribution (Crawley, 2005) in R version 2.11.1 (R Development Core Team, 2010). For co-infections we tested whether specific combinations of endosymbionts were observed more or less frequently than expected using Fisher's exact test in R. Geographic correlations between the frequencies of different secondary symbionts within species and geographic correlations between the frequencies of the same symbiont in different species were analyzed with Pearson correlations in SPSS version 19 (IBM SPSS Statistics).
2. Facultative symbionts in natural populations of aphids

**Table 1:** Reverse labeled primers used in the PCR multiplex for symbiont detection

<table>
<thead>
<tr>
<th>Symbiont species</th>
<th>Reverse primer</th>
<th>Dye</th>
<th>Reverse primer sequence (5’→3’)</th>
<th>Length (bp)</th>
<th>Volume in µl per 10 µl reaction (primer concentration = 2 µM)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Buchnera aphidicola</em></td>
<td>Buch16S1R</td>
<td>ATTO565</td>
<td>CTTCTGCGGGTACACGTCACGAA</td>
<td>~ 490</td>
<td>0.1</td>
<td>Tsuchida et al. (2002)</td>
</tr>
<tr>
<td><em>Hamiltonella defensa</em></td>
<td>TO419R</td>
<td>FAM</td>
<td>AAATGGTATTGGCATTTATCG</td>
<td>~ 470</td>
<td>0.4</td>
<td>Ferrari et al. (2011)</td>
</tr>
<tr>
<td><em>Rickettsia</em></td>
<td>Rick16S1R</td>
<td>ATTO565</td>
<td>CATCCATCAGCATAAATCTTTC</td>
<td>~ 210</td>
<td>0.2</td>
<td>Fukatsu et al. (2001)</td>
</tr>
<tr>
<td><em>Regiella insecticola</em></td>
<td>U443R</td>
<td>Yakima</td>
<td>GGTAACGTCATCGATAAGCA</td>
<td>~ 480</td>
<td>0.1</td>
<td>Ferrari et al. (2011)</td>
</tr>
<tr>
<td><em>Serratia symbiotica</em></td>
<td>R443R</td>
<td>ATTO550</td>
<td>CTTCTGCGAGTACACGTCATG</td>
<td>~ 485</td>
<td>0.1</td>
<td>Ferrari et al. (2011)</td>
</tr>
<tr>
<td>X-type</td>
<td>X420R</td>
<td>ATTO565</td>
<td>GCAACACTCTTTGCATTG</td>
<td>~ 470</td>
<td>0.1</td>
<td>Ferrari et al. (2011)</td>
</tr>
<tr>
<td><em>Spiroplasma</em></td>
<td>TKSSspR</td>
<td>FAM</td>
<td>TAGCCGTGGCTTTCTGGAT</td>
<td>~ 495</td>
<td>0.2</td>
<td>Fukatsu &amp; Nikoh (2000)</td>
</tr>
</tbody>
</table>
2. Facultative symbionts in natural populations of aphids

Results

Secondary endosymbionts in the five *Aphis* species

The average number of secondary symbionts per individual varied significantly between aphid species (GLM, family = Poisson, df=4, Deviance=193.8, p<0.001): *A. fabae cirsiiacanthoidis* carried on average 0.33 secondary symbionts per individual, *A. hederae* carried on average 0.74, *A. fabae fabae* 0.75, *A. ruborum* 1.04 and *A. urticata* 1.26 (Fig. 2). The most common symbionts were *H. defensa*, *S. symbiotica*, *Rickettsia* and *R. insecticola* with infection rates over all individuals tested of 36%, 30%, 8% and 6%, respectively. These symbionts were the only ones present in all five species (Fig. 2). *Spiroplasma* was found at a frequency of only 1% overall and was absent in *A. urticata*, while X-type symbionts were found only in *A. hederae* at a frequency of 0.7% (Fig. 2). *Hamiltonella defensa* was the most common secondary symbiont in *A. fabae fabae*.

![Sampling localities in Switzerland (1 - 14) and southern France (15 - 17) for the aphids analyzed in this study. Numbers correspond to locality numbers defined in Table S1, supporting information.](image)

**Fig. 1**: Sampling localities in Switzerland (1 - 14) and southern France (15 - 17) for the aphids analyzed in this study. Numbers correspond to locality numbers defined in Table S1, supporting information.
(52%), A. hederae (27%) and A. ruborum (75%) and the second most common in A. urticata with a frequency of 22% (Fig. 2). The most common secondary symbiont in A. urticata was S. symbiotica, which was found in 95% of individuals. It also occurred frequently in A. hederae (20%) and A. ruborum (24%). Rickettsia was common in A. hederae only with a frequency of 24% and R. insecticola occurred mainly in A. fabae cirsiiacanthoidis and A. fabae fabae with frequencies of 12% and 13%, respectively (Fig. 2). Aphid species and site had a significant effect on the frequency of all symbionts except for Spiroplasma (Table 3), highlighting the importance of the geographic origin as well as the aphid species for the frequency of infection with secondary symbionts. We also found a significant interaction between aphid species and site for H. defensa, Rickettsia and S. symbiotica (Table 3), indicating that the frequencies of these symbionts in different geographic regions varied independently in each aphid species.

Multiple infections

We found double infections in all aphid species: 0.03% of A. fabae cirsiiacanthoidis individuals carried two facultative symbionts of various combinations, 5% of A. fabae fabae, 10% of A. hederae, 14% of A. ruborum and 27% of A. urticata. We also found a few triple infections with H. defensa, Rickettsia and S. Symbiotica in both A. hederae (2 individuals in 258) and A. urticata (3 in 328), and one individual from A. hederae was even harboring four secondary endosymbionts with R. insecticola in addition to H. defensa, R. insecticola and Rickettsia. Three pairwise combinations of endosymbionts occurred less often than expected by chance: H. defensa with R. insecticola in A. fabae fabae and A. ruborum, as well as H. defensa with S. symbiotica in A. ruborum (Table 4).

Correlations between symbionts within aphid species and between aphid species

To investigate possible mutualism or exclusion between different symbionts, we tested for correlations between the frequencies of different symbionts among sites within the same aphid taxa. In A. fabae cirsiiacanthoidis, we found a positive correlation between S.
symbiotica and both H. defensa (r=0.517; p=0.04) and Rickettsia (r=0.765; p<0.001). For A. fabae fabae, we found a negative correlation (r=-0.50; p=0.039) between H. defensa and Regiella insecticola (Fig. 3). We did not find any other significant correlations and did not test for correlation with X-type endosymbionts or Spiroplasma sp., as their frequencies were very low.

We also tested for correlations of local infection frequencies with each symbiont species across the different aphid taxa as a possible sign of frequent horizontal transfer of symbionts between different aphids and/or similar selection shaping symbiont frequencies. We found a positive geographic correlation for R. insecticola between A. urticata and A. fabae cirsiacanthoidis (r=0.619; p=0.008), between A. urticata and A. hederae (r=0.695; p=0.002) and between A. urticata and A. ruborum (r=0.541; p=0.025). However, these correlation are driven by the single site at which R. insecticola occurred in A. urticata and should therefore be regarded with caution (site 16, Montélimar, France; Figs. 1 & 3). The only other significant result was a positive correlation for Rickettsia between A. fabae cirsiacanthoidis and A. fabae fabae (r=0.694, p=0.02). However, this endosymbiont was very rare in both aphid species and the correlation is determined by only two sites at which Rickettsia occurred in A. f. cirsiacanthoides as well as A. f. fabae (Fig. 3).
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Fig. 2: Secondary endosymbiont frequencies in the different aphid species analyzed in this study.
2. Facultative symbionts in natural populations of aphids

![Fig. 3: Secondary endosymbionts in five aphid species by location.](image)


Table 3: Results of generalized linear models for the presence of the six secondary symbionts included in the screen. The models used a logit link and binomial errors.

<table>
<thead>
<tr>
<th>Symbiont species</th>
<th>Factors</th>
<th>df</th>
<th>Deviance</th>
<th>$P$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Aphid species</td>
<td>4</td>
<td>396</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>Site</td>
<td>16</td>
<td>88</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>Aphid species*site</td>
<td>62</td>
<td>215</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>Residual</td>
<td>1511</td>
<td>1384</td>
<td></td>
</tr>
<tr>
<td>H. defensa</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Aphid species</td>
<td>4</td>
<td>114</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>Site</td>
<td>16</td>
<td>56</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>Aphid species*site</td>
<td>62</td>
<td>107</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>Residual</td>
<td>1511</td>
<td>628</td>
<td></td>
</tr>
<tr>
<td>Rickettsia</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Aphid species</td>
<td>4</td>
<td>95</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>Site</td>
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<td>100</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>Aphid species*site</td>
<td>62</td>
<td>76</td>
<td>0.107</td>
</tr>
<tr>
<td></td>
<td>Residual</td>
<td>1511</td>
<td>481</td>
<td></td>
</tr>
<tr>
<td>R. insecticola</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Aphid species</td>
<td>4</td>
<td>881</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>Site</td>
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<td>56</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>Aphid species*site</td>
<td>62</td>
<td>111</td>
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</tr>
<tr>
<td></td>
<td>Residual</td>
<td>1511</td>
<td>913</td>
<td></td>
</tr>
<tr>
<td>S. symbiotica</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Aphid species</td>
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<td>9</td>
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<td>Aphid species*site</td>
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<td>0</td>
<td>1.000</td>
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<td></td>
<td>Residual</td>
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<td>16</td>
<td></td>
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<tr>
<td>Spiroplasma</td>
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<tr>
<td></td>
<td>Aphid species</td>
<td>4</td>
<td>13</td>
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<td>Site</td>
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<td>38</td>
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<td></td>
<td>Aphid species*site</td>
<td>62</td>
<td>26</td>
<td>0.999</td>
</tr>
<tr>
<td></td>
<td>Residual</td>
<td>1511</td>
<td>120</td>
<td></td>
</tr>
</tbody>
</table>
2. Facultative symbionts in natural populations of aphids

**Table 4** Comparisons of observed and expected frequencies of co-infections with two different secondary symbionts for all cases with an expected frequency of co-infection > 5 in an aphid species. We used Fisher’s exact tests and show significant differences in bold.

<table>
<thead>
<tr>
<th>Aphid species</th>
<th>Symbiont 1</th>
<th>Symbiont 2</th>
<th>observed</th>
<th>expected</th>
<th>$P$-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>A. fabae fabae</em></td>
<td><em>H. defensa</em></td>
<td><em>Rickettsia</em></td>
<td>6</td>
<td>8.2</td>
<td>0.312</td>
</tr>
<tr>
<td><em>A. fabae fabae</em></td>
<td><em>H. defensa</em></td>
<td><em>R. insecticola</em></td>
<td>1</td>
<td>27.7</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td><em>A. fabae fabae</em></td>
<td><em>H. defensa</em></td>
<td><em>S. symbiotica</em></td>
<td>9</td>
<td>10.8</td>
<td>0.504</td>
</tr>
<tr>
<td><em>A. hederae</em></td>
<td><em>H. defensa</em></td>
<td><em>Rickettsia</em></td>
<td>14</td>
<td>19.6</td>
<td>0.098</td>
</tr>
<tr>
<td><em>A. hederae</em></td>
<td><em>H. defensa</em></td>
<td><em>S. symbiotica</em></td>
<td>11</td>
<td>16.2</td>
<td>0.107</td>
</tr>
<tr>
<td><em>A. hederae</em></td>
<td><em>Rickettsia</em></td>
<td><em>S. symbiotica</em></td>
<td>11</td>
<td>14.6</td>
<td>0.246</td>
</tr>
<tr>
<td><em>A. ruborum</em></td>
<td><em>H. defensa</em></td>
<td><em>R. insecticola</em></td>
<td>0</td>
<td>5.2</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td><em>A. ruborum</em></td>
<td><em>H. defensa</em></td>
<td><em>S. symbiotica</em></td>
<td>30</td>
<td>45.6</td>
<td>&lt;0.001</td>
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<tr>
<td><em>A. urticata</em></td>
<td><em>H. defensa</em></td>
<td><em>Rickettsia</em></td>
<td>3</td>
<td>6.0</td>
<td>0.225</td>
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<tr>
<td><em>A. urticata</em></td>
<td><em>H. defensa</em></td>
<td><em>S. symbiotica</em></td>
<td>69</td>
<td>69.4</td>
<td>0.852</td>
</tr>
<tr>
<td><em>A. urticata</em></td>
<td><em>Rickettsia</em></td>
<td><em>S. symbiotica</em></td>
<td>26</td>
<td>25.7</td>
<td>1.000</td>
</tr>
</tbody>
</table>
Discussion

Previous studies of natural populations revealed the presence of a large variety of secondary endosymbionts in aphids, particularly the pea aphid. The most common are *H. defensa*, *Rickettsia*, *R. insecticola* and *S. symbiotica* (Tsuchida *et al.*, 2002; Russell *et al.*, 2003; Tsuchida *et al.*, 2006; Ferrari *et al.*, 2012). Here we presented an extensive survey of bacterial endosymbionts in five taxa of the genus *Aphis* across Switzerland and the south of France. We detected *H. defensa*, *Rickettsia*, *R. insecticola* and *S. symbiotica* in all of the aphid species tested, *Spiroplasma* in four out of five species and X-type symbionts in *A. hederae* only (Fig. 2). Note that by using diagnostic PCR, we could only detect what we screened for. We would not have detected any previously unknown endosymbionts of aphids with this approach, and such symbionts may well exist. A very recent study by Augustinos *et al.* (2011) suggests that *Wolbachia*, a very widespread endosymbiont of insects that was thought to be absent or extremely rare in aphids, may have been overlooked due to low titres and high divergence of aphid-infecting strains.

Although the majority of secondary endosymbionts we screened for occurred in all aphid species analyzed, we found striking differences in their frequencies between species (Fig. 2). Also the average number of secondary symbionts per aphid varied markedly, ranging from 0.33 symbionts in *A. fabae cirsiiacanthodis* up to 1.26 in *A. urticae*. Thus, aphid species was clearly the most important factor structuring the secondary symbiont communities represented in our samples. This is likely to reflect historical events of symbiont acquisition coupled with faithful vertical transmission as well as ecological differences among aphid species. We cannot separate the effects of aphid species and host plant in the present study, but work by Ferrari *et al.* (2012) on a single species, the pea aphid, revealed ample variation in the symbiont communities harboured by different, host-associated subpopulations. This suggests that at least some of the secondary symbionts may influence host plant adaption, possibly by exhibiting a nutritional function. So far, experimental evidence supporting this hypothesis is restricted to one study on pea aphids, in which *R. insecticola* appears to improve aphid performance on red clover (Tsuchida *et al.*, 2004).
In addition to the differences among aphid species, we found significant geographic variation within species for the prevalence of five bacterial endosymbionts. However, this variation did not show many parallels for the different species, as indicated by the highly significant site × species interactions for the three most common symbionts and by the lack of any strong between-species correlations in their frequencies across sites. Hence, the communities of heritable symbionts appear to evolve largely independently in the five aphid species we analyzed. This is not to say that no lateral transfer of facultative endosymbionts may occur. Low 16S rDNA divergences between facultative symbionts of various aphid species indicate that symbionts have jumped between species in the past and that horizontal transfer may be an ongoing process (Oliver et al. 2010; Peccoud et al., 2009; Russell et al., 2003). Moreover, two natural routes of horizontal transfer are known to exist. Parasitoid wasps have been shown to act as vectors for lateral transmission of heritable symbionts between aphids (Gehrer & Vorburger, 2012), and male-to-female transfer during the sexual generation in autumn has also been demonstrated (Moran & Dunbar, 2006). In the present case, the latter route could only be called upon for A. f. fabae and A. f. cirsiicanthoides, which mate on the same primary host plant, the spindle tree. The former route might be more generally available, because all five aphid species can be attacked by the same parasitoid species. On the other hand, parallels were found between the genetic structure of facultative symbiont H. defensa and that of their host, the pea aphid, with populations adapted to different host plants carrying distinct strains of the bacteria (Ferrari et al. 2011). This suggests very little horizontal transfer between aphids specialized on different host plants, possibly because parasitoid wasps show little movement between hosts on different plant species. The main parasitoid of the aphids studied here, L. fabarum, does indeed exhibit host-associated genetic differentiation, which is indicative of limited movement between hosts (Sandrock et al. 2011). Whatever the underlying reasons, our data clearly indicate that despite occurring in close physical proximity, horizontal transmission is not frequent enough and/or local selection on symbiont-mediated phenotypic traits not similar enough to homogenize the local composition of symbiont communities between species of the genus Aphids.
Because of its known effect of providing protection against parasitoids (Oliver et al. 2003; Vorburger et al. 2009), the frequencies of *H. defensa* deserve particular attention. It was very prevalent in both *A. ruborum* and in *A. fabae fabae* with more than half of the individuals being infected. It was also the most common endosymbiont in *A. hederae* and the second most common in *A. urticata* with infection frequencies of 27% and 22%, respectively, but it was rare in *A. f. cirsiiacanthoides* (Fig. 2). It is of course possible that the risk of parasitism and therefore the net benefit of harbouring *H. defensa* varies among aphid species. However, parasitoids must impose substantial selection on all of these aphids, making it worth considering if *H. defensa* may also have other functions, e.g. in host plant adaptation, that could explain the large differences among aphid species. This remains to be investigated. Unfortunately, the ecological roles of the other facultative symbionts are even less well known, except for some data on *R. insecticola* and *S. symbiotica* in the pea aphid. While *S. symbiotica* can also provide some moderate protection against parasitoids (Oliver et al., 2003) and may influence thermal tolerance (Montllor et al., 2002), *R. insecticola* was shown to increase resistance to fungal pathogens (Scarborough et al., 2005). *Regiella insecticola* was the most common facultative endosymbiont in *A. fabae cirsiiacanthoidis* (11.7%) and the second most common in *A. fabae fabae* (13%) but was very rare in the three other aphid species (Fig. 2). Interestingly, co-infections with both *H. defensa* and *R. insecticola* were occurring less often than expected in *A. fabae fabae* and *A. ruborum*, and we found a negative geographic correlation between the frequencies of *H. defensa* and *R. insecticola* in *A. fabae fabae*. Possibly, these two symbionts cannot coexist well within the same hosts, similar to the observation made by Oliver et al. (2006) for *H. defensa* and *S. symbiotica* in the pea aphid. If protection against parasitoids and fungal pathogens are general effects of *H. defensa* and *R. insecticola*, respectively, this could result in an interesting trade-off. Their mutual exclusion would constrain the ability of aphids to respond to selection by parasitoids and fungal pathogens simultaneously by acquiring the appropriate symbionts.

A remarkable result was the high prevalence of *S. symbiotica* in *A. urticata*, with an overall infection level of 95% (Fig. 2). Considering the strong effect of host plant on the endosymbiont community in pea aphids (Ferrari et al., 2012), *S. symbiotica* could play a role in *A. urticata*’s adaptation to the host plant from which it was collected, the
2. Facultative symbionts in natural populations of aphids

stinging nettle *U. dioica*. Another, non-exclusive explanation is that *S. symbiotica* is in the process of evolving from a facultative towards an obligate endosymbiont in *A. urticata*. This hypothesis would need to be tested separately, but such a scenario would not be without precedence. In the cedar aphid *Cinara cedri*, *S. symbiotica* showed a transition to an obligate symbiont by providing the host with tryptophan and riboflavin, which are no longer synthesized by *B. aphidica* in this aphid (Lamelas et al., 2011). *S. symbiotica* has also been reported to increase fitness under heat stress in *A. pisum* (Montllor et al., 2002; Russell & Moran, 2006), but this explanation is unlikely in the present case, because all five aphids are exposed to similar temperatures in their natural environment.

In summary, we report strong differences in the facultative endosymbiont communities harboured by five congeneric aphid species occurring in close proximity within a similar habitat. Sampling site also explained a substantial part of the variation in symbiont frequencies, albeit with little congruence among aphid species. The interpretation of this variation is still hampered by limited knowledge about the ecological role of many bacterial endosymbionts. In order to explain the differences in symbiont frequencies between aphid species, further investigation of their phenotypic effects, particularly in relation to aphid host plant specialization, are urgently needed.

Acknowledgements

We thank Alexandre Gouskov for help with field sampling. We are very grateful to Julia Ferrari for providing us with DNA samples of *S. symbiotica* and X-type symbionts and for sharing the sequences of the specific reverse primers for *H. defensa, S. symbiotica, R. insecticola* and X-type before publication. Last but not least, we thank Ryuichi Koga for providing us with DNA samples of *Rickettsia* and *Spiroplasma* and Marek Kwiatkowski for helpful comments on our paper. This work was supported by the Swiss National Science Foundation.
References


2. Facultative symbionts in natural populations of aphids


2. Facultative symbionts in natural populations of aphids


2. Facultative symbionts in natural populations of aphids

**Table S1** Details of the sampling sites and the number of aphids analyzed for each species per locality.

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<th><em>A. f. fabae</em></th>
<th><em>A. hederae</em></th>
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Chapter 3

Are aphid parasitoids locally adapted to the prevalence of defensive symbionts in their hosts?

Romain Rouchet & Christoph Vorburger

Abstract:

Parasites are under strong selection to overcome their hosts' defences. In aphids, resistance to parasitoids is largely determined by the presence or absence of protective endosymbionts such as *Hamiltonella defensa*. Hence, parasitoids may become locally adapted to the prevalence of protective symbionts in their host populations. To address this, we collected aphid parasitoids of the *Lysiphlebus fabarum* group from 17 sites in Switzerland and France, at which the frequency of infection with *H. defensa* had been quantified for five important aphid host species. We estimated the parasitoids' ability to overcome *H. defensa*-mediated defences and related this ability to the frequency of infection with *H. defensa* in their local host populations. The experiment was done by measuring parasitoid infectivities on four sublines of a single clone of *Aphis fabae* that were either uninfected or harbouring one of three different isolates of *H. defensa*. Although parasitoids from sites with high prevalences of *H. defensa* in their aphid hosts tended to be more infective on the sublines possessing *H. defensa*, this relationship was not significant, thus providing no conclusive evidence that parasitoid are locally adapted their hosts' defensive symbionts. We found a significant variation among the host species from which parasitoids were collected on their ability to overcome *H. defensa*-mediated defences, but no indication that this variation was related to the frequency of the symbiont in the hosts they came from. Importantly, we observed a very strong interaction between parasitoid line and *H. defensa* isolate on parasitism success. This genetic specificity in the interaction suggests that it may be more important for parasitoids to adapt to the particular strains of *H. defensa* in their host populations than to the general prevalence of this symbiont, and it highlights the important role symbionts can play in mediating host-parasite coevolution.

Keywords:

*Aphis*, facultative endosymbiont, local adaptation, *Lysiphlebus*, geographic structure.
3. Local adaptation of parasitoids to *H. defensa*

**Introduction**

For many insects, parasitoids are important natural enemies that cause substantial mortality, thus exerting intense selection for host resistance (Godfray, 1993). The hosts' defences, in turn, impose strong selection on parasitoid infectivity. This sets the scene for antagonistic coevolution by reciprocal adaptation, which requires genetic variation for traits involved in the outcome of their interaction (Thompson, 1994). Such variation was described for a number of insect host-parasitoids interactions (Henter, 1995; Ferrari *et al.*, 2001; Kraaijeveld *et al.*, 2002; Dubuffet *et al.*, 2007; Sandrock *et al.*, 2010). The potential for ongoing coevolution is also expected to vary geographically (Thomson, 1994). Selective environments in different locations may favour different traits and interacting populations may exhibit variable degrees of connectivity between them (Thompson, 1999). Hence, an investigation of coevolutionary processes must seek for intraspecific variation of the traits involved in the species interactions. Despite of its importance for the dynamics of coevolution, studies of local adaptation in host-parasitoids system are restricted to a few studies (Kraaijeveld & Godfray, 2001; Pannebakker *et al.*, 2008; Gibert *et al.*, 2010). For example, Dupas *et al.* (2003) reported strong evidence for local adaptation in the interaction between the parasitoid *Leptolina boulardi* and its host *Drosophila melanogaster*.

Aphids rely strongly on protective endosymbionts for resistance against parasitoid wasps, with individuals that harbour the endosymbiotic bacterium *Hamiltonella defensa* exhibiting a much higher resistance to parasitoids than uninfected aphids (Oliver *et al.*, 2003; Vorburger *et al.*, 2009; Oliver *et al.*, 2010; Vorburger *et al.*, 2010). Mechanistically, *Hamiltonella defensa* provide aphids with protection against parasitoids by producing eukaryote-targeted toxins encoded by lysogenic bacteriophages called APSE, found within the *H. defensa* genome (Moran *et al.*, 2005; Degnan & Moran, 2008; Oliver *et al.*, 2010). These toxins appear to prevent the development of the egg or early larval stages of the parasitoid. The black bean aphid, *Aphis fabae fabae*, and its most important parasitoid, *Lysiphlebus fabarum*, have become a useful model to investigate the role of *H. defensa* in mediating aphid host-parasitoid interactions (e.g. Vorburger *et al.*, 2009, Vorburger & Gouskov, 2011). It could be shown that different lines of *L. fabarum*
vary in their ability to parasitize aphids harbouring \textit{H. defensa} (Schmid et al., 2012; Rouchet & Vorburger, in press), indicating that parasitoid populations possess genetic variation for overcoming symbiont-conferred resistance. We could further show (Chapter 2) that the frequency of infection with \textit{H. defensa} exhibits significant geographic variation in \textit{A. f. fabae} as well as four other \textit{Aphis} species that are alternative hosts of \textit{L. fabarum}, namely \textit{A. fabae cirsiiacanthoidis}, \textit{A. hederae}, \textit{A. urticata} and \textit{A. ruborum} (Stáry, 2006). Moreover, Dion et al. (2011) showed, using an experimental evolution procedure, that the parasitoid wasp \textit{Aphidius ervi} can adapt quickly to the presence of \textit{H. defensa} in its host \textit{Acyrthosiphon pisum}. Taken together, these results suggest that parasitoid wasps might have the potential to locally adapt to the prevalence of \textit{H. defensa} in their hosts.

In this study we addressed this possibility by testing for correlations between the frequencies of \textit{H. defensa} in natural populations of aphids and the ability of parasitoids originating from these host populations to overcome \textit{H. defensa}-mediated defences.

\section*{Materials and methods}

\textit{Parasitoid collection}

Aphid parasitoids belonging to the \textit{L. fabarum} group were collected from May to early July 2009 at fourteen locations in Switzerland and three locations in France (Table S1). From the same locations and the same time period we had estimates of the frequency of infection with \textit{H. defensa} for five important hosts of \textit{L. fabarum}: \textit{A. fabae fabae}, \textit{A. fabae cirsiiacanthoidis}, \textit{A. hederae}, \textit{A. urticata} and \textit{A. ruborum}. These estimates were obtained from a comprehensive screen of these species’ facultative bacterial endosymbionts with molecular methods and are reported elsewhere (Chapter 2). Parasitized aphid colonies are readily recognized by the presence of mummies. We collected visibly parasitized colonies of the five focal aphid species into ventilated plastic tubes (5x10cm) and brought them back to the laboratory. Tubes were checked every second day for emerged adult parasitoid wasps. We used acetyl acetate vapour to lightly anesthetize the wasps before determining the species and sex. The two first \textit{L. fabarum} group females to emerge per
aphid colony were individually transferred to a caged colony of a H. defensa-free clone of A. fabae fabae growing on broad bean, Vicia faba to establish isofemale lines of parasitoids for later testing. Additional parasitoids emerging from the same aphid colonies were conserved in 96% ethanol. Unfortunately, a substantial fraction of parasitoids failed to establish in the laboratory. Of 223 samples from which L. fabarum group parasitoids emerged, we managed to establish at least one line in 103 cases.

Note that the taxonomy of parasitoids from the L. fabarum group is contentious and further complicated by the occurrence of sexual as well as asexual lineages. According to the existing taxonomic literature (Marshall, 1896; Tremblay & Eady, 1978), three species are distinguished within this group based on subtle morphological differences: L. fabarum, L. cardui and L. confusus. However, more recent molecular and experimental work provided no support for this distinction and showed that sexual and asexual lineages show only incomplete genetic isolation (Belshaw et al., 1999; Sandrock & Vorburger, 2011; Sandrock et al., 2011a). Therefore, we retained sexual as well as asexual lines from all three morphotypes for use in the experiments.

Aphid lines

To meaningfully estimate the parasitoids' ability to overcome symbiont-conferred resistance, it is important to distinguish the protection by H. defensa from any underlying genetic variation in the aphid host. This is achieved by using genetically identical aphids with and without H. defensa. Here we used a single clone of A. fabae fabae, A06-407, collected in July 2006 on Chenopodium album in St. Margrethen (Switzerland) and maintained in the laboratory on Vicia faba since its collection. This clone was naturally uninfected with any known facultative endosymbiont of aphids (Vorburger et al., 2009). We generated H. defensa-infected sublines of this clone by microinjection of hemolymph from donor clones that naturally harboured an infection, following the protocol described in Vorburger et al. (2010). To cover at least some of the variation present in H. defensa, we used three different donor clones which, based on previous experiments, exhibit different levels of resistance to L. fabarum (Vorburger et al., 2009; Rouchet &
Vorburger, in press). The donor clones were also obtained in 2006 and included A06-323, collected from *Vicia faba* in Aesch (Switzerland), A06-402 collected from *Chenopodium album* in St. Margrethen (Switzerland), and A06-76 collected from *C. album* in La Grande Motte (France). The transfections were carried out between June 2008 and March 2009 and resulted in stable heritable infections with *H. defensa*. The experimentally infected sublines were labelled A06-407^H323, A06-407^H402, and A06-407^H76. The presence of *H. defensa* in these sublines was checked again by diagnostic PCR two weeks prior to the beginning of the experiments described below as well as at the end of the experiments.

*Parasitoid infectivity test*

To measure their ability to overcome symbiont-mediated protection, we determined the infectivity of all field-collected parasitoid on the symbiont-free and the *H. defensa*-infected sublines of clone A06-407. As in Henter & Via (1995), the assay consisted of exposing a group of aphid nymphs to wasps for a fixed period of time and in later counting the number of individuals mummified by the parasitoids. The proportion of mummified aphids was used as an estimate of parasitoid infectivity. The parasitoid lines were tested at the second, third, or fourth generation after establishment in the laboratory. If we obtained a laboratory line from both females originally taken from the same field sample, i.e. the same aphid colony, only one of the two lines was tested because it is likely that two females emerging from the same aphid colony are sisters. Each of the 103 wasp lines was tested on all four aphid sublines in five replicate assays, unless a line yielded fewer than 20 female wasps at the time of testing (mean number of replicates = 4.24). For each replicate, three mature aphid females were placed for 24 hours on a *V. faba* seedling to reproduce. Plants were grown in a 0.07-L-plastic pot and covered with a cage. Two days after adult removal, the aphid nymphs (48 to 72h old) were counted on each plant (mean colony size 21.5 ± 6.8 SD). One female wasp was then introduced into the aphid colony for 12 hours. Ten to eleven days after wasp exposure, all successfully parasitized aphids had turned to mummies and were counted.
Microsatellite genotyping of parasitoid lines

Because a large proportion of *L. fabarum* group parasitoids reproduce asexually, it was possible that we collected multiple parasitoids belonging to the same asexual lines either from the same or even from different geographic locations. We determined to what extent this was the case by genotyping each line with 12 microsatellites (Fauvergue *et al.*, 2005; Sandrock *et al.*, 2007). When a parasitoid line was tested, one of the female wasps was collected into a 1.5ml tube and stored at -80°C until use. DNA extractions and microsatellite genotyping followed protocols published in Sandrock *et al.* (2007). Fragment sizes were determined on an ABI 3730 sequencer and allele scoring was done with the software GeneMapper® version 3.7. Asexual lines of the parasitoid with identical genotypes are reported in Table S2.

Statistical analysis

For the infectivity tests, analyses were carried out in R version 2.11.1 (R Development Core Team, 2010), and the proportion of aphids exposed to parasitoids that were mummified was taken as the response variable. Overdispersion prevented us from analyzing these success-failure data with a generalized linear models and binomial errors. Hence, the proportions of aphids mummified were arcsine square-root transformed and analyzed with linear mixed models (LMM). We used the lmer function from the lme4 package (Bates *et al.*, 2011), and tested for the effects of aphid subline (fixed), parasitoid line (random), the aphid subline x parasitoid line interaction (random), host aphid species (fixed), the aphid subline x aphid host species interaction (fixed), site (random), and aphid subline x site (random). *P*-values for the fixed effects were calculated using *F*-tests with Satterthwaite’s approximation and *p*-values for the random effects were calculated based on Chi square test with the function totalAnalysis from the MixMod package (Kuznetsova & Brockhoff, 2012). Sexual and asexual lines were treated equally, as preliminary analysis indicated no differences in their infectivities depending on their reproductive mode. The correlations between the average parasitoid infectivity on *H. defensa* bearing aphids by location and the average frequency of *H. defensa* in aphids
from the same locations was tested with a Pearson’s correlation in SPSS version 19 (IBM SPSS Statistics). The correlations between the average infectivity of parasitoid lines on the different aphid sublines harbouring *H. defensa* were also tested with a Pearson’s correlation in SPSS.

**Results**

There was significant variation among the four aphid sublines in the proportion of individuals mummified by parasitoids (Table 1). The three *H. defensa*-protected sublines were much more resistant than the subline without *H. defensa* (Fig. 1). The parasitoids' site of origin did not significantly affect the proportion of aphids mummified, nor was there a significant aphid subline x site interaction (Table 1). On the other hand, the aphid host from which parasitoids were collected did have a significant effect on mummification, and there was also a significant interaction between aphid subline and the hosts from which parasitoids came (Table 1). These effects largely reflect that parasitoids collected from *A. hederae* had low infectivity overall and were particularly ineffective at parasitizing the *H. defensa*-protected sublines compared to parasitoids collected from other hosts (Fig. 1). The strongest effects in the analysis were the variation among parasitoid lines and particularly the aphid subline x parasitoid line interaction (Table 1). The different lines of *L. fabarum* not only differed strongly in their ability to overcome *H. defensa*-conferred resistance, they also differed in their relative infectivities on aphids protected by different sublines of *H. defensa* (Fig. 2), as indicated by the strong interaction. Interestingly, *H. defensa* isolate H76 appeared to represent a very different challenge to parasitoids than isolates H323 and H402 (Figs 1 & 2). This was supported by the fact that we found a significant positive correlation between parasitoid infectivity on line A06-407\textsuperscript{H323} and A06-407\textsuperscript{H402} (r=0.83, p<0.001), whereas parasitoid infectivity on line A06-407\textsuperscript{H76} was not significantly correlated with infectivity on either line A06-407\textsuperscript{H323} (r=0.14; p=0.135) or A06-407\textsuperscript{H402} (r=0.07, p=0.47). Thus, different isolates of *H. defensa* provide different levels of protection to aphids depending on which parasitoid line they are attacked by.
In seven cases, we had collected and tested parasitoid lines with identical microsatellite multilocus either twice or three times (see Appendix table S2). In these cases, lines with identical genotypes produced very similar patterns of infectivity, for example lines 09-258 and 09-260 collected in Zurich (CH), or lines 09-348 and 09-381 collected in Geneva (CH) and Orbe (CH), which are depicted in Fig. 1. Hence, the ability of parasitoids to successfully infect aphids protected by a particular isolate of *H. defensa* appears to be a genetic trait in *L. fabarum*, and this leads to a high degree of specificity in the interaction between *H. defensa* and *L. fabarum* in this system.

To test for local adaptation of parasitoids, we compared the mean infectivity among parasitoid lines per site on the *H. defensa*-infected aphid sublines per site with the average prevalence of *H. defensa* across the five aphid species collected at the same sites (see chapter 2). In four cases, missing data for the frequency of *H. defensa* in one of the aphid species were replaced by the average frequency of *H. defensa* over all sites for the aphid species. Although we detected a relationship in the expected direction (Fig. 3), this correlation was not statistically significant (Fig. 3). Hence, there is no conclusive evidence that parasitoid ability to overcome *H. defensa*-mediated defences has evolved to match the local prevalence of this symbiont in their host populations.
Table 1: Results of the linear mixed model on the proportion of aphids mummified by parasitoids. Proportions were arcsine square-root transformed before analysis. *P*-values of random effects are based on likelihood ratio tests and *P*-values of fixed effects on F tests with Satterthwaite’s approximation.

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<td>213.88</td>
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Fig 1: Parasitoid infectivity on the different aphid lines depending on their aphid host species.
3. Local adaptation of parasitoids to *H. defensa*

![Graphs showing infectivity of parasitoid lines from four different locations on the different aphid sublines. Parasitoid lines with similar genotypes are shown in bold with identical symbols and lines.](image)

**Fig. 2:** Infectivity of parasitoid lines from four different locations on the different aphid sublines. Parasitoid lines with similar genotypes are shown in bold with identical symbols and lines.
3. Local adaptation of parasitoids to *H. defensa*

Fig. 3: Correlation between average parasitoid infectivity per location on *H. defensa*-bearing aphid and the average frequency of *H. defensa* in aphids per location.

Discussion

In the present experiment, we found enormous variation in the ability of different parasitoid lines of the *L. fabarum* group to overcome *H. defensa*-mediated defences in *A. fabae*. The parasitoids came from multiple locations with different prevalences of *H. defensa* in their aphid hosts, but we did not find a significant effect of location on parasitoid infectivity on *H. defensa*-bearing hosts. However, when averaged over the three *H. defensa*-bearing aphid sublines and location, parasitoid infectivities exhibited a non-significant trend towards higher infectivities in sites with a high average frequency of *H. defensa*. For example, the three parasitoid populations originating from France exhibited high infectivities on symbiont-protected aphids (e.g. Romans in Fig. 2). The same sites also showed a high average frequency of infection with *H. defensa* in host aphids (>40%, see chapter 2). Nevertheless, the overall correlation was not significant (Fig. 3).
A possible explanation for the lack of significant evidence for local adaptation in this study is the strong *H. defensa* genotype-by-parasitoid genotype interaction we observed. We only used three different isolates of *H. defensa*, yet the rate of successful parasitism depended strongly on the combination of *H. defensa* isolate and parasitoid genotype. More specifically, parasitoids well adapted to *H. defensa* isolate H323 were also well adapted to isolate H402, but not to isolate H76, and vice-versa. These results indicate that parasitoids would have to adapt not simply to the prevalence of *H. defensa* in their host populations, but to the specific strains of *H. defensa* harboured by their hosts. Unfortunately, very little is known about the geographical distribution of different genotypes of *H. defensa* in aphid populations. To elucidate this issue, parasitoids would have to be tested on sympatric and allopatric isolates of *H. defensa* within the same aphid genetic backgrounds, which would be a very challenging experiment.

Another potential explanation for the lack of parasitoid local adaptation is related to the relative migration rates of hosts and parasitoids. Studies on parasites with higher migration rates than their hosts report local adaptation, as measured by infection success, significantly more often than studies of parasites with relatively low migration rates (Greischar & Koskella 2007). Although the dispersal rate of *L. fabarum* is unknown, other species of this genus were found to be relatively poor dispersers (Weisser & Völk, 1997; Nyabuga *et al.*, 2010). *Aphis fabae fabae*, on the other hand seems to exhibit rather high dispersal rates (Sandrock *et al.*, 2011b). Hence, the evolution of parasitoid local adaptation may also be hampered by *L. fabarum* having a low dispersal rate compared to its hosts.

Although parasitoids collected from different aphid hosts exhibited significant differences in their ability to overcome *H. defensa*-mediated defences, there was no indication that parasitoids collected from host species with a high frequency of infection by *H. defensa* were better adapted to symbiont-conferred resistance. For example, despite the very low frequency of *H. defensa* in *A. fabae cirsiiacanthoides* (5%, Chapter 2), parasitoids collected from this host exhibited the highest infectivity on the *H. defensa*-bearing aphids in our experiment (Fig. 1). This outcome should be interpreted with caution, however, because of the strong parasitoid-by-*H. defensa* specificity reported
above. In the pea aphid, *Acyrthosiphon pisum*, hosts exploiting different host plants tend to harbour different genotypes of *H. defensa* (Ferrari *et al.*, 2012). From our study system it is known that parasitoids of the *L. fabarum* group exhibit genetic differentiation among different aphid hosts (Sandrock *et al.*, 2011a). The three isolates of *H. defensa* used in the present study all originated from *A. fabae fabae*, and it is unknown whether the five aphid species from which parasitoids were collected harbour similar or different genotypes of *H. defensa*. If different, parasitoids may be adapted to strains of *H. defensa* specific to their host aphid, which would have been impossible to detect with the design of the present experiment.

This study is the first, to our knowledge, to test for local adaptation of parasitoids to the frequency of protective symbionts in their hosts. Our results provide, at best, only very limited evidence that *L. fabarum* from sites with a high prevalence of *H. defensa* in their aphid hosts tend to be more infective on protected hosts. We also found a significant effect of the hosts species from which parasitoids were collected on their infectivity on *H. defensa*-protected aphids, but the observed differences were unrelated to the prevalence of *H. defensa* in the respective aphid hosts. Considering the strong parasitoid line-by-symbiont isolate interaction we observed in our experiment, it becomes clear that to better address these questions, the variation of *H. defensa* genotypes among geographic locations and aphid species would have to be investigated. Finally, by using different isolates of *H. defensa* within a single aphid clone, we showed that the high genetic specificity between aphids harbouring *H. defensa* and their parasitoids, as reported in Rouchet & Vorburger (in press), is mediated by the symbiont's genotype, rather than the host’s.

**Acknowledgments**

This study was founded by the Swiss National Foundation. We are very thankful to Sämi Schär for genotyping all the parasitoids tested in this study and to Alexandre Gouskov for the great help in the numerous tests of parasitoid infectivity.
3. Local adaptation of parasitoids to *H. defensa*

**Bibliography**


3. Local adaptation of parasitoids to *H. defensa*


3. Local adaptation of parasitoids to *H. defensa*

**Table S1** Details of the sampling sites and the number of parasitoid lines sampled, for Map ID see Chapter 2.

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<th>Long.</th>
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3. Local adaptation of parasitoids to *H. defensa*

**Table S2** Details of the parasitoid lines presenting identical genotypes, for Map ID see Chapter 2.

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Chapter 4

Experimental evolution of parasitoid infectivity on symbiont-protected hosts leads to the emergence of genotype-specificity

Romain Rouchet & Christoph Vorburger

Abstract:

Host-parasitoid interactions may lead to intense reciprocal selection for traits involved in host resistance and parasitoid counter-defences. In aphids, individuals harbouring the facultative endosymbiont Hamiltonella defensa exhibit enhanced resistance against their parasitoid wasps, and different strains of the bacteria provide different levels of protection to aphids. We used an experimental evolution approach to investigate the ability of the parasitoid wasp Lysiphlebus fabarum to adapt to the presence of H. defensa in its aphid host Aphis fabae. Sexual populations of the parasitoid were exposed for eleven generations to a single clone of A. fabae, either free of H. defensa or harbouring artificial infections with different isolates of H. defensa. Parasitoids adapted rapidly to the presence of H. defensa in their hosts, but this adaptation was in part specific to the symbiont isolate they were evolving against and did not result in an improved infectivity on all symbiont-protected hosts. Comparisons of life-history traits among the evolved lines of parasitoids did not reveal any evidence for costs of adaptation to H. defensa in terms of correlated responses that could constrain such adaptation. These results confirm that heritable defensive symbionts impose strong selection on aphid parasitoids and show that these symbionts mediate the host-parasite interaction by inducing line-by-line genetic specificity.

Keywords:

Aphis fabae, experimental evolution, facultative symbionts, Hamiltonella defensa, host parasitoid coevolution, trade-off.
4. Experimental evolution of parasitoid infectivity

Introduction

Parasitism is the most common lifestyle on Earth and affects all living organisms (Windsor, 1998). Host-parasite interactions may lead to intense and continuous processes of reciprocal adaptation for the traits involved in the outcome of the interaction, such as host resistance and parasite virulence (Thompson, 1994). These antagonistic coevolutionary dynamics have been widely studied according to their importance in diverse fields such as evolutionary biology, agricultural science, conservation biology or medical science (Woolhouse et al., 2002; Ebert, 2008; Laine, 2009; Brown & Tellier, 2011). Because the outcome of their interaction is always fatal for one of the antagonists, host-parasitoid systems exhibit particularly strong reciprocal selection and thus offer great scope for the study of coevolution (Godfray, 1993; Kraaijeveld et al., 1998).

Aphids frequently rely on facultative bacterial endosymbionts for defence against natural enemies (Oliver et al., 2010). In addition to their primary symbiont Buchnera aphidicola, which provides them with essential nutrients missing from their phloem diet, aphids may harbour a large variety of facultative or secondary endosymbionts. These are generally not essential for survival and reproduction (Oliver et al., 2010), but they can provide aphids with important ecological benefits. For example, Regiella insecticola increases the resistance to fungal pathogens in the pea aphid (Scarborough et al., 2005), and Hamiltonella defensa has been shown to provide different aphid species with protection against parasitoid wasps (Ferrari et al., 2004; Oliver et al. 2005; Vorburger et al. 2009). Hamiltonella defensa is one of the most common facultative endosymbionts in natural populations of aphids (Ferrari et al., 2012; Chapter 2). The protection against parasitoids afforded by H. defensa is influenced by the presence of lysogenic bacteriophages called APSE that are found within the H. defensa genome (Moran et al., 2005; Degnan & Moran, 2008; Oliver et al., 2008, Oliver et al., 2009). Different strains of H. defensa are associated with different variants of this bacteriophage encoding for a variety of toxins targeting eukaryotic cells (Moran et al., 2005), and there is evidence that different strains of H. defensa provide different levels of protection against parasitoids (Oliver et al., 2005). Moreover, some studies suggest that resistance conferred by H. defensa acts more specifically than the (limited) defences of H. defensa-free hosts.
4. Experimental evolution of parasitoid infectivity

(Vorburger et al., 2009; Schmid et al., 2012; Chapter 1). Hence, the presence of *H. defensa* in aphids may impose strong and potentially multifarious selection on parasitoids to adapt to symbiont-conferred resistance. In chapter 1 and 3, we found that different lines of the parasitoid *Lysiphlebus fabarum* exhibited large differences in the ability to overcome *H. defensa*-mediated defences of their host, *Aphis fabae*, suggesting that parasitoid populations possess the necessary genetic variation. Indeed, Dion et al. (2011) showed, using an experimental evolution procedure, that the parasitoid wasp *Aphidius ervi* can adapt very quickly to the presence of *H. defensa* in the pea aphid *Acyrthosiphon pisum*. Still unknown is whether parasitoid adaptation to one *H. defensa* strain generally improves the ability to overcome symbiont-conferred defences, whether such adaptation reduces infectivity on *H. defensa*-free aphids, or whether it is constrained by other trade-offs, e.g. with life-history traits. In this study, we addressed these questions with an experimental evolution approach, monitoring the adaptation to symbiont-conferred protection in sexual populations of the parasitoid *L. fabarum* over eleven generations. As hosts we used four sublines of a single clone of *A. fabae* that were artificially infected with one of three different isolates of *H. defensa* or symbiont-free as a control. We also tested whether adaptation to symbiont-mediated defences comes at costs by comparing life-history traits among the evolved populations.

**Material and Methods**

*Insects*

The black bean aphid, *Aphis fabae*, is a widely distributed agricultural pest reproducing by cyclical parthenogenesis. In Europe, *A. fabae* uses mainly the spindle tree *Euonymus europaeus* as a primary hosts for the sexual generation that lays the overwintering eggs and many species of herbaceous annual plants as secondary host for the viviparous, parthenogenetic generations throughout the growth season. It is particularly damaging on broad beans, *Vicia faba*, and sugar beets, *Beta vulgaris* (Blackman and Eastop 2006). By keeping it under summer-like conditions with a long photoperiod, it is possible to maintain *A. fabae* clonally for any period of time.
To test for the adaptation of parasitoids to *H. defensa*-mediated defences, we used genetically identical aphids with and without different isolates of the symbiont. The common genetic background was a single clone of *A. fabae*, A06-407, that was collected in July 2006 on *Chenopodium album* in St. Margrethen (Switzerland) and found to be uninfected with any known secondary symbiont of aphids. Since then it was maintained in the laboratory on *Vicia faba*. Between June 2008 and March 2009 we artificially created three *H. defensa*-infected sublines of this clonal host, namely A06-407^H323^, A06-407^H402^ and A06-407^H76^, by microinjecting hemolymph from three different *A. fabae* clones (nrs. A06-76, A06-323 and A06-402) harbouring the symbiont and exhibiting different level of resistance to the parasitoid *L. fabarum* (Vorburger et al. 2009; Chapter 1; Chapter 3). Using the same genetic background enabled us to separate the protection conferred by *H. defensa* from genetic variation in the aphids' own defences. Microinjections followed the protocol described in Vorburger et al. (2010) and resulted in stable, heritable infections that were re-confirmed by diagnostic PCR prior to the lines' use in the experiment.

*Lysiphlebus fabarum* is the main parasitoid of *A. fabae*, and together they constitute an ideal system for the study of host-parasitoid interactions mediated by *H. defensa* (Vorburger et al. 2009; Vorburger and Gouskov 2011; Schmid et al. 2012; Chapter 1). *Lysiphlebus fabarum* has the particularity of comprising both sexual (arrhenotokous) and asexual (thelytokous) lineages (Sandrock and Vorburger 2011). Here we worked with sexual *L. fabarum*. Between May and July 2009, sexuals from eight different locations in Switzerland and in France were collected (Table S1). They were maintained as mass cultures for 6 months on caged colonies of a *H. defensa*-free clone of *A. fabae* on *Vicia faba*. Prior to the experiment, 30 females and 20 males from each of the 8 sexual populations were mixed in a large polyester mesh cage (47.5cm x 47.5cm x 47.5cm, BugDorm 44545F, MegaView Science, Taichung, Taiwan) and allowed to interbreed for three generations. This created the common, genetically variable stock populations that we used for the experiment.
4. Experimental evolution of parasitoid infectivity

Experimental evolution procedure

The experimental evolution procedure consisted of four aphid treatments (i.e. evolution treatments): the *H. defensa*- free clone A06-407 and the three *H. defensa*-infected sublines of the same clone: A06-407\textsuperscript{H323}, A06-407\textsuperscript{H402} and A06-407\textsuperscript{H76}. Each evolution treatment was replicated four times (evolution lines) in independent polyester mesh cages (25 cm x 25 cm x 25 cm, BugDorm 4020F, MegaView Science, Taichung, Taiwan) placed on random position on an illuminated bench in a climatized room (20°C). Every cage contained four pots with three broad bean plants colonized by large numbers of aphids from the respective sublines. At the beginning of the experiment, 34 females and 20 males from the stock population were introduced in each cage. Time from oviposition to the emergence of the next generation of wasps is approximately two weeks. When the next generation had emerged, 30 females and 15 males (sometimes fewer, if only low numbers emerged) were transferred into a fresh cage with new aphid colonies from the corresponding treatment sublines. This process was repeated for 11 generations. Through re-stocking the cages with fresh aphids every parasitoid generation, the hosts remained a 'static target', allowing us to observe parasitoid adaptation independent of host or symbiont counter-adaptations.

We first estimated the infectivity of the parasitoids in the mixed parasitoid population at the beginning of the experiment. The infectivity test consisted in exposing a parasitoid female to a colony of 48-72h old aphid nymphs from one of the aphid sublines for a period of 12h, and 11 days later counting the number of mummified hosts, as described by Henter and Via (1995). The proportion of mummified aphids was used as a direct measure of parasitoid infectivity on each of the aphid subline. Each aphid subline x parasitoid combination was replicated 12 times. At generation 5 and 11, parasitoid infectivity tests were realized the same way with 3 replicates for each aphid subline x evolution line. Moreover, once the experiment was terminated, we mixed the remaining evolution lines within treatment and maintained them for two generations on the *H. defensa*-free clone 407. The mixing of the evolution lines aimed to reduce the potential effects of bottlenecks and inbreeding in the different evolution lines during the
experimental evolution generations. Parasitoid infectivity was then measured based on 8 replicates for each aphid subline x evolution treatment combination.

Correlated responses

At the end of the experimental evolution procedure, all evolution lines were placed on *H. defensa*-free clones for two generations before estimating several phenotypic traits to test for correlated responses. The individuals to be assayed were produced by placing one parasitoid female in an aphid colony of the *H. defensa*-free clone A06-407 for 12 hours. This was replicated eight times for each evolution line. Eleven days after the test, the number of mummies per colony was counted. Starting from that date, colonies were checked daily and all hatched wasps were collected, providing us with an estimate of development time. All emerging individuals were sexed, weighed to the nearest microgram on a Mettler MX5 microbalance (Mettler Toledo GmbH, Greifensee, Switzerland) and then stored at -20°C for later measurement of the length of both tibiae with an ocular micrometer at under a Nikon Eclipse 80i microscope (Nikon, Tokyo, Japon). Thirty-seven females in total were dissected fresh to remove the ovaries and measure the length of 10 mature eggs per female under the microscope to have an estimate of egg size. Apart from egg size, all these traits were measured in the exact same way on parasitoids hatching from the *H. defensa*-free aphid sublines in the infectivity test on the mixed evolution lines (see above). As for infectivity, the mixing of the evolution lines was expected to reduce the potential effects of bottlenecks and inbreeding on these different traits.

Statistical analysis

All statistical analyses were carried out in R version 2.11.1 (R Development Core Team, 2010). For the infectivity tests, the proportion of aphids exposed to parasitoids that were mummified were taken as the response variable. Overdispersion prevented us from analyzing these success-failure data with a generalized linear models and binomial errors.
Hence, the proportions of aphids mummified were arcsine square-root transformed and analyzed with a linear model (LM) or linear mixed models (LMM). First we compared the proportion of aphids mummified by the original stock of parasitoids at generation 0 among aphid sublines with a LM. For the tests after 5 and 11 generations of experimental evolution we used LMMs, employing the lmer function from the lme4 package (Bates et al., 2011). We tested for the fixed effects of aphid subline, evolution treatment, and the subline x treatment interaction, as well as for the random effects of evolution line (nested within evolution treatment) and the evolution line x aphid subline interaction. P-values for the fixed effects were calculated using F-tests with Satterthwaite’s approximation and p-values for the random effects were calculated based on Chi square test with the function totalAnalysis from the MixMod package (Kuznetsova & Brockhoff, 2012). After mixing the evolution lines within treatment at the end of the experiment, we tested for the effects of aphid subline, evolution treatment and the interaction of aphid subline and evolution treatment with a LM.

To test for the effect of evolution treatment on parasitoid infectivity on *H. defensa*-free hosts, we used the results of the infectivity test on the clone A06-407 after mixing evolution lines within evolution treatment at the end of the experiment. We tested for the effect of evolution treatment on parasitoid infectivity (proportion of mummified aphids) with a LM.

To test for potential correlated responses to evolution on symbiont-protected hosts we compared life-history traits (weight, tibia length, development time and egg size) at the end of the experiment with LMMs, using the lme function from the nlme package (Pinheiro et al., 2012). We used a mixed model because replicate within evolution line and evolution line (nested within evolution treatment) were considered as random effects. We analyzed the effects on each measured trait of replicate, evolution line, evolution treatment, sex, and the interaction of evolution treatment and sex. P-values for the fixed effects were calculated using a type III test and P-values for the random effects were calculated using a likelihood ratio test. A similar analysis was applied to the measurements of life-history traits after mixing of the evolution lines within evolution treatment.
4. Experimental evolution of parasitoid infectivity

Results

Evolution of infectivity

The infectivity assays at generation 0 showed that the stock of parasitoids used as the starting population for the experiment was poorly adapted to *H. defensa*-protected hosts. The mean proportion of hosts mummified differed significantly among aphid sublines (LM, $F_{3, 44} = 39.80, P < 0.001$) and was 0.54 on *H. defensa*-free aphids, but only 0.05 on A06-407$^{H323}$, 0.06 on A06-407$^{H402}$ and 0.00 on aphid line A06-407$^{H76}$ (Fig. 1). Not surprisingly, therefore, selection by *H. defensa*-protected aphids was very strong in the first generations of the experiment, and three evolution lines went extinct within 5 generations (two from the A06-407$^{H76}$ treatment, one from the A06-407$^{H402}$ treatment).

At the next assay in generation 5, an evolutionary response to this selection was already evident in the remaining evolution lines. Infectivity differed significantly among aphid sublines as well as among evolution treatments, and there was a significant aphid subline \( \times \) evolution treatment interaction (Table 1), indicating that the infectivities on aphids with or without different isolates of *H. defensa* depended on which aphids the parasitoids had evolved on. Parasitoids from all evolution treatments were able to parasitize the unprotected aphids at similar rates of approximately 40% (Fig. 1). The parasitoids that had evolved on unprotected aphids (control evolution lines) achieved very low rates of parasitism on all aphid sublines harbouring *H. defensa*, similar to the starting population.

Parasitoids that had evolved on aphid sublines A06-407$^{H323}$ and A06-407$^{H402}$ showed a strongly improved ability to parasitize A06-407$^{H323}$ as well as A06-407$^{H402}$ compared to the control lines, but were still near-unable to parasitize A06-407$^{H76}$ (Fig. 1). The most infective parasitoids on A06-407$^{H76}$ were from the two remaining evolution lines that had evolved on these strongly protected aphids, but the proportion of aphids mummified of approximately 0.1 was still relatively low, and they also showed low infectivity on the aphids harbouring the other two isolates of *H. defensa* (Fig. 1). The evolutionary responses observed at generation 5 were consistent among evolution lines of parasitoids in the same evolution treatment, which was reflected in the non-significant variation among these evolution lines and the lack of an interaction between evolution lines from the same treatment and the aphids they were tested on (Table 1).
Unfortunately, two more evolution lines (both from the A06-407<sup>H402</sup> treatment) went extinct between generations 5 and 11, probably due to the outbreak of a fungal pathogen in their cages. This left us with just a single line of this treatment at the next assay in generation 11, which exhibited rather low infectivity on all aphid sublines except the one it evolved on (Fig 1). The results for the other evolution treatments were very similar to those obtained in generation 5. Wasps evolved on aphids without <i>H. defensa</i> still exhibited low infectivities on all aphid sublines possessing <i>H. defensa</i>. Wasps evolved on A06-407<sup>H323</sup> had elevated infectivities on A06-407<sup>H323</sup> as well as A06-407<sup>H402</sup>, but still very low infectivity on A06-407<sup>H76</sup>. Only the wasps evolved on A06-407<sup>H76</sup> performed well on these aphids (meanwhile approx. 20% infectivity), but not on the other two aphid sublines harbouring <i>H. defensa</i> (Fig. 1). Apart from a significant effect of aphid subline, the analyses revealed again a significant aphid subline x parasitoid treatment interaction (Table 1), confirming that line x line specificity evolved in the experiment as a consequence of parasitoid adaptation to different isolates of the host's symbionts. As in generation 5, there was no significant variation among evolution lines of the same evolution treatment and no evolution line x aphid subline interaction, showing that the evolutionary response was similar in the different evolution lines of the same treatment. Repeating the infectivity assays after the mixing of the evolution lines from the same evolution treatment, to account for possible inbreeding effects, provided very similar results (Fig. 2). In particular we also found a significant interaction of aphid subline with evolution treatment (Table 1), confirming the strong genotype-by-genotype interaction observed at generation 5 and 11. Importantly, we did not find a significant effect of the evolution treatment when just analyzing infectivity on <i>H.defensa</i>-free aphids (LM, $F_{3,93} = 0.78$, $P = 0.514$), indicating that parasitoid adaptation to <i>H.defensa</i>-mediated defences did not decrease parasitoid infectivity on unprotected hosts.
4. Experimental evolution of parasitoid infectivity

**Table 1.** Results of linear mixed effects models on the proportion of aphid mummified by parasitoids after 5 and 11 generations of experimental evolution. Proportions were arcsine square-root transformed before analysis. *P*-values of random effects are based on likelihood ratio tests and *P*-values of fixed effects on F tests with Satterthwaite’s approximation.

<table>
<thead>
<tr>
<th>Source</th>
<th>ndf for fixed effects</th>
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<th><em>P</em></th>
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<td>Generation 5</td>
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<td>20.82</td>
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<td>4.45</td>
<td>0.005</td>
</tr>
<tr>
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<tr>
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<td></td>
<td></td>
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<td>11.97</td>
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<td>2.18</td>
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**Table 2.** Results of a linear model on the proportion of aphid infection by parasitoids after the mixing of evolution lines within evolution treatment. Proportions were arcsine square root transformed before analysis.

<table>
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<tr>
<th>Source</th>
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<td>Evolution treatment</td>
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<td>Aphid subline x Evolution treatment</td>
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<tr>
<td>Residuals</td>
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Figure 1. Mean infectivity on the four aphid sublines of the original parasitoid population (n= 12), and of the parasitoids from each evolution treatment at generation 5 and 11 (n = 3).
Figure 2. Mean infectivity of the parasitoids from the different evolution treatments after the mixing of evolution lines within evolution treatment. Infectivity is represented as the proportion of mummified aphids on the four aphid sublines (n = 8).
**Correlated responses**

There was no significant effect of evolution treatment, nor any significant variation among evolution lines within treatment on parasitoid weight or tibia length at the end of the experiment (Fig 3, Table 3). Differences between male and female wasps were not significant, albeit nearly so for tibia length, and there were no sex x evolution treatment interactions (Table 3). Egg size did not differ significantly among wasps from the different evolution treatments either (Table 3). The only trait that exhibited significant variation among evolution treatments was development time (Table 3). However, this result has to be interpreted with caution, because it is entirely due to a longer development time of wasps evolved on evolution treatment A06-407H402 (Fig. 3). This is the one evolution treatment that was unreplicated at the end of the experiment because three of the four lines in this treatment died out during the experiment (see above). There was also a significant evolution treatment x sex interaction because particularly the males of the line evolved on A06-407H402 emerged from mummies very late. When the analysis was repeated on females only, the variation among evolution treatments was lower, but still nearly significant \( (F_{3,6} = 4.36, P = 0.059) \).

The second experiment to compare wasp weight, tibia length and development time took place after mixing the remaining evolution lines to account for potential effects of inbreeding. It revealed no significant effect of evolution treatment on any of the three traits (Table 4), supporting that parasitoid adaptation to symbiont-conferring resistance did not entail any marked correlated responses in terms of altered development time or body size.
### Table 3. Results of the linear mixed model for the correlated traits measured in the different evolution lines. $P$-values for the fixed effects were calculated using a type III test and $P$-values for the random effects were calculated using a likelihood ratio test.

<table>
<thead>
<tr>
<th>Trait</th>
<th>Source</th>
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<td>Evolution treatment</td>
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<td>0.331</td>
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<td></td>
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<td>Egg size</td>
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4. Experimental evolution of parasitoid infectivity

Table 4. Results of the linear mixed model for the life-history traits measured after mixing evolution lines within treatment. *P*-values for the fixed effects were calculated using a type III test and *P*-values for the random effects were calculated using a likelihood ratio test.

<table>
<thead>
<tr>
<th>Trait</th>
<th>Source</th>
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<td>0.01/0.998</td>
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<td>Sex</td>
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<td>0.17/0.686</td>
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<td>57</td>
<td>0.12/0.950</td>
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</table>
4. Experimental evolution of parasitoid infectivity

**Figure 2.** Mean adult mass (a), tibia length (b), development time (c) and egg size (d) per evolution line measured on *H. defensa*-free aphids at the end of the experimental evolution procedure.
4. Experimental evolution of parasitoid infectivity

Discussion

Experimental evolution is a powerful tool for the study of adaptation. In the present experiment, we found that sexual populations of the parasitoid wasp *Lysiphlebus fabarum* can adapt quickly to the presence of the defensive endosymbiont *H. defensa* in their aphid host, *A. fabae*. They increased their infectivity on protected aphids within very few generations. These results are concordant with the results of a study by Dion *et al.* (2011), in which the parasitoid *Aphidius ervi* experienced a fast increase of infectivity over time when maintained on pea aphids harbouring *H. defensa*. By evolving the wasps on aphids harbouring different isolates of *H. defensa* in the same genetic background, we could further show that this adaptation was in part specific to the symbiont isolate. The two isolates H323 and H402 represented a similar challenge to the parasitoids. Adaptation to aphid subline A06-407\(^{H323}\) increased infectivity also on A06-407\(^{H402}\) and *vice versa*, but did not improve the ability to parasitize the host subline A06-407\(^{H76}\). This subline was extremely resistant to the starting population of parasitoids and more difficult for the parasitoids to adapt to. Nevertheless, after 11 generations of experimental evolution on host subline A06-407\(^{H76}\), the parasitoids showed a clear increase of infectivity on their own subline, but this did not entail an improved ability to parasitize sublines A06-407\(^{H323}\) and A06-407\(^{H402}\). Thus, different adaptations appear to be required to overcome host protection by different symbiont strains. The overall outcome was a significant line x line interaction of the outcome of infection – the hallmark of a genotype-specific host-parasitoid interaction (Lambrechts *et al.*, 2006). This confirms earlier observations from experiments with asexual lines of *L. fabarum* and *H. defensa*-protected aphids which suggested that symbionts mediate the genotype-specificity in this host-parasitoid interaction (Vorburger *et al.*, 2009; Schmid *et al.*, 2012; Chapter 1). The presence of *H. defensa* adds an additional, more specific layer of defence in addition to the aphids' own, less specific defences (Sandrock *et al.*, 2010). The mechanistic basis underlying the observed specificity is still unknown. Differences between *H. defensa* strains in the level of protection provided to the host have been proposed to be due to differences between APSE bacteriophage variants harboured by the symbiont and known to encode for a variety of toxins (Degnan & Moran, 2008). Hence, the strong specificity between
symbiont strain and parasitoid line revealed by our study could be explained by differences between symbiont strains in not only the amount of toxins produced, but also in the nature of the toxins.

We found no strong evidence that the adaptation of parasitoids to *H. defensa*-mediated defences comes at costs. Wasps evolved on hosts harbouring *H.defensa* experienced no decrease in their ability to parasitize symbiont-free hosts. Moreover, we found no consistent differences between evolution treatments in individual mass, size, development time and egg size at the end of the experiment. This contrasts with the experimental evolution experiment by Dion *et al.* (2011), who reported a decrease in the body size of wasps evolving on *H. defensa*-bearing aphids. However, they compared body sizes of wasps emerging from the hosts they had evolved on and not from a common stock of *H. defensa*-free aphids as in the present experiment. Thus the decreased body size reported in Dion *et al.* (2011) may have been a direct environmental consequence of developing in aphids with *H. defensa* rather than a correlated genetic response of adaptation to such hosts. It is known that wasps developing in aphids harbouring *H. defensa* can indeed suffer from negative effects such as delayed development, reduced emergence from mummies and smaller body size (Nyanguba *et al.*, 2010, Schmid *et al.*, 2012). In a selection experiment for increased infectivity of *Asobara tabida*, a parasitoid of *Drosophila melanogaster*, Kraaijeveld *et al.* (2001) also found no differences in the parasitoids' adult size, fat content, egg load and infectivity on susceptible hosts, but they did detect an increase in the duration of the egg stage in the selected lines. This remains one of the sole convincing examples for a cost of increased infectivity in a parasitoid.

Although there are now two studies showing that parasitoids are able to adapt rapidly to *H. defensa*-mediated defences in aphids (Dion *et al.*, 2011, this work), little is known about how protective symbionts in aphids affect parasitoid populations in the field, e.g. in terms of local adaptation. There is some limited evidence for *L. fabarum* that at sites with a high prevalence of *H. defensa* in the aphid hosts, parasitoids tend to be more infective on protected hosts, but this relationship was not significant (Chapter 3). The results reported here suggest that parasitoid local adaptation might be more difficult
to evolve than envisaged, because rather than just to the presence of *H. defensa* in their hosts, parasitoids may have to adapt to the very strains of symbionts their hosts are harbouring. Unfortunately, not much is known yet about the genetic variability within *H. defensa* and the geographical distribution of different genotypes of *H. defensa* in aphids. The little information there is suggests that factors not considered in our study may also contribute to structuring this variability: in the pea aphid, *Acyrthosiphon pisum*, host races exist that exploit different plants and tend to harbour different genotypes of *H. defensa* (Ferrari *et al.*, 2012). This raises interesting prospects for future research on eco-evolutionary interactions in such systems, e.g. on the relative roles of host ecology and of the coevolution with parasitoids for symbiont diversity in aphids. The high genetic specificity of the resistance conferred by *H. defensa* observed here and elsewhere certainly suggests that coevolutionary interactions with parasitoids may be highly dynamic, because genotype-specificity readily leads to negative frequency-dependent selection and thus to a rapid turnover of genotypes under antagonistic coevolution.

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Bibliography


4. Experimental evolution of parasitoid infectivity


### Table S1. Details of the parasitoid lines used in the experiment.

<table>
<thead>
<tr>
<th>Parasitoid line</th>
<th>Parasitoid species</th>
<th>Date of sampling</th>
<th>Site of origin</th>
<th>Host aphid</th>
<th>Host plant</th>
</tr>
</thead>
<tbody>
<tr>
<td>09-46</td>
<td><em>L. fabarum</em></td>
<td>21/05/2009</td>
<td>Remouins (FR)</td>
<td><em>Aphis hederae</em></td>
<td><em>Hedera helix</em></td>
</tr>
<tr>
<td>09-241</td>
<td><em>L. fabarum</em></td>
<td>01/06/2009</td>
<td>Sierre (CH)</td>
<td><em>Aphis hederae</em></td>
<td><em>Hedera helix</em></td>
</tr>
<tr>
<td>09-242</td>
<td><em>L. fabarum</em></td>
<td>01/06/2009</td>
<td>Sierre (CH)</td>
<td><em>Aphis hederae</em></td>
<td><em>Hedera helix</em></td>
</tr>
<tr>
<td>09-243</td>
<td><em>L. fabarum</em></td>
<td>01/06/2009</td>
<td>Sierre (CH)</td>
<td><em>Aphis hederae</em></td>
<td><em>Hedera helix</em></td>
</tr>
<tr>
<td>09-360</td>
<td><em>L. fabarum</em></td>
<td>25/06/2009</td>
<td>Geneva (CH)</td>
<td><em>Aphis hederae</em></td>
<td><em>Hedera helix</em></td>
</tr>
<tr>
<td>09-372</td>
<td><em>L. fabarum</em></td>
<td>25/06/2009</td>
<td>Orbe (CH)</td>
<td><em>Aphis hederae</em></td>
<td><em>Hedera helix</em></td>
</tr>
<tr>
<td>09-418</td>
<td><em>L. fabarum</em></td>
<td>09/07/2009</td>
<td>Alpnach (CH)</td>
<td><em>Aphis hederae</em></td>
<td><em>Hedera helix</em></td>
</tr>
<tr>
<td>09-501</td>
<td><em>L. fabarum</em></td>
<td>14/06/2009</td>
<td>Neunkirch (CH)</td>
<td><em>Aphis hederae</em></td>
<td><em>Hedera helix</em></td>
</tr>
</tbody>
</table>
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Romain Rouchet

Birth: 13th of April 1984, Colmar
Nationality: French
Languages: English, French, Spanish

Ph. +41 (0)78 888 20 74
E-mail: romain.rouchet@eawag.ch; romain.rouchet@gmail.com

Education

2009 - present: PhD candidate.
Department of Aquatic Ecology, Eawag, Dübendorf, &
Institute of Integrative Biology, ETH Zürich, Switzerland.

University of Rennes 1, France (with honours).

University of Rennes 1, France.

2004: DEUG in Biology (two-years university degree).
University Louis Pasteur, Strasbourg, France.

Research experience

2009 - 2012: PhD student at the Institute of Integrative Biology, ETH Zürich, &
Department of Aquatic Ecology, Eawag, Dübendorf, Switzerland. “Host-
parasitoid coevolution: The role of parasitoid adaptation to endosymbiont-
mediated defences in aphids.” Supervisor: Prof. Dr. Christoph Vorburger.

2008: Master project at UMR 6553 Ecobio, University of Rennes 1, France. “Plant-
phytophagous insect interactions: Reproductive success of the weevil  Exapion ulicis in relation with the phenology of its host plant, Ulex europaeus.”
Supervisors: Dr. Michèle Tarayre and Dr. Joan van Baaren.
Curriculum vitae


2006: Bachelor project at the Laboratory of Ecobiology of Parasitoid Insects, University of Rennes 1, France. “Influence of dimethyl disulfide (DMDS) on the location of cabbage root flies (*Delia radicum* L.) by two predatory and parasitoid Staphylinidae species (*Aleochara bilineata* and *A. bipustulata*).” Supervisor: Prof. Dr. Anne-Marie Cortesero.

Publications


Presentations at conferences and seminars

2011: Meeting of the European Society for Evolutionary Biology (ESEB), Tübingen, Germany. Oral presentation: Adaptation of parasitoid wasps to protective endosymbionts in aphids under experimental evolution.

2011: Biology’11, Zürich, Switzerland. Oral presentation: Adaptation of parasitoid wasps to protective endosymbionts in aphids under experimental evolution.


2009: Meeting of the European Society for Evolutionary Biology, Torino, Italy. Oral presentation: Reproductive success of *Exapion ulicis* in relation to the phenology of its host plant.