The facultative endosymbionts of grain aphids and the horizontal transfer of ecologically important traits

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Abstract

1. Insects are often infected with facultative endosymbiotic bacteria, which can have a range of important ecological effects. The grain aphid, *Sitobion avenae*, harbours diverse facultative symbionts, which suggests their importance in grain aphid biology.
2. This thesis attempts to explain the ecological roles of the facultative endosymbionts in *S. avenae*. It also examines the question of whether the horizontal transmission of symbionts between aphid clones and species can be important for shaping the ecology and evolution of multi-species aphid communities. Novel techniques developed for research with the grain aphid study system are presented.
3. Grain aphid clones vary in their tolerance to low temperatures, but this trait is not affected by their facultative endosymbionts.
4. Strains of a symbiont *Hamiltonella defensa* do not protect grain aphids from hymenopterous parasitoids, regardless of the host genotype. However, experienced parasitoid females preferentially oviposit in aphids which do not harbour symbionts.
5. Comparison of the fitness consequences of infection with the same *Hamiltonella* strains in their original and in novel grain aphid host clones reveal no consistent differences.
6. Symbiont strains establish easily following artificial transfer between clones of the grain aphid, but the symbionts transferred from other aphid species form less stable infections. *Hamiltonella* strains do not affect the fecundity of their grain aphid host clones regardless of their host species of origin, but also do not generally confer protection against parasitoids.
7. There are no clear patterns in the distribution of parasitoid-resistant phenotypes across phylogenetic trees of *Hamiltonella* and its bacteriophage APSE.
8. Strains of four unrelated species of endosymbionts, *Rickettsia*, *Spiroplasma*, *Rickettsiella* and *Regiella*, confer the same pathogen-resistant phenotype to a single pea aphid clone. The same symbiont strains can confer resistance to clones of two different aphid species. Some strains in multiple infections may compensate for the costs of infections with other symbionts.
9. The importance of these results for understanding the ecological and evolutionary role of facultative endosymbionts in aphids and other insects are discussed, and directions for further research are proposed.

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Contents

Chapter 1

General Introduction

Symbioses, defined for the purpose of this thesis as long-term, but not necessarily mutually beneficial associations between different organisms (de Bary, 1879; Wilkinson, 2001), are increasingly being regarded as shaping the evolution of life. Out of many examples of successful and important symbioses, the most spectacular may be the very existence of eukaryotes, traced back to an acquisition of an aerobic alphaproteobacterium by an anaerobic protoeukaryote approximately 2 billion years ago. The resulting association, benefitting from the combined metabolic properties of the two very different organisms, in the course of evolution became a single cell, the common ancestor of all protozoans, animals, plants and fungi. The alphaproteobacterium within a eukaryotic cell remained distinct, though, and is now referred to as mitochondrion (Embley & Martin, 2006; Lang et al., 1999; Margulis, 1981; Sagan, 1967). Similarly, acquisition of an autotrophic cyanobacterium by a heterotrophic protist 1.5 billion years ago has resulted in another extremely stable association, which became the ancestor of all eukaryotic primary producers on land and in the water (Gould et al., 2008; Reyes-Prieto et al., 2007; Rodriguez-Ezpeleta et al., 2005). These two cases of successful symbioses determined the course of evolution on the planet. However, other examples of long-term associations between unrelated organisms, either mutually benefitting from each other's distinct properties or one taking advantage of the other, have been reported from all major taxa (Bright & Bulgheresi, 2010, and references therein; Herre et al., 1999). While these associations are not as ancient, they have had a tremendous effect on the evolution of both partners (Moran, 2006; Moran et al., 2008).

Over recent years, symbioses between insects and bacteria living within their haemocoel and cells (further referred to as endosymbionts) have become a very active area of research in ecology and evolutionary biology (Baumann, 2005; Moran et al., 2008; Oliver et al., 2010; Werren et al., 2008). In fact, the occurrence of

symbiotic microbes living within haemocoel of their insect hosts has been known for more than forty years (Buchner, 1965), but the lack of cultivability of these bacteria hindered conventional microbiological experiments. It was the development and expansion of molecular biology techniques in 1990s, and particularly within the last decade, which enabled researchers to realize the ubiquity and diversity of endosymbiotic bacteria, and to get an insight into their biology and evolution (Moran et al., 2008). Additionally, the opportunity to reliably (and inexpensively) assess the presence and type of endosymbiotic bacteria in large samples of insects encouraged experimental studies on their ecological roles, resulting in a series of reports on the effects of infection on their hosts' fitness (Haine, 2008; Oliver et al., 2010; Werren et al., 2008).

In their recent review, Moran and colleagues (2008) classified the endosymbiotic bacteria of insects into functional groups based on their effects on their hosts and on the evolutionary history of the host-symbiont associations. The first group, primary (or obligatory) endosymbionts, supply amino acids and vitamins deficient in the diets of insects specializing on unbalanced foods such as blood, wood or plant sap (Baumann, 2005; Douglas, 1998; Moran et al., 2008). Thus, these bacteria are essential for the survival and reproduction of their hosts, but are themselves dependent on the hosts for maintenance, unable to survive outside of them or invade novel hosts. Phylogenetic data reveal that they have been transmitted strictly maternally (vertically), co-speciating with their hosts, for millions of years, and as much as 260 million years in the case of the oldest such known symbiosis (Moran et al., 2008; Moran et al., 2005c). This has resulted in a very close adaptation between the bacteria and the hosts, as reflected by the extreme reduction of symbiont genomes and retention of only the genes most essential for their cells' functioning and for the hosts' nutrition (McCutcheon et al., 2009; McCutcheon & Moran, 2010;

Moran et al., 2008; Nakabachi et al., 2006; Wernegreen, 2002), as well as by the insects having developed specialized organs (bacteriomes) for hosting the symbionts (Baumann, 2005; Buchner, 1965; Douglas, 1998).

Endosymbiotic bacteria of the second category, referred to as secondary or facultative symbionts, are also primarily vertically transmitted, but have had a much shorter co-evolutionary history with their hosts. This is revealed by the incongruence of their phylogenies with those of their hosts, and by their ability to invade and establish themselves in novel hosts as demonstrated by experimental studies (Oliver et al., 2010; Russell & Moran, 2005; Werren et al., 2008). Facultative endosymbionts are not essential for their hosts, and as they rely on hosts for energy and nutrients, their upkeep is believed normally to carry some costs to the hosts. Thus, in order to persist and spread in insect populations, facultative symbionts employ one of two distinct, but not mutually exclusive strategies. They can either manipulate reproduction of their hosts in order to increase maternal transmission, or provide fitness benefits which allow their carriers to increase in frequency in populations (Moran et al., 2008). The ability to manipulate the host's reproduction has independently evolved several times (Engelstadter & Hurst, 2009), and the mechanisms range from induction of cytoplasmic incompatibility or parthenogenesis, to feminisation or killing of males. The best-known example of a reproductive manipulator is an alphaproteobacterium *Wolbachia pipientis* (Stouthamer et al., 1999; Werren et al., 2008), estimated to reside in as many as two-thirds of all arthropod species (Hilgenboecker et al., 2008). The second functional group of non-essential endosymbionts, facultative mutualists, is also represented by bacteria from a range of phyla. These symbionts can have diverse effects on their hosts (Moran et al., 2008; Oliver et al., 2010), but their ability to confer protection against natural enemies has received the most attention (Brownlie & Johnson, 2009; Haine, 2008).

As pointed out by Moran et al. (2008), there are no firm borders between these categories of endosymbionts, and there are frequent apparent shifts and intermediate cases. For example, facultative mutualists have became fixed in some insect lineages and co-speciate with them, as is the case of *Hamiltonella defensa* in the aphid genus *Uroleucon* (Degnan & Moran, 2008b). Also, reproductive manipulators may affect other fitness-related traits of their hosts, and provide them with a competitive edge over non-infected conspecifics (Himler et al., 2011). The ability to manipulate the host's reproduction can also be replaced in evolutionary time by the ability to confer fitness benefits (Hedges et al., 2008; Teixeira et al., 2008) or *vice versa* (Majerus & Majerus, 2010). To make matters more complicated, different strains, species and functional groups of endosymbionts can stably co-exist within the same hosts (e.g., Haynes et al., 2003; McLean et al., 2011; Skaljac et al., 2010; Thierry et al., 2011), and differ in their effects on the hosts' life history traits. In evolutionary time, there is much potential for co-evolution between symbionts within a host (Vautrin & Vavre, 2009), which can lead to shifts in the symbionts' roles, or evolution of novel functions (Gosalbes et al., 2008; Perez-Brocal et al., 2006). However, the nature, incidence and importance of these processes are not yet well understood.

A single insect whose symbioses may have received the most attention is the pea aphid, *Acyrthosiphon pisum* Harris 1776. Like almost all aphids, it harbours a primary endosymbiont *Buchnera aphidicola*, which provides it with amino acids deficient in sap of legumes that it feeds on (Douglas, 1998). Elimination of *Buchnera* typically results in sterilization of the aphid or its offspring, even though these effects may be mitigated to a certain degree by other bacteria (Koga et al., 2003). In addition to *Buchnera*, seven species of secondary endosymbionts have so far been reported to infect pea aphids, including five species of gammaproteobacteria: *Hamiltonella defensa*, *Regiella insecticola*, *Serratia symbiotica*, "X-type", and *Rickettsiella* (Guay

et al., 2009; Moran et al., 2005b; Tsuchida et al., 2010), an alphaproteobacterium *Rickettsia* (Chen et al., 1996), and a representative of the class Mollicutes, *Spiroplasma* (Fukatsu et al., 2001). These symbionts can infect their hosts singly, but natural double and even triple infections have been reported (Ferrari et al., accepted; Frantz et al., 2009; McLean et al., 2011; Oliver et al., 2006; Tsuchida et al., 2002). Facultative endosymbionts are distributed nonrandomly across pea aphid host plant races (Ferrari et al., 2004; Ferrari et al., accepted; Frantz et al., 2009) and geographic locations (Tsuchida et al., 2002), but the reasons for these distribution patterns are not well understood (Ferrari et al., accepted; Oliver et al., 2010). A likely explanation is that certain symbionts provide fitness benefits particularly important to aphids utilizing certain environmental niches - and indeed, a range of beneficial effects of infection have been reported. Different facultative endosymbionts can protect their pea aphid hosts against hymenopterous parasitoids (Nyabuga et al., 2010; Oliver et al., 2005; Oliver et al., 2003), pathogenic fungi (Ferrari et al., 2004; Scarborough et al., 2005), high temperatures (Chen et al., 2000; Montllor et al., 2002; Russell & Moran, 2006), and affect host plant specialisation (McLean et al., 2011; Tsuchida et al., 2004). Symbiont influence on aphid body colour, with its likely pleiotrophic effects, has also been reported (Tsuchida et al., 2010). Symbionts can also affect induction of sexual reproduction in their pea aphid hosts (Leonardo & Mondor, 2006; Simon et al., 2007), which typically reproduce parthenogenetically under long-day conditions and only produce a sexual generation in response to an increase in night lengths and a decrease in temperature. During sexual reproduction, facultative endosymbionts are transmitted to eggs by mothers, but paternal symbionts can also be transferred, and even replace the maternal symbiont in clonal lines started from embryos (Moran & Dunbar, 2006); paternal transmission of facultative endosymbionts in other systems is rare (Chafee et al., 2011; Damiani et al., 2008;

Yamauchi et al., 2010). However, during the parthenogenetic part of the aphid life cycle natural infections with single endosymbionts are extremely stable over numerous aphid generations (Chen & Purcell, 1997; Darby & Douglas, 2003; Oliver et al., 2009), even though there have been reports on loss of some symbionts from multiple infections (Chen & Purcell, 1997). At the same time, symbionts can be artificially transferred between pea aphid host clones following haemolymph microinjection (Chen & Purcell, 1997; Oliver et al., 2003; Russell & Moran, 2005) or feeding on contaminated diet (Darby & Douglas, 2003), and form stable associations with the novel hosts. There are reasons to believe that such transfers occur under natural conditions as well (Darby & Douglas, 2003; Moran & Dunbar, 2006; Oliver et al., 2008).

Detailed reviews of the ecology and importance of facultative symbionts in pea aphids are available (Douglas, 1998; Oliver et al., 2010), but the brief overview presented here makes it clear that interactions with endosymbiotic bacteria have had a major effect on the ecology and evolution of this species. These data complement the large amount of information available on pea aphid ecology, evolution and genetics (Brisson & Stern, 2006). The publication of its complete genome (The International Aphid Genomics Consortium, 2010) has further facilitated research on the biology of *A. pisum*. However, the pea aphid clearly stands out from other aphids, but also other insects, in having its endosymbiotic microbial flora so well characterized. There are only limited reports on the diversity and distribution of facultative symbionts in other aphid species (Haynes et al., 2003; von Burg et al., 2008; Vorburger et al., 2009), or on their roles (Oliver et al., 2005; Vorburger et al., 2010; Vorburger et al., 2009). Thus, it is not possible to state how universal the observations made on the pea aphid symbionts are without researching other systems. However, the ecology, evolution and economic importance, but not

symbioses, of numerous other aphid species are well understood. For example, 70% more articles published between 2001 and 2010 referred to the peach-potato aphid (Web of Science search conducted on 20th July 2011, with key words: "Myzus persicae"; 1755 records) than to "Acyrthosiphon pisum" (1039 records), and much data have also been published on important aphid pests such as *Aphis gossypii* (977 records), *Rhopalosiphum padi* (674) or *Sitobion avenae* (450). Studies on symbiotic bacteria in these species would not only increase our knowledge of their biology, but also would generate data complementary to those gathered on the pea aphid model system, helping to extend our understanding of the role of facultative endosymbionts to other aphids, but also to insects in general.

The grain aphid, *Sitobion avenae* Fabricius 1775, is a medium-sized, variably coloured aphid found in Europe, Northern Africa, Middle East, but also in central Asia, South Africa and both Americas (Blackman & Eastop, 2000). Grain aphids feed on cereals and grasses, and are regarded as major pests on wheat, moderate pests on barley and oats, and minor pests on maize (Dedryver et al., 2010; Dewar & Carter, 1984; Watt et al., 1984). This is due to direct feeding damage they cause, but also their role as vectors of the barley yellow dwarf virus and other pathogens (Dedryver et al., 2005). Aphid clonal genotypes may vary in their performance across host plant species and varieties (Caillaud et al., 1995; Markkula & Roukka, 1972; Migui & Lamb, 2003). There are differing reports about the degree of specialisation of grain aphids to feeding on particular host plants. It may be possible to genetically differentiate host plant races of *S. avenae* (DeBarro et al., 1995b; Sunnucks et al., 1997; Vialatte et al., 2005), but the local movement of clonal genotypes between different host plants has been reported (DeBarro et al., 1995a). Also, the same common obligatorily asexual genotypes have been collected from across host plants (Figueroa et al., 2005; Haack et al., 2000; Vialatte et al., 2005).

This is in marked contrast to the pea aphid, in which sympatric host plant races are highly specialized to feeding on different legumes (Ferrari et al., 2008; Ferrari et al., accepted; Peccoud et al., 2009).

Grain aphid population structure is largely determined by geographical differences in reproductive strategies: in areas with mild winters most genotypes reproduce parthenogenetically throughout the year and overwinter as active stages on vegetation (anholocycly), but in regions with cold winters most genotypes are holocyclic and induce a sexual generation, producing frost-hardy overwintering eggs, in autumn (Dedryver et al., 2001; Llewellyn et al., 2003; Newton & Dixon, 1988b; Papura et al., 2003; Simon et al., 1999). Some populations of *S. avenae* are largely composed of only a few asexual genotypes (Haack et al., 2000; Llewellyn et al., 2003; Llewellyn et al., 2004), which can be widespread across geographic areas and common across years (Figueroa et al., 2005). Not as much data are available on the reproductive structure of pea aphid populations (Frantz et al., 2006; Peccoud et al., 2008), but the general patterns in that species appear broadly similar.

Grain aphids are attacked by a wide range of natural enemies, including many types of predators, at least 17 species of primary parasitoids, and numerous pathogens (Dean & Wilding, 1971; Feng et al., 1990; Kavallieratos et al., 2004; Kröber & Carl, 1991). There is much information on their abundance, diversity and distribution, as well as on their effects on populations of *S. avenae* (Dean et al., 1981; Feng et al., 1991; Holmes, 1984; Jensen et al., 2008; Muller et al., 1999; Schmidt et al., 2003; Traugott et al., 2008). Again, many of these natural enemies, including an entomopathogenic fungus *Pandora neoaphidis* and a parasitoid *Aphidius ervi*, both used in previous studies on symbiont-conferred resistance in *A. pisum*, are shared

between grain aphids and pea aphids (Daza-Bustamante et al., 2003; Kavallieratos et al., 2004; Shah et al., 2004).

This brief overview of the grain aphid biology suggests many ways in which facultative endosymbionts might influence the life history traits of their grain aphid hosts. Unravelling these effects would obviously expand our understanding of the ecology and evolution of this important pest. At the same time, the many similarities, but also the differences in biology of the grain aphid and the pea aphid indicate the potential for comparative studies between these two sympatric and related species (the same tribe Macrosiphini within the subfamily Aphidinae). However, the study of the grain aphid symbionts provides the opportunity to ask more general ecological and evolutionary questions as well.

As mentioned previously, facultative endosymbionts, although primarily transmitted maternally (=vertically), have a potential of invading and establishing in novel hosts unrelated to their original carriers. Such horizontal transfers probably occur the most frequently between matrilines within species, but there are several examples of successful transmission of symbionts between host species, including species from distantly related taxa (Degnan & Moran, 2008b; Majerus & Majerus, 2010; Raychoudhury et al., 2009). In novel hosts the symbionts can be transferred maternally with high fidelity (Chen et al., 2000; Russell & Moran, 2005), and can also express similar phenotypic effects as in their original hosts, that way having important effects on their novel hosts' biology (Vorburger et al., 2010). Thus, the facultative symbionts in insect communities can be viewed as a horizontal gene pool from which populations adapting to novel environmental challenges may be able to recruit beneficial genes (Ferrari & Vavre, 2011; Oliver et al., 2010). It is clear that such horizontal transfer of symbionts and their genes encoding ecologically

important traits within insect communities could have important consequences for the evolution of species. However, there are virtually no data on the incidence of the horizontal transmission in natural populations of insects. Thus, data on the diversity, distribution and roles of facultative symbionts in *S. avenae*, compared against the data available for *A. pisum*, could also provide valuable information on the potential of the symbionts for the horizontal transmission between aphid species, and its likely ecological and evolutionary consequences in multi-species communities.

In this thesis, I present a series of experiments designed to unravel the role of facultative endosymbiotic bacteria in the ecology and evolution of the grain aphid, *Sitobion avenae*. I place particular emphasis on the symbionts' ability to transfer horizontally between host clones and species. Much of the reported work has been conducted on a new model study system which I have established, consisting of grain aphids, their facultative endosymbionts and their natural enemies. However, I have also taken advantage of the wealth of resources and information available on pea aphids.

In Chapter 2 of this thesis, I present some of the culturing, molecular and symbiont manipulation techniques which I have developed or adapted for work on the grain aphid system. While much of the data I present in that chapter are based on informal observations and assays, and may be of limited applicability in other systems, it could be a useful reference for other workers setting up new study systems, or seeking to modify or improve the existing ones.

In Chapter 3 I present data on the diversity and distribution of facultative endosymbionts in a population of *S. avenae*, and discuss the results in relation to grain aphid biology and population structure. Small sample sizes made it impossible to draw species-wide conclusions, but the results revealed a high diversity of

facultative endosymbionts and suggested the importance of symbiosis in ecology and evolution of grain aphids, justifying further research on the grain aphid system.

Chapter 4 of this thesis describes the results of a short study testing whether facultative endosymbionts may influence survival of grain aphids following exposure to sub-zero temperatures, or affect life history traits of the survivors.

I then focus on the effects of secondary symbionts on aphid interactions with natural enemies. In Chapter 5, I tested whether strains of the facultative endosymbiont *Hamiltonella defensa* confer resistance to two species of hymenopterous parasitoids in the grain aphid hosts. After transferring endosymbionts between aphid clonal genotypes, I measured the effects of infection on parasitoid mummification and emergence rates, life history traits of the emerging wasps, and on the parasitoid oviposition choice.

In Chapter 6, I test whether the fitness effects of infection with facultative endosymbionts differ between clones originally symbiont-infected and symbiont-free. I measured the effects of infection with the same strains of endosymbionts on aphid fecundity and susceptibility to parasitoids both in the original and in novel hosts, and then developed and tested an additional series of lineages in order to assess whether the observed effects are due to the host or symbiont genotypes.

In my largest project, described in Chapter 7, I studied the potential of aphid facultative endosymbionts for establishing themselves in novel hosts. I introduced seventeen strains of facultative symbionts originating from five aphid species into two genotypes of the grain aphid, both cured from their original infections. I then measured the successful establishment rate of the novel symbionts, their effects on host fecundity, as well as on susceptibility to two categories of natural enemies. I

also compared the fitness consequences of infection with the same set of parasitoid resistance-conferring symbionts in a novel pea aphid and a grain aphid host.

In Chapter 8, I attempted to trace the evolution of the symbiont-conferred parasitoid-resistant phenotype across phylogenies of *Hamiltonella* and its bacteriophage APSE, regarded as the main factor determining the resistant phenotype conferred by the bacterium. For more than 20 APSE-carrying *Hamiltonella* strains I combined the existing and new sequence data for several bacterial and phage loci with the experimental data on phenotypic effects of infection. Then, I discussed the distributions of defensive phenotypes across phylogenetic trees of *Hamiltonella* and its bacteriophage.

In Chapter 9 of this thesis, I attempted to unravel the role of individual facultative endosymbionts in multiple infections in their effects on aphids. I separated the endosymbionts originally co-infecting two pathogen-resistant pea aphid clones by introducing them into five novel host clones of two aphid species, either separately or together, and then measured the effects of infection on aphid resistance to an entomopathogen, as well as on fecundity. Additionally, in order to assess how universal pathogen-resistant phenotype is across symbiont species, I introduced several additional strains into one of the novel pea aphid host clones and tested whether they confer resistance to the fungus.

Finally, in Chapter 10 of this thesis, the General Discussion, I summarize the conclusions of projects described in previous chapters of this thesis, and propose additional research which could further expand our understanding of the role of facultative endosymbiotic bacteria in the ecology and evolution of their aphid hosts.

Chapter 2

Grain aphids, their endosymbionts and parasitoids as a new model study system

Abstract

Most of the data presented in this thesis resulted from my research on the new model system which I had established, focused on the grain aphid *Sitobion avenae*, its facultative endosymbiotic bacteria, and its natural enemies. An important part of my work was to establish this study system, and design efficient ways of working with it. In this chapter I present and discuss some of the culturing, symbiont detection and manipulation and molecular techniques which I developed or adapted for work with grain aphids and their symbionts. These informally presented observations and results could make a useful reference for other workers setting up new study systems, or seeking to modify or improve the existing systems.

Introduction

By the time I started my D.Phil. research, members of my group had developed considerable expertise in working with the pea aphid, *Acyrthosiphon pisum*, but not with other aphid species. Thus, the essential first phase of my research was identifying or designing techniques suitable for working with grain aphids, including culturing them and their parasitoids, distinguishing between aphid clonal genotypes, or identifying facultative endosymbionts and manipulating them, and then optimizing them. Many of the methods were easily adapted from the pea aphid system, for example, molecular techniques for detecting facultative endosymbionts. However, some other methods required more experimenting and fine-tuning.

I believe that during my D.Phil. course I developed a powerful study system suitable for testing a wide range of ecological and evolutionary hypotheses. I feel that some of the techniques which I used for work with grain aphids and their facultative endosymbionts might find their applications in the future. In this chapter I describe the techniques for aphid culturing, for identifying facultative endosymbiont by Gram staining of samples of cornicle secretion, for manipulating endosymbionts in aphids, and for microsatellite genotyping aphid clones using a multiplex microsatellite PCR. More details of particular experimental protocols are presented in other chapters of this thesis.

Aphid culturing

In most previous studies on aphid ecology and evolution, aphid lines used for experiments were kept on plants grown in pots and covered with transparent plastic bags, transparent pots, or modified plastic drinks bottles (e.g., Caillaud et al., 1995; Ferrari et al., 2001; Oliver et al., 2003; von Burg et al., 2008; see also

http://insected.arizona.edu/gg/resource/default.html). That way, potted plants continue growing while being fed on by the aphids. However, within approximately three weeks at 14°C the numbers of aphids are becoming too high for the plants to support, and thus some of the aphids have to be transferred to fresh plants before the colony crashes. However, while transferring aphids from these large cultures it is difficult to contain them within old pots and prevent them from spreading around the workspace, with a risk of contaminating other cultures. Also, transferring large number of aphid clones between bottle cages fixed on pots with plants is very labour-intensive. In response to this, a post-doc in our lab, Dr Margriet van Asch, started keeping pea aphids in non-vented Petri dishes on individual bean leaves with their petioles inserted into agar, which kept them fresh for approximately a week at 14°C. This technique of keeping stock cultures, which may have been previously used by other groups, required more frequent transfers but much less effort associated with each transfer. It also considerably reduced the risk of contamination. Due to its convenience, the method has been widely adopted in our lab (e.g., McLean et al., 2011).

I found Petri dishes even more suitable for culturing grain aphids, for several reasons. First, hundreds of wheat plants sufficient for culturing large number of clones can be grown in only a few pots, greatly reducing the amount of time and effort required for planting enough wheat for culture of the whole large stock. Also, the plants were becoming usable in as little as a week from planting (when grown in a greenhouse during summer time), which greatly facilitated planning the experimental work. Second, at 14°C wheat plants with their stems inserted into agar typically remain suitable for aphids for at least 14 days, twice as long as bean leaves, halving the effort associated with culturing. Grasses such as cocksfoot, *Dactylis glomerata*, which I found to be a suitable food plant for all of my grain aphid clones,

under such conditions remained fresh for even longer, typically for at least three weeks. Finally, Petri dishes allowed me to easily assess the condition of each individual culture, and react accordingly if needed, for example in case of pathogen infection. These advantages, combined with the ease of making large numbers of highly uniform dishes, persuaded me to use this way of keeping aphids also during experiments.

Initially, I kept stock culture aphids in 90-mm non-vented Petri dishes (to begin with manufactured by Sterilin, later by VWR), with two approximately 10-days-old (12-15 cm high) wheat plants in each dish. Later, for stock cultures I started using Petri dishes with one or two wheat plants and additionally a single four-week-old (approx. 10 cm high) cocksfoot plant. As stated previously, this modification was justified by the aphids preferentially feeding on wheat but surviving on both plant species, while the fact that cocksfoot was suitable to them for longer assured colony survival for at least 18 days after a transfer. Regardless of the type of dishes, the procedure for making them was the same:

1. Add sufficient amount of agar powder to boiling water to make a 2% solution, boil it again to dissolve the powder.

2. Pour 8-10 ml of agar gel into a 90-mm non-vented Petri dish, placed at an angle - so that gel collects in one place, next to a wall.

3. Wait until agar cools down, but not too long because once it becomes solid, it is difficult to insert plants. Typically, I waited until the gel stopped flowing when a dish was moved.

4. In the meantime, cut stems of wheat/cocksfoot plants just above the ground. Rinse off any soil. Keep the stems immersed in water when waiting for agar to cool down.

5. Insert plants into agar. Then, wait for the agar to solidify and cool down - but not longer than 15-20 minutes, as after that time the plants start wilting

6. Wrap leaves inside a dish. Close the dish with a lid.

Typically, in one hour I was able to make approximately 60 dishes. Transferring stock or experimental aphids from old to fresh dishes also took approximately one hour per 60 dishes. When transferring aphids, I typically used four or five fine (size '00') paintbrushes; I used a different paintbrush for transferring aphids from each culture, sterilizing it in 70% ethanol between uses. This procedure reduced the risk of transmitting pathogens, but also transferring young nymphs entangled in paintbrush hairs, between cultures of different clones. In controlled temperature rooms, I stored the dishes in piles of four, as I found that in larger piles not enough light reaches the bottom dishes, negatively affecting the condition of aphids.

The dishes which I was using for building up aphid numbers prior to experiments, or for keeping aphids following exposures to parasitoids or pathogens, were made the same way, with two wheat plants per dish. Dishes used for the fecundity experiments described in Chapters 4, 6, 7 and 9 of this thesis were made with a single plant per dish. At the experimental temperature of 20°C, wheat plants in the dishes remained fresh for at least seven days. However, in order to offer the best conditions for the aphids, the dishes and plants were generally exchanged every three days.

I also developed and used other culturing techniques. During any extended absences, keeping aphids on wheat growing in 14cm non-vented Petri dishes (VWR)

proved a good alternative to the standard culturing technique described above. Four days before planned transfers, in a handful of compost placed in each dish I was planting four wheat seeds, and then watering them abundantly. After four days at 20-25°C, the wheat sprouts were typically large enough to support aphids. After transferring four or five young nymphs onto the plants I closed the dishes and fixed the lids with Scotch tape. At 14°C, this type of culture would support aphids for a very long time: nearly always the dishes contained healthy aphid populations four weeks from inoculation, and in many cases aphids survived for as long as seven weeks. Preparing dishes was, however, labour-intensive and required advance planning.

I also cultured aphids in constant temperature rooms at 20°C and relative humidity of approximately 70% in 30x30x30cm transparent plastic cages, which fitted up to six pots with plants. The plants needed to be watered twice a week, and replaced every two or three weeks, depending on the size of the aphid populations. The technique permitted the simultaneous maintenance of very large numbers of aphids. However, insects cultured under these conditions were typically small and not very fecund, most likely as a consequence of overcrowding, higher age of plants and lower humidity. Despite this, it was a convenient way culturing aphids for stock cultures of parasitoids, which were maintained in identical cages and supplied with aphids every two weeks.

An important point to consider before starting aphid cultures was selecting the plant variety. Cereal varieties differ in their suitability to aphids, but there are also differences between aphid clones in performance on resistant plant varieties (e.g., Caillaud et al., 1995). I found it worth investing time in selecting a variety suitable for the experimental clones, but also displaying favourable growth patterns. It is also

important to identify a supplier that can guarantee supplying seeds of the same variety for the duration of the planned research. I initially used wheat seeds provided by an Italian manufacturer Bavicchi; however, halfway through my research I was provided with a different variety, on which the aphids displayed slower growth and higher mortality. This caused severe disruptions to my work, forcing me to rapidly identify another suitable wheat variety. After comparing aphid performance on plants grown from seeds obtained from a few sources (data not shown), I started using seeds of "an ancient Polish wheat variety" purchased from Brow Farm, Ormskirk, Lancashire, UK (http://www.browfarm.co.uk/online_store/wheatgrass_seed.htm).

Identification of aphid endosymbionts by Gram staining of cornicle secretion

While the first facultative endosymbionts of aphids were detected using microscopy (Buchner, 1965), research on these bacteria was greatly facilitated by advancement in molecular ecology techniques. Chen and colleagues (1996) were probably the first to sequence a gene of a facultative endosymbiont of an aphid, and all following studies on the ecology and evolution of these bacteria have relied on molecular techniques for their detection and identification. Of these, diagnostic polymerase chain reaction using primers specific for 16S and 23S ribosomal RNA genes of the bacteria, combined with sequencing of the product, has been the most commonly used (e.g., Chen et al., 1996; Ferrari et al., accepted; Fukatsu et al., 2001; Sandstrom et al., 2001; Tsuchida et al., 2010). This is also the approach which I adopted when setting up my study system. For detecting and identifying facultative endosymbionts of aphids I was usually extracting DNA from single insects with DNeasy kit (Qiagen) following the manufacturer's protocol, and then using PCR primers and reaction conditions similar to those used in other projects in our

laboratory (Ferrari et al., accepted; McLean et al., 2011) or described by other authors (Tsuchida et al., 2010). The identity of the symbionts was confirmed with sequencing of the PCR product cleaned following ExoSAP-IT protocol (USB), using Big Dye Terminator v. 3.1 reaction mix. However, I also developed and successfully used a very different method of assessing endosymbiont infection status of live aphids, based on Gram staining of aphid cornicle secretion.

Disturbed aphids of many species produce droplets of secretion from their cornicles (or siphunculi) (Mondor & Messing, 2007; Xiangyu et al., 2002), which harden rapidly upon contact with rough surfaces such as parasitoid cuticle (Edwards, 1966; Wynn & Boudreaux, 1972). While this can disrupt, and in rare cases even kill the parasitoid, the direct fitness benefits to an individual producing cornicle secretion have been disputed (Dixon, 1958; Edwards, 1966; Wu et al., 2010). However, such individual can benefit indirectly by reducing predation risk in nearby clonal kin which is warned by the aphid alarm pheromone released together with droplets of cornicle secretion (Mondor & Messing, 2007; Montgomery & Nault, 1977; Xiangyu et al., 2002). At the same time, the costs associated with production of the secretion are not large, even though age-dependent (Mondor & Roitberg, 2003). Cornicle secretion consists of liquid lipids suspended in haemolymph (Edwards 1966), which is known to contain secondary symbionts (Chen & Purcell, 1997; Darby & Douglas, 2003; Oliver et al., 2003). Indeed, Darby and Douglas (2003) used diagnostic PCR to detect *Hamiltonella* in all five samples of cornicle secretion collected from infected pea aphids.

A traditional method of visualizing bacteria in liquid or semi-liquid media has been Gram staining (Beveridge, 2001), previously used with success for visualizing facultative endosymbionts in pea aphid haemolymph (Scarborough, 2006). I tested

whether Gram staining of cornicle secretion of grain aphids can be used as a reliable method of identifying endosymbiont infection status of live aphids.

I found that grain aphids feeding on wheat plants often produce cornicle secretion when they are prodded gently on the abdomen with a glass needle. I succeeded in increasing the rate of cornicle secretion production by using dead parasitoids for inducing aphid defensive responses: tapping grain aphids with the antennae of freeze-killed wasps held by their wings resulted in the production of secretion in the majority of cases, regardless of aphid genotype or age. Pea aphids were less likely to produce secretion while continuing feeding, but also frequently they started rolling immediately after being touched, thus making the collection of haemolymph more difficult. However, with some experience, I succeeded in holding juvenile pea aphids in place and collecting the secretion which they then produced. Because cornicle secretion hardens rapidly upon contact with rough surfaces, I started collecting the secretion with glass needles which had their tips melted into balls. Using such a tool, in most cases, I was able to transfer a sufficient volume of secretion to a microscope slide and smear it in a previously designated space before it solidified. Then, I stained slides with 10-12 secretion samples on each following a protocol similar to the general method of Gram staining described by Beveridge (2001). I used Gram's crystal violet solution, Gram's iodine solution and Gram's safranine solution, all manufactured by Sigma-Aldrich. The stained slides were observed using immersion oil under bright field microscope at a magnification of 1300x.

Bacteria were typically found only in a small part of the area originally smeared with cornicle secretion, but were usually numerous. *Hamiltonella*, *Regiella* and *Buchnera* cells are Gram-negative and thus stain pink (Scarborough, 2006). *Buchnera*, found occasionally in the samples, had large, round cells. Cells of

Hamiltonella strain Ha-3 (as defined in Chapter 3 of this thesis) were small and rod-shaped, with darker spots at both ends of the cell, and very numerous. Cells of other grain aphid *Hamiltonella* strains were much larger and elongated bacilli, but with considerable differences in the length of individual cells within and between samples. Cells of the two grain aphid *Regiella* strains were finer, very elongated, straight bacilli, but those of a single pea aphid *Regiella* strain that I looked at had large and strongly bent cells. I was soon able to identify endosymbiont species and many of the haplotypes based on their morphology. Unfortunately, I was not able to access a high-magnification microscope with attached camera and document the variety of symbiont shapes and sizes.

The method of identifying endosymbiont infections using Gram staining of cornicle secretion was not easy to master. However, once I perfected the method, I found it reliable, convenient and inexpensive. When calibrating the technique on a sample of grain aphid clones of known infection status, I stained 32 randomized cornicle secretion samples collected from 15 clones symbiont-free or infected with one of five symbiont strains. I correctly assessed symbiont presence/absence in 31 samples, and identified 4/4 *Regiella* Re-2 samples, 5/6 *Hamiltonella* Ha-3 samples and 9/9 Ha-4 samples, but failed to distinguish between Ha-1 and Ha-2. The total processing time was 155 minutes, or less than 5 minutes per sample, and the total cost (including glass slides, plastics and chemicals) approximately £1.10, or £0.035 per sample, a hundredth of the cost of assessing infection status using standard molecular techniques. Previously, when testing pea aphids for the first time, I correctly scored the presence/absence of symbionts in 18/21 cornicle secretion samples, and distinguished categories which later proved to correspond to symbiont 16S haplotypes.

I found the method particularly useful when testing and calibrating endosymbiont manipulation techniques, as it allowed me identify the successful manipulations early. Typically, in order to assess symbiont infection using molecular techniques, I waited until isolated offspring of injected or antibiotic-treated aphids reproduces, and only then extracted DNA from individual first-generation offspring and run diagnostic PCRs. Gram staining of cornicle secretion allowed me to test offspring of the treated aphids when they were 2nd or 3rd instar, and further process only the ones of the required infection status. I later confirmed the results of Gram staining using diagnostic PCRs, and found that my identification of symbiont presence in aphids being infected was correct in 100% cases, and of symbiont absence in aphids being cured in approximately 70% cases, likely as a consequence of a drop in symbiont titre in offspring of antibiotic-treated aphids. Using Gram staining saved much time, as well as costs and effort of testing dozens of additional aphids using molecular techniques. However, later I found the method less useful, largely due to my very high success rate when introducing novel symbionts into aphids (Chapter 7 of this thesis).

While I found Gram staining of cornicle secretion a useful technique and benefited from having developed it, the method is not likely to become widely adopted, for several reasons. First, the costs and effort associated with using molecular techniques are dropping rapidly, reducing the advantage of screening aphids using alternative techniques. Secondly, mastering the technique takes considerable time, effort and patience, and to make it efficient a high degree of precision is required. Many of the workers potentially interested in using the technique would not have the necessary patience, precision and enthusiasm, or will not be able to invest time in learning it. Thirdly, it could be difficult to use the technique on a larger scale due to limitations in the amount of time which can be

spent performing high-precision tasks and staring into a microscope on a single day. However, the method may well find its smaller-scale applications.

Manipulating endosymbionts in aphid clones

From a symbiosis researcher's perspective, one of the major advantages of working on an aphid-endosymbiont system is the opportunity to measure the consequences of manipulating endosymbionts in controlled host genetic background. This is possible thanks to the reproductive biology of aphids, which typically reproduce parthenogenetically under long-day conditions, and only induce the sexual generation in response to decreasing temperatures and shortening photoperiod (Moran, 1992; Simon et al., 2002). In most cases, during the asexual part of the life cycle aphids transmit their facultative endosymbionts to offspring with near-complete fidelity (Chen & Purcell, 1997; Darby & Douglas, 2003; Russell & Moran, 2005). Therefore, manipulating bacteria in a pre-reproductive aphid often results in it producing some offspring of the novel infection status, which can be used for starting the manipulated lines.

Other researchers studying aphid symbioses have taken two distinct approaches to the problem of manipulating endosymbionts in aphids. These were 1) introducing bacteria into clones free from infection with facultative symbionts; and 2) removing facultative endosymbionts by treating naturally infected clones with mixtures of antibiotics specifically targeting facultative endosymbionts without harming primary symbionts. Chen and Purcell (1997) were the first to describe the procedure of microinjection of symbiont-containing haemolymph into non-infected hosts. The same approach was later successfully employed by Oliver and colleagues (2003), and in a series of consecutive studies in Nancy Moran's group at the University of Arizona (Oliver et al., 2009; Oliver et al., 2005, 2006; Russell & Moran, 2005,

2006), but also Charles Godfray's group at Imperial College (Ferrari et al., 2007; Scarborough et al., 2005), and in a series of studies in Japan (Fukatsu et al., 2001; Koga et al., 2003; Sakurai et al., 2005; Tsuchida et al., 2004; Tsuchida et al., 2010). Darby and Douglas (2003) successfully infected aphids by feeding them on artificial diets containing previously isolated cells of facultative endosymbionts. Symbiont elimination by antibiotic treatment was an alternative approach, first described by Koga and colleagues (2003), and subsequently used by several other authors (Douglas et al., 2006; Koga et al., 2007; Leonardo, 2004; McLean et al., 2011; Tsuchida et al., 2004). These authors were administering antibiotics to aphids either by injection, through artificial diets, or through plants with their stems immersed in antibiotic solutions.

During my research, I tried all these different approaches and techniques of manipulating symbiotic bacteria. The technique I used for infecting aphids by microinjection of haemolymph was loosely based on the protocols described previously (Chen & Purcell, 1997; Oliver et al., 2003). Fourth- or fifth-instar donor aphids were mounted upside down on sticky tape, and had a part of a leg removed. Clear droplets of haemolymph oozing from the wound were collected with a fine glass needle, previously pulled with Narishige PN-30 magnetic puller from a Harvard Apparatus borosilicate glass capillary (product GC120TF-10), and connected with a thin rubber tube to a 20ml syringe. Haemolymph collected from one or two donors was injected into the body cavities of a few first-instar recipient aphids, turned upside down and held in place with a paintbrush. I did not attempt to quantify volumes of haemolymph injected, but it was usually possible to see the injected aphids swell during the injection. Following injections, and every three days afterwards, the aphids were transferred in groups of approximately six individuals into Petri dishes with fresh plants, which were kept at the temperature of 20 degrees.

Fourteen days after injections, the survivors were isolated in individual dishes, and some of the first generation (F1) offspring they produced were retained, and kept under the same conditions in Petri dishes. Once the F1 aphids reached adulthood, they were in turn isolated, allowed to reproduce, and then tested for the presence of facultative endosymbionts using diagnostic PCRs. Second generation (F2) offspring produced by symbiont-positive F1 mothers were used for starting the experimental lines, which were tested at least twice more in consecutive generations prior to use in experiments. As discussed previously, assessing symbiont infection status using Gram staining of cornicle secretion expedited the process of assessing the success rate of injections, as the F1 aphids could be assessed for infection status when they were at the second or third instar stage, with only the ones testing positive being processed further.

The injection technique required time to master, and initially the survival rate of the injected aphids and the proportion of the survivors producing infected offspring were low. However, my later injections resulted in a 14-day survival rate of the injected aphids, including *S. avenae* or identically treated *A. pisum*, of over 80%, with all or nearly all survivors producing infected offspring (Chapters 7 and 9 of this thesis). This technique for manipulating facultative endosymbiotic bacteria in aphids has proven to be a very powerful tool. The total number of novel aphid host-endosymbiont combinations which I successfully developed by haemolymph microinjection and described in this thesis is 69, rivalling, I believe, the number of such novel symbioses developed and published together by all research groups worldwide.

I found curing aphids considerably more challenging, despite trying a range of concentrations of different antibiotics which were administered in different ways.

Administering antibiotics through artificial diets (Douglas et al., 2006; Hansen, 2006) was time-consuming, survival of aphids feeding on the diets highly variable, and most importantly, administering ampicillin at concentrations of 200 µg or 500 µg per ml of diet did not eliminate endosymbionts in any of the cases. Injections with antibiotics (5% ampicillin, 2% ampicillin, or a mixture of 1.5% ampicillin, 1.5% gentamicin and 1.5% cefotaxime; Koga et al, 2007) either failed to eliminate facultative endosymbionts from offspring of the treated aphids, or eliminated both facultative and primary symbionts, preventing offspring of the treated aphids from reproducing. The method which I found the most useful was administering antibiotics through wheat plants (McLean et al., 2011). Greenhouse-grown, approximately one-week-old wheat plants were cut off, and had their stems inserted into 600µl Eppendorf tubes containing antibiotic solutions. 2% ampicillin solution was used to cure symbionts (*Hamiltonella defensa* and *Regiella insecticola*) from clones Co26 and Co21, respectively, and a mixture of 0.5% ampicillin + 0.7% gentamicin + 0.3% cefotaxime succeeded in eliminating strains of *Hamiltonella* from clones Co08, Co23 and Co37. After four days of feeding on antibiotic plants, the aphids were transferred to Petri dishes, and later treated and tested the same way as following the injections. The method resulted in high mortality of the treated aphids, and only approximately 30% of the survivors tested negative for the symbionts. The actual rate of successful curing was lower, though: in some lineages which I initially (after testing generation F1) identified as successfully cured, facultative bacteria were apparently not completely eliminated but rather had their densities reduced, and they tested positive three generations later. In each of the five clones listed above the successful elimination of the facultative endosymbionts was confirmed several times afterwards, though. There is, however, definitely space for further improvement of the curing protocol. Particularly, as it was shown in another project conducted in our

lab (McLean et al., 2011), administering antibiotics through plants does not typically affect infections with non-gammaproteobacterial symbionts *Rickettsia* and *Spiroplasma*.

Microsatellite genotyping of grain aphid clones

Microsatellite genotyping has become one of the most powerful tools in population biology (Jarne & Lagoda, 1996). It has also proved very useful in research on the biology of aphids (Foottit et al., 2008), including the grain aphid. The ecology, evolution and structure of populations of this species have been understood largely thanks to the application of microsatellite markers (Dedryver et al., 2001; Figueroa et al., 2005; Haack et al., 2000; Llewellyn et al., 2003; Llewellyn et al., 2004; Simon et al., 1999; Sunnucks et al., 1997). It was clear that distinguishing between microsatellite genotypes of the grain aphids which I collected in the field was an essential step in my research.

Initially, I tried amplifying four microsatellite loci, S23, S24, S30 (Wilson et al., 2004) and Aph-08M (Caillaud et al., 2004) routinely used in our laboratory for genotyping pea aphids (Ferrari et al., 2008). Locus Aph-08M could not be amplified in *S. avenae*, and therefore I looked for additional published loci. The loci originally isolated from *Sitobion miscanthi*, S16B, S19, S49 and Sm10, were selected based on the results of Wilson and colleagues (2004), who found them highly variable in that species. The primers were then ordered, with one of the four fluorescent labels, 6-FAM, NED, PET or VIC, attached. I successfully amplified all loci in my *S. avenae* clones in a series of simplex reactions using cycling conditions as described by Wilson et al. (2004), followed by mixing of the products with different labels. All loci were variable, and proved useful in distinguishing between my experimental clones; the summary of these loci is presented in Table 1 in Chapter 3. However,

setting up seven separate PCR reactions was time-consuming, and thus I developed and optimized multiplex reactions. The final protocols allowed for testing all seven microsatellite loci in two reactions, saving much time and considerably reducing costs associated with genotyping compared to seven simplex reactions. Throughout my research project, I used the first of these multiplex reactions for genotyping my grain aphid clones. I found it equally useful for typing pea aphid clones.

Reaction 1 - loci S23+S24+S30+S16B. For each sample, use:

BioMix - 12.5 µl (pre-mixed *Taq* polymerase, dNTPs and buffer; Bioline Ltd.)

S23 - primers F and R: 0.2 µl + 0.2 µl (primer concentrations 20 pmol/µl)

S24 - primers F and R: 0.4 µl + 0.4 µl

S30 - primers F and R: 0.2 µl + 0.2 µl

S16B - primers F and R: 0.5 µl + 0.5 µl

dd H20 - 8.9 µl

DNA - 1.0 µl

Reaction 2 - loci S19+S49+Sm10+S16B. For each sample, use:

BioMix - 12.5 µl

S19 - primers F and R: 0.6 µl + 0.6 µl (primer concentrations 20 pmol/µl)

S49 - primers F and R: 0.3 µl + 0.3 µl

Sm10 - primers F and R: 0.3 µl + 0.3 µl

S16B - primers F and R: 0.3 µl + 0.3 µl

dd H20 - 7.5 µl

DNA - 1.0 µl

For both reactions, PCR cycling conditions were as follows: initial denaturation 94°C 2 mins, followed by one cycle of 62°C 30 sec, 72°C 45 sec, 94°C 15 sec; one cycle of 61°C 30 sec, 72°C 45 sec, 94°C 15 sec; one cycle of 59°C 30 sec, 72°C 45

sec, 94°C 15 sec; one cycle of 57°C 30 sec, 72°C 45 sec, 94°C 15 sec; 30 cycles of 55°C 30 sec, 72°C 45 sec, 94°C 15 sec; final extension 72 °C for 2 mins (Program PMS1 - Wilson et al., 2004).

The main problem associated with using this set of primers for genotyping grain aphids was that it did not allow me to directly relate to the results of other workers who had studied grain aphid populations in the UK, France, Denmark and Chile (Dedryver et al., 2001; Figueroa et al., 2005; Haack et al., 2000; Jensen et al., 2008; Llewellyn et al., 2003; Llewellyn et al., 2004; Simon et al., 1999; Sunnucks et al., 1997). Generally, these authors used different sets of primers.

Conclusions

The data presented in the consecutive chapters of this thesis demonstrate that the grain aphid study system can be successfully used for testing a range of important hypotheses regarding the ecological and evolutionary roles of the facultative endosymbiotic bacteria. I have no doubts that the techniques presented in this chapter have considerably facilitated my research on this system, and I would recommend them to other workers researching the ecology and evolution of grain aphids. Some of these methods may not be suitable for other study systems, or impractical for implementation on a larger scale, though. Despite this, I feel that their description could be a useful reference for other workers setting up new study systems, or seeking to modify or improve the existing systems.

Chapter 3

Diversity of facultative endosymbionts in a population of the grain aphid

Abstract

Aphids are known to harbour a wide range of facultative endosymbiotic bacteria, but there are important differences in the diversity and frequency of infections amongst species, host plant races and geographic locations. However, research to date has largely focused on a single species, the pea aphid, and data on the distributions of facultative endosymbionts in other aphid species is scarce.

In this study, we assessed the diversity of facultative endosymbionts in a population of the grain aphid, *Sitobion avenae*, from Oxfordshire, UK. Aphids collected from three plant species at two sites and representing 22 multilocus genotypes harboured four distinct 16S haplotypes of the symbiont *Hamiltonella defensa*, three haplotypes of *Regiella insecticola*, as well as *Serratia symbiotica*. Several of the aphid genotypes were represented by multiple insects collected from more than one host plant species, and in most of these cases, there were differences in endosymbiont infection status within genotypes. Our data indicate a high diversity of endosymbionts in grain aphids compared to the pea aphid populations, and also the possibility of horizontal transsmission of facultative endosymbionts between clonal genotypes, with a potential for symbiont-mediated adaptation to host plants in obligatorily asexual clones.

Introduction

Insects harbour a diversity of endosymbiotic bacteria, which can have a range of important effects on the life history traits of their hosts (Moran et al., 2008). Aphids (Hemiptera: Aphididae) are one of the groups in which the roles of endosymbionts have been studied the most extensively (Oliver et al., 2010). Virtually all aphids harbour a primary endosymbiont, *Buchnera aphidicola*, which resides in specialized cells surrounding insect gut, synthesises essential amino acids and nutrients deficient in aphid diet, and is thus essential for aphid growth and reproduction (Baumann, 2005; Douglas, 1998; Moran et al., 2008). *Buchnera* has been transferred strictly vertically and co-diversified with its hosts for at least 100 million years, since it established in a common ancestor of aphids (Moran et al., 1993; Moran et al., 1995). The second category of endosymbiotic bacteria are secondary, or facultative, endosymbionts. They are transferred maternally with high fidelity (Chen & Purcell, 1997; Darby & Douglas, 2003; Oliver et al., 2009), but are patchily distributed across aphid clones and species, and phylogenetic analyses and experimental symbiont transfers revealed that they are capable of horizontal transmission between unrelated hosts (Burke et al., 2009; Oliver et al., 2010; Russell et al., 2003; Sandstrom et al., 2001). Facultative endosymbionts are not essential for aphids, but may confer important fitness benefits under certain environmental conditions, including protection against parasitoids (Oliver et al., 2005; Oliver et al., 2003; Vorburger et al., 2010), pathogens (Scarborough et al., 2005) and heat (Chen et al., 2000; Montllor et al., 2002; Russell & Moran, 2006), or improved performance on certain host plants (Tsuchida et al., 2004; but see McLean et al., 2011).

Most of the effects listed above have been studied in the pea aphid *Acyrthosiphon pisum*, a model insect species (Brisson & Stern, 2006), in which the distribution and

diversity of facultative endosymbionts have also been extensively studied. Not less than seven species of facultative endosymbionts have been reported from pea aphids (Oliver et al., 2010; Tsuchida et al., 2010), and there seem to be important differences in the type and frequency of infections across host plant races and geographical regions. For example, infections with *Regiella insecticola* are very common in aphids specialized on clover (*Trifolium spp.*) but sporadic in other aphid host plant races, and more frequent in colder, drier parts of Japan (Ferrari et al., 2004; Ferrari et al., accepted; Frantz et al., 2009; Tsuchida et al., 2002). Much less information is available on the diversity of facultative endosymbionts in other aphid species. Four species (including *A. pisum*) collected at a single site in England differed considerably in incidence of infections with *Hamiltonella defensa*, *Regiella insecticola*, *Serratia symbiotica* and *Rickettsia*, and in the average number of endosymbiont species per host (Haynes et al., 2003). 24 clonal genotypes of black bean aphid collected from different host plants at a range of sites in Switzerland harboured either *Hamiltonella defensa, Regiella insecticola*, or no gammaproteobacterial secondary symbionts (Vorburger et al., 2009), and a single case of infection with *Regiella* was reported by von Burg et al. (2008) among 17 Australian *Myzus persicae* clones. In the Chinese populations of the grain aphid *Sitobion miscanthi*, two endosymbiont species unknown from pea aphid were reported (Li et al., 2011; Wang et al., 2009), but these populations were not assessed for infection with other known aphid symbionts. Studies which scanned large numbers of species have often only looked at a single individual per species (Burke et al., 2009; Russell et al., 2003). Thus, while existing data clearly demonstrate the diversity of endosymbionts in Aphididae and the differences between aphid species, limited information is available on the genetic diversity of endosymbionts and its associations with aphid population structure beyond the pea aphid system. Such data

is essential for understanding the ecology of facultative symbionts, and particularly their potential for horizontal transmission, as well as their role in aphid evolution.

Grain aphid, *Sitobion avenae* Fabricius 1775*,* is a serious pest of cereals worldwide, and its economic importance has led to a large number of studies on the ecology, biotic interactions and population structure of this species (e.g., Kröber & Carl, 1991; Markkula & Roukka, 1972; Vickerman & Wratten, 1979). Grain aphids employ a range of reproductive strategies (Simon et al., 2002). Holocycylic genotypes reproduce parthenogenetically during spring and summer and have a single sexual generation, producing overwintering eggs, in autumn. Anholocyclic genotypes reproduce clonally throughout the year, and overwinter as active stages on vegetation. In addition to these two strategies, intermediate genotypes, overwintering as active stages but also capable of producing sexual forms, have been reported (Dedryver et al., 2001; Simon et al., 2002; Wilson et al., 2003). There are important geographical differences in the frequency of these strategies: in regions with mild winters anholocyclic clones make up a significant proportion of populations, but in regions experiencing low sub-zero temperatures populations are primarily composed of sexual genotypes capable of producing frost-hardy eggs. Such gradients in the incidence of sexual reproduction have been reported from continental Europe (Papura et al., 2003) and France in particular (Dedryver et al., 2001; Simon et al., 1999), as well as from Britain (Llewellyn et al., 2003; Newton & Dixon, 1988a). In contrast, in Chile populations of *S. avenae* are largely composed of only a few asexual genotypes, some of which are widespread geographically, distributed relatively evenly across host plants, and common across years (Figueroa et al., 2005).

Grain aphids feed on a wide range of species from the grass family, Poaceae, including most cereals and temperate pasture grasses (Blackman & Eastop, 2000),

but their performance can vary across plant species and varieties (e.g., Caillaud et al., 1995; Di Pietro et al., 1998; Markkula & Roukka, 1972; Migui & Lamb, 2003). There are differing reports about the degree of specialisation of grain aphids to feeding on particular host plants. English *S. avenae* clones collected from wheat (*Triticum sp.*) and cocksfoot (*Dactylis glomerata*) were shown to form three distinct groups with high levels of recombination within each: wheat-specific, cocksfoot-specific, and 'typical *S. avenae*', the least specialized (DeBarro et al., 1995b; Sunnucks et al., 1997). Similarly, grain aphid clones collected from cultivated and wild grasses in France formed three distinct tribes, with genetic divergence between populations feeding on plants from tribe Triticeae (incorporating barley, wheat and triticale) and Poaeae (perennial grasses, including *Dactylis*, *Agrostis* and *Holcus*) falling within the range usually encountered for host races of phytophagous insects (Vialatte et al., 2005). Oats (tribe Aveneae) occupied an intermediate position, and were suggested as favourable hosts also for other host races of *S. avenae*. However, local movement of clonal genotypes from cocksfoot to wheat was reported during the year (DeBarro et al., 1995a). Also, common anholocyclic genotypes making up significant proportions of populations were sampled from across host plant species both in France and in Chile (Figueroa et al., 2005; Haack et al., 2000), and were thought to be generalists.

Despite a considerable amount of information being available on various aspects of grain aphid biology, there is no published data on the facultative endosymbiotic bacteria that they harbour. Considering the importance of secondary symbionts in the biology of pea aphids, but also the large differences in frequency of infections across aphid populations and species, studying microbial associates of grain aphids would clearly contribute to understanding their ecology and evolution, with potential applications in pest control. Furthermore, data on the diversity of facultative

endosymbionts in a further aphid species whose ecology is well understood would provide valuable information on whether the effects reported from pea aphids are representative for other species.

In this chapter, we present the results of screening of a population of the grain aphid for facultative endosymbionts. We provide data on the diversity of endosymbiont haplotypes across aphid microsatellite genotypes collected from wheat on two neighbouring fields, but also from two other host plant species in the same and an additional site.

Material and methods

Aphids were collected in June 2008 at two sites in Oxfordshire. The primary sampling location was Colleymore Farm, an organic farm near Coleshill, where on 24th and 25th June from two nearby fields we collected 38 aphids from wheat and five aphids from cocksfoot. Previously, on 15th June, five aphids were collected from oat (*Avena sativa*) and two aphids from cocksfoot near the village of Lower Radley, approximately 30 km east from the first site. In all cases, aphids were collected from flowering plants not less than 5m apart, in order to reduce the risk of sampling the same genotype multiple times. Usually, we collected single reproducing wingless adults, but occasionally third- or fourth-instar nymphs were collected. Single parthenogenetic females were allowed to reproduce in Petri dishes on young wheat plants which were kept fresh by having their stems immersed in 2% agar. Afterwards, they were used for DNA extractions, while their offspring was used to start clonal lines. Females collected at both sites which were killed by parasitoids or entomopathogenic fungi before they reproduced were not included in the counts given above.

DNA from field-collected adult aphids used to establish laboratory clonal lines was extracted with DNeasy Blood & Tissue Kit (Qiagen). All aphid samples were typed at seven microsatellite loci – S16B, S10, S23, S24, S30, S49 and Sm10 (Simon et al., 1999; Wilson et al., 2004). Then, the samples were screened for the known aphid secondary endosymbionts, including *Hamiltonella defensa*, *Regiella insecticola*, *Serratia symbiotica*, X-type, *Rickettsia*, *Spiroplasma* and *Rickettsiella*, with a series of polymerase chain reactions (PCR) using symbiont-specific primers for the ribosomal 16S gene (McLean et al., 2011; Tsuchida et al., 2010). The PCR products were sequenced from both ends using Big Dye Terminator v 3.1 sequencing mix (Applied Biosystems), and the sequences assembled and edited with Codon Code v. 2.06 were compared against each other, as well as against reference sequences of the identified symbionts (Moran et al., 2005b). Also, for 26 aphids representing most genotypes we amplified and sequenced a part of the cytochrome c oxidase I (COI) gene, a genomic region that can be used to confirm the identity of the aphid species, using primers LepF and LepR (Foottit et al., 2008).

Many of the clonal lineages started from field-collected females were maintained in culture for several generations, in some cases as much as three years, and re-tested, often more than once, for the presence of facultative endosymbionts. These additional data provided information on the stability of the infections.

Results

COI sequences of all tested aphids were at least 99.4% identical to each other and to the reference sequence of *S. avenae*, confirming our identification based on morphological features (Blackman & Eastop, 2000; Foottit et al., 2008). In all 50 field-collected aphids we successfully amplified the full set of seven microsatellite loci (Table 1), which permitted identification of 22 distinct microsatellite genotypes

(Table 2). 14 of these genotypes were represented by single insects, others by multiple individuals. All genotypes found in Radley were present also on Colleymore Farm. 20 of the genotypes were found on wheat. Out of 12 aphids collected from oat and cocksfoot, 11 belonged to genotypes found on at least 2 host plant species, and aphids representing the most common genotype Co21 were found on the three plant species (Table 3).

Grain aphids were infected with a diversity of facultative endosymbionts. 13 of the field-collected aphids harboured no facultative endosymbiotic bacteria, 23 were infected with *Hamiltonella defensa*, 17 with *Regiella insecticola*, and 3 with *Serratia* species. Some multiple infections were detected. Sequencing of 16S rRNA gene revealed three distinct haplotypes of *Regiella* and four haplotypes of *Hamiltonella*, all at least 99% identical to the reference sequences of the two symbiont species (Moran et al., 2005b) (Table 4). Sequences of *Serratia* in two of the samples were identical to each other, and 99.7% identical to the reference sequence of *Serratia symbiotica* (Moran et al., 2005b), but the third sequence was 4.3% divergent, and identical to the sequence of *S. proteamaculans*, a bacterium found in a range of habitats, including arthropod guts (Kwak et al., 2006). Notably, five out of six aphid microsatellite genotypes which were represented by aphids collected from more than one species of plant differed in endosymbiont infection status (Tables 2 and 3).

Our re-testing of aphid lineages retained in culture revealed that the natural single infections were stable: we have not recorded a single case of loss of any of the symbionts, in several cases for over 30 months. In contrast, triple infections in aphids collected from oat were unstable during culturing on wheat. By the sixth generations after collection the lineages established from both oat-collected triple-infected aphids had lost *Serratia*, and within a year, only *Hamiltonella* remained in a lineage of clone

Co39, and only *Regiella* in a lineage of clone Co21. Unfortunately, the loss of symbionts from these multiple infections was not assessed in a more systematic manner.

Discussion

We have presented data on seven-locus microsatellite profiles and facultative endosymbiont infection statuses for 50 grain aphids collected from three plant species at two sites in Oxfordshire. Most of the microsatellite loci studied had high allelic diversity (Table 1), and were useful in distinguishing between aphid clones. It was suggested that four or five moderately diverse loci are sufficient for distinguishing between multilocus genotypes in grain aphid populations (Haack et al., 2000; Wilson et al., 1999), and indeed, any four of the assessed loci would have sufficed to distinguish between the 22 genotypes reported in this study. However, as discussed in Chapter 2 of this thesis, the seven microsatellite loci used in this study were selected based on their immediate availability in our laboratory and allelic diversity as reported by Wilson et al. (2004), but apart from locus Sm10 were different from loci typed by other authors who studied grain aphid population structure in Britain and in France (Haack et al., 2000; Llewellyn et al., 2003; Llewellyn et al., 2004; Simon et al., 1999; Sunnucks et al., 1997; Vialatte et al., 2005). Therefore, our results cannot be directly compared with theirs.

Because *S. avenae* reproduces asexually for at least a part of the year, individuals with the same multilocus genotype across seven variable loci are almost certain to have a common asexual ancestor, as the probability of the same genotype arising *de novo* is very low (Sunnucks et al., 1997). In Southern England, populations of *S. avenae* are believed to be largely anholocyclic, as demonstrated by Newton and Dixon (1988b) who were unable to induce sexual reproduction in English aphids.

This is further confirmed by numerous genotypes being represented across samples collected in different years at different sites (Llewellyn et al., 2003; Llewellyn et al., 2004). In agreement with this work, we detected aphids sharing multilocus genotypes at two sites separated by a distance of 30 km. Aphid genotypes collected in replicates on a single field may well have descended from a single fundatrix which hatched from a sexually-produced egg the same spring. However, it is unlikely that aphids collected from two sites 30 kilometers apart, and found feeding on different plant species (Lushai et al., 1997), represented a genotype which had only arisen a few generations earlier. Thus, they should be regarded as representatives of anholocyclic clones overwintering as active stages. Interestingly, genotype Co21, the most common in our sample, had the same rare alleles at microsatellite locus Sm10 as the second most common clone in suction trap samples and in field collections in Britain in 1997-1998, which had also been found in France (Llewellyn et al., 2003; Llewellyn et al., 2004; Simon et al., 1999). These data indicate the possibility of a long history and considerable ecological success of this clone since the last incidence of sexual reproduction. However, no confirmation of the identity of the clone can be made based on data for a single locus.

Among the 50 field-collected aphids I detected four distinct 16S haplotypes of *Hamiltonella*, three haplotypes of *Regiella*, and one of *Serratia symbiotica*. Each of these haplotypes was detected in at least two aphid genotypes (including *Hamiltonella* Ha-2, found in an aphid collected at a different site - P. Łukasik, unpublished data). These results demonstrate a surprising diversity of endosymbionts in grain aphid populations, revealed despite a relatively low sampling intensity. In comparison, in 297 pea aphid clones belonging to eight divergent host plant races and collected in two countries, five 16S haplotypes of *Hamiltonella*, three haplotypes of *Regiella* and one haplotype of *Serratia* were detected, usually associated with

particular host plant races (Ferrari et al., accepted). Only single haplotypes of *Hamiltonella* and *Regiella* were reported from 24 microsatellite genotypes of *Aphis fabae* collected from a range of host plants (Vorburger et al., 2009). The reasons for the diversity of endosymbionts in the grain aphid population merit attention.

Possibly the most interesting finding of this study is the diversity of endosymbionts within microsatellite genotypes of their hosts (Table 3). In five of six aphid genotypes detected on more than one host plant species, at least one of the aphids harboured different symbionts than the others. In particular, both cases of triple infections and all three cases of infection with *Serratia* were reported from aphids collected from oat, despite their low number. The triple infections were not stable during culturing on wheat, suggesting strong competition for host resources between endosymbionts in multiple infections under these conditions. We lack verification that *Serratia*, particularly *S. proteamaculans*, is a vertically transmitted endosymbiotic mutualist in *S. avenae*. However, these data appear to indicate that long-established asexual lineages may acquire novel strains of facultative endosymbionts, and suggest that these bacteria may be influencing host plant specialisation (Leonardo, 2004; McLean et al., 2011; Tsuchida et al., 2004). This study may be the first indication of the ability of anholocyclic clones to adapt to local conditions through acquisition of facultative endosymbionts. Previous authors failed to demonstrate spontaneous transmission of facultative endosymbionts between aphid clones (Chen et al., 2000; Darby & Douglas, 2003; Oliver et al., 2008), but the numbers of aphids which can be assessed during laboratory experiments are small compared to the numbers of individuals in widespread anholocyclic clones in natural aphid populations (Figueroa et al., 2005; Haack et al., 2000; Llewellyn et al., 2004). Even if successful horizontal transfers of facultative endosymbionts are very rare, they could be expected to happen occasionally in such widespread genotypes. Then,

if a newly established bacterium provides net fitness benefits to an already successful host genotype in a part of the ecological niche it occupies, the prevalence of the infection in the host population could well increase. Potentially, it could lead to symbiont-mediated adaptation to local conditions (Loxdale, 2008; Wilson et al., 2003) in an asexual clone. Such processes could explain the presence of *Serratia* in grain aphids we collected from oat. On a larger scale, the reason why certain anholocyclic aphid clones are so common across habitats and host plants (Figueroa et al., 2005; Haack et al., 2000) could be their harbouring of different symbiont complements in different environments. A comprehensive assessment of endosymbiont infections in widespread asexual clones, followed by experimental studies, would be necessary to understand the potential of symbionts to mediate local adaptation in anholocyclic aphid clones.

An alternative explanation for the diversity of endosymbionts within our clones could be that the most recent incidences of sexual reproduction has resulted in clones unstably multiply infected with maternal and paternal symbionts (Moran & Dunbar, 2006), and that different bacteria were lost in different matrilines established from offspring produced by the same multiply infected fundatrix. However, such processes are unlikely to explain all reported cases.

The work presented in this chapter has several limitations as a population-wide assessment of aphid endosymbionts, which necessarily affect the conclusions. Most importantly, numbers of aphids collected from cocksfoot and oat were low, and did not allow for a formal comparison between wheat and these two plant species. A sufficiently large aphid sample was collected from only a single area, which makes it possible that the observed effects are population-specific. Comparisons between aphids collected from oat and from two other plant species are further complicated

by the geographical distance between the sampling sites, confounding the effect of host plant with the effect of location. Also, the microsatellite loci analyzed in this study, while suitable for typing grain aphid populations, do not allow for direct comparisons with data collected by other authors who previously studied this species (Llewellyn et al., 2003; Llewellyn et al., 2004; Simon et al., 1999; Sunnucks et al., 1997). Therefore, the data presented in this chapter, while indicating high diversity of facultative endosymbionts in *S. avenae* and the occurrence of horizontal transmission of endosymbionts between anholocyclic aphid clones, and suggesting symbiont-mediated local adaptation, cannot be regarded as a comprehensive assessment of endosymbiont complements across multilocus genotypes or host plants. However, the project fulfilled its original purpose - the identification and collection of grain aphid clones infected with diverse facultative endosymbionts, which could become the subject of a series of experiments regarding the significance of symbioses in *S. avenae*. These experiments are described in the consecutive chapters of this thesis.

Tables and Figures

Locus	N alleles	Size range	H_O
S16B	13	157 - 268	0.957
S19	14	122 - 261	0.826
S23	4	132 - 147	0.478
S24	7	165 - 185	0.783
S30	5	161 - 173	0.609
S49	10	91 - 135	0.826
Sm10	5	152 - 168	0.348

Table 1. Characteristics of microsatellite loci used in the study. For each locus, a number of alleles detected, their size range (in bp), and observed heterozygosity among 22 distinct genotypes of *S. avenae* (H_O) are provided. Locus Sm10 was first described by Simon et al. (1999), other six loci by Haack et al (2000) and Wilson et al. (2004).

Genotype	Count			Symbiont	Microsatellite profile													
	wheat	cocksfoot	oat		S16B		S19		S23		S24		S30		S49		Sm10	
Co05	1			Ha-4	171	207	235	235	132	132	177	185	161	163	117	131	164	168
Co07	1			Ha-3 + Re-2	171	228	122	235	132	147	181	181	163	171	117	131	164	164
Co08	1			Ha-2	157	171	229	231	132	147	165	181	161	173	91	115	164	164
Co09	1			Ha-3	228	251	182	235	132	132	165	177	161	161	117	131	164	164
Co12	1			Ha-1	171	205	122	199	132	140	165	165	163	171	91	129	160	164
Co16	1			none	205	268	223	235	132	132	177	185	163	163	117	119	164	164
Co19	1			Ha-4	175	205	122	235	132	132	167	185	161	163	117	117	164	168
Co21	7	1 #	2 #	Re-2 *	171	205	122	223	132	140	165	175	171	171	117	125	152	166
Co23	2			Ha-3	171	258	135	235	132	132	165	185	163	163	117	117	164	164
Co26	1			Ha-4	258	268	207	223	132	132	183	185	163	163	117	119	164	164
Co28	1			none	171	175	223	261	132	142	177	181	163	171	117	131	168	168
Co29	1			none	220	237	182	182	140	147	165	181	161	163	119	125	164	164
Co31	1			none	211	239	150	235	132	147	165	181	163	163	117	125	166	166
Co32	4	1 #		none *	171	258	148	235	132	132	181	185	161	163	111	119	160	164
Co34	3			Re-1	157	171	122	135	132	147	165	165	161	163	91	91	164	166
Co37	1		1 #	Ha-3	258	268	153	199	132	132	181	183	163	171	117	127	164	164
Co39		1	1 #	none *	171	171	156	199	132	132	175	183	163	171	111	135	164	166
Co41	1			Ha-4	171	205	223	235	132	140	181	183	163	163	117	117	164	164
Co45	1			Ha-4	171	205	223	235	132	147	181	185	163	163	117	135	164	164
Co47		1		Ha-4	171	205	235	235	132	132	177	185	161	163	119	127	164	164
Co49	4	3		Ha-4 *	258	268	223	235	132	132	183	185	163	163	117	119	164	164
Co50	4		1 #	none *	171	228	223	235	132	132	165	165	165	171	117	119	166	166

Table 2. Characteristics of the grain aphid microsatellite genotypes collected in June 2008. For each genotype, we provided the numbers of aphids collected from three host plants, its symbiont complement, and the profile at seven microsatellite loci. The abbreviations of the symbiont names are Ha (*Hamiltonella defensa*) and Re (*Regiella insecticola*), followed by a number identifying a distinct 16S haplotype (see Table 4). The five clones which we found polymorphic in symbiont infection status are highlighted, and the symbiont infection status is provided for the most common type collected from wheat, or for clone Co39 - from cocksfoot (see Table 3 for other variants). Collections marked with hash (#) were made in Lower Radley; all other aphids were collected on Colleymore Farm.

Clone	Collection plant		
	Wheat	Cocksfoot	Oat
Co21	Re-2 (7)	Re-2 (1)	Re-2 + *Serratia symbiotica* (1) Re-2 + Ha-3 + *Serratia proteamaculans* (1)
Co32	None (3) Re-1 (1)	None (1)	
Co39		None (1)	Re-3 + Ha-4 + *Serratia symbiotica* (1)
Co49	Ha-4 (4)	Ha-4 (2) Re-3 (1)	
Co50	None (4)		Ha-1 (1)

Table 3. Microsatellite genotypes of *S. avenae* which we found to be naturally polymorphic for endosymbiont infection status. For each of the five genotypes and each of the collection plants, we provided information on complement and the number of individuals harbouring these particular symbionts. The abbreviations of the symbiont names are Ha (*Hamiltonella defensa*) and Re (*Regiella insecticola*), followed by a number identifying a distinct 16S haplotype (see Table 4).

Hamiltonella strains

Haplotype / Base #	35	77	80	95	99	217	470	686	716	817	1316
S. avenae Ha-1	G	T	G	T	T	G	G	C	G	T	A
S. avenae Ha-2	G	C	G	C	C	G	G	C	G	C	G
S. avenae Ha-3	G	T	T	T	T	G	G	C	G	T	G
S. avenae Ha-4	G	T	G	T	T	T	C	T	G	T	G
A. pisum clone 6-1	A	T	G	T	T	G	G	C	C	T	G

***Regiella* strains**

Haplotype / Base #	150	208	226	329	649	678	840	1009	1010	1138	1143	1290	1291	1371	1382
S. avenae Re-1	G	T	A	A	G	T	G	G	A	G	C	ø	ø	T	T
S. avenae Re-2	A	C	G	G	T	T	A	T	G	A	T	T	A	C	C
S. avenae Re-3	A	T	A	G	T	.	.	.	.	.	.	.	.	.	.
A. pisum clone 2a	G	T	A	A	G	C	G	G	A	G	C	ø	ø	T	C

Table 4. 16S haplotypes of strains of *Hamiltonella defensa* (based on sequences of 1407 bp) and *Regiella insecticola* (1414 bp) identified in aphids collected during this study, aligned against reference sequences of the two symbiont species (Moran et al., 2005b). Base numbers are based on the reference sequences. Only partial sequence was obtained for strain Re-3.

Chapter 4

Grain aphid clones vary in frost resistance, but this trait is not influenced by facultative endosymbionts

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Abstract

1. Facultative endosymbiotic bacteria of insects are known to affect life history traits of their hosts, and can provide important fitness benefits under certain environmental conditions. While several distinct endosymbiont-induced effects have been reported, there are no data on whether heritable facultative endosymbionts in any species affect their hosts' performance at low temperatures, something that could have a major effect on insect physiology and survival, and thus population structure and distribution.

2. The original facultative endosymbionts were experimentally removed from five clonal genotypes of the grain aphid, *Sitobion avenae*, which were then exposed to frost.

3. Aphid genotypes differed considerably in survival following the exposure and in fecundity of the survivors. However, the presence of the facultative symbionts had no overall effect on the studied traits.

4. The results suggest that the facultative symbionts have limited effects on the cold hardiness of their grain aphid hosts.

Introduction

Facultative endosymbionts of insects have been shown to have a wide range of effects on the life history traits of their hosts, and there is a growing understanding of their role in insect ecology and evolution (Moran et al., 2008; Oliver et al., 2010). In aphids, a model system for the study of this type of symbiosis, the benefits of infection with facultative endosymbionts include resistance to natural enemies, protection against abiotic stressors such as heat shock, and improved performance on particular host plants (reviewed by Oliver et al., 2010). At the same time, the costs of infection in clones naturally carrying symbionts appear to be low or absent (e.g., Leonardo, 2004; McLean et al., 2011), making it unclear why endosymbionts are not more widespread across aphid species and clones. However, with the exception of work on heat shock resistance (Montllor et al., 2002; Russell & Moran, 2006), most studies on aphid endosymbionts have been conducted under benign laboratory conditions and the costs and benefits of carrying endosymbionts under testing and fluctuating natural conditions are not known.

One of the most important factors determining the population structure and distribution of insects in temperate zones is periodic exposure to sub-zero temperatures (Bale, 2002). Frost can lead to tissue damage resulting from ice crystal formation within cells with major effects on insect survival and fecundity (Parish & Bale, 1993). Insects have evolved numerous mechanisms to enable them to withstand low temperatures (Bale, 2002; Doucet et al., 2009), but their cold hardiness can also be influenced by the microorganisms with which they are associated. Gut and surface bacteria have been shown to act as ice nucleating agents increasing the freezing point of tissue in overwintering insects (Lee et al., 1993; Lee et al., 1992) and therefore leading to higher mortality in frost-susceptible species. Facultative endosymbionts

may also be capable of producing, or affecting the production of, compounds such as antifreeze proteins, polyols and sugars, ice nucleating proteins or heat shock proteins, which help to prevent or control ice crystal growth and reduce frost-related damage, or aid their hosts' recovery following freezing (Bale, 2002; Burke et al., 2010; Doucet et al., 2009; Neelakanta et al., 2010). Insect fitness can be reduced if their obligate nutritional endosymbionts are damaged by extremes of hot or cold (Ohtaka & Ishikawa, 1991; Parish & Bale, 1991), and genetic changes in the obligate symbionts can increase their susceptibility to heat shock so affecting the thermal tolerance of their insect hosts (Dunbar et al., 2007). However, facultative endosymbionts can provide some protection following heat shock (Montllor et al., 2002; Russell & Moran, 2006). Symbionts can also affect their hosts' survival during the cold season indirectly – by altering their behaviour, or influencing the induction of the sexual generation which produces frost-resistant eggs in aphids (Leonardo & Mondor, 2006). Finally, the beneficial effects of infection with facultative endosymbionts in aphids, such as provision of resistance to natural enemies, are known to vary with temperature (Bensadia et al., 2006; Guay et al., 2009). Regardless of the mechanisms involved, the prevalence of the symbiont *Regiella insecticola* in pea aphids collected in colder, drier parts of Japan suggests that the infection might be beneficial under cooler conditions (Tsuchida et al., 2002). However, despite the many ways in which responses to temperature can be influenced by symbiont infection status, we are not aware of any study that has specifically asked whether maternally transmitted facultative endosymbionts affect their insect host's sensitivity to low temperatures.

Frost sensitivity has received considerable attention in the grain aphid *Sitobion avenae* Fab. 1775, a serious pest of cereals in Europe (e.g., Knight & Bale, 1986; Parish & Bale, 1991; Powell & Bale, 2004). In regions with severe winters,

populations of *S. avenae* are largely holocyclic (i.e., alternating parthenogenesis with sexual reproduction) and overwinter as frost-resistant eggs (Papura et al., 2003). However, in regions with milder winters anholocyclic (obligatorily asexual) clones overwinter as feeding nymphs and adults, though they can frequently be exposed to sub-zero temperatures and suffer high mortality (Dedryver et al., 2001; Dewar & Carter, 1984; Knight & Bale, 1986). In the south of England, grain aphid populations are mostly composed of anholocyclic clones (Llewellyn et al., 2003; Newton & Dixon, 1988b), which vary in their susceptibility to cold (Griffiths & Wratten, 1979). Grain aphids in this region frequently harbour two species of facultative endosymbionts, *Hamiltonella defensa* and *Regiella insecticola* (P. Łukasik et al., unpublished data), but the symbionts' effects on ecologically important traits in this species are unknown. The present study tested whether the cold hardiness of *S. avenae* can be affected by their facultative endosymbionts. Five genetically distinct clones of *S. avenae* that were naturally infected with *H. defensa* or *R. insecticola* were selected and lineages cured of the infection were created using antibiotics. The mortality due to freezing and the fecundity of aphids which survived the exposure to frost were compared in each pair of infected and cured lineages.

Materials and methods

The clonal genotypes of *S. avenae* used in this study were collected from wheat on an organic farm near Faringdon, Oxfordshire, southern England, where the grass temperatures drop below -10°C during most winters (Radcliffe Meteorological Station, 2011). The aphids were cultured in 90-mm non-vented Petri dishes at 20±1°C and L16:D8 light regime on regularly exchanged, approximately 10-day old wheat plants with their stems placed in 2% agar. These conditions ensured indefinite asexual reproduction. Five clones of *S. avenae*, distinct at seven microsatellite loci

(Chapter 3 of this thesis) were selected for the study, including four genotypes (code numbers Co08, Co23, Co26 and Co37) originally infected with *Hamiltonella defensa* and one (Co21) originally infected with *Regiella insecticola*. Polymerase chain reactions with diagnostic primers revealed none of the additional facultative microbial symbionts reported from other aphid species, including *Serratia symbiotica*, X-type, *Rickettsia*, *Spiroplasma* and *Rickettsiella* (McLean et al., 2011; Tsuchida et al., 2010), and confirmed the presence of the essential primary endosymbiont *Buchnera aphidicola*. Not less than 12 generations before the experiment, facultative symbionts from the experimental genotypes were removed by the oral administration of antibiotics (2% ampicillin for Co21 and Co26, or a mixture of 0.5% ampicillin, 0.7% gentamicin and 0.3% cefotaxine for Co08, Co23 and Co37) through young wheat plants, at doses which did not eliminate *Buchnera* (McLean et al., 2011). Successful elimination of facultative symbionts was confirmed with PCR in not less than four separate generations for each genotype. The resulting five pairs of lineages, sharing genotype and primary symbiont but differing in the presence of secondary symbionts, were used for assessing the effects of symbionts on frost resistance.

The post-freezing mortality of 200 nymphs from each of the ten experimental lineages was assessed. First-instar experimental aphids aged 36±12 hours were transferred in groups of 20 to dishes containing young wheat plants with their stems inserted into parafilm-sealed Eppendorf tubes containing water. After the aphids settled on the plants, the dishes were randomly arranged inside an incubator (Model 1200, LMS Ltd, Sevenoaks, Kent, UK), which was cooled from 20°C to 0°C at a rate of 0.25°C/min and then maintained at that temperature for three hours before being cooled at the same rate to -10°C. Six hours later (after 5hr 20min at -10°C), the dishes were re-warmed to 20°C, again at a rate of 0.25°C/min. Fresh plants were then

added to each dish and after 48 hours at 20°C the proportion of aphids surviving was assessed. All insects found feeding or walking around the dish were classified as surviving (Powell & Bale, 2004).

To assess the effect of freezing on fecundity, groups of aphids aged 36±3 hours were subjected to a similar treatment to the above, except that the exposure to -10°C lasted for only 2hr 20min. After the end of the cold treatment the aphids were kept for 48 hours and then 12 survivors from each experimental lineage were randomly selected from the full set of dishes and kept isolated in Petri dishes on wheat plants which were exchanged every three days, with the offspring counted after each exchange. Simultaneously, the fecundity of the same number of control aphids, kept at 20°C and not exposed to frost, was measured. The number of offspring produced in the first 15 days of life by females surviving until the end of the study was used as a measure of fecundity. Only data for wingless individuals was used. There was no clear pattern of the occurrence of winged individuals across treatments or aphid lines.

Data were analyzed using the statistical package R v. 2.9.2 (R Development Core Team, 2011) using generalized linear modelling techniques with aphid genotype, symbiont infection status and treatment (fecundity assay only) as explanatory variables. In order to correct for overdispersion in the data, quasibinomial (survival) or quasipoisson (fecundity) error variances were assumed.

Results and discussion

The mortality following freezing differed significantly among the five experimental genotypes ($F_{4, 90} = 22.23$, $p < 0.001$). However, the presence of the facultative symbionts did not affect aphid survival ($F_{1, 86} = 0.59$, $p = 0.44$), and there was no aphid genotype by symbiont presence interaction ($F_{4, 85} = 1.09$, $p = 0.37$)

(Figure 1). Similarly, the aphid genotypes differed significantly in their overall fecundity ($F_{4, 164} = 9.21$, $p < 0.001$) and in the effect freezing had on their fecundity (genotype by treatment interaction: $F_{4, 158} = 5.12$, $p < 0.001$), but aphid fecundity was not significantly affected by the presence of endosymbionts ($F_{1, 159} = 2.10$, $p = 0.15$) and there was no significant interaction between endosymbiont presence and treatment ($F_{4, 154} = 1.93$, $p = 0.11$) (Figure 2).

Significant differences were found amongst the experimental clones of *S. avenae* in their susceptibility to frost, as revealed both by differences in post-freezing mortality and in the survivors' fecundity. Such clonal variation has previously been reported in *S. avenae* and in other aphid species (Griffiths & Wratten, 1979; Vorburger, 2004). However, no evidence was found that any of the facultative endosymbiont strains used in this study had a significant effect – either positive or negative – on the fitness of their aphid hosts following this brief exposure to sub-zero temperatures. These results indicate that the facultative symbionts infecting grain aphids, *Hamiltonella defensa* and *Regiella insecticola*, are not a major force shaping the structure of anholocyclic aphid populations in response to freezing.

The absence of apparent costs of carrying symbionts in naturally infected clones under permissible conditions has been noted previously in the pea aphid (Leonardo, 2004; McLean et al., 2011) but not in other aphid species. However, facultative symbionts rely on nutrients provided by the hosts for their upkeep, incurring some costs to their carriers, which need to be compensated for in order for the infection to persist in a population. The five experimental symbiont strains do not appear to protect grain aphids against their natural enemies (P. Łukasik et al., unpublished data). Thus, the prevalence and diversity of facultative endosymbionts in grain aphid populations need to be explained by other as yet unknown beneficial effects on host

fitness. Further work on the effects of facultative endosymbionts on thermal sensitivity of their hosts (Montllor et al., 2002; Russell & Moran, 2006) and on temperature-dependency of other fitness effects of infection (Bensadia et al., 2006; Guay et al., 2009) will be essential for understanding how symbiosis in insect species and communities varies across climatic zones.

Figures

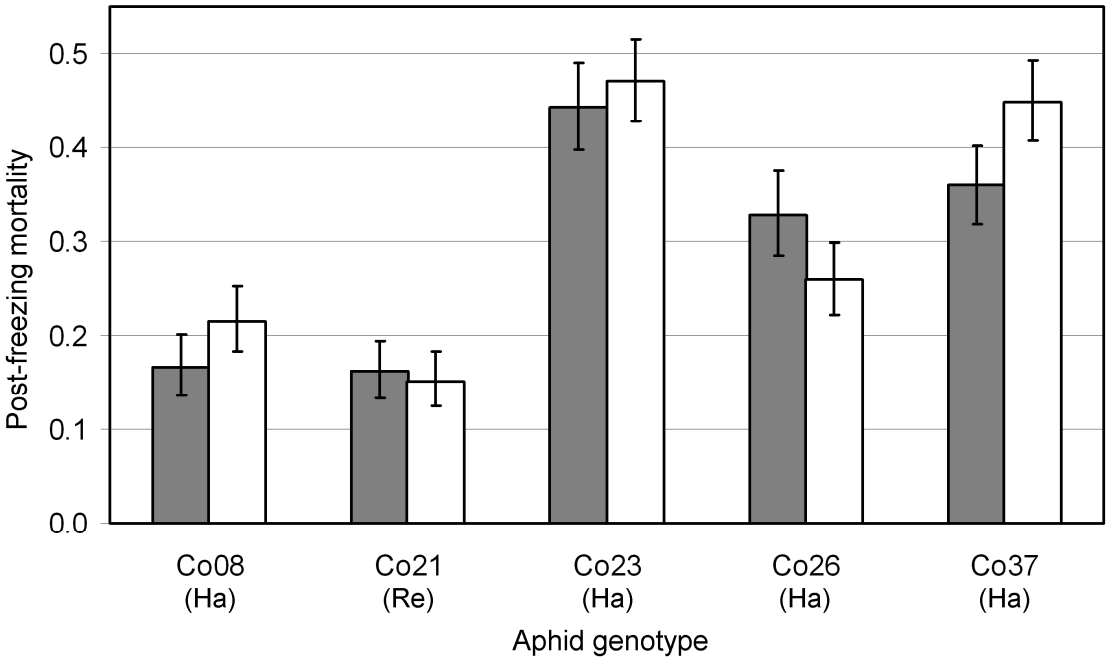

Figure 1. Mortality (mean ± standard error) of juveniles from five genotypes of *Sitobion avenae*, originally infected with facultative symbionts *Hamiltonella defensa* (Ha) or *Regiella insecticola* (Re) (grey bars) or cured from the infection (white bars), recorded 48 hours after the end of a 6-hour exposure to -10°C.

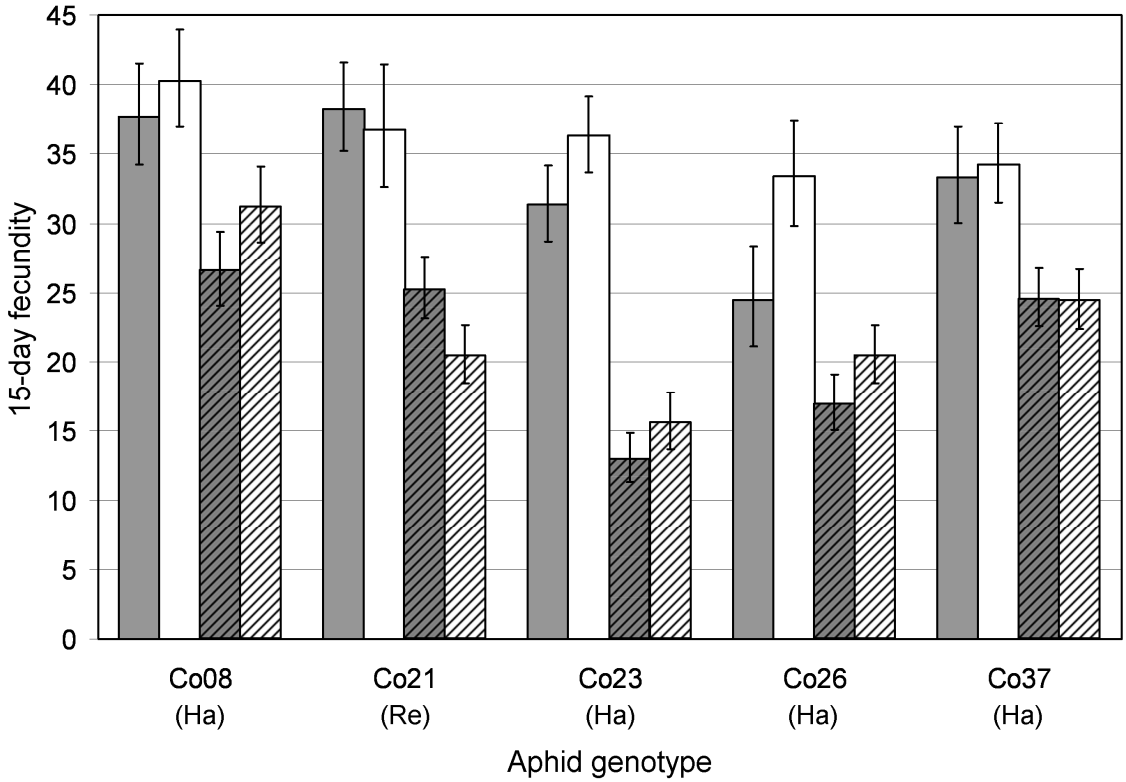

Figure 2. Numbers of offspring (mean ± standard error) produced within 15 days by wingless females from five genotypes of *Sitobion avenae*, originally infected with facultative endosymbionts *Hamiltonella defensa* (Ha) or *Regiella insecticola* (Re) (grey bars) or cured from the infection (white bars), and either kept at a constant temperature of 20°C throughout the study (uniform bars), or exposed as juveniles for 3 hours to -10°C (diagonally striped bars).

Chapter 5

Facultative endosymbionts of aphids influence oviposition choice of parasitoids

Abstract

The biology of many insects is influenced by mutualisms with facultative endosymbiotic bacteria, which may provide their hosts with important fitness benefits under certain environmental conditions. One of the best-known examples is a facultative endosymbiont *Hamiltonella defensa*, which has been shown to provide resistance to hymenopterous parasitoids to the pea aphid. However, several aspects of this defensive symbiosis, for example the effect of symbionts on parasitoid oviposition behaviour, are poorly understood. Furthermore, while *H. defensa* is widespread within and beyond Aphidoidea, there are little data on its defensive properties in species other than the pea aphid.

We studied the effects of infection with *H. defensa* in the English grain aphid, *Sitobion avenae*. After transferring symbionts between aphid clonal genotypes, we measured the effects of infection on aphid susceptibility to two species of aphidiine parasitoids, *Aphidius ervi* and *Ephedrus plagiator*, and on parasitoid developmental traits. We also tested whether symbiont presence in aphids affected the oviposition decisions of the wasps.

Contrary to the expectations, the symbionts did not reduce the rate of successful parasitism in the grain aphids exposed to parasitoids, and had no negative effects on life history traits of wasps developing in symbiont-infected hosts. Despite this, experienced females of both parasitoid species when given a mixture of symbiont-infected and non-infected aphids preferentially oviposited in the latter. These results suggest that *H. defensa* can provide some indirect protection to its grain aphid hosts by making them appear less attractive to searching parasitoids. While the fitness consequences of parasitoid selectivity are likely to vary between study systems,

habitats and seasons, such effect of the endosymbionts on the outcome of encounters between hosts and parasitoids may have important consequences for the evolution of arthropod symbioses and host-symbiont-parasitoid interactions.

Introduction

Parasitoids are a major source of mortality for many insects, and have had a large influence on their ecology and evolution (Godfray, 1994). Aphids are a good example of a group subject to a strong parasitoid pressure. For example, as many as 17 species of hymenopterous primary parasitoids attack a single species, the English grain aphid *Sitobion avenae* in Europe (Kavallieratos et al., 2004; Muller et al., 1999; Powell, 1982), and regularly decimate its populations (Schmidt et al., 2003; Sigsgaard, 2002; Tomanovic et al., 2008). After hatching from single eggs oviposited into the haemocoel of their aphid hosts, the parasitoid larvae develop within the body cavities of aphids while they continue feeding and growing. Ultimately, however, the parasitoids kill their hosts and pupate inside their remains, thus acting as functional predators. Aphids are under strong selection pressure to evolve defenses against these important natural enemies, whose biology offers opportunities for escaping parasitoid-induced mortality in at least two ways: by avoiding oviposition, or by killing larvae developing within the aphid haemocoel.

When they sense the presence of parasitoids, aphids exhibit a range of defensive behaviours, including attempts to evade them such as walking away, jumping, or dropping off host plants, and attempts to drive them away by kicking, striking with wings or antennae, or jerking movements (Gross, 1993). Additionally, disturbed aphids of many species produce cornicle secretion containing rapidly hardening liquid lipids (Edwards, 1966; Wynn & Boudreaux, 1972) which they attempt to smear over attacking parasitoids. That can make the parasitoids abort the attack, stop to remove the lipid plaque, leave the colony prematurely, or even die, unable to extricate themselves from the lipid cast (Dixon, 1958; Edwards, 1966; Wu et al., 2010). The aphid alarm pheromone released together with droplets of cornicle

secretion warns nearby clonal kin of an attacked aphid of the presence of danger, and elicits their escape or defensive responses (Montgomery & Nault, 1977; Xiangyu et al., 2002), increasing the inclusive fitness of an individual producing cornicle secretion (Dixon, 1958; Edwards, 1966; Goff & Nault, 1974; Wu et al., 2010).

It has been shown that aphid clones vary considerably in the mortality they suffer following exposure to parasitoids (Ferrari et al., 2001; Henter & Via, 1995; Nyabuga et al., 2010; von Burg et al., 2008; Vorburger et al., 2009), and some of the variation in resistance may be explained by genetic differences in behavioural defences. Clones may also vary in the functioning of their immune system, even though it does not appear to play an important role in aphids (Gerardo et al., 2010). However, the major role in aphid resistance to parasitoids has been attributed to infection with facultative endosymbiotic bacteria, or secondary symbionts – microbes found within the haemocoel of some clones of many aphid species. These bacteria are not essential for growth or reproduction of their hosts, but can have important effects on their life history traits (see Oliver et al., 2010 for review). The best known of them, a gammaproteobacterium *Hamiltonella defensa* (Moran et al., 2005b), has been associated with resistance to parasitoids in naturally infected clones of the pea aphid, *Acyrthosiphon pisum* (Ferrari et al., 2004; Nyabuga et al., 2010), and the black bean aphid, *Aphis fabae* (Vorburger et al., 2009). Studies involving artificial transfer of *H. defensa* strains between pea aphid clones, and in one case between *Aphis craccivora* and the pea aphid, confirmed that the increase in resistance to the parasitoid *Aphidius ervi* is associated with the infection by this symbiont (Oliver et al., 2005; Oliver et al., 2003). More specifically, the defensive properties of *H. defensa* in the pea aphid have been attributed to diverse toxins encoded by its lysogenic lambdoid bacteriophage known as APSE - phage from *A. pisum* secondary endosymbiont (van der Wilk et al., 1999), detected in isolates of *H. defensa* from several pea aphid

clones and from other aphid and homopteran species (Degnan & Moran, 2008a, b; Moran et al., 2005a; Oliver et al., 2009). Strains of other species of secondary endosymbionts, including *Serratia symbiotica* and *Regiella insecticola*, have also been associated with parasitoid resistance (Ferrari et al., 2004; Nyabuga et al., 2010; Oliver et al., 2003; Vorburger et al., 2010), but little is known about the possible mechanisms, or about how common their defensive properties are. Also, while *H. defensa* and other secondary symbionts are common and widespread among aphids other than *A. pisum* (Burke et al., 2009; Degnan & Moran, 2008b; Haynes et al., 2003; Russell et al., 2003; Sandstrom et al., 2001), there are little experimental data on their fitness effects, including provision of resistance to parasitoids, in these other species (Vorburger et al., 2010; Vorburger et al., 2009).

While aphids are under selection to evolve ways of escaping parasitism, parasitoids are strongly selected for their abilities to locate and identify suitable hosts and overcome their defences (Godfray, 1994). Following location of plants colonized by aphids, parasitoid females rely for the identification and selection of hosts for oviposition on visual cues such as size, shape, colour, and movement, but also on volatile and contact chemical cues (Battaglia et al., 1995; Battaglia et al., 2000; Hatano et al., 2008; Mackauer et al., 1996). The final decision on accepting a host and ovipositing appears to be based on the hosts' internal chemistry, and is only made after the host is probed with the ovipositor, which has chemoreceptors at its tip (Larocca et al., 2007). Parasitoids are known to discriminate between species of suitable aphid hosts and between host sizes (Daza-Bustamante et al., 2003; Henry et al., 2005; Henry et al., 2009; Mackauer et al., 1996; Powell et al., 1998), and they can identify hosts which have already been parasitized (Chow & Mackauer, 1986; Outreman et al., 2001). The ability to detect resistance-enhancing secondary symbionts in aphids, and to avoid infected aphids when selecting hosts for

oviposition, is likely to be advantageous at least at times when abundant potential hosts of variable infection statuses are available. Selection for the ability to signal the resistance-enhancing infection to searching parasitoids and possibly discourage them from ovipositing could also be expected in aphid–bacterial symbioses, since defending against parasitoid larvae developing within haemocoel can be very costly even in resistant aphids (Ferrari et al., 2004; Nyabuga et al., 2010; Vorburger et al., 2008). There is no information on whether the symbionts may affect the composition of the cuticular chemicals of aphids that are essential for host recognition by parasitoids (Muratori et al., 2006). However, symbiont-induced changes to the aphid metabolome (Burke et al., 2010) may offer the opportunity to detect the infection. Also, the effects that some aphid endosymbionts have on their hosts' body colour (Tsuchida et al., 2010) may be perceived by parasitoids. However, the effects of symbionts infecting aphids on oviposition decisions of parasitoids have not been explicitly tested. Henter and Via (1995) detected no differences in the number of eggs oviposited by *A. ervi* into pea aphids from a susceptible and a highly resistant clone when they were exposed together to parasitoid wasps, but the comparison they made was between two visually distinct genotypes of unknown infection status. Later, Oliver et al. (2003) found similar numbers of eggs laid by *A. ervi* in symbiont-free pea aphid lines and those artificially infected with resistance-enhancing strains of *H. defensa* or *S. symbiotica*, but the parasitoid females used in their study had no experience handling aphids of different infection statuses and were given no alternative hosts. In contrast, our preliminary data suggested that *A. ervi* may be able to discriminate between infected and non-infected grain aphid lineages when they are exposed to the wasps in mixed groups, and preferentially oviposit in aphids which do not harbour symbionts.

In the present study, we measured the effects of infection with each of the four strains of *H. defensa* originating from field-collected clonal genotypes of the grain aphid *Sitobion avenae* Fab. 1775 and introduced into four originally non-infected clones of the same species. We compared the susceptibility of the originally non-infected and artificially infected lineages to two species of aphidiine parasitoids commonly attacking grain aphids in England, *Aphidius ervi* Haliday 1834 and *Ephedrus plagiator* Nees 1811 (Muller et al., 1999; Powell, 1982). We measured the rates of successful parasitism and the development times and sizes of the parasitoids emerging from the exposed aphids. Furthermore, we measured the effects of infection on parasitoid oviposition choice by comparing the numbers of eggs laid in aphids of infected and non-infected lineages after they were exposed together to parasitoid females. Finally, we assessed the behaviour of parasitoids and infected and non-infected aphids during the exposure.

Material and Methods

Experimental aphids and symbionts

The clonal genotypes of *S. avenae* used in this study were collected from wheat on an organic farm near Faringdon, Oxfordshire, UK in June 2008 (Chapter 3 of this thesis). After collection, the aphids were cultured in 90-mm non-vented Petri dishes at 14±1°C and L16:D8 light regime on regularly exchanged, approximately 10-day old wheat plants with their stems placed in 2% agar. The plants and the dishes were exchanged approximately every 10 days. These conditions ensured indefinite asexual reproduction. Before any experiments, the aphids were kept at the experimental temperature (20±2°C), on plants exchanged every 3-4 days, for at least three generations.

DNA from field-collected adult aphids that were used to establish laboratory clonal lines was extracted with DNeasy Blood & Tissue Kit (Qiagen) following a protocol provided by the manufacturer. All aphid samples were typed at seven microsatellite loci – S16B, S10, S23, S24, S30, S49 and Sm10 to ensure that they were distinct genotypes (Chapter 3 of this thesis). Then, the samples were screened for the known aphid secondary endosymbionts, *Hamiltonella defensa*, *Regiella insecticola*, *Serratia symbiotica*, X-type, *Rickettsia*, *Spiroplasma* and *Rickettsiella*, with polymerase chain reaction (PCR) using symbiont-specific primers for the ribosomal 16S gene (Ferrari et al., accepted; McLean et al., 2011; Tsuchida et al., 2010). The PCR products were sequenced and compared with the resources available in Genbank in order to verify the symbiont identity (Chapter 3 of this thesis). Four grain aphid clones infected with *H. defensa* only and four clones free from infection with any known secondary symbionts were selected for further work (Tab 1).

The novel *S. avenae – H. defensa* associations were created by injecting haemolymph containing symbionts from four naturally infected "donor" aphid clones into four symbiont-free "recipient" clones (Oliver et al., 2003). The offspring produced by each of the surviving injected aphids after the 14^{th} day from injection were isolated, and after they reproduced themselves, they were checked for successful infection using diagnostic PCR. Successfully infected lines were reassessed with diagnostic PCRs in the eighth generation from injection, and the identity of the originally non-infected and artificially infected experimental lineages was confirmed with microsatellites and diagnostic PCRs shortly before the experiments.

Parasitism assay

We compared the susceptibility of the eight experimental lineages (i.e., the originally non-infected and the artificially *Hamiltonella*-infected lineage from each of the four experimental clones) to two species of aphidiine parasitoids, *Aphidius ervi* and *Ephedrus plagiator*. *A. ervi* was obtained from Syngenta Bioline Ltd (Little Clacton, Essex, UK) in 2007, and cultured on a secondary symbiont-free pea aphid clone until September 2008, when we established a mass culture on a symbiont-free *S. avenae* clone Co50. A stock culture of *E. plagiator* was established in September 2008 from parasitoids emerging from mummies of unidentified cereal aphids collected from *Holcus* sp. near the village of Radley, Oxfordshire, UK. The identity of both parasitoid species was confirmed by sequencing a fragment of the mitochondrial cytochrome oxidase subunit I (COI) gene (Traugott et al., 2008). Both parasitoid species had been in culture on Co50, maintained on young potted wheat plants enclosed in 27 dm^3 transparent cages, for at least 8 months, or 15 generations, before they were used for the experiments.

The resistance assay for each parasitoid species was conducted in two blocks. Experimental parasitoid females for the first block emerged from mummies isolated directly from the stock culture, but for the second block they were obtained by exposing groups of synchronised Co50 aphids to wasps from a stock culture under conditions identical to those in the experiment (described below). In both experimental blocks, parasitoid cocoons ("mummies") were transferred to a large cage with an abundance of honey water-soaked tissue, and mated females taken directly from that cage were used for the assay. Groups of thirty 72-96 hour-old aphids (late 2^{nd} or early 3^{rd} instar) of each of the eight experimental lineages were transferred to 90mm Petri dishes with two young wheat plants whose stems were inserted into agar; the Petri dishes also contained some honey water-soaked tissue.

After the aphids settled on the plants, naïve parasitoid females were introduced into each dish. Exposure to parasitoids was for eight hours in the case of *A. ervi* and 10 hours for *E. plagiator*. After this the parasitoids were removed from the dishes and the aphids were cultured for 15 days at 20±2°C, being transferred every 3 days to fresh dishes. During each transfer we counted any parasitoid mummies, as well as reproducing and dead aphids in the experimental dishes. In the second block, aphids and mummies were discarded once classified. In the first block, newly forming parasitoid mummies were transferred singly to individually labelled 0.68ml gelatine capsules (Value Healthcare, Sheffield, UK) on day 8 and every 24 hours afterwards. Capsules were then monitored every 12 hours. For each emerging wasp, we noted its sex and development time, and then transferred the capsule to an incubator set to 50°C, with relative humidity below 10%. After not less than seven days in the incubator, the desiccated wasps were weighted on an AD-4 microbalance (Perkin-Elmer Inc., Waltham, MA, U.S.A.) to an accuracy of 0.1 µg.

As a measure of susceptibility, we used the proportion of exposed aphids in which the parasitoids successfully pupated. The dishes in which we recorded no cases of successful parasitism were excluded from analyses, with the absence of parasitism attributed to low activity of some parasitoid females (Oliver et al., 2003). These excluded dishes contained exposed aphids from across clones and symbiont infection statuses.

Parasitoid oviposition choice

For the two parasitoid species, *A. ervi* and *E. plagiator*, we measured their host choice by comparing the numbers of eggs oviposited in *Hamiltonella*-infected and non-infected aphids after the two lineages representing each of the four experimental aphid clones were exposed together to experienced parasitoid females.

Soon after emergence, parasitoids were transferred in groups of four females and four males to 140mm non-vented Petri dishes containing several wheat plants with their stems inserted into agar, and approximately 500 aphids of different ages. Each of the dishes contained an equal mixture of the two aphid lineages representing the same clonal genotype, one of which was *Hamiltonella*-infected and another non-infected. The same two lineages were later used for the choice assay with these particular wasps. We predicted that after 24 hours in dishes with abundant hosts, the parasitoid females would have experienced both non-infected and infected aphids and would have partly depleted their egg reserves, thus becoming more selective. After they were removed from the "experience" dishes, the females were allowed to rest for six (*A. ervi*) or nine (*E. plagiator*) hours in Eppendorf tubes with honey water-soaked filter paper before they were used for the oviposition choice assay.

The choice experiment was conducted in 90mm Petri dishes containing two young wheat plants with their stems placed in agar. Into each dish, we transferred 15 second-instar aphids (60-72 hours old) from a *Hamiltonella*-infected lineage, and 15 equally-aged aphids from a non-infected lineage of the same clone. The aphids were marked by having a part of the last segment of their left or right antenna clipped off not less than 6 hours before the experiment. Single parasitoid females, with previous experience as in the protocol described above, were added to dishes once the aphids settled on plants. The exposure period lasted 9 hours for *A. ervi* and 12 hours for *E. plagiator*; as determined during a pilot study, these were times sufficient for the wasps to parasitize approximately half of the aphids available. After the specified time, the dishes were frozen and maintained at -26°C until the aphids were dissected, with symbiont infection status and the number of parasitoid eggs oviposited into each aphid noted (Oliver et al., 2003). The length of a subsample of 2nd-instar aphids

exposed to *A. ervi* (two dishes per clone) was measured under a binocular microscope before dissection.

Behavioural assay

We observed the behaviour of naïve females of *A. ervi* which were simultaneously presented with aphids from two lineages of clone Co31: the infected and the non-infected with *Hamiltonella*. Synchronised 3rd instar (96-108 hours old) aphids from each lineage were transferred to a Petri dish and allowed to settle on small pieces of wheat leaves. Then, we selected leaf pieces with four aphids feeding within a body length from each other, and placed two pieces, one with aphids of each lineage, next to each other in a transparent arena 4mm high and of 45mm diameter. The parasitoid females, which were allowed into the arenas through small openings in one of the walls, were thus presented with two neighbouring colonies of four aphids of different infection statuses. For each parasitoid we recorded the number of contacts with infected and non-infected aphids before the first oviposition attempt as well as the sequence of oviposition attempts. We also made notes on the aphids' behaviour, particularly on the production of cornicle secretion and their tendency to walk away. The observations were continued until the majority of aphids from both lineages started dispersing and the two groups became mixed and indistinguishable, or until the parasitoid stopped searching and had no contact with the aphids for a period of five minutes.

Statistical analyses

All analyses were conducted using the statistical package R version 2.13.0 (R Development Core Team, 2011), separately for each of the two parasitoid species. Proportional data on successful parasitism was analysed with generalized linear

modelling (GLM) techniques, with overdispersion being accounted for through the use of quasibinomial error variance, and with block, aphid genotype and symbiont infection status treated as fixed factors. Quasibinomial error variance was also assumed when comparing sex ratios among parasitoids emerging from aphids exposed during the first block of the resistance assay. The proportions of infected and non-infected aphids parasitised during the choice assay were compared with GLM assuming binomial error variance, with the effect of exposure dish fitted in addition to aphid genotype and symbiont infection.

Log-transformed data on parasitoid development times and dry weights were analyzed separately for the two sexes of each wasp species, with the effect of the exposure dish from which the parasitoid originated included. The same transformation was applied before estimating the effect of genotype and symbiont infection on the size of aphids exposed to *A. ervi*. When comparing the numbers of *Aphidius* eggs per parasitized aphid, quasipoisson error variance was assumed, again to account for overdispersion in the data.

In the behavioural assay, data on frequencies of parasitoid or aphid behaviours were analyzed using a series of chi-squared tests.

Results

New host-symbiont associations

Following injections, the symbionts established easily in the novel hosts. In all clones, at least half of the injected aphids reproduced after the 14th day from the injection, and not less than half of them produced infected offspring. Both values tended to increase with my experience in doing the injections. Novel aphid-symbiont

associations were stable: in the two years following the first injections (i.e., for at least 50 aphid generations), we did not record a single case of symbiont loss.

Parasitism assay

We gathered data on successful parasitism in 87 dishes, each containing 30 aphids exposed to *A. ervi*, and in 63 dishes with 30 aphids exposed to *E. plagiator*. There was no effect of infection with *H. defensa* on the proportion of the exposed aphids within which *A. ervi* successfully pupated ($F_{1, 79} = 0.15$, $p = 0.70$), with no differences in the effect of symbionts between aphid clones (genotype x symbiont interaction: $F_{3, 78} = 0.94$, $p = 0.42$) (Figure 1). Also, *Hamiltonella* infection did not have an overall effect on the proportion of aphids successfully parasitized by *E. plagiator* ($F_{1, 54} = 0.01$, $p = 0.91$), although the consequences of infection with *H. defensa* varied significantly across aphid clones (genotype x symbiont interaction: $F_{3, 53} = 5.55$, $p = 0.002$) (Figure 1).

The mean rate of successful emergence of *A. ervi* from a mummy was 97.2%, and of *E. plagiator* 94.5%, with no differences in the emergence rate between aphid clones or symbiont infection statuses (in both species, $p > 0.25$). Among 404 emerging *A. ervi*, the mean proportion of females was 41.8%, and among 377 *E. plagiator* it was 50.2%, with no significant differences between host clones or symbiont infection statuses in either species (in all cases, $p > 0.60$). The parasitoid developmental times differed considerably between wasp species and sexes, but in neither sex of *A. ervi* or *E. plagiator* it was significantly affected by the presence of symbionts, and there were no differences between clones in the effect of symbionts (in all cases, $p > 0.20$).

The overall effect of *H. defensa* infection on the size of emerging *A. ervi* varied significantly across aphid clones (genotype x symbiont interaction: $F_{1, 209} = 4.59$, $p = 0.033$ for males, and $F_{1, 179} = 6.81$, $p = 0.010$ for females), but the effect of symbiont alone was not significant ($p > 0.10$ in both sexes) (Figure 2a). In contrast, in *E. plagiator* infection with *Hamiltonella* had a significant negative effect on the size of females ($F_{1, 184} = 6.60$, $p = 0.011$), but tended to affect positively the size of males ($F_{1, 184} = 3.57$, $p = 0.060$), with no differences between clones in the effect of symbionts in either sex ($p > 0.50$) (Figure 2b).

Parasitoid oviposition choice

In 4 out of 37 exposure dishes, we found no parasitoid eggs in the experimental aphids; these dishes were excluded from analysis. Otherwise, the mean proportion of aphids which contained at least one parasitoid egg was 0.46 in 16 dishes with aphids exposed to *A. ervi*, and 0.64 in 17 dishes with aphids exposed to *E. plagiator*. The analysis across dishes and clones revealed a significant negative effect of symbionts on the probability of parasitism in both *A. ervi* ($\chi^2 = 8.52$, df = 1, $p = 0.004$) and *E. plagiator* ($\chi^2 = 7.87$, df = 1, $p = 0.005$). The aphid clones differed significantly in the effect of symbionts on the oviposition decisions of *E. plagiator* ($\chi^2 = 8.83$, df = 3, $p = 0.032$), and tended to differ in *A. ervi* ($\chi^2 = 6.22$, df = 3, $p = 0.101$), with similar patterns observed across clones in both parasitoid species (Figure 3).

In 91% of cases, *E. plagiator* females laid only a single egg into each aphid they parasitized, and we never found more than two eggs per aphid. *A. ervi* superparasitized in 41.5% of cases and we found up to six eggs within a single dissected aphid. The mean number of *Aphidius* eggs in parasitized aphids was not significantly affected by the presence of the symbiont, and there were no differences between genotypes in the effects of infection ($p > 0.15$). In the eight *Aphidius*

exposure dishes for which we collected data on the size of the exposed aphids, parasitoid females tended to oviposit more eggs into larger aphids ($F_{4, 211} = 2.08$, $p = 0.084$). However, there were no differences between the average sizes of the infected and non-infected aphids from the same dishes, or between aphid genotypes in the effects of symbionts on aphid size ($p > 0.50$).

Behavioural assay

Our observations on the behaviour of 52 *A. ervi* females did not suggest parasitoid bias towards one of the aphid lineages, or differences in the defensive behaviour of the aphids. Usually, a newly introduced wasp walked quickly around the arena, often getting in contact with aphids from one or both colonies but not attacking them. Only after a delay did the wasp start to search for hosts, first encountering an infected or a non-infected aphid with the same probability ($\chi^2 = 0.02$, d.f. = 1, $p = 0.88$). The parasitoid stung the aphid it first encountered in 62% cases (n = 45), regardless of its infection status ($\chi^2 = 0.18$, d.f. = 1, $p = 0.67$), then generally moved away a few body lengths and quickly returned to the colony to sting another aphid, although sometimes it encountered the second colony on the way and attacked one of these aphids instead. In 50% cases (n = 30), the wasps which had antennal contact with both infected and non-infected aphids before the first oviposition attempt stung an infected aphid first. The second attacked aphid had the same infection status as the first one in 69% cases (n = 29), significantly more than expected by chance ($\chi^2 = 4.24$, d.f. = 1, $p = 0.04$), but with no effect of infection status of the first stung aphid ($\chi^2 = 0.08$, d.f. = 1, $p = 0.78$). Out of the total number of 131 oviposition attempts recorded across the 30 replicates with the wasps which had antennal contact with both infected and non-infected aphids before the first

attack, in 51.9% cases a non-infected aphid was stung. This value is not significantly different from 50% ($\chi^2 = 0.19$, d.f. = 1, $p = 0.66$).

37% of feeding aphids stung by a parasitoid (n = 73) produced cornicle secretion, and 70% stopped feeding and walked away after being stung. There were no differences in the frequency of these behaviours between infected and non-infected lineages (in both cases, $p > 0.40$). Typically, once a droplet of cornicle secretion was produced, most aphids from the same colony, and often from the nearby second colony, ceased feeding and started walking, while the remaining aphids started waving their antennae and kicking. In that excited state, all were more likely to produce cornicle secretion when stung, or even touched, by the wasp. While these aspects of the aphid defensive behaviour were more difficult to quantify, we observed no apparent differences between the two lineages.

Discussion

We found no effect of infection with either of the four *H. defensa* strains on resistance of grain aphids to either of the two parasitoid species. Neither the rate of successful parasitism, parasitoid development times nor sizes were affected by the presence of symbionts in the aphid hosts. While we later identified other symbionts which under identical experimental conditions protected their grain aphid hosts from *A. ervi* (Chapter 7 of this thesis), the lack of defensive properties of the four experimental *Hamiltonella* strains was later confirmed in other grain aphid clones, including the original hosts (Chapters 6 and 7 of this thesis). Our results are in marked contrast to the findings of other workers, who reported defensive properties of *H. defensa* in populations of two other aphid species (Ferrari et al., 2004; Nyabuga et al., 2010; Oliver et al., 2005; Oliver et al., 2003; Vorburger et al., 2009). The multi-locus sequence typing revealed that the four experimental symbiont strains,

representing three different genotypes, were very similar to the *Hamiltonella* strains providing resistance to *A. ervi* in pea aphids, and all harboured the bacteriophage APSE (Chapter 8 of this thesis), which is known to encode various eukaryotic toxins, and which has been associated with the resistant phenotype of *Hamiltonella*-infected pea aphids (Degnan & Moran, 2008a, b; Moran et al., 2005a; Oliver et al., 2009). While parasitoids may evolve partial resistance to the symbiont-encoded toxins (Dion et al., 2011), the wasps used in the experiment originated from cultures maintained on a highly susceptible, secondary symbiont-free aphid clone. The fact that infection with none of the experimental strains affected aphid susceptibility to *A. ervi* or to *E. plagiator* suggests that the APSE-infected strains of *H. defensa* in grain aphids, common and diverse in populations of *S. avenae* (Chapter 3 of this thesis), do not universally provide resistance to hymenopterous parasitoids in this species. Some of the possible explanations for this phenomenon are provided in Chapters 7 and 8 of this thesis.

We found no overall effect of *H. defensa* on the development times of the parasitoids developing within infected aphids, but the presence of secondary symbionts within aphid hosts had an effect on the size of those parasitoids which successfully completed development, albeit not consistent across wasp species and sexes (Figure 2). *A. ervi* females emerging from aphids of three of the experimental genotypes were larger when the hosts harboured no symbionts, but the effect was reversed in the fourth genotype, and absent in males. *E. plagiator* females were on average larger when emerging from non-infected aphids (regardless of the host genotype), but the trend was reversed in males of the same species. It was previously shown that parasitoids completing development in resistant hosts take significantly longer to develop and are smaller upon emergence than the wasps developing in susceptible hosts, regardless of their sex (Li et al., 2002; Nyabuga et al., 2010). Thus,

our results support the view that the experimental symbiont strains do not have a direct negative effect on parasitoids developing within aphids. The relatively small differences detected in our study could be explained by the effects of symbionts on aphid productivity detected in some aphid clones (Chapter 6 of this thesis) and leading to changes in the amount of resources available to the developing parasitoid larvae. Also, if parasitoids perceive symbiont infections as causing changes in aphid quality, they could potentially alter their oviposition decisions, for example regarding dependency between host size and sex of oviposited eggs, or the amount of venom injected (Godfray, 1994).

Despite the lack of direct detrimental effects of the four *H. defensa* strains on the life history traits of the developing parasitoids, experienced *A. ervi* and *E. plagiator* females parasitized a significantly higher proportion of symbiont-free aphids when given the choice (Figure 3). The 12% mean difference in proportions of parasitized infected and non-infected aphids can be regarded as relatively low, considering the pre-treatment which was thought to increase the selectivity of the parasitoids. However, under natural conditions grain aphids representing the same genotype and infection status typically form distinct clonal colonies, each on a distinct plant. Thus, the wasps can assess the suitability of hosts in a patch after sampling only a few of them. In our choice experiment, the infected and non-infected aphids were mixed and had to be individually assessed by the parasitoids, which has likely increased the costs of conducting the assessment and decreased the wasp selectivity. Therefore, we believe that the significant difference in the proportion of parasitized infected and non-infected experimental aphids reflects a real effect of the experimental strains of *H. defensa* on parasitoid oviposition decisions, resulting in a certain degree of protection from parasitoids for their grain aphid hosts. We observed no differences in size, appearance or behavioural defences of the infected or non-infected aphids,

suggesting that these were not the factors affecting parasitoid host choice in our study. However, symbiont infection has an effect on the aphid metabolome (Burke et al., 2010), and there is much evidence that the chemicals present on aphid cuticle or in aphid haemolymph, and detectable by parasitoids either during the initial assessment with antennae or after probing the prospective hosts using the ovipositor, are important for host recognition and acceptance by aphid parasitoids (Hatano et al., 2008; Larocca et al., 2007; Mackauer et al., 1996). Therefore, we propose symbiont-induced changes in the chemical profile of the aphids, and the parasitoids' ability to detect and react to these changes, as the most likely mechanisms for parasitoid discrimination between the types of hosts in the choice experiment. The effects of endosymbionts on the composition of semiochemicals of their insect hosts, and on the ability of natural enemies to perceive and react to any changes, definitely merits further study.

In our study system, the parasitoids' preference for non-infected aphids appears to be without direct benefits to the parasitoids since the four experimental *H. defensa* strains had no negative effects on the fitness of the developing wasps. However, both *A. ervi* and *E. plagiator* are polyphagous and capable of parasitizing pea aphids (Daza-Bustamante et al., 2003; Kavallieratos et al., 2004; Muller et al., 1999; Tomic et al., 2005), and thus can regularly encounter symbionts which confer resistance to their aphid hosts. Yet, the degree of protection provided by strains of *H. defensa* and other endosymbionts to their aphid hosts varies from none to complete immunity even within species (Nyabuga et al., 2010; Oliver et al., 2009; Oliver et al., 2003; Vorburger et al., 2010; this study), and depends both on parasitoid genotype (Dion et al., 2011; Vorburger et al., 2009) and on environmental conditions such as temperature (Bensadia et al., 2006; Guay et al., 2009). Given this variation, the evolution of the ability to discriminate reliably between hosts based on their level of

symbiont-associated resistance is unlikely. Also, while resistant aphid-symbiont associations could potentially benefit from chemically signalling their unsuitability to the wasps, such signalling could perhaps be mimicked by "cheater" strains not providing resistance, and thus the reliability of any such signal would be questionable.

The ability to assess host quality based on their symbiont complement should be expected to carry costs to the parasitoids, associated with developing and maintaining the physiological mechanisms enabling detection of symbionts, as well as the time and energy required for conducting the assessment and the opportunity cost of unnecessarily rejecting suitable hosts. Yet, the mean level and pattern of symbiont-associated resistance in a multi-species host community is just one of several factors which parasitoid females could benefit from taking into account when making oviposition decisions. The optimal host selection model assumes that in order to maximize their fitness, a parasitoid selecting hosts should consider the quality of the host as food and shelter for the offspring, but also the amount of time needed to locate and handle the next host in relation to the number of eggs available, and any risks involved (Godfray, 1994). From a parasitoid's perspective, the quality of a host depends on its species (Daza-Bustamante et al., 2003; Henry et al., 2008), on its relative size to the parasitoid (Henry et al., 2009; Henry et al., 2006), and on the presence of other natural enemies of aphids nearby (Meisner et al., 2011; Nakashima et al., 2004), in addition to their symbiont complement. In *A. ervi* females, which are capable of parasitizing approximately 80 aphids per day for several days (He et al., 2006), and processing even large hosts within a minute (Henry et al., 2009), the egg availability may be the limiting factor at the time when host densities are high. However, the sizes of aphid populations change dramatically over the seasons (Dewar & Carter, 1984), and for much of the year egg availability is

unlikely to be the limiting factor. Under such conditions, selectivity would be disadvantageous, as even oviposition into marginally suitable hosts could result in fitness gain. Then, the discrimination between infected and non-infected lineages should be conditional and depend on the individual experience of a particular female, and be more pronounced in females that are highly successful in locating suitable hosts. These conditions were fulfilled for the parasitoids used for the choice experiment, which were kept for a day in dishes with high densities of both infected and non-infected aphids prior to being used for the assay, but not for wasps whose selectivity was studied by other authors (Henter & Via, 1995; Oliver et al., 2003), or those used in the behavioural assay. This could explain the differences between the results of these studies and of the choice experiment presented in this chapter. While these observations seem to fit theoretical predictions, further research on the ecology of host-parasitoid interactions is needed in order to understand the fitness consequences of selectivity in parasitoids.

In this chapter we have shown that the facultative symbiont *Hamiltonella defensa* in grain aphids does not universally provide resistance to parasitoids, in marked contrast with the pea aphid and some other aphid species. Our results demonstrate that the defensive effects of endosymbiont infection vary between aphid species, and stress the need to exercise caution when making taxon-wide generalisations based on results obtained from a single system. This study is also the first description of the effects of microbial endosymbionts of aphids on the oviposition choice of their hymenopterous parasitoids, which under certain conditions may reduce parasitism in symbiont-infected aphids. There is no reason why parasitoids should not discriminate between infected and non-infected hosts in other systems, particularly those where the symbionts actually reduce the suitability of their hosts to parasitoids. However, it is clear that further work on chemicals involved in decision-making and on fitness

costs and benefits of selectivity in parasitoids across fluctuating natural conditions is required. Such studies on the mechanisms and ecological and evolutionary consequences of symbiont influence on parasitoid oviposition choice would definitely improve our understanding of the arthropod symbioses and host-symbiont-parasitoid interactions.

Tables and Figures

Clone	Colour	Symbiont	Microsatellite profile														Role in the study
			S16B		S19		S23		S24		S30		S49		Sm10		
Co08	yellow	Ha	157	171	229	231	132	147	165	181	161	173	91	115	164	164	Donor for Co32
Co23	brown	Ha	171	258	135	235	132	132	165	185	163	163	117	117	164	164	Donor for Co28
Co26	brown	Ha	258	268	207	223	132	132	183	185	163	163	117	119	164	164	Donor for Co50
Co37	brown	Ha	258	268	153	199	132	132	181	183	163	171	117	127	164	164	Donor for Co31
Co28	red-brown	none	171	175	223	261	132	142	177	181	163	171	117	131	168	168	Experimental clone
Co31	brown	none	211	239	150	235	132	147	165	181	163	163	117	125	166	166	Experimental clone
Co32	green	none	171	258	148	235	132	132	181	185	161	163	111	119	160	164	Experimental clone
Co50	green	none	171	228	223	235	132	132	165	165	165	171	117	119	166	166	Experimental clone, stock for parasitoid culture

Table 1. Characteristics of the eight *Sitobion avenae* clones used in the study. The clones were either infected with a facultative endosymbiont *Hamiltonella defensa* (Ha) or carried no symbionts, and were all typed at seven microsatellite loci.

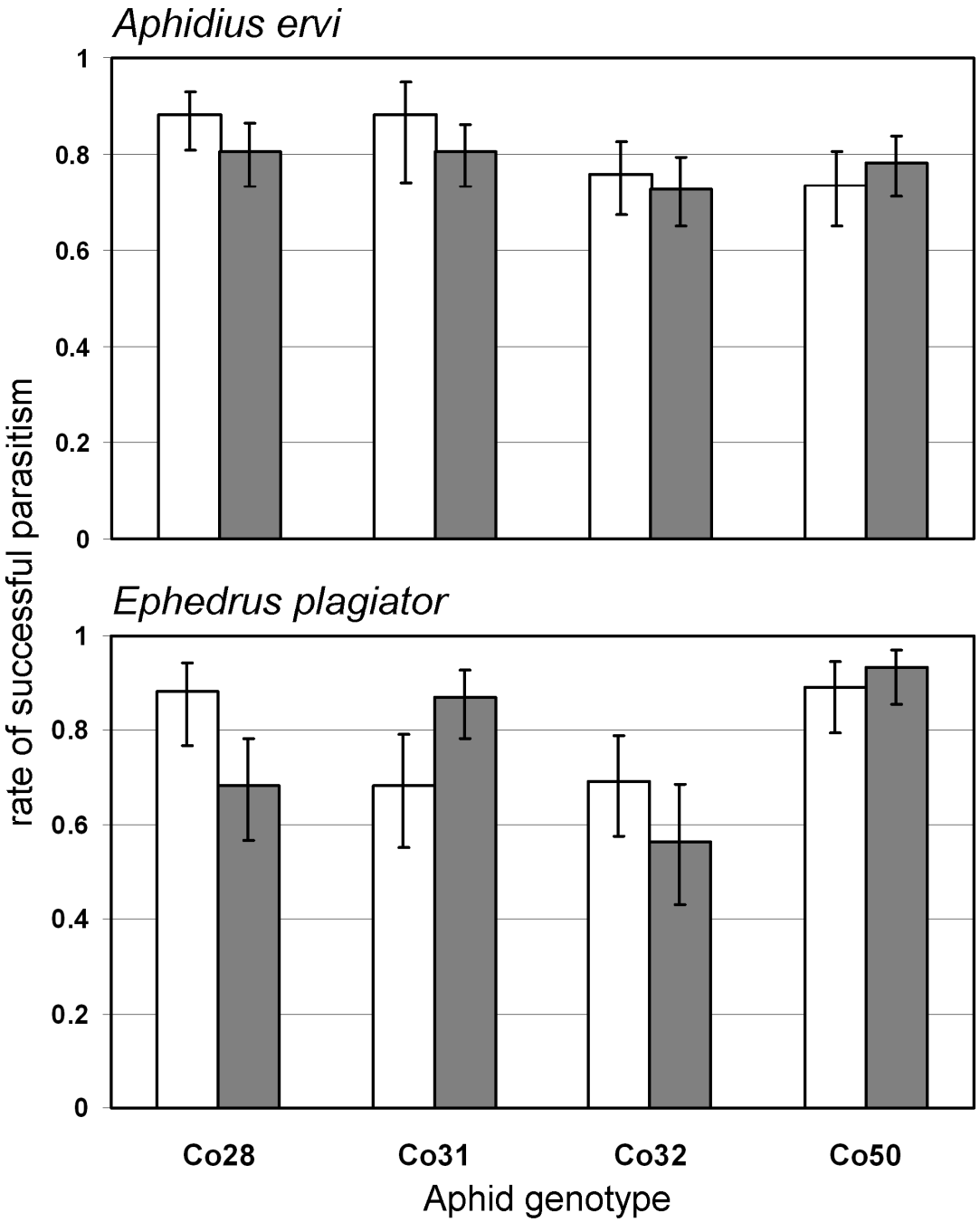

Figure 1. Rate (mean ± standard error) of successful parasitism by two parasitoid species in four clones of the grain aphid, originally free from infection with secondary symbionts (white bars) and artificially infected with *Hamiltonella defensa* from other grain aphid clones (grey bars). The means for the larger second experimental block are shown, but the errors were calculated for data from both experimental blocks.

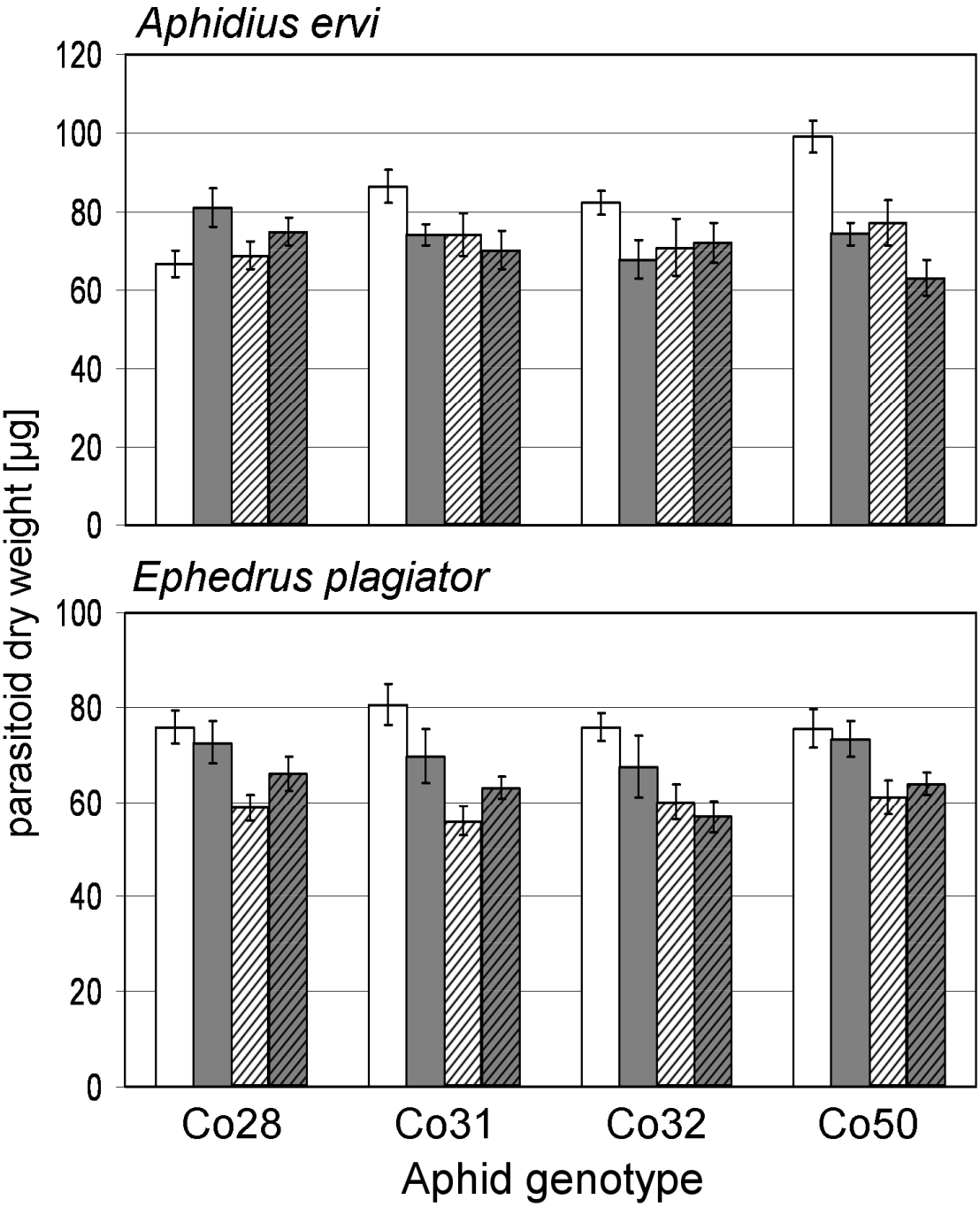

Figure 2. Dry weights (mean ± standard error) of two species of parasitoids emerging from four clones of grain aphids, originally not infected with secondary symbionts (white bars) or artificially infected with *Hamiltonella defensa* (grey bars). Data is shown for females (empty bars) and males (diagonally striped bars). Errors were computed separately for each sex of each parasitoid species.

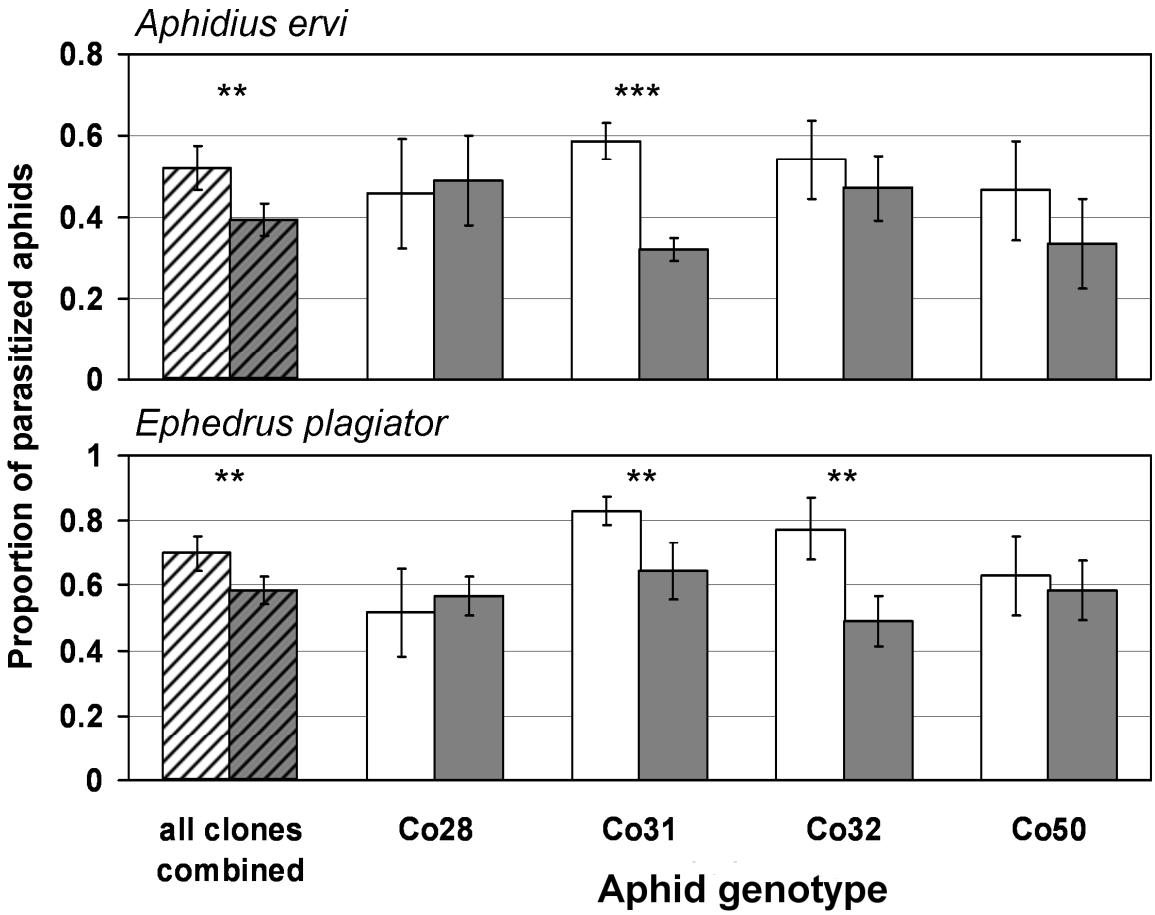

Figure 3. Proportions (mean ± standard error) of secondary symbiont-free (white bars) and *Hamiltonella*-infected (grey bars) grain aphids of four genotypes, which were found to contain at least one parasitoid egg after mixed groups of infected and non-infected aphids were exposed to single females of either parasitoid species. Data is shown for all experimental clones combined (diagonally striped bars), as well as for each of the clones. Significant differences between infected and non infected lineages are indicated with asterisks: ** ($p < 0.01$) or *** ($p < 0.001$)

Chapter 6

Mutual adaptation between hosts and symbionts in aphid symbioses

Abstract

Facultative endosymbionts of aphids have evolved very efficient modes of vertical transmission within clonal lineages of their hosts, and have limited potential for horizontal transmission between unrelated hosts. We hypothesised that the potentially long-established host-symbiont associations in naturally infected aphid clones may have resulted in mutual adaptation between host and symbiont genotypes. We tested this assumption in the grain aphid, *Sitobion avenae*, which is frequently infected with a symbiont *Hamiltonella defensa*. For each of the four experimental strains of *H. defensa*, we compared its effects on fecundity and on susceptibility to parasitoids in its original host genotype and in a novel host genotype, originally free from infection.

The symbionts did not affect the fecundity of the originally *Hamiltonella*-infected clones, but in two of the originally symbiont-free clones they had a significant effect, in one case positive and in another case negative. These effects appeared to result from the intrinsic properties of the particular symbiont and host genotypes. The presence of *H. defensa* did not affect aphid resistance to a parasitoid *Aphidius ervi*, neither in the original, nor in the novel hosts. Our results did not indicate systematic differences in response to infection between the original and novel hosts, and thus did not suggest the presence of adaptation to symbiosis in naturally symbiotic aphid genotypes. The variation in response to infection between aphid genotypes does not appear to limit the horizontal transmission of facultative endosymbionts in grain aphid populations.

Introduction

Facultative endosymbiotic bacteria of insects are not typically necessary for growth or reproduction of their hosts (Moran et al., 2008; Oliver et al., 2010). At the same time, they gather nutrients and energy from their hosts, incurring certain costs to their carriers, and do not easily transmit horizontally between host lineages (Moran et al., 2008). Thus, they can only persist and spread in host populations and communities by maximizing vertical transmission to next generations, generally by adopting one of two strategies. Firstly, facultative endosymbionts, which in most cases can be transmitted to offspring through mothers, but not through fathers (aphids are a notable exception - Moran & Dunbar, 2006), can increase the female hosts' reproduction through daughters at the expense of reproduction through sons. Such symbionts are described as reproductive manipulators (Engelstadter & Hurst, 2009; Werren et al., 2008). Alternatively, symbionts can confer fitness benefits upon their hosts, allowing their carriers to increase in frequency in populations; such symbionts are referred to as facultative mutualists (Moran et al., 2008; Oliver et al., 2010). These two strategies are not necessarily mutually exclusive (Himler et al., 2011). Symbiont strains adopting either of them are selected for reduction in virulence, or for increase in benefits to their hosts, but also for increase in transmission rate from mothers to daughters.

Indeed, as numerous studies on aphids have shown, their facultative (or secondary) endosymbionts have developed very efficient modes of vertical transmission between clonal generations of their hosts. There have been numerous reports on stability of infections over tens or hundreds of aphid generations (Chen et al., 2000; Darby & Douglas, 2003; Oliver et al., 2009). Also, there have been no reported cases of spontaneous symbiont loss in asexual genotypes originally infected

with a single symbiont strain, even though one or more symbionts in multiple infections may become lost over time (Chen & Purcell, 1997; Moran & Dunbar, 2006; Chapter 3 of this thesis), and some newly introduced symbionts may not form stable associations with novel hosts (Russell & Moran, 2005; Chapter 7 of this thesis). While phylogenetic studies indicate that facultative symbionts have successfully invaded novel aphid species (Burke et al., 2009; Russell et al., 2003; Sandstrom et al., 2001) or widespread obligatorily parthenogenetic clones of some species (Leonardo, 2004; Chapter 3 of this thesis) it appears that such spontaneous transfers are infrequent (Chen et al., 2000; Chen & Purcell, 1997; Darby & Douglas, 2003; Oliver et al., 2008). The incidence of sexual reproduction in aphids (Moran, 1992), during which symbionts can be transferred to offspring by both mothers and fathers (Moran & Dunbar, 2006), can disrupt long-term associations between host and symbiont genotypes. However, there are important differences among aphid populations and species in the incidence of sex, and in regions with mild winters sexual reproduction may be infrequent (Dedryver et al., 2001; Figueroa et al., 2005; Papura et al., 2003; Peccoud et al., 2008). Symbionts themselves can affect the induction of sexual reproduction in aphids (Leonardo & Mondor, 2006). Furthermore, aphid host plant races which may be largely reproductively isolated during the sexual generation (Peccoud et al., 2009) can be dominated by certain symbiont species and genotypes (Ferrari et al., 2004; Ferrari et al., accepted; Frantz et al., 2009), and the effects of symbionts on mate choice (Miller et al., 2010) may be further limiting the mixing between host and symbiont genotypes. Thus, there are reasons to believe that many of the naturally occurring associations between facultative endosymbiont and host genotypes have had a long history.

If host-symbiont associations are indeed stable over several generations, the partners may evolve adaptations to symbiosis with a particular clonal genotype of

host or symbiont, leading to an increase in the overall fitness of the host-symbiont assemblage (Koga et al., 2007; Vautrin & Vavre, 2009). For example, facultative symbionts may evolve the ability to produce or detoxify chemical compounds important in host biology (Koga et al., 2003), modify metabolism or nutrient uptake to cope better with nutritional requirements of the host, hide from the hosts' immune responses (Siozios et al., 2008) or even replace some of their hosts' functions, allowing them to reduce investment in costly traits such as the immune system (Gerardo et al., 2010; Oliver et al., 2010). Parallel adaptations can simultaneously evolve in clonal hosts, although at a slower rate corresponding to a much longer generation time. In such coevolved aphid-symbiont associations, the costs of infection should be limited and balanced by the benefits, and the disruption of the system by artificial removal of the symbiont should not typically benefit the host. Indeed, experimental studies revealed that the elimination of facultative endosymbionts from naturally infected aphid clones does not typically increase, and may decrease aphid fecundity, although the effects vary across host and symbiont species and genotypes, as well as across experimental host plants (Koga et al., 2007; Leonardo, 2004; McLean et al., 2011; Sakurai et al., 2005; Tsuchida et al., 2004; Chapter 4 of this thesis).

At the same time, if symbiont losses are indeed rare, then aphid clonal genotypes which are found not to harbour symbionts naturally may not have maintained, or maybe not have developed at all, potentially costly adaptations to symbiosis. Following an introduction of a symbiont into such host genotype, its potential maladaptation to coping with symbionts' metabolism, nutritional requirements and immune reactions it triggers may incur fitness penalties. The negative effects of artificial infection on fecundity of the originally symbiont-free clones under benign conditions have been reported by several authors (Chen et al., 2000; McLean et al.,

2011; Sakurai et al., 2005), even though these costs may be outweighed by the benefits symbionts can confer under stressful conditions, for example at high temperatures (Chen et al., 2000; Montllor et al., 2002; Russell & Moran, 2006) or under pressure of natural enemies (Oliver et al., 2008; Oliver et al., 2005; Oliver et al., 2003; Scarborough et al., 2005). While this has not been demonstrated, these benefits could also vary among host genotypes.

The studies listed above suggest a potential for the evolution of adaptation between host and symbiont genotypes, likely affecting symbiont dynamics within aphid populations and species, as well as the ecology and evolution of the insects. However, we are not aware of any explicit comparisons of the fitness consequences of infection with facultative endosymbionts in host genotypes they naturally originated from, and in novel, originally symbiont-free and thus supposedly non-adapted host genotypes.

In this chapter we explore whether the fitness consequences of infection with facultative endosymbionts differ between host and symbiont strains, or depend on the age of the associations. Four separate hypotheses were tested in two experiments. In Experiment 1, we tested the hypothesis A) that there are differences in the effects of infection with the same *Hamiltonella* strains in original and in novel hosts; we predicted that the fitness benefits of carrying the symbiont will be higher in the original and potentially co-adapted hosts. We also tested B) whether antibiotic treatment followed by reintroduction of the same symbiont has an effect on the fitness benefits of infection in the original host clone. We did this by introducing strains of *Hamiltonella defensa* originating from four clonal genotypes of the grain aphid, *Sitobion avenae*, into four originally non-infected clones of the same species,

as well as reintroducing them into the original host clones cured from the infection. Then, we measured fecundity and susceptibility to parasitoids of all these lineages.

As in Experiment 1 we detected significant fecundity effects of infection in two of the novel host-symbiont associations, we then asked additional questions about their nature. In Experiment 2, we tested C) whether the effects of symbionts on fecundity of their hosts change with the age of association; we predicted that fitness benefits of infection will be higher in older associations between the same host and symbiont genotypes, as the symbionts had more time to adapt to the hosts. We also tested D) whether the fecundity effects observed in the novel host-symbiont associations are specific to host genotype, symbiont genotype or result from an interaction between the two. We did this by comparing fecundities of two host clones infected with three different symbiont strains, and including in the study isofemale lines of the same host-symbiont associations, but developed either 2 months or 20 months before the experiment.

Material and Methods

Study organisms

For the study we used eight clonal genotypes of the grain aphid, *Sitobion avenae*, collected from wheat on an organic farm near Faringdon, Oxfordshire, UK in June 2008. All clones had different profiles at seven microsatellite loci (Chapter 5 of this thesis). As described in Chapters 3 and 5 of this thesis, a series of diagnostic PCRs with primers specific for different facultative symbionts revealed that four of these clones (code numbers Co08, Co23, Co26 and Co37) were infected with *Hamiltonella defensa*, and four others (Co28, Co31, Co32, Co50) were not infected with any known aphid facultative endosymbionts. All these clones originated from a

population which is regarded as composed of obligatorily asexual genotypes, and three of them (Co32, Co37 and Co50) represented microsatellite genotypes collected in multiple copies at two distant locations, and thus almost certainly obligatorily asexual (Chapters 3 and 4 of this thesis). All clones were cultured at 14±1°C in 90-mm non-vented Petri dishes with wheat leaves, which were kept fresh by inserting their stems in 2% agar (Chapter 2 of this thesis). Culturing newly injected aphids and all experimental work took place in identical dishes at 20±2°C.

The aphidiine parasitoid *Aphidius ervi*, originally obtained from Syngenta Bioline Ltd., was maintained in mass culture on a *Hamiltonella*-free grain aphid clone Co50 for not less than 15 months, or 30 parasitoid generations, before the first assays. For the experiments, synchronised parasitoids were obtained by exposing groups of 72-96 hour old Co50 aphids to individual females in Petri dishes. Then, the aphids were cultured on plants exchanged every three days for 12 days, and all parasitoid mummies were transferred to a large cage with an abundance of honey water. Parasitoid females were collected from the cage and used for the experiments typically two to four days after emergence.

The experimental aphid clones, the parasitoid culture and the culturing methods are characterized in more detail in Chapter 5 of this thesis.

Development of experimental lineages

The eight experimental genotypes, characterized in Table 1 in Chapter 5 of this thesis, were divided into four pairs, so that each *Hamiltonella*-infected clone was paired with a non-infected clone. The four pairs of clones were Co28-Co23, Co31-Co37, Co32-Co08 and Co50-Co26, with the first clone in each pair originally symbiont-free, and the second clone originally harbouring *Hamiltonella*. The

originally-infected clones from all pairs were cured from *Hamiltonella* infection by oral administration of antibiotics (Chapter 4 of this thesis). Then, in each pair, the *Hamiltonella* strain originating from the originally infected clone was introduced into the non-infected clone from the same pair (Chapter 5 of this thesis), as well as re-introduced into the original host genotype, previously cured (Figure 1). That way in each pair of clones we established five distinct lineages: 1) originally symbiont-free, not subjected to manipulations; 2) originally symbiont-free with *Hamiltonella* artificially introduced; 3) originally *Hamiltonella*-infected, not manipulated; 4) cured from the original *Hamiltonella* infection; and 5) cured from the original infection, and reinfected with the same symbiont strain. In Experiment 1, the four groups of five lineages had their fecundity, as well as their susceptibility to parasitism by *A. ervi* measured.

For Experiment 2, three *Hamiltonella* strains, originating from Co08, Co23 and Co26, were introduced into the facultative symbiont-free clones Co28 and Co50. Six generations from injection, two isofemale lines originating from different injected females from each host-symbiont association had their fecundity measured. Together with the lines representing the six newly developed host-symbiont associations, we re-tested the lines developed for Experiment 1 (including two isofemale lines of clone Co28 infected with *Hamiltonella* from Co23 and two lines of clone Co50 infected with *Hamiltonella* from Co26) approximately 20 months, or not less than 45 generations, before the experiment.

The presence/absence of *Hamiltonella* within each experimental lineage was confirmed with diagnostic PCRs on not less than three separate occasions, and the identity of all clones was also confirmed with microsatellites prior to the experiments (Chapter 5 of this thesis).

Bioassay procedures

In the fecundity assay of Experiment 1, we measured the numbers of nymphs produced within 16 days from birth by individual females from the experimental lineages. We followed a protocol described in Chapter 4 of this thesis. Briefly, the experimental aphids produced in Petri dishes within 8 hours by synchronised high-quality wingless mothers were placed in separate Petri dishes. They were transferred to fresh dishes with new plants on day 4, 7, 10, and 13 since birth, and the offspring produced during each three-day interval by each individual aphid were counted. As a measure of fecundity, we used the total number of offspring produced within 16 days. Each group of five lineages was tested in one block, but separately from other groups. Due to uneven proportions of winged morph across groups, for the analysis we used data for winged aphids only from the pair of clones Co31-Co37, and for wingless aphids only from the other three pairs of clones.

In the fecundity assay of Experiment 2, isofemale lines of each of the two clones were tested in a separate block, and for the analysis we used data for winged aphids of clone Co50, and wingless aphids of clone Co28. Unfortunately, in two lines of Co28 the proportion of wingless aphids was very low, and therefore these lines had to be excluded from analysis.

In the parasitoid resistance assay of Experiment 1, conducted following a modified protocol described in Chapter 5 of this thesis, groups of 30 juvenile (72-96 h old) aphids were exposed to synchronised naïve females of *A. ervi* in 90-mm Petri dishes with wheat plants, identical to dishes used for culturing. Parasitoids which did not start stinging aphids within 15 minutes from introduction to the experimental dishes were replaced with fresh wasps. The exposure lasted for 8 hours, and afterwards the aphids were transferred every three days to fresh plants in new Petri

dishes. During each transfer, parasitoid mummies (i.e., aphids successfully parasitized), reproducing aphids (which have avoided or resisted parasitism) and dead aphids were counted. The proportion of exposed aphids which became successfully parasitized was used as a measure of resistance. As in the fecundity assay, each group of five lineages was tested in a separate block.

Statistical analysis

The hypothesis A) that in the originally infected clones the fitness benefits of carrying symbionts are higher was tested using data collected during Experiment 1. The fecundity and parasitoid susceptibility of the experimental lineages were compared. The fecundities of the non-infected and artificially infected lineages of the two clones in each of the four groups were compared using ANOVA, as their distributions did not depart from normal. The rates of successful parasitism in the same set of lineages were compared with generalized linear modelling (GLM) assuming quasibinomial error variance in order to account for overdispersion in the data. In both analyses, the group, the original infection status, and the present infection status were included as factors. The aphid genotypes were nested within the interaction of the original infection status and group, and not included separately in the analysis. Also, each of the four groups was tested in a separate block, so that the block effect was not distinguishable from the effect of a symbiont strain tested in a particular group.

The data collected during Experiment 1 were also used for testing the hypothesis B) that the elimination and reintroduction of symbionts affect fitness of the originally infected clones. Fecundity and parasitoid susceptibility of the manipulated and non-manipulated lineages of the same four originally infected clones were compared with ANOVA and GLM, respectively, with clone and manipulation included as factors.

Data collected during Experiment 2 were used for testing the hypothesis C) that the older associations between the same hosts and symbionts (clone Co28 – *Hamiltonella* from Co23, and clone Co50 – *Hamiltonella* from Co26) would have higher fecundity. We compared fecundities of one or two isofemale lines per age (2 or 20 months) of each of the two host-symbiont associations, separately for each association. We used ANOVA with isofemale line nested within the age of the association as factors.

Data collected during Experiment 2 were also used for testing the hypothesis D) that there are differences between the three symbiont strains in their effect on fecundity of their hosts of the two experimental genotypes, Co28 and Co50. The fecundities of isofemale lines of all novel host-symbiont associations, in most cases two per association, were analyzed with ANOVA. The analysis was run separately for each clone and with symbiont strain as a factor, and line nested within symbiont strain. In cases of significant differences, the effects of different symbiont strains on fecundity of their hosts were compared with Tukey's HSD test.

All data was analyzed using the statistical package R v. 2.13.0 (R Development Core Team, 2011).

Results

A) Differences between the original and novel host clones

The comparison of the effects of symbiont infection on fecundity of the experimental clones (nested within interaction of the original infection status and group) revealed significant differences in the effect of infection on fecundity between aphid clones, as well as differences in fecundity of the clones (Table 1, Figure 2). These effects were largely driven by significant effects of infection in two clones: a

21% increase in fecundity of the originally uninfected clone Co28 following introduction of *H. defensa* originating from clone Co23, and a 27% decrease in fecundity of the originally uninfected clone Co50 following infection with the symbiont from clone Co26. Symbiont infection did not significantly increase fecundity of the other six clones, and the mean effect of infection on fecundity across all experimental clones was not significantly different from 0. The effects of *Hamiltonella* presence on fecundity did not differ between originally infected and originally uninfected clones, but interestingly the fecundity of clones originally infected with *H. defensa*, regardless of their present infection status, was significantly higher. The highly significant difference between groups of lineages resulted largely from the fact that in the group composed of clones Co31 and Co37 we measured fecundity of winged females, and in other groups - of wingless females.

Infection with *H. defensa* had no effect on the rate of successful parasitism in the same set of experimental lineages ($F_{1,90} = 1.00$, $p = 0.32$), there were also no differences between genotypes originally infected and non-infected, or between symbiont strains, in susceptibility to parasitoids ($p > 0.10$) (Figure 3).

B) Effects of manipulating symbionts in the original hosts

A comparison of lineages of the four originally infected clones which were either re-infected with the original symbiont following curing, or not subjected to manipulations, revealed no effect of manipulation on aphid fecundity ($F_{1,90} = 0.42$, $p = 0.52$), and no differences between clones in the effect of manipulation on fecundity ($F_{3,87} = 0.66$, $p = 0.58$). Similarly, symbiont manipulation had no effect on aphid susceptibility to parasitoids ($F_{1,43} = 0.42$, $p = 0.40$), again with no differences between clones ($F_{3,46} = 1.03$, $p = 0.39$).

C) Effects of the age of host-symbiont associations

We found no differences in fecundity between isofemale lines representing the same host-symbiont associations, but established either 2 months or 20 months before the experiment, neither in genotype Co28 infected with *Hamiltonella* from Co23 ($F_{2,44} = 0.36$, $p = 0.70$) nor in genotype Co50 infected with *Hamiltonella* from Co26 ($F_{3,63} = 1.03$, $p = 0.38$).

D) Comparison of the effects of the host and symbiont genotypes in novel associations

For testing hypothesis D) we used all isofemale lines representing particular host-symbiont associations, regardless of the age of association. We found no significant differences in fecundity between isofemale lines representing the same associations either in clone Co28 ($F_{5,126} = 1.01$, $p = 0.42$) or Co50 ($F_{3,103} = 0.40$, $p = 0.75$). However, there were significant differences in the effect of infection with different symbiont strains both in clone Co28 ($F_{3,106} = 3.84$, $p = 0.012$) and in Co50 ($F_{3,129} = 7.01$, $p < 0.001$) (Figure 4). Tukey's HSD tests revealed that in Co50 infection with *Hamiltonella* strains originating from Co26 and from Co08 significantly decreased aphid fecundity. Infection with neither of the symbiont strains significantly affected fecundity of clone Co28, but there were differences between lineages infected with different strains. Notably, in both host clones a lineage infected with *Hamiltonella* strain from clone Co23 had significantly higher fecundity than a lineage infected with *Hamiltonella* from Co26, and a strain from clone Co08 had an intermediate effect. This suggests that the symbiont strains may differ in their overall virulence to aphids. In clone Co50, the average effect of infection with one of the three symbiont strains on fecundity was -10.0%, as opposed to +1.2% in Co28; this difference was highly significant according to Student's paired t-test ($t = 15.80$, d.f. = 2, $p = 0.004$),

suggesting that the clonal genotypes differ in their generalized response to infection. However, the fact that the lineages of clones Co28 and Co50 were assessed in separate blocks, and that we looked at wingless females in clone Co28 but at winged females in Co50, may have contributed to the effect.

Discussion

We found no consistent effects of infection with secondary symbionts on fecundity of the experimental grain aphid clones (Hypothesis A; Figure 2). In the four originally *Hamiltonella*-infected clones, reintroducing symbionts into cured lineages had no effect on aphid performance, either in comparison to non-manipulated or to cured lineages. No costs of infection were also detected in the previous study (Chapter 4 of this thesis). This suggests that the fitness costs of infection with secondary symbionts are generally low, but also that they provide limited benefits under benign experimental conditions. However, in two out of four originally symbiont-free genotypes we detected a strong effect of infection with *H. defensa* on aphid fecundity. These effects were not consistent across clones, and included a strong decrease in fecundity in one combination of host and symbiont genotypes and an increase in another. The effects of infection with the same symbiont strains in the same hosts were less pronounced in Experiment 2 (Hypothesis D; Figure 4), indicating that their magnitude is dependent on the experimental conditions, but the direction remained the same. Also, we previously reported differences in weight of females of the parasitoid *A. ervi* emerging from infected and non-infected lineages of clones Co28 and Co50, consistent with findings of the present study: the wasps were larger when their Co28 hosts harboured symbionts, but smaller when their Co50 hosts were infected (Chapter 5 of this thesis). These results further confirm that the symbionts affect aphid productivity,

reflected either in offspring production, or in the amount of resources available to parasitoids.

In both clones tested in Experiment 2 the relative effects of infection with the three symbiont strains were similar: the lineages infected with *Hamiltonella* from Co23 had significantly higher fecundity than lineages infected with the strain from Co26, and those infected with symbiont from Co08 had intermediate fecundity (Hypothesis D; Figure 4). These results suggest inherent differences between symbiont strains in their effects on novel hosts. At the same time, the host clones differed in their response to novel infections, indicating that the fitness consequences of infections in non-adapted hosts are a resultant of symbiont properties and host tolerance. However, lineages of the two experimental clones were tested in two separate blocks, and due to large differences in the proportion of the winged morph across blocks, we were forced to use data for a different morph from each block. Thus, the interpretation of the results of Experiment 2 is complicated by the possibility of an interaction between symbiont infection and block, and particularly between infection and aphid morph. It was shown that facultative symbionts may affect the induction of the winged morph (Leonardo & Mondor, 2006), and some strains can cause developmental abnormalities specifically decreasing fitness of the winged aphids (Chapter 7 of this thesis). While no such apparent effects were observed in the present study, at this stage no definite conclusions can be made regarding inherent differences between clones, and further work is required on the responses of different genotypes to infection.

We did not detect differences in the effects of symbionts introduced into the experimental clones 20 months or 2 months prior to the fecundity assay (Hypothesis C). This may have resulted from the culturing protocol, imposing little selective

pressure on host-symbiont assemblages: every two weeks, few randomly selected aphids from each culture in stock were used to produce the next generation, and thus drift was more important than selection. Culturing the experimental lineages under conditions enforcing competition (Oliver et al., 2008) could have been more likely to lead to decrease in symbiont-related costs of infection (or increase in benefits) over time. Indeed, Koga et al. (2003; 2007) reported a 50% reduction in fecundity when a strain of *Serratia* was introduced into a symbiont-free pea aphid clone, but only a marginally significant cost when the symbiont was later removed. Their experimental aphids were cultured on potted plants, which are typically exchanged less frequently than plants in Petri dish cultures (Chapter 2 of this thesis), allowing for a build-up of aphid numbers and a stronger selection against deleterious effects of infection. Measuring changes in the fitness consequences of infection with secondary symbiont strains during culturing under different regimes, description of mechanisms causing any such changes and study of the transferability of these effects across clones could considerably extend our understanding of the evolution of arthropod symbioses. Also, we have shown that a comparison of the effects of the introduction of different symbiont strains into the same naturally infected clones can be a powerful tool for the study of the degree of symbiont and host specificity. We did not detect significant differences in fecundities of clones Co23 and Co26 cured from the original infection after they were re-infected with one of 11 *Hamiltonella* strains (Chapter 7 of this thesis). These data suggested that clonal genotypes which originally harboured facultative endosymbionts may be more tolerant to nutritional requirements of bacteria, or other negative consequences of infection, and that this tolerance is not limited to specific symbiont genotypes. However, no formal comparison of responses to infection with several different symbiont strains has yet been made between originally infected and non-infected clones.

We found no differences in fecundity between lineages originally infected with *H. defensa* and artificially reinfected with the same symbiont strain following its removal with antibiotic treatment (Hypothesis B). This was anticipated, given that previous studies demonstrated limited effects of removing gammaproteobacterial symbiont infection from their original hosts (Leonardo, 2004; McLean et al., 2011; Tsuchida et al., 2004; Chapter 4 of this thesis), and have not suggested any mechanisms for long-term residual effects of curing and infecting treatments (Koga et al., 2007; Oliver et al., 2003). However, our study is the first test of the effects of such manipulation *per se*, adding to the body of evidence for the absence of such effects and therefore validating the conclusions on the role of aphid symbionts drawn by other manipulation studies.

There are no data on the mechanisms underlying the effects of the symbiont strains on fecundity of the experimental clones. Previous studies suggested that an introduction or removal of a secondary symbiont strain can affect the densities of other endosymbionts within the same host, particularly the primary symbiont *Buchnera* (Koga et al., 2007; Montllor et al., 2002; Oliver et al., 2006; Sakurai et al., 2005), potentially harming the treated aphids. However, Oliver et al. (2003) found no differences in densities of facultative symbionts between a naturally infected and an artificially infected aphid clone, concluding that symbionts behave in a similar manner in naturally symbiotic and artificially inoculated aphids. A comparison of *Hamiltonella* and *Buchnera* densities between the experimental grain aphid clones could provide valuable information on the processes involved.

No strain of *Hamiltonella defensa*, the symbiotic bacterium named for its defensive properties reported in other aphid species (Moran et al., 2005b; Oliver et al., 2005; Oliver et al., 2003; Vorburger et al., 2009), provided any degree of

protection against the parasitoid *A. ervi*, either in its original, or novel host (Hypothesis A, Figure 3). These results confirm our previous findings on the absence of any effects of infection with the four experimental *H. defensa* strains in the novel host backgrounds on aphid susceptibility to any of the two aphidiine parasitoid species tested (Chapter 5 of this thesis). However, they also confirm that the lack of defensive properties of symbionts in the artificially infected grain aphid clones did not result from disruptions between co-adapted host and symbiont genotypes (Oliver et al., 2005). It appears that the high incidence of infections and the diversity of *H. defensa* strains in natural populations of *S. avenae* (Chapter 3 of this thesis) cannot be attributed to the provision of resistance to hymenopterous parasitoids.

In the present study, neither aphid fecundity nor susceptibility to parasitoids - two of the most important traits determining the ecological performance of an aphid clone (Oliver et al., 2008) - were consistently affected by the introduction of *H. defensa* strains. Also, our results did not indicate differences between naturally symbiotic and naturally symbiont-free clones of the grain aphid in the fitness consequences of infection with the same symbiont strains. A wider set of host genotypes, ideally of more than one species, needs to be studied before definitive conclusions regarding differences between clones in response to infection can be drawn. Such work would benefit from including different species of symbionts. A symbiont *Serratia symbiotica*, which incurs dramatic fitness costs in some of the novel hosts (Chen et al., 2000; Chen & Purcell, 1997; Koga et al., 2003) but is widespread among aphid species and host races (Burke et al., 2009; Ferrari et al., accepted; Frantz et al., 2009; Russell et al., 2003) could be a particularly useful organism for such a study. A wide-scale survey of the characteristics of different genotypes of hosts and symbionts, the assessment of the symbiont specificity, and the experimental studies of the potential for mutual adaptation between host and

symbiont genotypes are essential for understanding the dynamics of the horizontal transmission of facultative endosymbionts, which seems to play a major yet still poorly understood role in insect ecology and evolution.

Tables and Figures

Factor(s)	d.f.	S.S.	% Var.	F	p
Group (incl. Block)	3	9728.9	54.51	100.1741	**< 0.001**
Original infection	1	753.3	4.22	23.2702	**< 0.001**
Present infection	1	10.3	0.06	0.3179	0.573
Original infection × Present infection	1	0.2	0.00	0.0049	0.944
Group × Present infection	3	175.6	0.98	1.8078	0.147
Original infection × Group (= Clone)	3	619.8	3.47	6.3822	**< 0.001**
Original infection × Group × Present infection	3	798.6	4.47	8.2225	**< 0.001**
Residual	178	5762.4			

Table 1. Effects of infection with *Hamiltonella* on the number of offspring produced by isolated aphids over 16 days. Experimental clones were divided into groups, each consisting of a *Hamiltonella*-infected and a non-infected lineage of each of the two clones, one of which was naturally symbiotic and another was originally symbiont-free. Terms were added in the order shown in the table and the change in degrees of freedom and the sum of squares (the raw figure and as a percentage of the total) are given along with the F statistic and its associated probability (in bold when < 0.05).

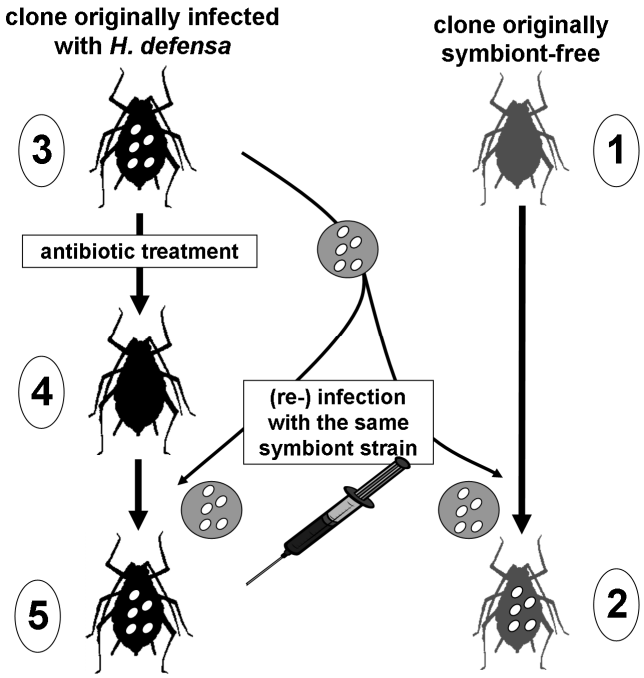

Figure 1. Development of groups of five experimental lineages in each of the four pairs of clones. Symbiont strain originating from the originally infected clone in each pair was introduced into the originally symbiont-free clone, as well as into the

original host clone previously cured from infection. Numbers next to aphid icons correspond to numbers in the text.

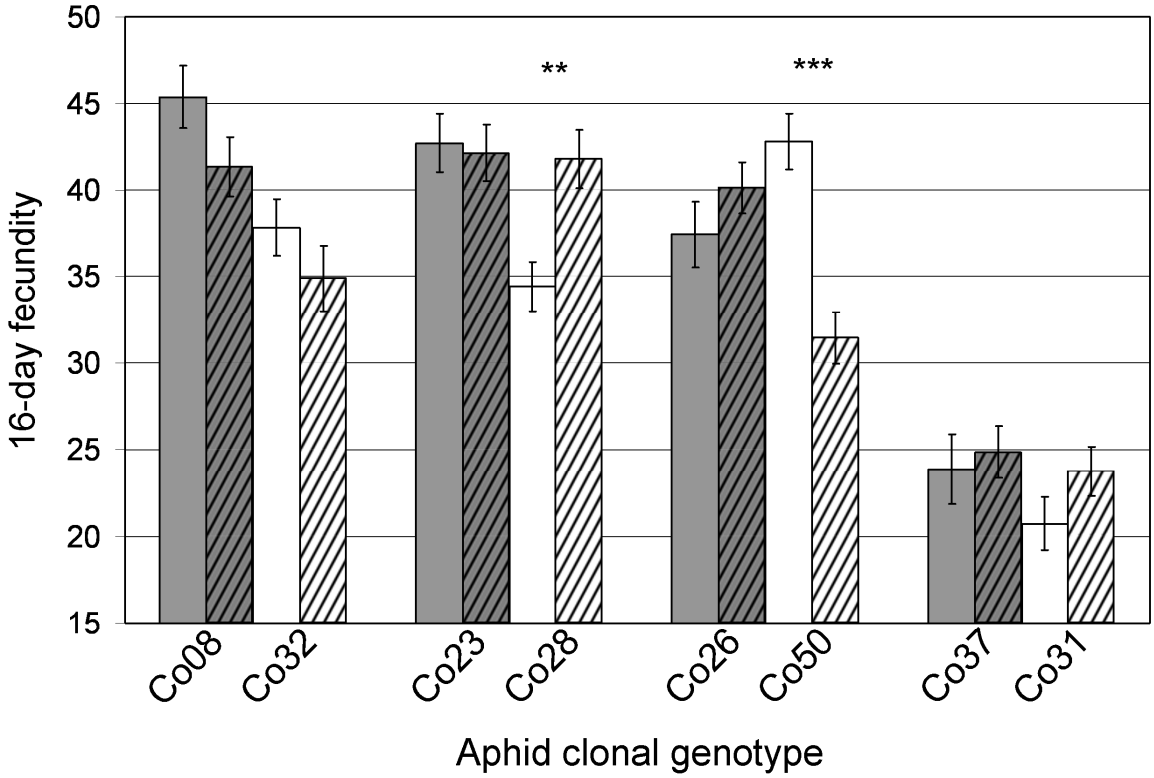

Figure 2. Fecundity (mean ± S.E.) of lineages of eight grain aphid clones divided into four groups. Each group consist of a lineage free from infection with secondary symbionts (empty bars) and a lineage *Hamiltonella*-infected (diagonally striped bars) of each of the two clones, one of which originally harboured *Hamiltonella* (grey bars) and another was symbiont-free (white bars). In each group, the same symbiont strain, originating from the originally infected clone, was tested in both genetic backgrounds. Significant differences between uninfected non infected lineages of the same clone, according to a post-hoc HSD test, are marked with asterisks: ** ($p < 0.01$) or *** ($p < 0.001$).

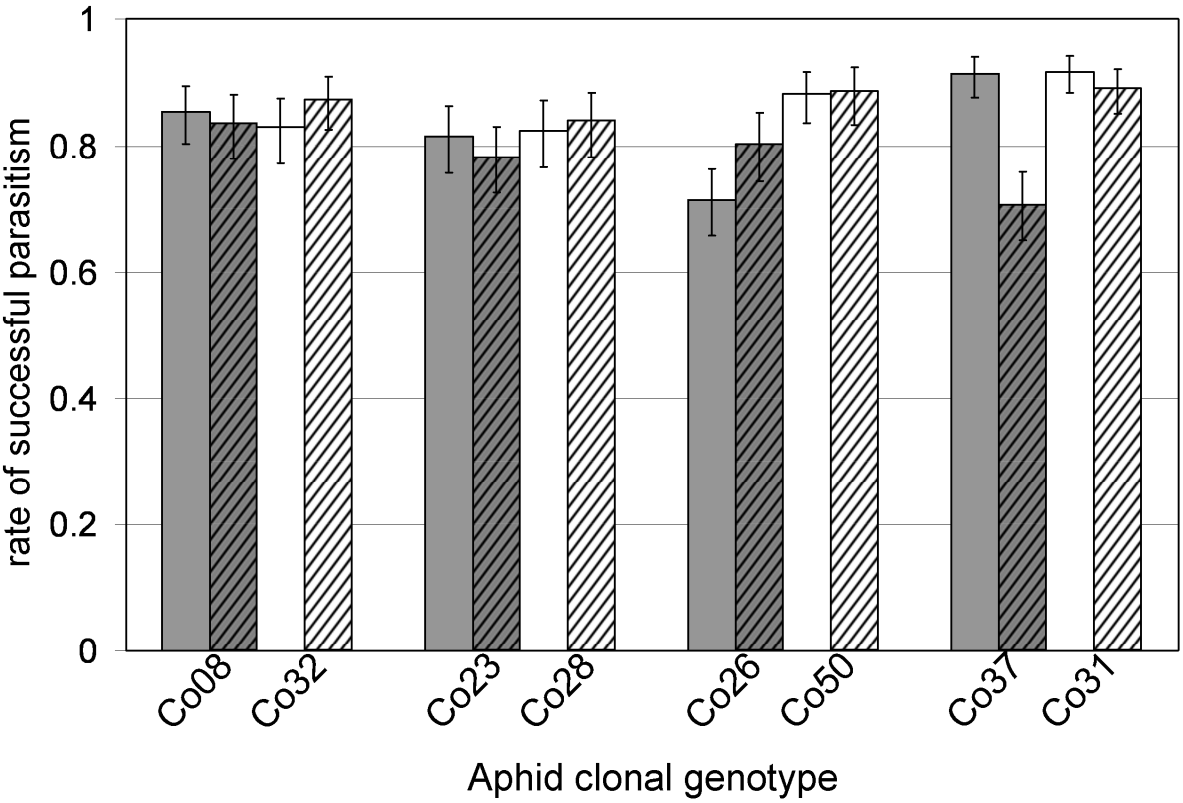

Figure 3. Susceptibility (mean ± S.E.) to a parasitoid *Aphidius ervi* of lineages of eight grain aphid clones divided into four groups. Each group consist of a lineage free from infection with secondary symbionts (empty bars) and a lineage *Hamiltonella*-infected (diagonally striped bars) of each of the two clones, one of which originally harboured *Hamiltonella* (grey bars) and another was symbiont-free (white bars). In each group, the same symbiont strain, originating from the originally infected clone, was tested in both genetic backgrounds.

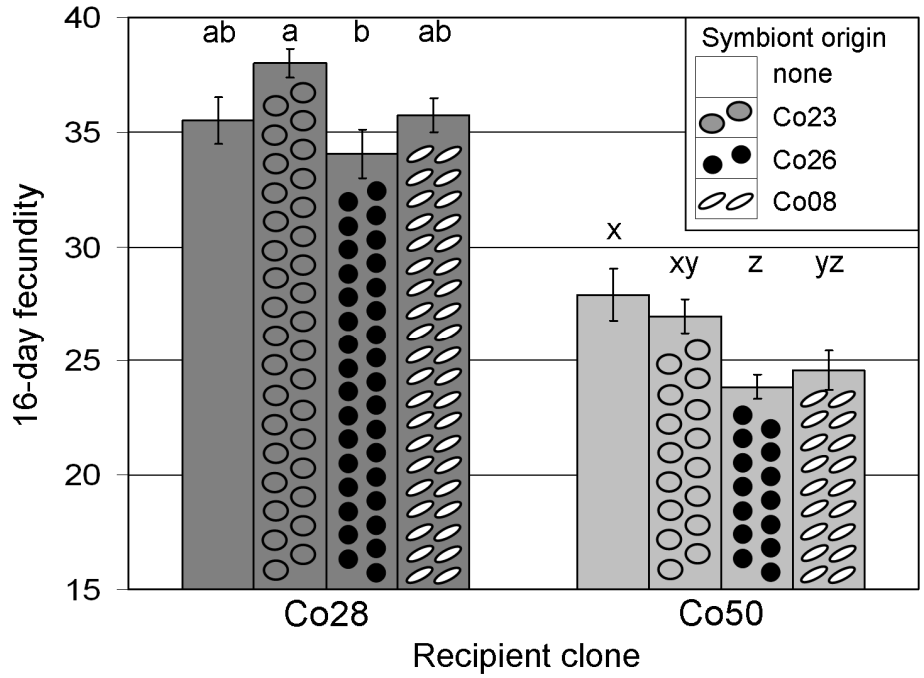

Figure 4. Fecundity (mean ± S.E.) of two aphid clones, either symbiont-free or infected with one of three Hamiltonella strains. Letters identify lineages not significantly different from others within the same comparison according to Tukey's HSD test with 95% confidence intervals.

Chapter 7

Assessing the potential for horizontal transmission of facultative endosymbionts of aphids

Abstract

Facultative endosymbionts of aphids can have important effects on the life history traits and fitness of their hosts. Phylogenetic and experimental studies have revealed that these symbionts are capable of transferring to and establishing successfully in novel host clonal genotypes, including those distantly related to their original hosts, and that the beneficial effects of infection can be expressed in the novel hosts. However, there have been no attempts to measure the barriers in symbiont transmission, or systematically compare the fitness effects of infection following symbiont transmission within or between species.

We tested whether the establishment rate of symbionts artificially introduced into new aphid genetic backgrounds and their effects on aphid life history traits vary with phylogenetic distance between the original and the novel host. We did this by introducing seventeen strains of the facultative endosymbionts *Hamiltonella defensa* and *Regiella insecticola*, originating from five aphid species, into two clonal genotypes of the grain aphid, *Sitobion avenae*. For all newly introduced symbiont strains, we quantified their establishment rate in the novel hosts and measured the effects of the novel infections on aphid fecundity and resistance to two species of natural enemies.

Symbiont strains originating from conspecific donors established easily and formed stable infections in the novel hosts, but strains originating from more distantly related donor aphids tended to establish less easily, and in some cases formed less stable infections. Once established, most symbionts had no effect on aphid fecundity, regardless of their origin. Unexpectedly, none of the symbiont strains significantly decreased the rate of successful parasitism in aphids exposed to

parasitoids, an effect for which pea aphid *Hamiltonella* is renowned. However, three of the symbiont strains conferred resistance against a pathogenic fungus.

Our data indicate the presence of barriers to symbiont transmission, while suggesting that a strain which succeeds in establishing in a novel host may well persist and spread in a host population. These results support the view of aphid secondary symbionts as a eukaryote horizontal gene pool.

Introduction

The biology of all animals is influenced by interactions with bacteria (Moran, 2006; Moran et al., 2008). Microbes can form a wide range of associations with their hosts, ranging in their incidence from obligatory to occassional, and in their effects from mutualisms indispensable for both partners to pathogenicity. Endosymbiosis, the most intimate of these host-symbiont associations, is defined as a condition when one organism stably resides within the body cavity or cells of another organism (Wernegreen, 2004). Endosymbioses have received much attention in insects, and particularly in aphids (Baumann, 2005; Moran et al., 2008; Oliver et al., 2010).

Two main categories of endosymbionts, differing considerably in their effects and in co-evolutionary history with their hosts, can be distinguished in aphids, but also in many other insects (Baumann, 2005; Moran et al., 2008). Nearly all aphids harbour the primary (or obligatory) endosymbiont *Buchnera aphidicola*, which synthesises essential nutrients deficient in plant sap, the sole aphid diet, and is thus essential for the survival and reproduction of their hosts (Douglas, 1998). *Buchnera* originally established in the common ancestor of the Aphididae not less than 100 millions years ago, and since then it has been transmitted strictly vertically, from mothers to daughters, coevolving with their hosts and enabling their adaptation and diversification onto host plants (Moran et al., 1993; Moran et al., 1995).

Aphid endosymbiotic bacteria from the second group, referred to as secondary or facultative endosymbionts, are not essential to their hosts, and are patchily distributed across clones and species (Burke et al., 2009; Oliver et al., 2010; Russell et al., 2003; Sandstrom et al., 2001; Tsuchida et al., 2002). For their growth and multiplication, facultative endosymbionts require nutrients from their hosts, and thus

infection with them is believed to carry certain costs. In order to persist and spread in insect populations (Moran et al., 2008), facultative endosymbionts compensate for these costs by providing fitness benefits which allow their carriers to increase in frequency in populations (Oliver et al., 2010). The best-known example of an aphid facultative mutualist is a gammaproteobacterium *Hamiltonella defensa*, which has been reported from clones of many species of aphids and allied groups (Degnan & Moran, 2008b; Haynes et al., 2003; Moran et al., 2005b; Russell et al., 2003; Sandstrom et al., 2001), and has been shown to confer protection against hymenopterous parasitoids to their pea aphid and black bean aphid hosts (Oliver et al., 2005; Oliver et al., 2003; Vorburger et al., 2009; but see chapter 5 of this thesis). A closely related bacterium, *Regiella insecticola*, also found in a wide range of aphid species, can protect pea aphids against an entomopathogenic fungus *Pandora neoaphidis* (Ferrari et al., 2004; Scarborough et al., 2005), another important natural enemy of aphids (Feng et al., 1991). Some *Regiella* strains also confer resistance to parasitoids (Nyabuga et al., 2010; Vorburger et al., 2010), contribute to host plant specialisation (Tsuchida et al., 2004; but see McLean et al., 2011), and affect induction of winged morph and of sexual generation (Leonardo & Mondor, 2006). Not less than eight other species of facultative endosymbionts have been reported from aphids; the fitness consequences of infection with some of them are already known (Oliver et al., 2010 and references therein).

Facultative endosymbionts are transferred maternally with very high fidelity (Darby & Douglas, 2003; Oliver et al., 2009; Russell & Moran, 2005). However, phylogenies of facultative endosymbionts are generally incongruent with phylogenies of their hosts, indicating that under natural conditions the symbionts are capable of transferring horizontally between matrilines and species and establishing successfully in novel hosts (Burke et al., 2009; Russell et al., 2003; Sandstrom et al.,

2001; Chapter 3 of this thesis). The mechanisms of these horizontal transfers have not been unequivocally determined. Aphids may acquire endosymbiont infections orally, by feeding on artificial diets with bacterial cells added (Darby & Douglas, 2003), but attempts to induce spontaneous transfer of endosymbionts between clones feeding on common host plants proved unsuccessful (Chen et al., 2000; Chen & Purcell, 1997; Darby & Douglas, 2003). While Oliver et al. (2008) noted one possible case of such transfer in a high-density population cage, they were unable to provide conclusive evidence. Symbionts could also be transmitted by natural enemies, possibly on mouthparts or on the tip of ovipositor of a predator or a parasitoid which injure, but do not kill, novel hosts (Jaenike et al., 2007). More complex mechanism may also be involved, including symbionts invading parasitoids developing within, or feeding on, infected hosts and subsequently being introduced into novel hosts (Chiel et al., 2009). Finally, symbionts may be transferred during the sexual generation by males, which occasionally attempt matings with heterospecific females (Moran & Dunbar, 2006). While all these and potentially other processes may play a role in symbiont horizontal transmission in natural populations, there are no data regarding their incidence. However, it is clear that facultative symbionts can be easily transferred artificially under laboratory conditions. Microinjections of haemolymph collected with glass microcapillary from infected donor aphids into juveniles of a recipient clone have been repeatedly shown to result in a highly successful introduction of the symbionts into novel host, which can then be maternally transferred to offspring of the injected aphid (e.g., Chen & Purcell, 1997; Russell & Moran, 2005).

Entry into the haemocoel of a new host is only the first step of a successful invasion of the novel clone by a symbiont (Bright & Bulgheresi, 2010). Newly introduced symbionts need to avoid immune response of the host, increase in

numbers and migrate to and invade embryos developing within the mothers. While the aphid immune system is not well developed and may not respond strongly to the introduction of bacteria (Gerardo et al., 2010), short lifespans and high mortality of aphids in the field mean that the symbionts may not be given enough time to invade the embryos. Even following successful transmission to the second generation of a novel host clone, symbionts face the challenge of transmission to further generations. While artificially introduced symbionts originating from donors conspecific or closely related to the recipient clone are generally transferred vertically with high fidelity (Chen et al., 2000; Chen & Purcell, 1997; Darby & Douglas, 2003; Oliver et al., 2005; Oliver et al., 2003; Russell & Moran, 2005), symbionts originating from more distantly related hosts may form associations less stable over the consecutive generations (Russell & Moran, 2005).

Under natural conditions, any host newly infected with a symbiont which is primarily vertically transmitted needs to remain competitive against other lineages of the same and of other species if the infection is to persist. Thus, novel endosymbionts which are costly to maintain would not normally spread in the population, and soon go extinct. Maintenance costs can be offset by symbiont-conferred benefits, though, some of which have been discussed in the previous paragraphs. The value of many of these benefits depends on the environment, and is bound to vary across habitats and seasons (Chen et al., 2000; Oliver et al., 2008). For example, resistance to high temperatures conferred by *Serratia symbiotica* and *Rickettsia* symbionts (Chen et al., 2000; Montllor et al., 2002; Russell & Moran, 2006) would be particularly beneficial during a hot summer, and the value of symbiont-conferred protection to natural enemies (Oliver et al., 2005; Oliver et al., 2003; Scarborough et al., 2005) is determined by the enemies' abundance and diversity. Fitness consequences of infection can also vary across symbiont genotypes (Chapters 6 and 9 of this thesis).

Thus, a lineage newly infected with a vertically transmitted symbiont would only be able to increase in frequency in a population if the short-term net fitness gain resulting from infection is greater than zero, and persist if the long-term gain, measured across the seasons, is at least zero. Studies reporting the effects of artificial introduction or removal of symbionts tended to focus on individual life history traits measured under carefully controlled conditions (Oliver et al., 2005; Oliver et al., 2003; Russell & Moran, 2006; Scarborough et al., 2005). However, there has been little comprehensive data on how symbionts influence various life history traits of their aphid hosts, and how those changes affect the host fitness under highly variable natural conditions (Chen et al., 2000; Darby et al., 2003; Oliver et al., 2008; Tsuchida et al., 2002).

As outlined above, symbionts need to succeed at several steps before they successfully invade a novel host clone or a novel host species (Bright & Bulgheresi, 2010). These barriers to symbiont establishment in novel hosts can be classified as (i) ecological - resulting from limited opportunities for symbionts to enter into haemocoels of novel hosts, (ii) physiological - determined by the symbiont's ability to multiply in novel hosts and to be transmitted vertically across host generations, and (iii) fitness - determined by the effects of the novel infection on life history traits of the host, and on its competitive abilities. The first of these barriers should largely depend on the ecology of aphids, and possibly of their natural enemies, but could also be affected by symbiont traits (Chiel et al., 2009). The physiological barrier and the fitness effects of novel infections should be largely determined by the symbiont genotype, but also the host genotype and the interaction between the two (Chapter 6 of this thesis). Generally, the change in the internal environment experienced by a symbiont transferred between host clones should be correlated with the phylogenetic distance between the original and the novel host, and it can be expected that a

symbiont would perform better in a more familiar environment. Thus, it is possible that the physiological barrier in establishment for a new symbiont to overcome is less following a transfer between more closely related host species. Also, the net fitness benefits of the novel infection could be typically higher in a more closely related novel host (Chen et al., 2000; Russell & Moran, 2005). However, this has not been tested. There are virtually no data on how often symbionts succeed in overcoming these barriers, and succeed in establishing and spreading in novel host populations under natural conditions. Such information is crucial for understanding the ecology and evolution of facultative symbionts and their aphid hosts.

The present study attempted to explain some of the processes limiting the horizontal transmission of facultative endosymbionts of aphids. We tested the hypothesis that symbionts could be transferred more easily between conspecific hosts than between more distantly related ones, and that the net fitness gain of infection with a novel symbiont would be higher when the symbiont originates from a more closely related host. We did this by injecting haemolymph from 13 *Hamiltonella*-infected and 4 *Regiella*-infected clones of five aphid species into two common genotypes of the grain aphid, *Sitobion avenae*, which had had their original symbionts removed. We quantified the symbiont establishment rate and assessed the stability of infections in the new host clones. We also measured the effects of the symbionts on their novel hosts' fecundity and susceptibility to natural enemies.

Material and Methods

Experimental aphid clones

The aphid clones used in the study are listed and briefly characterized in Table 1. All cereal aphid and pea aphid clones were collected in the United Kingdom between

2003 and 2009, and identified to species level under a binocular microscope using dichotomous keys (Blackman & Eastop, 2000; Heie, 1994). The two black bean aphid clones were originally collected in Switzerland in 2006 (Vorburger et al., 2009). In order to confirm the identifications of all aphid clones used in the experiment we amplified and sequenced the mitochondrial cytochrome oxidase c subunit I (COI) gene, the standard DNA barcode region for animals, shown to be a powerful tool for the discrimination of aphid species (Foottit et al., 2008). DNA from field-collected adult aphids used to establish laboratory clonal lines was extracted with DNeasy Blood & Tissue Kit (Qiagen) following a protocol provided by the manufacturer. Then, primers LepF and LepR were used to amplify a part of the COI gene (Foottit et al., 2008). The amplicons were sequenced using Big Dye Terminator v. 3.1. (Applied Biosystems) Sequence traces for each sample were individually assembled and edited using Codon Code v. 2.06. The alignment, which in addition to COI sequences of the experimental clones included the sequence of *Eulachnus rileyi* (subfamily Lachninae; Genbank reference EU701667) as an outgroup, was generated using ClustalW algorithm and tested to find the best-fitting models of evolution, GTR with gamma distribution, using software MEGA version 5 (Tamura et al., 2011). Phylogeny was built using phyML v. 3.0 (Guindon et al., 2010), and bootstrap analysis carried out with 1000 replications. The sequences were also compared using BLAST search with sequences available in Genbank.

The aphids were cultured under conditions ensuring indefinite asexual reproduction, at 14±1°C, 70±15% humidity and at L16:D8 light regime. *S. avenae* clones were kept in 90-mm Petri dishes with approx. 10-day old wheat plants and 4-week old cocksfoot (*Dactylis glomerata*) plants, which were kept fresh by having their stems placed in 2% agar. *S. fragariae* and *Utamphorophora sp.* were cultured in dishes with cocksfoot only, while *A. pisum* and *A. fabae* clones were cultured on

leaves of broad beans, *Vicia faba* (cv. The Sutton), which also had their petioles immersed in agar. The dishes were typically exchanged every fourteen days for cereal aphids and every seven days for aphids feeding on beans. All experiments were conducted at a temperature of 20±2°C, to which the aphids had been acclimatized for at least three generations in Petri dishes exchanged every 3-4 days.

Experimental symbiont strains

DNA samples of the experimental aphid clones were screened for the presence of seven of the known aphid secondary symbionts, including *Hamiltonella defensa*, *Regiella insecticola*, *Serratia symbiotica*, X-type, *Rickettsia*, *Spiroplasma* and *Rickettsiella*. For the screening, we used polymerase chain reactions (PCR) using the Biomix reaction mix (Bioline Inc., USA) and symbiont-specific primers for the ribosomal 16S gene (McLean et al., 2011; Tsuchida et al., 2010). The identity of the symbionts was confirmed by sequencing of the PCR product.

For all *Hamiltonella*-infected donor clones, we also attempted to amplify five structural genes of APSE bacteriophage: P3, P35, P41, P45 and P51, using primers published by Degnan and Moran (2008b), as well as the four integration sites from integrated and non-integrated phages (Degnan & Moran, 2008a). All products were sequenced, but the molecular data will be discussed elsewhere (Chapter 8 of this thesis). Towards the end of the project, we also attempted to amplify APSE structural gene P3 and the attachment site *attP* from DNA samples of aphids isolated from the stock cultures of the donor clones and the experimental lineages. At the time, all of them have been in culture for at least 20 months.

Developing novel symbioses

The experimental symbiont strains were introduced into two genotypes of *S. avenae*, Co23 and Co26, which had been previously treated with antibiotics in order to remove their original infection with *H. defensa* (Chapter 4 of this thesis). Aphids from these two cured recipient lineages, labelled Co23X and Co26X, were injected with haemolymph from 17 *Hamiltonella*- or *Regiella*-infected clones of five aphid species (Table 1), including the original non-cured Co23 and Co26. Haemolymph, collected from 4th instar or adult donor aphids with a glass needle, was introduced into first-instar recipient aphids. We did not attempt to quantify the volume of haemolymph injected (Chapter 2 of this thesis). Typically, around 10 aphids from each clone were injected with haemolymph from each donor, although in some cases the number of injected aphids was considerably higher. Injections were generally done in series of three or four novel host-symbiont associations per day, separated by days or weeks. By the time the first injections were done, I had had considerable experience using this technique, and a record of introducing symbionts into novel hosts with success rate close to 100% (Chapters 5-6 of this thesis).

Following injections, the recipient aphids were cultured at 20±2°C on plants in Petri dishes, exchanged every three days. Fourteen days after the injections, the survivors were isolated, and the offspring they subsequently produced retained. When the first generation post-injection reached adulthood, two or three offspring of each injected mother were isolated. After they had reproduced, they were checked for the presence of symbionts using diagnostic PCRs. The second-generation offspring produced by mothers found to be infected were used to start the experimental lines. Generally, the lines were put through a single-female generation and reassessed for the presence of symbionts in the fourth generation from injection. On average, offspring of 7.7 (range 4-17) aphids reproducing after the 14th day from

injection from each novel clone-symbiont combination were assessed for infection. Typically, six lines were kept until reassessment in the 4th generation, and four lines for the following months. The lines were also put through a single-female generation and reassessed for the presence of symbionts, whose identity was additionally confirmed with partial sequencing of 16S rRNA gene, two or three generations before starting any experiments.

Effects of symbionts on aphid fecundity

The fitness effects of infections with novel symbiont strains were assessed not less than eight generations after successful introduction of the symbionts, and typically around the 15th-20th generation.

Fecundity assays were conducted following a protocol based on the one presented in Chapter 4 of this thesis. Initially, the aphids produced in Petri dishes within 8 hours by synchronised high-quality wingless mothers were kept in groups of six in dishes exchanged every three days. On the 7th day after birth, before they started reproducing, young winged adults or 4th instar juveniles with wing buds were randomly selected from across the dishes, and kept isolated in Petri dishes exchanged every three days. Offspring produced by each individual aphid during each three-day interval were counted, and the total number of offspring produced during the experiment was used as a measure of fecundity. Lineages of each clone were tested in a single block, with an average of 15.6 (range: 7-16) females from each lineage tested.

Resistance to natural enemies

The experimental lineages of two clones were tested for resistance to two species of important natural enemies of cereal aphids, a hymenopterous parasitoid *Aphidius ervi* and an enthomopathogenic fungus *Pandora neoaphidis*.

Females of *A. ervi* used in this study originated from a culture started from wasps obtained from a commercial supplier, and maintained in mass culture on a secondary symbiont-free grain aphid clone for 20 months before the first block of the resistance assay (Chapter 5 of this thesis). Females used for the experiments emerged from synchronised symbiont-free aphids exposed in Petri dishes under conditions resembling the experimental setup. Susceptibility of the experimental lineages to the parasitoid was assessed following a modified protocol presented in Chapter 5 of this thesis. Briefly, groups of thirty 72 to 96-hour-old nymphs were exposed in Petri dishes to synchronised parasitoid females. Following 8-hour exposure, the aphids were transferred to fresh Petri dishes every three days for 15 days, and parasitoids pupating within aphids (forming characteristic “mummies”), as well as reproducing and dead aphids, were counted during each transfer. All mummies were collected and kept in Petri dishes for two additional weeks, so the parasitoid emergence rate could be estimated. As a measure of susceptibility, we used the proportion of the exposed aphids in which the parasitoids successfully developed into pupae. We also compared across the experimental aphid lineages the proportions of parasitoids which successfully emerged from the cocoons. Lineages of clone Co23X were tested in two blocks, with an average of 6.6 (range 5-8) replicates of 30 aphids per lineage. Lineages of clone Co26X were tested in three blocks, with an average of 11.3 (range 5-16) replicates per lineage.

Strain X4 of *P. neoaphidis* was provided by Rothamsted Research, UK. It had been maintained *in vivo* on a secondary symbiont-free and highly susceptible pea aphid clone 145. Cadavers of adult aphids killed by the fungus were maintained refrigerated at low humidity conditions for not more than four weeks before they were used for the experiment (Chapter 9 of this thesis). The protocol for testing aphid susceptibility to *Pandora* was adapted from the study by Ferrari et al. (2001), and described in more detail in Chapter 9 of this thesis. Briefly, sporulating aphid cadavers were placed over cylinders approx. 25mm high and of 15mm diameter, each containing 20 young wingless adult aphids. After 90 minutes, the aphids were transferred in groups of 10 to Petri dishes, and kept at humidity approaching 100% for 24 hours, and then at reduced humidity for another week. Aphid survival was assessed daily, and any dead aphids were transferred to the surface of agar, where those successfully colonized and killed by the fungus sporulated within 24 hours. As a measure of fungal susceptibility we used the proportion of the exposed aphids which sporulated. We also analyzed the survival of the exposed aphids. Lineages of both clones were tested across three (Co23X) or five (Co26X) incomplete blocks, with an average of 5.8 (range 4-10) replicates of 20 aphids per lineage.

Fitness consequences of multiple infection with symbionts from clone N341

The main parasitoid susceptibility experiment did not indicate that any of the symbiont strains used conferred a substantial degree of protection to aphids. Therefore, in order to validate the protocol of the parasitoid resistance assay by obtaining a positive control, but also to compare the effects of infection with resistance-conferring symbionts in a pea aphid and in a grain aphid background, we made additional injections. As a donor, we used the pea aphid clone N341 stably infected with three distinct gammaproteobacterial symbionts, *Hamiltonella*, X-type

and *Rickettsiella*, and shown to be highly resistant to parasitoids (J. Ferrari et al., unpublished data). The symbionts from N341 were introduced into a facultative symbiont-free pea aphid clone 145 and into a grain aphid lineage Co26X. We monitored symbiont establishment rate, the stability of infections in the newly developed lineages and their viability, and measured their fecundity and susceptibility to *A. ervi* following the protocols described above.

Statistical analysis

Experimental data was analyzed using generalized linear modelling techniques in the statistical software R v. 2.13.0 (R Development Core Team, 2011). Analyzes were carried out separately for each of the two recipient clones, and with symbiont strain and block as factors. We assumed Poisson error variance for the analysis of fecundity data. To account for overdispersion in the proportional data, we assumed quasibinomial error variance when comparing symbiont establishment rates following intra- and interspecific transfers, proportions of aphids exposed to parasitoids which became mummified or from which adult parasitoids emerged, parasitoid mortality at mummy stage, or aphid sporulation rate or survival following exposure to the pathogenic fungus.

Results

The experimental aphid clones

The sequences of the COI gene of the experimental aphid clones yielded a well-resolved and well-supported phylogeny (Figure 1). The topology revealed that the experimental clones formed five distinct groups corresponding to species. Comparison of the sequences with reference sequences available in Genbank confirmed our morphology-based identifications of four species. However, the

sequence of clone Up16 did not match our identification, but was 98% identical to the sequence of *Utamphorophora humboldti*. At the time the stock culture of the clone had been lost, and as we were unable to attempt to re-identify the clone, we classified it as *Utamphorophora sp*.

Characterization of facultative endosymbionts

The list of symbiont strains infecting the experimental donor clones is provided in Table 1. 16S sequences of all symbiont strains used in this study were at least 99% identical to the sequences of other strains of the same symbiont species which infect aphids, which had been previously deposited in Genbank by other authors.

In all *Hamiltonella*-infected donor clones, we successfully amplified and sequenced the five structural genes of APSE. Furthermore, we succeeded in amplifying and sequencing the regions spanning the phage attachment sites on bacterial and APSE chromosome: loci *attP* and *attR* from all sixteen clones, *attL* from eleven clones, and *attB* from nine of the typed clones. These data indicate that the phages infecting all of the examined *Hamiltonella* strains exist both in the circular and integrated form in the same hosts, and are thus functional and capable of having the complete life cycle (Degnan & Moran, 2008a). This is discussed in Chapter 8 of this thesis, together with the sequence data and phylogenetic relationships between the symbiont strains. APSE genes P3 and *attP* were also successfully amplified, and in some cases sequenced, from DNA samples of all *Hamiltonella*-infected donor clones and experimental lineages extracted towards the end of the study. We have not recorded a single case of apparent loss of the phage.

Symbiont establishment rate and stability of novel infections

We introduced haemolymph from seventeen clones of five aphid species, all infected with one or two species of facultative endosymbionts, into two recipient clones of *S. avenae*, Co23X and Co26X (Table 1). The rate of successful initial infection was high: on average, 68% of the injected aphids reproduced after the 14th day from injection, and 81% of them produced infected offspring. Mortality of the injected aphids varied between different series of injections, but with no apparent differences between recipient clones, symbiont or donor species. In both recipient clones the proportion of females which produced infected offspring was significantly higher following injection with haemolymph from *S. avenae* than from singly infected heterospecific clones ($F_{1,13} = 14.97$, $p = 0.002$ in clone Co23X; $F_{1,13} = 14.86$, $p = 0.002$ in Co26X) (Figure 2), although the validity of such comparison can be disputed because of non-random division of injections into series.

The symbiont strains, once introduced into the novel hosts, differed in the stability of infections (Figure 2). We did not record cases of symbiont loss during at least 14 months of culturing in lineages infected with bacteria originating from *S. avenae* or *Utamphorophora sp.* There were single cases of symbiont loss in lineages injected with symbionts originating from singly-infected clones of *S. fragariae* and *A. pisum.* In contrast, infections with two *H. defensa* strains originating from *A. fabae* were unstable. Close monitoring of the second batch of aphids injected with symbionts from *Aphis fabae*, which included testing up to 30 individuals every second generation, allowed us to estimate the symbiont loss rate within one generation, or during a single transfer from mothers to daughters, at approximately 25% in aphids cultured at 20°C, and over 60% in aphids cultured at 14°C. The symbiont loss rate did not appear to decrease in later aphid generations from injections. We succeeded in maintaining symbionts in some aphid lineages for as

long as 10 generations, but ultimately all infections with *H. defensa* from *A. fabae* were lost.

Injections with haemolymph from doubly infected donor clones frequently led to only one of the symbionts forming stable infections (Figure 2). In aphids injected with haemolymph containing *Hamiltonella* and *Regiella* from clone Up11, *Hamiltonella* had a higher initial establishment rate, and *Regiella* disappeared from the doubly-infected lineages by the 4th generation from injections. Similarly, introduction of *Spiroplasma* and *Regiella* from clone 185 led to *Spiroplasma* initially establishing at a higher rate, and *Regiella* disappearing by 8th generation from all doubly infected lineages. In contrast, both *Hamiltonella* and *Rickettsia* from clone 208 successfully transmitted to all tested offspring of the injected aphids, and no cases of loss of either symbiont were recorded.

Effects of infection on aphid fecundity

In clone Co23X, there were significant differences between lineages in the effect of symbionts on fecundity (χ^2= 33.91, d.f. = 15, p = 0.004), and two symbiont strains significantly reduced the number of offspring produced: *Regiella* from pea aphid clone 313, and *Spiroplasma* from pea aphid clone 185 (Figure 3). In clone Co26X there were no significant differences in fecundity between lineages (χ^2= 11.96, d.f. = 15, p = 0.682).

The experimental symbiont strains did not generally affect morphology or behaviour of the novel hosts. The one exception was the *Regiella* strain originating from the pea aphid clone 313, which caused severe wing deformations in approximately 80% of winged females of the two recipient clones. The affected

individuals were unable to fly and frequently had problems with balance when walking.

Effects of symbionts on aphid susceptibility to natural enemies

We did not detect significant differences between lineages infected with different symbiont strains of either host genotype in the proportions of aphids successfully parasitized following exposure to the parasitoid *Aphidius ervi* ($F_{15,104} = 1.40$, $p = 0.170$ in clone Co23X; $F_{15,178} = 1.71$, $p = 0.055$ in Co26X) (Figure 4). However, in both clones there were differences between lineages in the rate of parasitoid emergence from successfully formed cocoons ("mummies") ($F_{15,104} = 2.25$, $p = 0.011$ in Co23X; $F_{15,178} = 3.61$, $p < 0.001$ in Co26X). In host genotype Co23X, *Hamiltonella* strains originating from two grain aphid clones, Co12 and Co26, tended to negatively affect parasitoid emergence rate ($p < 0.08$). In host genotype Co26X, *Hamiltonella* originating from one clone, Co12, significantly decreased the rate of parasitoid emergence. Comparison of the proportions of the exposed aphids from which parasitoids successfully emerged, thus combining data on parasitoid mortality both at a larva and at a mummy stage, revealed significant differences between lineages of genotype Co26X ($F_{15,178} = 2.15$, $p = 0.011$). In this clone, one symbiont complement, *Hamiltonella* and *Rickettsia* from pea aphid clone 208, significantly decreased parasitoid emergence rate compared to non-infected aphids ($p < 0.05$), while *Hamiltonella* from the grain aphid clone Co12 tended to decrease the emergence rate ($p < 0.08$).

In both novel host clonal genotypes, Co23X and Co26X, we found significant differences among the experimental aphid lineages in the susceptibility to *Pandora* ($F_{15,87} = 14.94$, $p < 0.001$ in Co23X; $F_{15,90} = 12.33$, $p < 0.001$ in Co26X) (Figure 4). In both clones, three symbiont complements significantly decreased aphid

susceptibility to the fungus: *Regiella* from grain aphid clone Co34, *Regiella* from pea aphid clone 313, and coinfection with *Hamiltonella* and *Rickettsia* from pea aphid clone 208 ($p < 0.003$ for each of these complements, in either host genotype). In neither clone there were significant differences in the effect of infection with different symbiont strains between blocks ($F_{25,72} = 1.62$, $p = 0.075$ in Co23X; $F_{34,75} = 0.78$, $p = 0.764$ in Co26X). The differences between experimental lineages carrying different symbionts in susceptibility to the entomopathogen were also reflected in the differences in aphid survival following the fungal exposure. In both host clones, there were significant differences between the lineages in aphid survival for 8 days after the exposure ($p < 0.001$), but with no differences between lineages across blocks ($p > 0.05$), and the same three symbiont complements - *Regiella* from grain aphid clone Co34, *Regiella* from pea aphid clone 313, and coinfection with *Hamiltonella* and *Rickettsia* from pea aphid clone 208 - significantly increased survival of both host genotypes ($p < 0.01$ in all cases).

Fitness consequences of multiple infection with symbionts from clone N341

Following injection of haemolymph from the triply-infected clone N341 into the pea aphid clone 145, the three symbionts - *Hamiltonella*, X-type and *Rickettsiella* - established in all recipient females tested. All newly established lineages were viable, and we did not record a single case of loss of either of the three symbionts over five months of culturing. In the grain aphid clone Co26X, 12 of the injected aphids reproduced after the 14th day from the injection. However, all offspring of 11 of them failed to reproduce; these aphids tested positive for each of the three endosymbionts. The 12th injected grain aphid produced a mixture of triple-infected offspring and offspring double infected with *Hamiltonella* and X-type. Most of the offspring of the 12th aphid, either doubly or triply infected, produced some live 2nd generation

offspring. However, the triple-infected lineages had extremely low fecundity, high juvenile mortality and high rate of sterility in adults, and we lost all of them over the following three generations. The fecundity of the doubly infected lineages was low, but sufficient for maintaining them in culture for several months. However, when building up aphid numbers for the experiments we had two cases when the large batches of high-quality synchronised adults produced only few offspring. The induction of sterility in infected grain aphids appeared to have been triggered by transfer from the culturing temperature of 14°C to the experimental temperature of 20°C, but we did not attempt to elucidate and quantify the mechanism involved.

In both novel host genotypes, the symbionts provided a very high degree of resistance to *A. ervi*. In clone 145, infection with the three symbionts reduced the rate of successful parasitism from 79% to 1%, and in Co26X *Hamiltonella* and X-type reduced the rate of successful parasitism by *A. ervi* from 64% to 0.

However, in both novel host genotypes the symbionts incurred dramatic fecundity costs. In the pea aphid clone 145 the three symbionts did not significantly decrease the number of offspring produced by the aphids over the first three days of reproduction ($F_{1,39}$=3.75, p = 0.060), but the fecundity of the infected aphids over the next three days was just 14% of the fecundity of the non-infected aphids ($F_{1,39}$=367.03, p < 0.001), and they produced no offspring afterwards, whereas the non-infected aphids continued reproducing at a steady rate for at least another week. In the grain aphid clone Co26X the mean number of offspring produced during the experiment by the doubly infected aphids was just 0.2, less than 1% of the fecundity of the non-infected aphids, and a fraction of fecundity of the infected lineage in any of the previous generations.

Discussion

We have demonstrated important differences between facultative endosymbiotic bacteria originating from conspecific and heterospecific donors in the ease of symbiont establishment in the novel grain aphid hosts (Figure 2). All strains of *Hamiltonella defensa* or *Regiella insecticola* originating from *Sitobion avenae* easily invaded the offspring of the injected novel host females and became permanently established within matrilines. In contrast, symbionts originating from other aphid species varied in their initial establishment rate, and tended to form less stable associations. Notably, the strain originating from *Utamphorophora sp.* clone Up16, one of the two heterospecific *Hamiltonella* strains maternally transmitted to offspring of the injected grain aphids with 100% fidelity was identical at 15 sequenced symbiont and APSE loci to two of the strains from *S. avenae* clones (Chapter 8 of this thesis). This indicates a recent successful horizontal transfer of this strain between aphid species, suggesting that this multilocus genotype should perform well in both cereal aphid species. At the opposite end of the spectrum, two *Hamiltonella* strains which failed to form stable infections in the grain aphid clones originated from *Aphis fabae*, the species most distantly related to *S. avenae* of all those used in this study (Figure 1), and were also the most divergent from other experimental strains (Chapter 8 of this thesis). There was no evidence that these strains adapted to the novel host environment, even after successful transmission for ten aphid generations. This may be seen as surprising, considering that the fitness of aphid endosymbionts is directly associated with their ability to infect embryos developing within infected mothers, and bacterial lines which repeatedly successfully transferred between generations of a novel host could be expected to have become at least partly adapted to the novel host environment. Other authors reported cases of both stable and unstable artificial transfers of secondary symbionts between aphid species from

tribes Aphidini (represented by *A. fabae*) and Macrosiphini (represented by *S. avenae*), and even between subfamilies within Aphididae (Darby & Douglas, 2003; Oliver et al., 2005; Russell & Moran, 2005; Vorburger et al., 2010). However, artificial transfers of symbionts between pea aphid clones and between species within the genus *Acyrthosiphon* have generally been stable, despite occasional losses of some newly introduced symbionts (Chen et al., 2000; Chen & Purcell, 1997; Oliver et al., 2005; Oliver et al., 2003; Russell & Moran, 2005). Taken together, the data presented in this chapter and published previously indicate that some physiological barriers in endosymbiont transmission between host species exist. While there seem to be few physiological limitations for symbiont genotypes to transfer between clones within species, both the symbiont genotype and the phylogenetic distance between the original and the novel host appear to play a role in symbiont establishment following its introduction into a novel host species.

This relationship can be complicated by the possibility of more than one species of facultative endosymbiont simultaneously infecting a single aphid host (Ferrari et al., 2004; Ferrari et al., accepted; Frantz et al., 2009; McLean et al., 2011; Nyabuga et al., 2010), and the potential for the distinct symbionts being transferred together to a novel host. Symbionts can interact within the host, and these interactions can affect the dynamics of their populations (Oliver et al., 2006; Vautrin & Vavre, 2009). Competition between endosymbiont species for limited space and host resources can be expected, and it can be potentially detrimental to the host. At the same time, symbionts can coexist within host genotypes or matrilines for evolutionarily significant periods of time as long as they induce different effects in their hosts or act synergistically (Vautrin & Vavre, 2009), offering potential for coevolution between symbiont genotypes within a particular host genetic background (Chapter 9 of this thesis). It can be expected that the balance between endosymbiont species in stable

co-infections can be disrupted by changes in the environmental conditions, and there is no doubt that a transfer into a novel host species can be regarded as such a change. While some symbiont combinations, like *Hamiltonella* and *Rickettsia* from clone 208, or *Hamiltonella*, X-type and *Rickettsiella* from N341, readily coexist in novel hosts, in other combinations one of the species can be eliminated from a host matriline (Chen & Purcell, 1997; Sandstrom et al., 2001; this chapter). There are little data on the factors which determine the fate of multiple infections in insects in general, and in novel hosts in particular. Further work is required for understanding how these interactions between bacteria in multiple infections affect the horizontal transmission of symbionts and fitness of novel hosts.

Under benign experimental conditions, none of the 15 symbiont complements has significantly affected the fecundity of the novel host clone Co26X, while two strains originating from pea aphids, a strain of *Regiella* and a strain of *Spiroplasma*, decreased fecundity of Co23X (Figure 3). Infection with *Spiroplasma* has been reported to negatively affect fitness of its novel hosts (Fukatsu et al., 2001), and so the effect observed here may well be symbiont species-specific. Interestingly, the costs of infection with the second of these symbionts, *Regiella* from pea aphid clone 313, appear to be morph-specific, as the majority of infected winged individuals had seriously deformed wings, preventing them from flying and disrupting walking. Under natural conditions, this would have been a seriously detrimental effect of infection, despite the lack of apparent negative effects of infection in the wingless morph. These results do not indicate that the fecundity costs of infection depend on the phylogenetic distance between the original and the novel host. They also suggest that a novel symbiont, once successfully introduced into a novel host, does not typically impose major fitness costs regardless of its origin, and could persist in the novel host population even without conferring fitness benefits. These effects may

vary between symbiont species, though (Chen et al., 2000; Koga et al., 2003; Russell & Moran, 2005; Sakurai et al., 2005), and may be affected by the host genotype (Chapter 6 of this thesis). Also, the fecundity as measured in this study is not a comprehensive measure of fitness, and the fitness consequences of infection with endosymbionts can vary dramatically across temperatures and host plant species (Chen et al., 2000; Montllor et al., 2002; Tsuchida et al., 2004), and also depend on factors such as food plant quality, population density or presence of natural enemies (Oliver et al., 2008).

In the pea aphid and the black bean aphid, *Hamiltonella* infection is associated with resistance against parasitoid wasps (Ferrari et al., 2004; Oliver et al., 2005; Oliver et al., 2003; Vorburger et al., 2009). This resistant phenotype appears to be associated with infection with a bacteriophage APSE, found in many *H. defensa* strains in field-collected aphids, and which encodes eukaryotic toxins that likely target parasitoid larvae (Degnan & Moran, 2008a, b; Oliver et al., 2009). Also, some *Regiella* strains can protect their aphid hosts against parasitoids (Nyabuga et al., 2010; Vorburger et al., 2010). However, in *S. avenae* we have not previously detected effects of infection with the four APSE-infected *Hamiltonella* strains on aphid susceptibility to either of the two tested species of parasitoid wasps (Chapters 5 and 6 of this thesis). Neither these, nor any of the eleven additional APSE-infected *Hamiltonella*, *Regiella* or *Spiroplasma* strains originating from four aphid species conferred protection against *A. ervi* in the novel host clones tested in the present study (Figure 4). Two of the newly introduced endosymbiont complements, *Hamiltonella* from the grain aphid clone Co12 and *Hamiltonella+Rickettsia* from the pea aphid clone 208, decreased the parasitoid emergence rate, but did not increase the survival of their aphid hosts, thus providing them with no direct benefits. These results indicate that the defensive properties of *H. defensa* are not as universal as it

was previously thought, and that APSE infection in *Hamiltonella* is not synonymous with resistant phenotype in aphids (Degnan & Moran, 2008a, b; Oliver et al., 2009). However, the assay with aphids multiply infected with symbionts from pea aphid clone N341 indicated that the parasitoid-resistant phenotype can be expressed in grain aphids, and that the negative results of the main experiment did not result from any particular properties of the host or parasitoid genotypes, or inherent problems with the experimental protocol. It needs to be noted, though, that the degree of protection conferred by endosymbionts depends on the parasitoid species and genotype (Dion et al., 2011; Ferrari et al., 2004; Vorburger et al., 2009) as well as on environmental conditions (Bensadia et al., 2006; Guay et al., 2009), and that symbionts can confer indirect protection by influencing parasitoid oviposition choice (Chapter 5 of this thesis).

Three of the strains of facultative endosymbionts used in this study affected resistance of the experimental clones to the pathogenic fungus *Pandora neoaphidis* (Figure 3), an important natural enemy of grain aphids (Dean & Wilding, 1973; Feng et al., 1991; Jensen et al., 2008). *Regiella* originating from grain aphid clone Co34 and from pea aphid clone 313, as well as co-infection with *Hamiltonella* and *Rickettsia* from clone 208 reduced the sporulation rate of the exposed aphids to less than a few percent, while significantly increasing their survival. *Regiella insecticola* has previously been shown to confer resistance to *Pandora* in pea aphids (Ferrari et al., 2004; Scarborough et al., 2005). The present study is the first demonstration that the pathogen-resistant phenotype conferred by a pea aphid symbiont can be expressed in a different host species, but also that *Regiella* originating from another host species can confer the same resistant phenotype. However, there are differences between *Regiella* strains originating from grain aphids, and the strain originating from clone Co21 did not protect aphids against *Pandora*. The ability to confer

protection against natural enemies has evolved more than once among aphid facultative endosymbionts, though (Chapter 9 of this thesis), and may be patchily distributed between strains of symbiont species (Chapter 8 of this thesis). As we have shown after separating the two symbionts from the pea aphid clone 208 in novel host clones, *Rickettsia*, but not *Hamiltonella*, also protects aphids from the fungus (Chapter 9 of this thesis). However, fungal isolates can vary in their specificity to host species (Shah et al., 2004) and some are able to infect clones immune to other isolates (Milner, 1982 and references therein). It is plausible that using other fungal strains or species (Feng et al., 1990) in the resistance experiment would have revealed different patterns of susceptibility across lineages infected with different strains of facultative endosymbionts.

Given fluctuating, but often high or very high pressure of natural enemies on aphid populations (Feng et al., 1991; Jensen et al., 2008; Schmidt et al., 2003), it is clear that the resistant phenotype conferred by facultative endosymbionts can increase aphid fitness. Our data indicate that two symbionts co-infecting a single host can cooperate in providing protection against a wider range of natural enemies. Two *Hamiltonella* strains originating from pea aphid clones 132 and 208, identical at 13 symbiont and APSE loci (Chapter 8 of this thesis), conferred the same moderate degree of protection against parasitoids in the pea aphid (van Asch et al., unpublished data), and *Rickettsia* co-infecting clone 208 protects the host against fungus (Chapter 9 of this thesis). *Rickettsiella*, another symbiont which can confer resistance against *Pandora* to the pea aphid (Chapter 9 of this thesis), was one of three species infecting the pea aphid clone N341 which was used as a donor in this study. Co-infection with two other symbionts from the same clone (*Hamiltonella* and X-type) made the recipient grain aphids highly resistant to parasitoids (Guay et al., 2009), and *Rickettsiella* may have protected them against pathogens as well. However, the

fecundity costs of multiple infections with some symbiont combinations in the novel hosts can be substantial, and it is unlikely that these novel host-symbiont associations would thrive under natural conditions. Similarly, Oliver et al. (2006) have shown that aphids infected with two species of defensive endosymbionts may not benefit from the increased protection against parasitoids. It needs to be remembered, though, that the fitness consequences of infection with defensive symbionts are context-dependent, and can vary dramatically between environmental conditions (Chen et al., 2000; Montllor et al., 2002; Oliver et al., 2008; Russell & Moran, 2006). The fine balance of environment-dependent costs and benefits likely explains the diversity of infections in natural populations of aphids.

The results presented in the current chapter, but also conclusions of the previously published studies, appear to suggest a coherent picture of the role and transmission dynamics of facultative endosymbionts in multi-species aphid communities over evolutionary time. Establishment of facultative endosymbionts in novel hosts representing the same aphid species appears largely unhindered, but across species it may be limited, to an unknown but likely highly variable degree, by ecological and physiological factors. Interspecific horizontal transmission of symbionts is thus likely to happen more frequently between certain subsets of aphid species within a community. This may have led to the establishment of distinct pools of endosymbionts transferred more frequently within groups of related aphid species sharing microhabitats and particularly host plants, as well as natural enemies, within particular geographic locations (Tsuchida et al., 2002). Such groups of aphid species are likely subject to different selection pressures, and as a result, facultative endosymbiont strains exchanged between clones and species within a group may be selected to provide certain benefits, potentially not the same across groups. Examples may include the widespread protection against heat shock in areas with high average

temperatures during summer months (Russell & Moran, 2006), or against fungal enthomopathogens in areas with high average humidity (Chapter 9 of this thesis). Transfers of endosymbiont strains between pools may happen, though, occassionally leading to establishment and spread of novel symbiont-conferred phenotypes. Only rarely do symbionts succeed in establishing in distantly related species, but this can then result in major innovations and may lead to selective sweeps in their host populations (Himler et al., 2011; Jaenike et al., 2010; Majerus & Majerus, 2010).

These ideas about large-scale symbiont dynamics in aphid communities require comprehensive testing. Most importantly, quantitative data on modes of transmission and on incidence of successful horizontal transmission of symbionts in natural populations of hosts are crucial for understanding the ecology of microorganisms associated with aphids (Darby & Douglas, 2003). Large-scale molecular studies on the diversity and evolution of facultative symbionts within and between aphid species, taking into account aphid ecology and distribution, could reveal the degree of structuring of endosymbionts within and across aphid species, as well as factors determining it. Finally, a comprehensive assessment of the fitness consequences of infections with original and newly introduced facultative endosymbionts in different aphid systems is crucial for fully understanding their ecological roles. Taken together, the results of these studies would tremendously improve our understanding of secondary symbionts as a eukaryote horizontal gene pool of aphids and of other animals.

Tables and figures

Clone	Aphid species	Symbiont	Collection site	Host plant	Date collected	Collector	Ref.
Co08	*Sitobion avenae*	Ha	Great Coxwell, Oxfordshire, UK	*Triticum aestivum*	24/06/2008	PŁ	
Co12	*Sitobion avenae*	Ha	Great Coxwell, Oxfordshire, UK	*Triticum aestivum*	24/06/2008	PŁ	
Co23	*Sitobion avenae*	Ha	Great Coxwell, Oxfordshire, UK	*Triticum aestivum*	24/06/2008	PŁ	
Co26	*Sitobion avenae*	Ha	Great Coxwell, Oxfordshire, UK	*Triticum aestivum*	24/06/2008	PŁ	
Co37	*Sitobion avenae*	Ha	Great Coxwell, Oxfordshire, UK	*Triticum aestivum*	25/06/2008	PŁ	
Co45	*Sitobion avenae*	Ha	Great Coxwell, Oxfordshire, UK	*Triticum aestivum*	25/06/2008	PŁ	
Up11	*Sitobion fragariae*	Ha, (Re)	Oxford, Oxfordshire, UK	*Dactylis glomerata*	21/05/2009	PŁ	
Sc02	*Sitobion fragariae*	Ha	Salcombe, Devon, UK	*Dactylis glomerata*	25/05/2009	PŁ	
101	*Acyrthosiphon pisum*	Ha	Westcott, Buckinghamshire, UK	*Ononis spinosa*	04/07/2003	JF	1, 2
208	*Acyrthosiphon pisum*	Ha, Ri	Silwood Park, Berkshire, UK	*Lotus pedunculatus*	29/05/2003	JF	1, 2
Up16	*Utamphorophora sp.*	Ha	Oxford, Oxfordshire, UK	*Dactylis glomerata*	08/06/2009	PŁ	
323	*Aphis fabae*	(Ha)	Aesch, Switzerland	*Vicia faba*	Jun/Jul 2006	CV	3
402	*Aphis fabae*	(Ha)	St. Margrethen, Switzerland	*Chenopodium album*	Jun/Jul 2006	CV	3
Co21	*Sitobion avenae*	Re	Great Coxwell, Oxfordshire, UK	*Triticum aestivum*	24/06/2008	PŁ	
Co34	*Sitobion avenae*	Re	Great Coxwell, Oxfordshire, UK	*Triticum aestivum*	24/06/2008	PŁ	
185	*Acyrthosiphon pisum*	(Re), Sp	Slough, Berkshire, UK	*Trifolium pratense*	04/06/2003	JF	1
313	*Acyrthosiphon pisum*	Re	Upper Slaughter, Gloucestershire, UK	*Trifolium pratense*	2008	MvA	
N341	*Acyrthosiphon pisum*	Ha, X, Rcl	Wexham, Buckinghamshire, UK	*Medicago sativa*	31/08/2007	JF	
145	*Acyrthosiphon pisum*	none	Windsor, Berkshire, UK	*Lathyrus pratensis*	2003	JF	1

Table 1. List of the aphid clones used in this study. *Sitobion avenae* clones Co23 and Co26, which had their original symbiont removed, were injected with symbiont strains from seventeen donor clones infected with different symbionts (including Co23 and Co26, but excluding N341). Symbionts from N341 were separately introduced into Co26-cured and into 145. Symbiont names were abbreviated as: Ha – *Hamiltonella defensa*; Re – *Regiella insecticola*; Ri – *Rickettsia*; Sp - *Spiroplasma*; X - X-type; Rcl - *Rickettsiella*, and the symbionts in brackets failed to establish in novel grain aphid hosts. Collectors were Piotr Łukasik (PŁ), Julia Ferrari (JF), Christoph Vorburger (CV) and Margriet van Asch (MvA). References are: 1 – Ferrari et al. 2008; 2 – McLean et al. 2011; 3 - Vorburger et al. 2009.

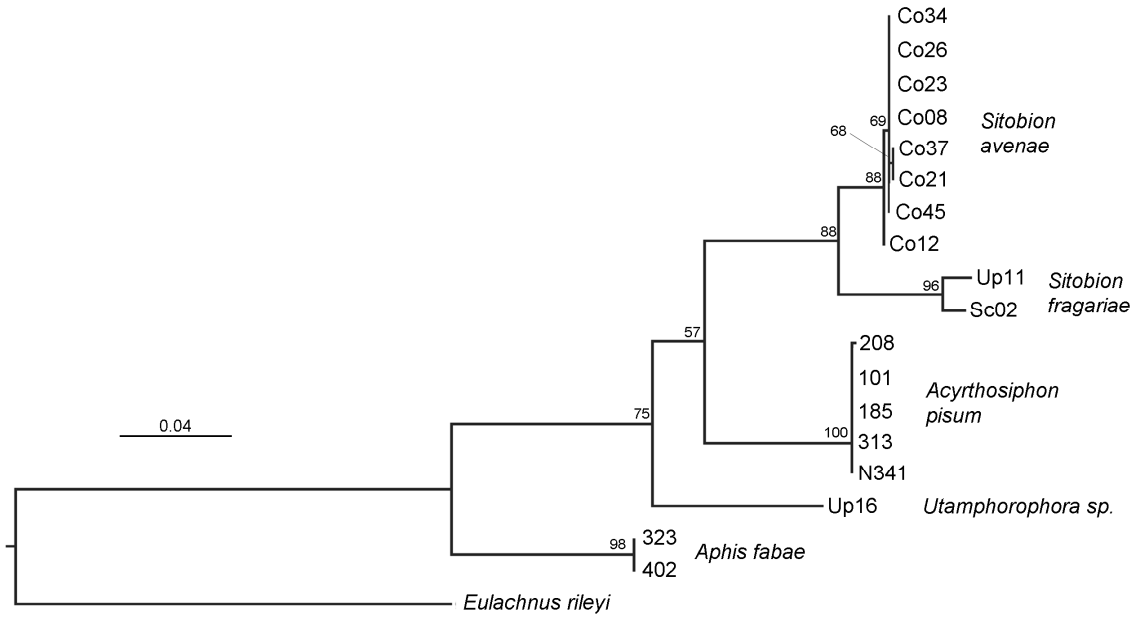

Figure 1. Maximum likelihood phylogeny of the experimental aphid clones based on partial sequence of cytochrome c oxidase I (COI). Bootstrap support values larger than 0.50 are presented as percentages above the nodes.

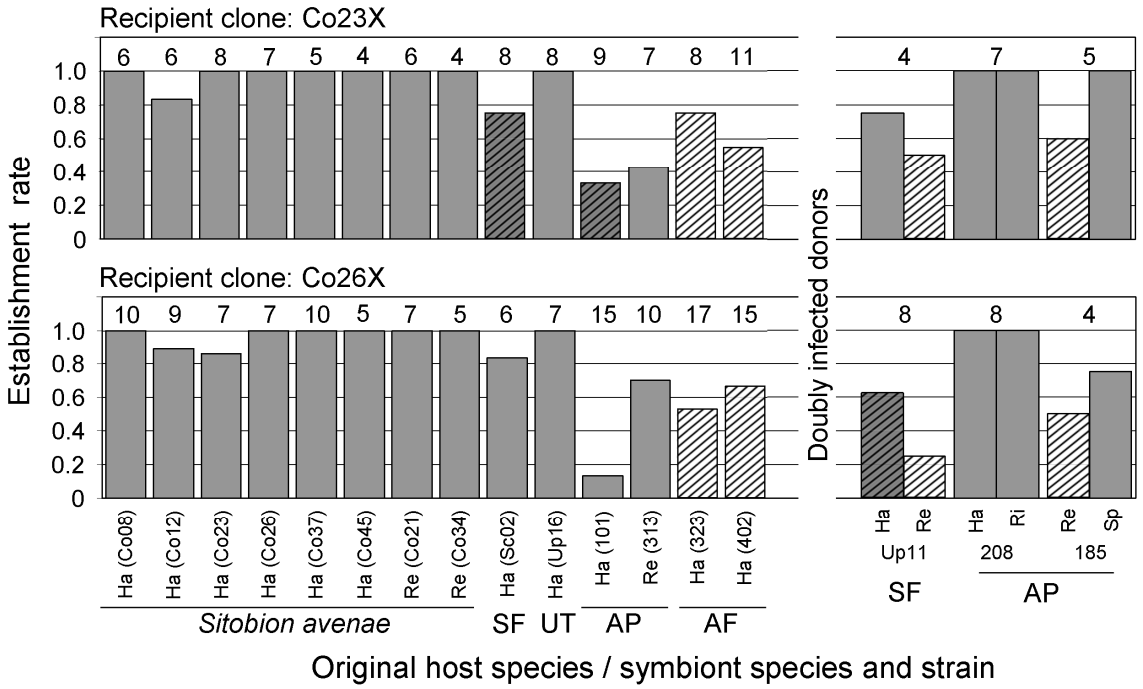

Figure 2. Proportions of females of two recipient clones reproducing after the 14th day from injection with haemolymph from one of the symbiont-infected donor clones, which produced offspring infected with the introduced symbiont. Numbers of the assessed females are shown above the bars. In most lineages, we recorded no cases of symbiont loss following their successful introduction into the novel hosts (grey uniform bars); in other lineages, we recorded single cases of symbiont loss (grey diagonally striped bars), or failed to establish stable infections (white diagonally striped bars). Symbiont specific names are abbreviated as Ha (*Hamiltonella defensa*), Re (*Regiella insecticola*), Ri (*Rickettsia*) and Sp (*Spiroplasma*). Host specific names are abbreviated as SF - *Sitobion fragariae*; UT - *Utamphorophora sp.*; AP - *Acyrthosiphon pisum*; AF – *Aphis fabae*

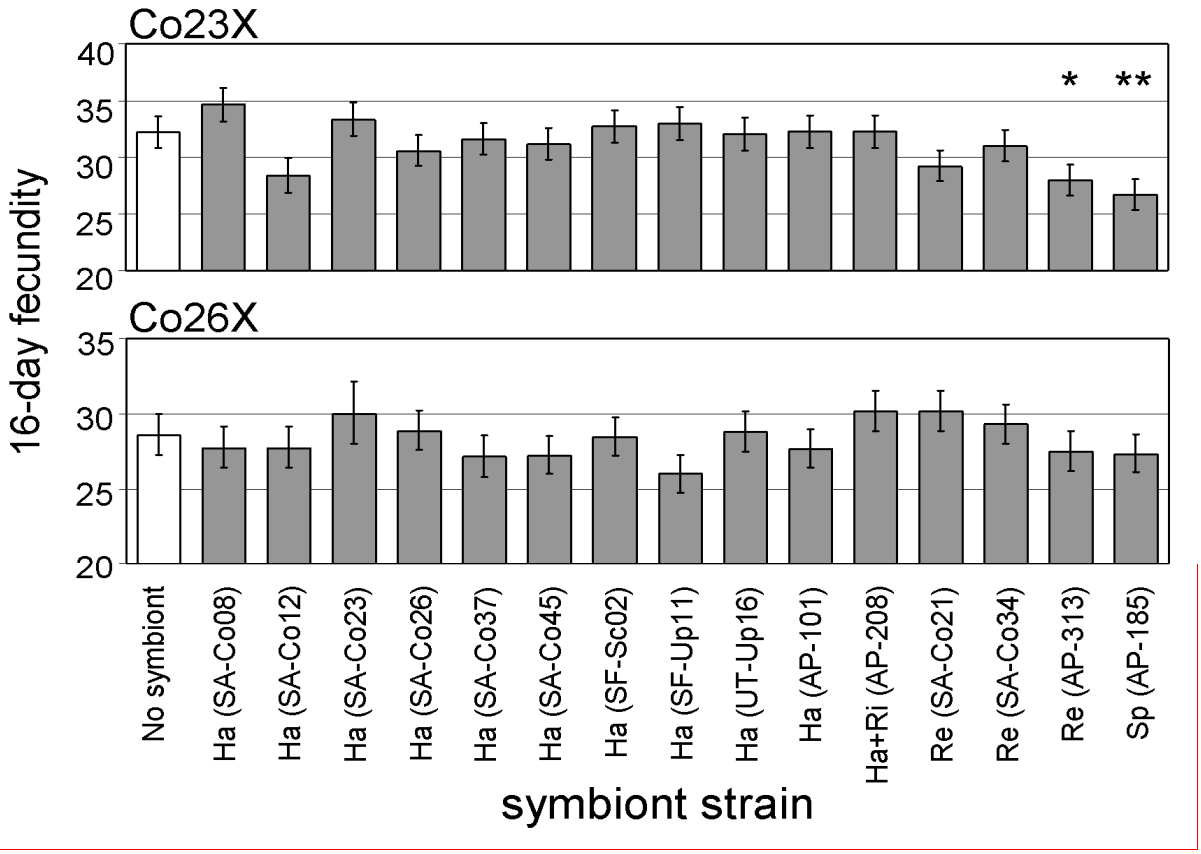

Figure 3. Fecundity (mean ± S.E.) of two grain aphid clones, either facultative symbiont-free (white bars) or infected with one of fifteen symbiont complements (grey bars). Symbiont specific names are abbreviated as Ha (*Hamiltonella defensa*), Ri (*Rickettsia*), Re (*Regiella insecticola*) and Sp (*Spiroplasma*), and followed by abbreviated specific name of the original host (SA - *Sitobion avenae*, SF - *Sitobion fragariae*; UT - *Utamphorophora sp.*; AP - *Acyrthosiphon pisum*) and the name of the host clone. Significant differences between a non-infected lineage and a particular infected lineage of the same clone are marked with asterisks: * ($p < 0.05$), ** ($p < 0.01$) or *** ($p < 0.001$)

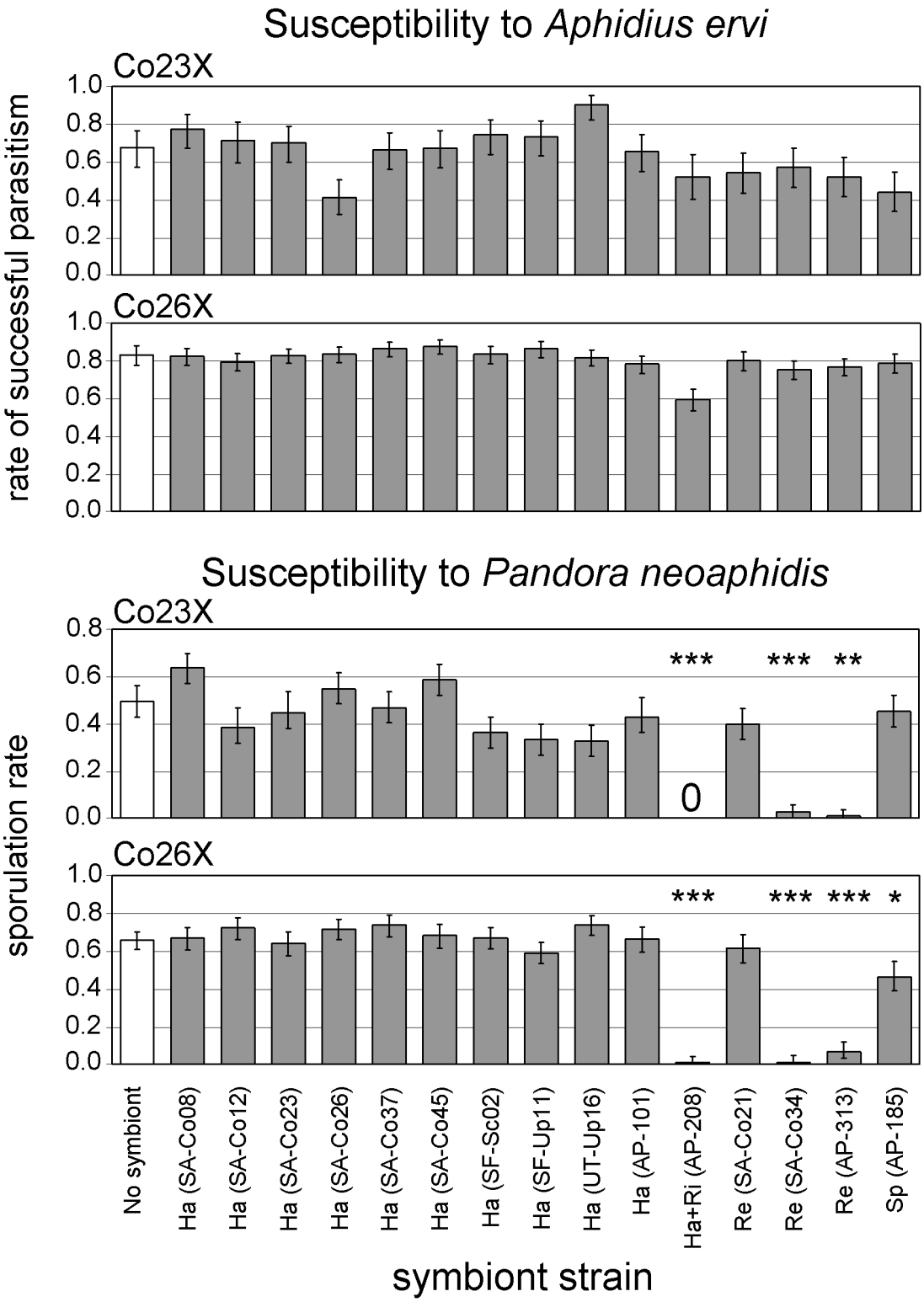

Figure 4. Susceptibility to two species of natural enemies (mean ± S.E.) of two grain aphid clones, either facultative symbiont-free (white bars) or infected with one of fifteen symbiont complements (grey bars). Abbreviations of symbiont names are explained in legend to Figure 3. For *A. ervi* bioassays, errors were calculated for data from across the experimental blocks, but only the means for the largest experimental block are shown. Means and errors for *P. neoaphidis* assays were calculated for data from across all blocks. Significant differences between a non-infected lineage and a particular infected lineage of the same clone are marked with asterisks: * ($p < 0.05$), ** ($p < 0.01$) or *** ($p < 0.001$)

Chapter 8

Symbionts, their bacteriophages, and parasitoid-resistant phenotype of aphids

Abstract

Aphid resistance to their hymenopterous parasitoids conferred by a bacterium *Hamiltonella defensa* is one of the best-understood effects of infection with a facultative mutualistic endosymbiont of an insect. The resistant phenotype appears to be associated with properties of a lambdoid bacteriophage called APSE, which infects some *Hamiltonella* strains. However, the evolution and distribution of this important trait among aphids and symbionts is not well understood.

In this chapter, we explore how the defensive properties of distinct strains of *Hamiltonella* are distributed across the phylogenies of the symbiont and its bacteriophage APSE. We did this by collecting data on the effects of infection with 19 strains of APSE-infected *Hamiltonella* representing 14 distinct haplotypes of the bacterium and the phage, on aphid resistance to hymenopterous parasitoids, from other chapters of this thesis and from studies by other authors. These data on resistant phenotypes conferred by endosymbionts were compared with a multilocus phylogeny of *Hamiltonella* and phylogenies of APSE genes.

There were no clear patterns of distribution of parasitoid-resistant phenotypes across the phylogenetic trees. However, the comparison of the phylogenies of bacteria and their phages suggests that the exchange of the bacteriophage between strains may be less frequent than previously thought. It is clear that further research, particularly focusing on the toxin genes carried by the phages, would provide us with a better insight into the evolution of defensive properties of aphid symbionts.

Introduction

Facultative endosymbionts can have a broad range of effects on life history traits and phenotypes of their insect hosts (Moran et al., 2008; Oliver et al., 2010; Werren et al., 2008), but different haplotypes of the same symbiont species may vary considerably in their effects on hosts (Oliver et al., 2009; Oliver et al., 2005; Werren et al., 2008; Chapters 7 and 9 of this thesis). One of the most intriguing, but also the best-studied phenotypic effects of infection with facultative endosymbiotic mutualists is the protection against hymenopterous parasitoids conferred by a gammaproteobacterium *Hamiltonella defensa* to its pea aphid hosts. Variation among clones of the pea aphid, *Acyrthosiphon pisum*, in resistance to a parasitoid *Aphidius ervi*, one of the most important natural enemies of this species (Henter & Via, 1995; Muller et al., 1999), was shown to be associated with the infection with facultative endosymbionts (Oliver et al., 2003). Different *Hamiltonella* strains were found to confer different levels of protection (Oliver et al., 2005). At the time, it was known that a lambdoid bacteriophage named APSE (for bacteriophage of the *A. pisum* secondary endosymbiont - van der Wilk et al., 1999), frequently infects *Hamiltonella* strains carried by pea aphids (Sandstrom et al., 2001). Moran and colleagues (2005a) were the first to note the diversity of bacteriophage-encoded eukaryotic toxins and the fact that the toxin-encoding genes can be highly expressed. They proposed the association between parasitoid-resistant aphid phenotype conferred by *Hamiltonella* and the diverse effects of APSE-encoded toxins. Later, it was shown that APSE infects only some of the *Hamiltonella* strains carried by pea aphids and by aphids representing other species (Degnan & Moran, 2008b; Oliver et al., 2009), but that each of the seven distinct APSE haplotypes identified (and assigned consecutive strain numbers from 1 to 7), carried genes encoding one of three distinct eukaryotic

toxins (Degnan & Moran, 2008a). Finally, Oliver et al. (2009) showed that the loss of the resistant phenotype in pea aphid lineages maintaining their infection with *Hamiltonella* was due to a spontaneous loss of infection with APSE from the *Hamiltonella* population still residing in its aphid hosts. The conclusions of these studies were straightforward: the variation in the protective phenotype conferred to their aphid hosts by *Hamiltonella* strains may be largely associated with the infection with APSE, and the expression and specificity of the APSE-encoded toxins.

Correlations between *Hamiltonella* infections and aphid resistance to parasitoids have also been reported by other groups studying pea aphids (Bensadia et al., 2006; Ferrari et al., 2004; Guay et al., 2009; Nyabuga et al., 2010), and also the black bean aphid, *Aphis fabae* (Vorburger et al., 2009). In contrast, our research on the grain aphid, *Sitobion avenae*, but also on the pea aphid, has revealed little effect of infection with *Hamiltonella* on aphid resistance to parasitoids, even though all the experimental *Hamiltonella* strains were infected with APSE (Chapter 7 of this thesis). Only one of fourteen *Hamiltonella* strains originating from different clones of four aphid species, tested in at least two host genetic backgrounds, conferred a high degree of resistance to parasitoids, and three further strains conferred a limited degree of resistance (Chapters 5-7 of this thesis; M. van Asch et al., unpublished data). These results seem to contradict the hypothesis that the presence of the bacteriophage may be the main determinant of the parasitoid-resistant phenotype in *Hamiltonella*-infected aphids.

Unlike the highly reduced and stable genomes of obligatory nutritional endosymbionts of insects (Baumann, 2005; Moran et al., 2008), genomes of facultative endosymbionts are dynamic and littered with mobile DNA including phage-derived genes, plasmids, insertion sequences and introns (Darby et al., 2010;

Degnan et al., 2010; Degnan et al., 2009; Ishmael et al., 2009; Moran et al., 2008). Genetic material may regularly be exchanged between cells of different symbionts co-infecting the same hosts (Chafee et al., 2010; Kent et al., 2011). Despite this, phylogenies for different genes of distinct *Hamiltonella* strains are similar, and the scale of recombination between different symbiont genes is limited (Degnan & Moran, 2008b; L. Henry, unpublished data). In contrast, APSE, like other bacteriophages, is expected to frequently engage in homologous and non-homologous gene exchange (Kent & Bordenstein, 2010; Weinbauer, 2004). This was confirmed by Degnan and Moran (2008b), who demonstrated recombination between APSE haplotypes by revealing major differences between phylogenies of single APSE genes, and their incongruence with the *Hamiltonella* phylogeny. These data suggested that the present distribution of the ability to confer parasitoid-resistant phenotype among *Hamiltonella* strains may be a result of a long series of gene exchange between symbiont genomes. However, this has not been verified due to the limited amount of information on the protective effects of carrying multi-locus haplotypes of *Hamiltonella* infected with APSE (Degnan & Moran, 2008b; Oliver et al., 2005), but also because only a limited number of *Hamiltonella*-APSE associations has been studied to date. This made it impossible to search for patterns between the aphid phenotypes and the genotypes of the symbiont and its phage. Thus, our new sequence data for the sixteen APSE-infected *Hamiltonella* strains, combined with information on their phenotypic effects of infection, and supplemented by the existing data on the gene sequences and phenotypic effects of infection with phage-carrying symbionts, offered us a unique opportunity for tracing the associations between the bacteria and their viruses, but particularly the evolution of parasitoid-resistant phenotype across *Hamiltonella* and APSE phylogenies.

In this paper, we explore how the defensive properties of the strains of the facultative endosymbiont of aphids, *Hamiltonella defensa*, are distributed across the phylogenies of the symbiont and its associated bacteriophage APSE. We did this by constructing the phylogenies of *Hamiltonella* and its APSE phage based on the published *Hamiltonella* and APSE sequences, as well as on novel sequence data for 16 strains representing 11 haplotypes. Then, the parasitoid-resistant phenotypes conferred by different *Hamiltonella* strains, as demonstrated in the previous chapters of this thesis and in published and unpublished studies by other authors, were mapped onto the phylogenies.

Material and methods

We identified 24 APSE-infected *Hamiltonella* strains originating from eight aphid and one whitefly species to be included in this study. Sequence data for APSE were available for eight of these strains (Degnan & Moran, 2008b; van der Wilk et al., 1999), and a number of *Hamiltonella* sequences for seven of them. The parasitoid-resistant phenotypes that four of them conferred were also known (Oliver et al., 2009; Oliver et al., 2005; Oliver et al., 2003). For sixteen other strains, data on their effect on parasitoid resistance of their aphid hosts were available (Vorburger et al., 2009; M. van Asch et al., unpublished data; Chapters 5-7 of this thesis), as well as DNA samples of their original aphid hosts, which enabled us to generate sequence data ourselves. The list of strains used in this study and their characteristics are provided in Table 1.

We assigned parasitoid-resistant phenotypes conferred by the symbiont to its aphid hosts to all APSE-infected *Hamiltonella* strains for which data were available. The classification was based on the results of comparisons of parasitoid susceptibility of aphids representing the same genotype, but either infected with a particular

Hamiltonella strain, or free from infection. Symbiont strains which reduced the rate of successful parasitoid pupation in infected aphids by 50% or more compared to non-infected aphids of the same clone were classified as conferring a high degree of resistance; strains reducing the rate of successful parasitism by less than 50% were classified as conferring partial resistance; and strains which did not significantly affect parasitoid pupation rate were classified as not conferring resistance. There were, however, exceptions to this classification. Two *Hamiltonella* strains originating from *Aphis fabae* were classified as conferring a high degree of resistance based on the results of a correlative study (Vorburger et al., 2009), which demonstrated that a *Hamiltonella*-infected clone 402 was immune to both experimental genotypes of a parthenogenetic parasitoid, and clone 323 was immune to one genotype and partly resistant to another one. The effects of infection with strains originating from the pea aphid clones 132 and 208, which were classified as conferring partial resistance, varied considerably between experimental blocks, host genetic backgrounds and parasitoid species (M. van Asch et al., unpublished data; Chapter 7 of this thesis). Also, a *Hamiltonella* strain originating from the grain aphid clone Co12 was classified as conferring partial resistance as it significantly increased wasp mortality at pupa stage, even though it did not reduce the parasitoid pupation rate (Chapter 7 of this thesis). Finally, three of the *Hamiltonella* strains occurred in co-infections with other symbiont species at the time of measuring parasitoid resistance of their hosts, which may have influenced the results of the assays (Table 1); however, herein it was assumed that the resistant phenotypes were due to infection with *Hamiltonella* only.

As already mentioned, APSE sequence data for eight of the strains, and *Hamiltonella* sequence data for seven strains had been published previously (Degnan & Moran, 2008b; Moran et al., 2005a; van der Wilk et al., 1999). DNA from the

original host clones of the other 16 symbiont strains was extracted from single adult aphids using DNeasy Blood and Tissue kit (Qiagen) following a standard protocol. Next, six *Hamiltonella* housekeeping genes, *accD*, *gyrB*, *hrpA*, *murE*, *recJ* and *rpoS*, as well as five APSE genes, P3, P35, P41, P45 and P51, were amplified using primers and following a protocol provided by Degnan & Moran (2008b), except that instead of adding *Taq* polymerase, dNTPs and reaction buffer separately to the reaction mixes, we used BioMix (Bioline), a pre-mixed solution. The PCR product was cleaned following an ExoSAP-IT protocol (USB), and sequenced from both ends using Big Dye Terminator v 3.1 reaction mix (Applied Biosystems).

Additionally, for the sixteen *Hamiltonella*-infected clones, we attempted to amplify and sequence the integration site boundaries from integrated and non-integrated phages, including the attachment site in APSE adjacent to the integrase gene P38 (*attP*), attachment site in *H. defensa* overlapping with the arginine tRNA gene (*attB*), and integrated boundaries (*attL* and *attR*) (Degnan & Moran, 2008a). The primers specified by Degnan and Moran (2008) were used, together with newly designed sequencing primers attR_seq (CGGCATTAAAACGAGCCGGAATAG) and attL_seq (CTCTTGGAACAGCGGACAAGAAGG).

Sequence traces for each sample were individually assembled, edited and manually inspected using Codon Code v. 2.06. Additional PCR and sequencing reactions were performed when necessary. All sequences will be deposited in Genbank by the time of the publication of this study.

The sequences of individual APSE genes were aligned using ClustalW algorithm and tested to find the best-fitting models of evolution using software MEGA version 5 (Tamura et al., 2011). Phylogenies were built using phyML v. 3.0 (Guindon et al., 2010), and bootstrap analysis carried out with 1000 replications in each case.

Following the example of Degnan & Moran (2008b), and given that the gene phylogenies for the five APSE genes were clearly different, we did not attempt to construct a single APSE tree based on concatenated phage sequences. However, a single phylogeny was computed using the same methods as described above for the six concatenated *Hamiltonella* genes, based on the results of L. Henry (unpublished data; see also Degnan & Moran, 2008b) on a much larger dataset, which validated this approach.

Once the *Hamiltonella* multi-locus phylogeny and the phylogenies for APSE genes were constructed, the parasitoid-resistant phenotypes were mapped onto the phylogenetic trees.

For the loci spanning the site of phage integration into *Hamiltonella* genome, presence or absence of the amplicons was noted, and their identity was confirmed by sequencing, trace assembly and alignment using Codon Code. These data will be processed further at a later date.

Results and Discussion

We successfully amplified and sequenced the six housekeeping genes of *Hamiltonella*, as well as the five structural genes of APSE from all sixteen symbiont strains which had not been previously genotyped (Table 1). Each *Hamiltonella* strain appeared to be infected with one primary APSE variant, although in a few cases sequence traces revealed polymorphisms at single nucleotides. In all cases we successfully amplified at least two of the four loci spanning the integration site of APSE into bacterial chromosome (Table 2), including locus *attP* amplifiable only when the phage genome is in a non-integrated, circular form, and locus *attL* spanning the attachment site of an integrated phage (Degnan & Moran, 2008a). Thus, all phage

variants appear to be functional and capable of entering both lysogenetic and lytic cycles in populations of *Hamiltonella* within single insects (St-Pierre & Endy, 2008; Weinbauer, 2004). Also, in most samples we successfully amplified and sequenced bacterial locus *attB* spanning the phage integration site, suggesting that in these aphids at least a part of the bacterial population was free from the phage infection. Degnan & Moran (2008b), who found APSE in only some *Hamiltonella* strains, argued that the amplifications of intergenic regions, or Southern hybridisations, are essential for confirming the presence of the bacteriophage. However, the fact that from each DNA sample we obtained at least seven high quality sequences highly similar to the APSE sequences published previously, but generally distinct from each other, indicates that our results were not due to contamination. It needs to be noted that during the course of research described in this thesis we have not detected a single aphid clone carrying a *Hamiltonella* strain but free from APSE infection, or any case of apparent APSE loss (Oliver et al., 2009). The reasons for the differences in the frequency and stability of infections with APSE between this project and the studies focusing on North American insects (Degnan & Moran, 2008b; Oliver et al., 2009) are unclear, but can possibly be explained by a sampling bias towards different aphid taxa.

The concatenated sequences of the six housekeeping genes of *Hamiltonella* yielded a well-resolved and well-supported phylogeny of the 23 symbiont strains (Figure 1). The topology revealed several groups of identical or very similar strains, as well as some more divergent haplotypes. In most cases, the identical symbiont strains originated from clones of the same aphid species, as was the case for *S. avenae* clones Co26 and Co45, *A. pisum* clones 132 and 208 or A1A and A2F, or the two *A. fabae* clones. Also, symbionts from the *S. avenae* clones Co23 and Co37 and the *Utamphorophora sp.* clone Up16 shared the same multilocus genotype. However,

in two other cases the clones hosting very similar symbionts represented distantly related aphid species. This, as well as the fact that the symbiont haplotypes originating from the two most widely represented aphid species, *A. pisum* and *S. avenae*, were widely distributed across the tree, is one of the more convincing pieces of evidence to date for the regular horizontal transfer of facultative endosymbionts between aphid species (Degnan & Moran, 2008b; Russell et al., 2003; Sandstrom et al., 2001).

Single-gene phylogenies for the APSE loci were less well supported (Figure 2). Particularly, the phylogeny of the locus P35 was poorly resolved as a consequence of less than 1% sequence divergence among all but one strains (data not shown), and will not be further discussed. The most divergent sequence for the multi-locus *Hamiltonella* phylogeny and four single-locus APSE phylogenies was always the one from the whitefly *Bemisia tabaci*. Also, for four loci the similar sequences isolated from *A. pisum* clone 5AT and *U. rudbeckiae* clone were positioned at the base of the trees for all remaining strains. Otherwise, there was little similarity between the phylogenies (Degnan & Moran, 2008b), although potentially partly due to limitations in the resolution of the phylogenetic analyses. However, some interesting patterns emerged. In all cases, *Hamiltonella* strains representing the same multi-locus haplotype hosted phage haplotypes identical at all sequenced loci, including the attachment sites. One of these examples included strains of *Hamiltonella* and the phage that were identical in two naturally infected, but distantly related aphid species, *Sitobion avenae* and *Utamphorophora sp.* (Degnan & Moran, 2008b). Also, phages infecting the very similar *Hamiltonella* strains hosted by *A. pisum* clone 5AT and the *U. rudbeckiae* clone were also similar at all loci. An interesting case is the *Hamiltonella* strain carried by the grain aphid clone Co12, which is very similar at the six bacterial loci to the strain from the pea aphid clone N341, but carries

bacteriophage identical at four loci (P35, P41, P45 and P51) to APSE-3 infecting two other pea aphid *Hamiltonella* strains. In addition, the phages infecting symbiont haplotypes originating from the same aphid species do not appear to cluster together on any of the phylogenies; given a higher chance for lateral transfer of phages between symbionts within populations or species (Chafee et al., 2010; Kent et al., 2011), such clustering should be expected if phage transfers were more frequent than interspecific transfers of symbionts. Thus, our data suggests that the lateral transmission and recombination of APSE bacteriophages between *Hamiltonella* strains, while both are clearly happening, may not be as common as previously thought (Degnan & Moran, 2008b; Kent & Bordenstein, 2010).

Mapping parasitoid-resistant phenotypes of aphids infected with particular *Hamiltonella* strains on symbiont and phage phylogenies (Figures 1 and 2) did not reveal any clear patterns. On all phylogenetic trees, symbiont strains conferring resistance were distributed across branches. In particular, none of the three strains conferring partial resistance (*A. craccivora* 5ATac, *A. pisum* 5AT or *A. pisum* 132 / 208) ever clustered together with other resistance-conferring strains. In some cases, distinct strains associated with resistance formed strongly supported groups, for example for *A. fabae* 323 / 402 - *A. pisum* APSE-1 / N341 strains for locus P51, or *A. pisum* APSE-1 - *A. pisum* APSE-3 for locus P41 (Figure 2). It has been revealed, however, that phage variants APSE-1 and APSE-3 encode different toxins (Degnan & Moran, 2008a), and thus similarity at that particular locus might be incidental. Different toxin genes were also carried by otherwise similar phage variants APSE-2 and APSE-5 infecting *A. pisum* clone 5AT and the *U. rudbeckiae* clone, respectively (Degnan & Moran, 2008a). The associations between the type, expression level and specificity of the toxin-encoding genes and phage or symbiont haplotypes are unknown. Given the lack of clear patterns detected in this study, it might be expected

that the ability to produce different toxin types should be as patchily distributed among the *Hamiltonella* and APSE phylogenies as the parasitoid-resistant phenotypes, but also that the aphid phenotypes may be more closely associated with the toxin-encoding genes than with other symbiont or phage genes. Knowledge of the toxin genes carried by the studied strains would greatly improve our understanding of the evolution of the protective phenotype among *Hamiltonella* strains.

It has been shown that in addition to *Hamiltonella* infection, aphid susceptibility to parasitoids can depend on a range of other factors, including aphid species (Daza-Bustamante et al., 2003; Henry et al., 2008), its age (Henry et al., 2005; Henry et al., 2009), co-infections with other endosymbionts (Guay et al., 2009; Nyabuga et al., 2010), parasitoid species and genotype (Dion et al., 2011; Ferrari et al., 2004; Vorburger et al., 2009), or environmental conditions (Bensadia et al., 2006; Guay et al., 2009). Indeed, many of these parameters are known to affect life cycles of bacteriophages, and thus the fates of phage-infected bacterial cells (Bordenstein et al., 2006; Chafee et al., 2011; Kent & Bordenstein, 2010; St-Pierre & Endy, 2008). APSE-induced lysis of *Hamiltonella* cells is a likely mechanism of delivering APSE-encoded toxins to parasitoids developing within resistant aphids (Moran et al., 2005a). Therefore, it can be expected that the fitness consequences of infection with a particular symbiont strain would vary between host genetic backgrounds, but also between experimental conditions associated with studying different systems (M. van Asch et al., unpublished data). This may have contributed to some of the uncertainty in our assigning different degrees of resistance to the strains used in this study. However, it has previously been shown that symbiont strains appear to have similar defensive properties in different aphid clones and species (Oliver et al., 2005; Vorburger et al., 2010; Chapters 6, 7 and 9 of this thesis), and that different strains of

the same symbiont species can vary in their effects on aphid hosts (Oliver et al., 2005; Chapters 7 and 9 of this thesis).

Other species of facultative endosymbionts of aphids can also protect their hosts from parasitoids (Ferrari et al., 2004; Nyabuga et al., 2010; Oliver et al., 2003; von Burg et al., 2008; Vorburger et al., 2010), even though no APSE infections have been reported from these bacteria. Similarly, strains of at least four unrelated species of endosymbionts can confer resistance against pathogens to their aphid hosts (Chapter 9 of this thesis). In any case, the mechanisms involved are unknown, and there is no data on whether genetic pathways associated with resistance have evolved independently, or have been transferred horizontally between species. It is clear that further work on the incidence of these defensive effects of infection, the fitness consequences of carrying the symbionts under fluctuating natural conditions, and the evolution and transferability of these important effects across endosymbiont strains and species is essential for our understanding of the role of facultative endosymbiotic mutualists in aphids and in other arthropod species.

Tables and Figures

Host species	Host clone	APSE	Co-infection	Parasitoid species tested against	Resistance level	References
Sitobion avenae	Co08			*Aphidius ervi, Ephedrus plagiator*	none	Ch5, Ch6, Ch7
Sitobion avenae	Co12			*A. ervi*	low	Ch7
Sitobion avenae	Co23			*A. ervi, E. plagiator*	none	Ch5, Ch6, Ch7
Sitobion avenae	Co26			*A. ervi, E. plagiator*	none	Ch5, Ch6, Ch7
Sitobion avenae	Co37			*A. ervi, E. plagiator*	none	Ch5, Ch6, Ch7
Sitobion avenae	Co45			*A. ervi*	none	Ch7
Sitobion fragariae	Up11		Re	*A. ervi* (SA)	none	Ch7
Sitobion fragariae	Sc02			*A. ervi* (SA)	none	Ch7
Acyrthosiphon pisum	101			*A. ervi* (AP, SA), *E. plagiator, Praon sp.*	none	Ch7, 1
Acyrthosiphon pisum	208		Ri*	*A. ervi* (AP, SA), *E. plagiator, Praon sp.*	low	Ch7, 1
Acyrthosiphon pisum	132			*A. ervi, E. plagiator, Praon sp.*	low	1
Acyrthosiphon pisum	161		Sp*	*A. ervi, E. plagiator, Praon sp.*	none	1
Acyrthosiphon pisum	N341		X*, Rcl	*A. ervi* (AP, SA)	high	Ch7
Utamphorophora sp.	Up16			*A. ervi* (SA)	none	Ch7
Aphis fabae	323			*Lysiphlebus fabarum*	high	2
Aphis fabae	402			*L. fabarum*	high	2
Acyrthosiphon pisum	5AT	APSE-2		*A. ervi*	low	3, 4, 6, 7, 8
Acyrthosiphon pisum	A1A	APSE-3		*A. ervi*	high	4, 5, 7, 8
Acyrthosiphon pisum	A2F	APSE-3		*A. ervi*	high	4, 5, 7, 8
Aphis craccivora	5ATac	APSE-4		*A. ervi* (AP)	low	4, 7, 8
Acyrthosiphon pisum		APSE-1			unknown	6, 7, 9
Chaitophorus sp.	N4	APSE-6			unknown	7, 8
Uroleucon rudbeckiae		APSE-5			unknown	7, 8
Bemisia tabaci (biovar B)		APSE-7			unknown	7, 8

Table 1. List of the *Hamiltonella defensa* strains used in this study, identified by their original host species and clone, and the associated APSE strain. Names of APSE strains follow Ref. 7. Symbionts originally found in co-infections with *Hamiltonella* are abbreviated as Re – *Regiella insecticola*; Ri – *Rickettsia*; Sp - *Spiroplasma*; X - X-type; Rcl - *Rickettsiella*, and the abbreviations followed by asterisks indicate symbionts which were present in experimental clones when they were tested for parasitoid resistance, and which thus may have influenced the results of the assays. If a strain has been tested against a certain parasitoid in a heterospecific host, this is indicated with letters SA (novel host = *Sitobion avenae*) or AP (*Acyrthosiphon pisum*) following a specific name of the parasitoid. Parasitoid resistance level is classified as high for symbionts reducing parasitoid pupation rate by more than 50%, low - less than 50%, or none - no significant effect. References are: Ch5-Ch7 - chapters of this thesis; 1 - van Asch et al. (unpublished data); 2 - Vorburger et al. (2009); 3 - Oliver et al. (2003); 4 - Oliver et al. (2005); 5 - Oliver et al. (2009); 6 - Moran et al. (2005a); 7 - Degnan & Moran (2008a); 8 - Degnan & Moran (2008b); 9 - van der Wilk et al. (1999)

Host clone	Resistance	*attP*	*attB*	*attR*	*attL*
Siave Co08	none	✓			✓
Siave Co23	none	✓	✓	✓	✓
Siave Co26	none	✓			✓
Siave Co37	none	✓	✓	✓	✓
Siave Co45	none	✓			✓
Sifra Up11	none	✓		✓	✓
Sifra Sc02	none	✓	✓	✓	✓
Acpis 101	none	✓		✓	✓
Acpis 161	none	✓	✓	✓	✓
Utsp1 Up16	none	✓	✓	✓	✓
Siave Co12	low	✓	✓	✓	✓
Acpis 208	low	✓			✓
Acpis 132	low	✓			✓
Acpis 5AT APSE-2	low	✓	✓	✓	✓
Apcra 5ATac APSE-4	low	✓			✓
Acpis N341	high	✓	✓	✓	✓
Apfab 323	high	✓	✓	✓	✓
Apfab 402	high	✓	✓	✓	✓
Acpis A1A APSE-3	high	✓	✓	✓	✓
Acpis A2F APSE-3	high	✓	✓	✓	✓
Acpis APSE-1	unknown	✓	✓	✓	✓
Chsp1 APSE-6	unknown		?		✓?
Urrud APSE-5	unknown	✓	✓	✓	✓
Betab APSE-7	unknown		?		

Table 2. The checklist of loci spanning the phage integration sites, which were successfully amplified in 24 APSE-infected *Hamiltonella* strains. *attP* is the attachment site in APSE genome, adjacent to the integrase gene P38. *attB* is the attachment site in *Hamiltonella* genome, overlapping with an arginine tRNA. *attL* and *attR* are integrated boundaries. *Hamiltonella* strains are identified by the first two letters of generic name and three letters of specific name of the host, followed by the clone name (if applicable) and APSE strain name (if assigned by Degnan & Moran, 2008a). Data for the strains with named APSE variants were published by Degnan and Moran (2008a), data for other strains is new with this study.

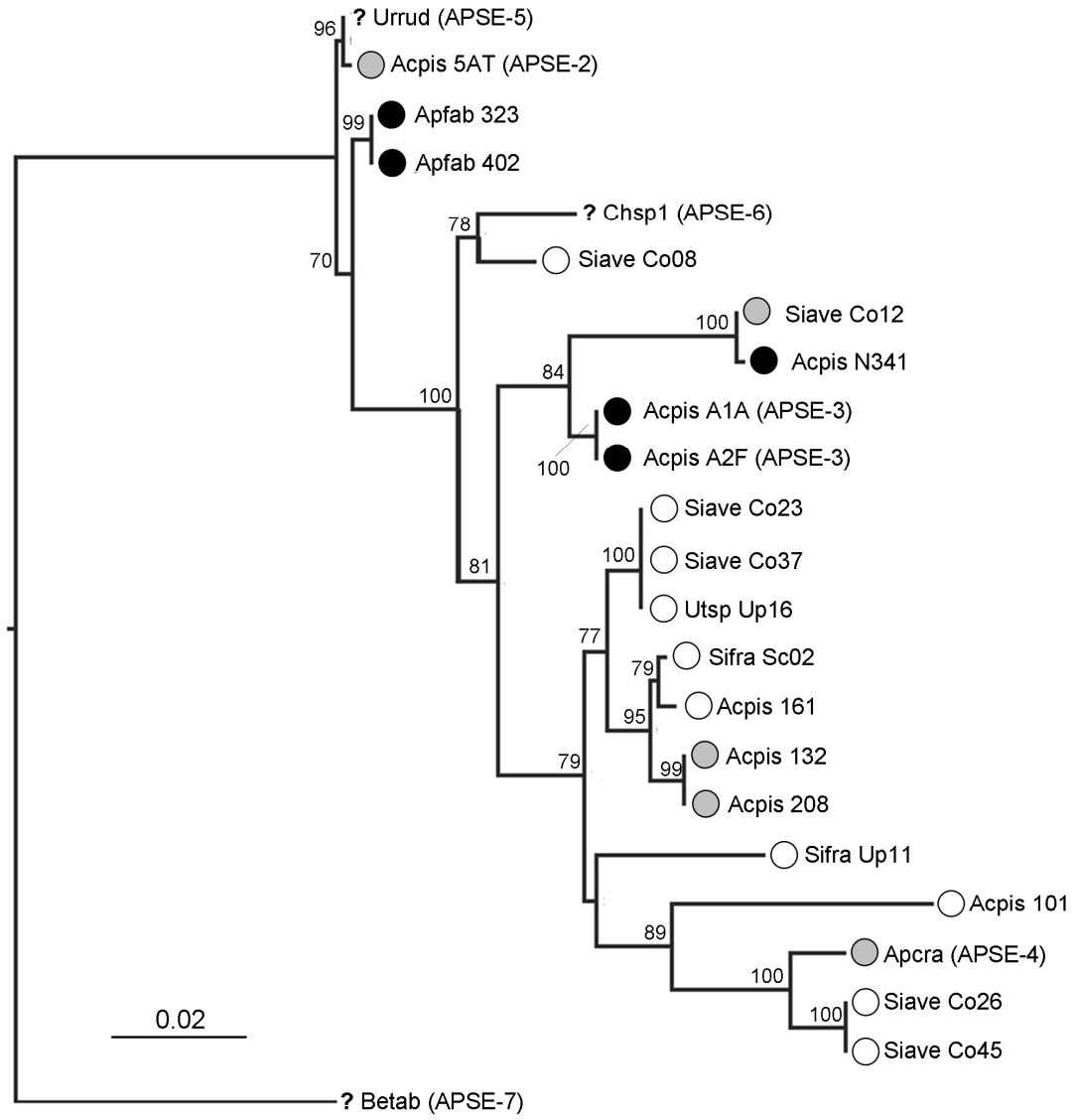

Figure 1. Maximum likelihood phylogenies based on nucleotide sequences of the six housekeeping genes for 23 strains of *Hamiltonella defensa*. Parasitoid-resistant phenotypes are shown as black (highly resistant), grey (partly resistant) or white circles (non-resistant) or question marks (no data). *Hamiltonella* strains are identified by the first two letters of generic name and three letters of specific name of the original host, followed by the clone name (if applicable) and APSE strain name (if assigned by Degnan & Moran, 2008a). Bootstrap support values larger than 0.50 are presented as percentages above the nodes.

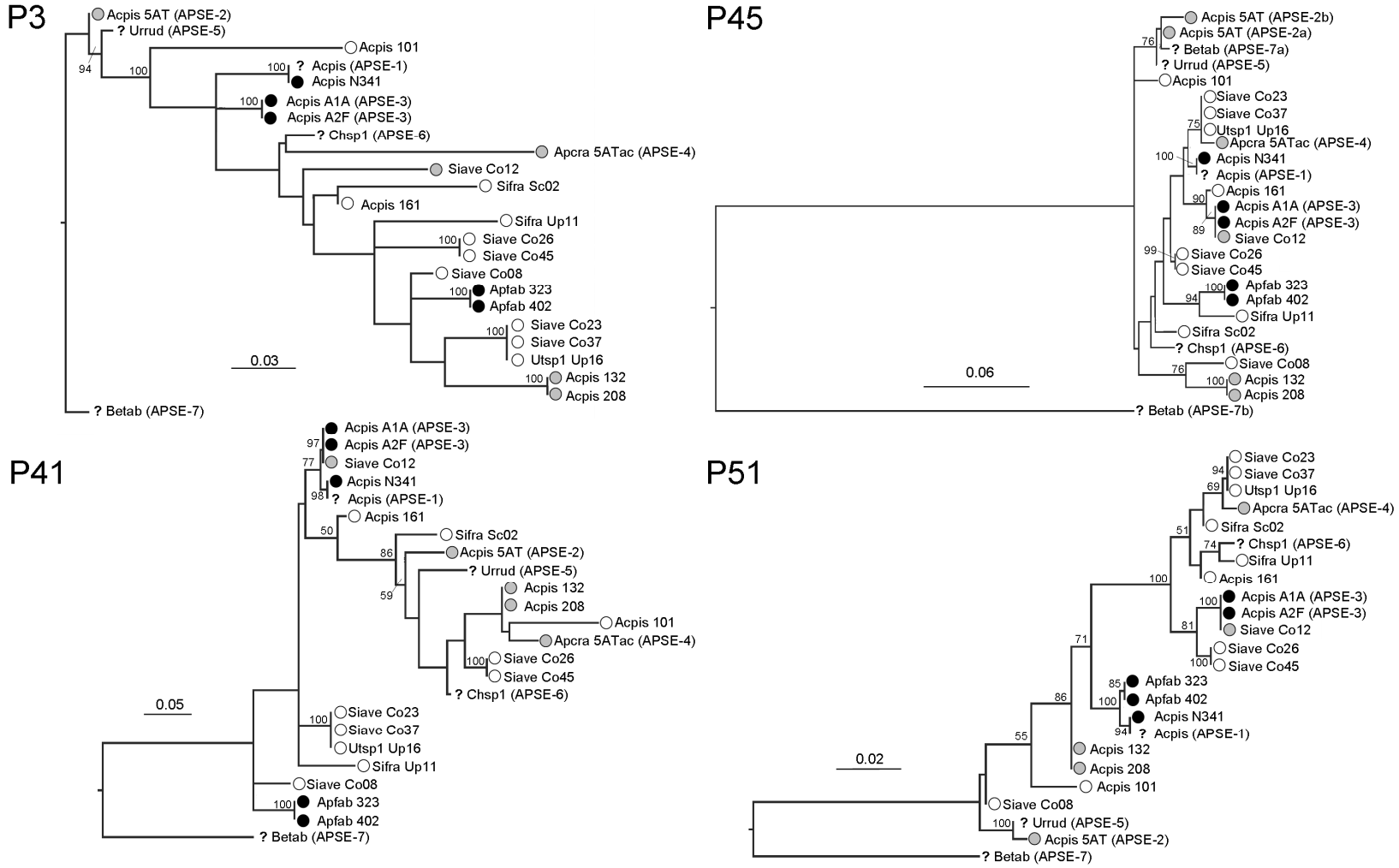

Figure 2. Maximum likelihood phylogenies for four APSE loci sequenced from the experimental strains. Parasitoid-resistant phenotypes are shown as black (highly resistant), grey (partly resistant), or white circles (non-resistant) or question marks (no data). Hamiltonella strains are identified by the first two letters of generic name and three letters of specific name of the host, followed by clone name (if applicable) and APSE strain name (if assigned by Degnan & Moran, 2008a). Bootstrap support values larger than 0.50 are presented as percentages

Chapter 9

Multiple species of facultative endosymbionts protect aphids from a fungal pathogen

Abstract

Facultative endosymbiotic bacteria confer a range of important effects on their insect hosts, including protection against natural enemies. The pea aphid can be protected by the symbionts against parasitoids and pathogenic fungi. However, there is limited understanding of how widespread these defensive effects are across symbiont strains and species, or across populations and species of hosts. Also, as different species of facultative symbionts frequently co-infect the same hosts, it is often not clear how the phenotypic effects of infection observed in multiply infected insects are determined, and how interactions between different bacteria within common hosts influence host fitness.

In the present study, we ask about the roles of facultative endosymbionts in two pea aphid clones, naturally co-infected with *Spiroplasma* and *Hamiltonella* in the first case, and with *Rickettsia* and *Hamiltonella* in the second. Both clones were resistant to a pathogenic fungus *Pandora neoaphidis* while harbouring both symbionts. We introduced the symbiont species from these co-infections, together or separately, into five novel host genotypes of two aphid species, and then tested their effects on aphid fecundity and on susceptibility to *Pandora*. Our data revealed that the experimental strains of *Rickettsia* and *Spiroplasma* confer resistance to the pathogen in all novel host genotypes, regardless of the presence of *Hamiltonella*. In some cases, co-infection with *Hamiltonella* increased fecundity in the pea aphid clones and never decreased it compared to single infections with *Spiroplasma* or with *Rickettsia*. These data suggest the presence of complex interactions between symbiont species, and a possible explanation for the frequency of multiple infections in nature.

We also introduced additional strains of five species of the pea aphid endosymbionts into one of the novel host clones of the pea aphid, and then tested these novel lineages for susceptibility to *P. neoaphidis*. Strains of four unrelated endosymbiont species: *Rickettsia*, *Spiroplasma*, *Rickettsiella* and *Regiella*, conferred a variable, but in all cases highly significant degree of resistance to the same strain of a fungal pathogen in the same host genetic background. These results, together with the conclusions of previous studies, reveal that all known facultative endosymbionts of pea aphids are involved in protecting their hosts against natural enemies, suggesting the importance of these defensive effects in the evolution of insect symbioses.

Introduction

The interactions with facultative endosymbiotic bacteria play an important role in the biology of their insect hosts (Moran, 2006; Moran et al., 2008). Facultative (or secondary) endosymbionts are using hosts as sources of nutrients and energy, thus imposing some costs to their carriers, but at the same time they are mostly transmitted maternally, and cannot easily invade and establish themselves in novel hosts which are unrelated to their original carriers (Chen et al., 2000; Chen & Purcell, 1997; Darby & Douglas, 2003; Chapter 7 of this thesis). However, there is evidence for the horizontal transmission of facultative symbionts, both between matrilines within a species and between species, on an evolutionary time-scale (Russell et al., 2003; Sandstrom et al., 2001; Weinert et al., 2009; Chapter 3 of this thesis). In order to persist and spread in insect populations facultative symbionts typically employ one of two distinct strategies: either manipulating reproduction of their hosts in order to increase maternal transmission, or providing fitness benefits which allow their carriers to increase in frequency in populations (Engelstadter & Hurst, 2009; Moran et al., 2008; Oliver et al., 2010; Werren et al., 2008). The facultative endosymbiotic mutualists have probably received the most attention in aphids, particularly in the pea aphid, *Acyrthosiphon pisum* Harris 1776.

In addition to the primary endosymbiont *Buchnera,* which produces nutrients essential to the aphids but deficient in their diet of plant sap and which is found in almost all Aphididae (Douglas, 1998), not less than seven species of secondary endosymbionts have been reported from different host races of the pea aphid. Many of the effects the facultative symbionts can have on biology of their hosts have already been described (Oliver et al., 2010). A gammaproteobacterium *Hamiltonella defensa* was shown to confer resistance against hymenopterous parasitoids in two

aphid species (Oliver et al., 2005; Oliver et al., 2003; Vorburger et al., 2009; but see Chapters 5-7 of this thesis), while a related symbiont, referred to as X-type, may extend the protection conferred by *Hamiltonella* to higher temperatures where it otherwise would be ineffective (Guay et al., 2009). Two other bacteria, *Serratia symbiotica* and *Regiella insecticola*, have also been linked to aphid resistance to parasitoids (Ferrari et al., 2004; Nyabuga et al., 2010; Oliver et al., 2003; Vorburger et al., 2010). *Serratia* can also protect aphids against heat shock (Chen et al., 2000; Montllor et al., 2002; Russell & Moran, 2006). *Regiella* is better known for its ability to confer protection against an entomopathogenic fungus (Ferrari et al., 2004; Scarborough et al., 2005), to influence host plant specialisation (Tsuchida et al., 2004), induction of the winged morph and switch to sexual reproduction (Leonardo & Mondor, 2006). The most recently described facultative endosymbiont, *Rickettsiella*, can alter the body colour of pea aphids, potentially influencing aphid interactions with visually-searching natural enemies (Tsuchida et al., 2010). The fitness benefits of infection with two other endosymbionts of the pea aphid, and the mechanisms they utilize to persist in aphid populations, are less well understood. *Rickettsia* was shown to protect its pea aphid hosts from moderately high temperatures (Chen et al., 2000), but at 18-20°C infection with this bacterium incurred significant fecundity costs in all tested clones (Chen et al., 2000; Montllor et al., 2002; Sakurai et al., 2005; Simon et al., 2007). Similarly, *Spiroplasma* negatively affects growth, reproduction and longevity of their pea aphid hosts (Fukatsu et al., 2001), but no fitness benefits of infections have been reported. Neither *Spiroplasma* nor *Rickettsia* appear to affect sexual reproduction in aphids (Simon et al., 2007; McLean et al., unpublished data). However, both these bacterial genera are widespread and common in pea aphid populations (Chen et al., 1996; Frantz et al., 2009; Simon et al., 2003; Tsuchida et al., 2002). Both have also been reported from a

range of arthropods beyond aphids, and while they have usually been regarded as pathogens and reproductive manipulators (Harris et al., 2010; Perlman et al., 2006; Weinert et al., 2009), they can provide important fitness benefits to their hosts (Himler et al., 2011; Jaenike et al., 2010; Xie et al., 2010).

Much of the data on the role of facultative endosymbionts have been gathered from studies correlating life history traits with infection statuses in naturally infected aphids originating from populations harbouring diverse bacteria (Ferrari et al., 2004; Nyabuga et al., 2010; von Burg et al., 2008; Vorburger et al., 2009), or by assessing the effects of manipulating symbiont strains within singly infected hosts (e.g., Oliver et al., 2003; Russell & Moran, 2006; Scarborough et al., 2005). These studies provided invaluable information about the importance and functions of symbioses. However, it is becoming clear that in aphids and other insects co-infections with multiple strains and species of facultative endosymbionts are common (Burke et al., 2009; Ferrari et al., accepted; Frantz et al., 2009; McLean et al., 2011; Skaljac et al., 2010). It is often difficult to determine the contributions of different bacteria within multiply infected hosts to the observed phenotypic effects of infection (Nyabuga et al., 2010; Chapter 7 of this thesis). Also, symbionts can interact within their hosts, and these interactions can dramatically affect the dynamics of the symbiotic populations and impact the biology of the hosts (Koga et al., 2003; Oliver et al., 2006; Sakurai et al., 2005). Furthermore, there is much potential for the symbionts stably co-residing in single hosts to co-adapt, likely with benefits to the hosts (Vautrin & Vavre, 2009). However, interactions between symbionts in multiple infections and the ecological consequences of these co-infections are not well understood. Dissecting the phenotypic effects of a co-infection with facultative endosymbionts in a single host requires separating the symbiont strains while controlling for the host genetic background, and only then assessing the effects of the

infections. This may be a complicated multi-step procedure, particularly in sexually reproducing species (Goto et al., 2006; Jaenike et al., 2010; White et al., 2011; White et al., 2009), but can be the only way to unequivocally identify symbionts responsible for manipulating the host reproduction or for other fitness effects of infection. An alternative approach, identifying hosts singly infected with strains identical or highly similar to those in multiply-infected hosts in the same population, and introducing them either separately or together into novel non-infected hosts, has been employed in aphids. For example, Oliver and colleagues (2006) reported large fitness costs of co-infection with *Hamiltonella* and *Serratia* in a pea aphid clone. In contrast, the effects of introducing *Rickettsia* and *Serratia* from a doubly infected donor on aphid fecundity across host plants and rearing temperatures did not differ from the effects of introducing *Rickettsia* alone from a singly infected donor into the same novel host genotype (Chen et al., 2000). While these studies have not controlled for differences between symbiont genotypes and the potential for co-adaptation between symbionts in long-term multiple infections, they provided evidence for interactions between symbionts in their effects on hosts, resulting in novel phenotypes.

In her review of symbiont-mediated defences in invertebrates, Haine (2008) provided seven examples of microbes protecting their arthropod hosts against pathogens, parasites or predators, and predicted that such effects should be more common. Indeed, additional examples of endosymbionts protecting their hosts against viruses, pathogenic fungi, parasites and parasitoids have been reported since (Jaenike et al., 2010; Sternberg et al., 2011; Teixeira et al., 2008; Vorburger et al., 2010; Xie et al., 2010), suggesting that provision of resistance to natural enemies may be widespread among endosymbionts and emphasising the need to further study the distribution, diversity and ecology of these protective effects of symbiont

infections. Pea aphids, which can harbour diverse endosymbiotic bacteria in different combinations, and whose general biology and ecology, including interactions with natural enemies, are well understood, make an ideal system for assessing the roles of facultative endosymbionts in resistance to natural enemies. Work on aphid endosymbionts is facilitated by the fact that some of these bacteria can be manipulated easily within aphid clones by antibiotic treatment or by haemolymph microinjection (Chen & Purcell, 1997; Koga et al., 2007; McLean et al., 2011; Chapter 7 of this thesis), and that any newly developed clonal host-symbiont associations can be maintained indefinitely in the laboratory without inducing sexual reproduction. That permits assessment of the fitness consequences of infections in controlled genetic backgrounds.

Among the most important natural enemies of aphids, including the pea aphid, are entomopathogenic fungi (Feng et al., 1991; Feng et al., 1990; Milner, 1982; Pickering et al., 1989). Fungal spores landing on aphid cuticle germinate, and fungal hyphae invade the aphid haemocoel eventually killing the aphids and overgrowing the cadaver. Under humid conditions, the fungus sporulates, dispersing the spores and thus closing the cycle. The Entomophthorales fungus *Pandora* (= *Erynia*) *neoaphidis* is among the pathogens most likely to cause natural epizootics in a wide spectrum of aphid species, and its impact on the biology of aphids and their natural enemies is being investigated with prospects of using the fungus as a biocontrol agent of temperate zone pest aphids (e.g., Ekesi et al., 2005). The pea aphid is among the species more susceptible to *Pandora* infection (Feng et al., 1991; Pickering et al., 1989; Shah et al., 2004), but clones very considerably in susceptibility to *Pandora* (Ferrari et al., 2001; Milner, 1982), and this variation is associated with infection with a facultative endosymbiont *Regiella insecticola* (Ferrari et al., 2004; Scarborough et al., 2005). However, when testing a collection of pea aphid clones

against a *Pandora* isolate we realized that two of them were resistant to the pathogen, despite not being infected with *Regiella* (M. van Asch et al., unpublished data). One of them, with code number 161, was double infected with *Hamiltonella* and *Spiroplasma*, and another one, with code number 208, harboured both *Hamiltonella* and *Rickettsia*. Later, we found that the resistance can be transferred to other clones together with the endosymbiont complement (Chapter 7 of this thesis), and that elimination of *Hamiltonella*, not affecting the presence of the second symbiont, does not decrease the degree of protection in the original hosts (M. van Asch et al., unpublished data). These results suggested a novel and important ecological function for the two species of aphid endosymbionts. Unfortunately, we did not succeed in removing *Spiroplasma* or *Rickettsia* from the original hosts, which could then be tested for fungal resistance in order to exclude the effects of other cytoplasmic factors on that important trait.

In this chapter we explore the effects of individual symbionts in natural multiple infections on life history traits of their aphid hosts, including fecundity and resistance to an entomopathogen *Pandora neoaphidis*. In particular, we ask whether resistance to the pathogen can be conferred by symbionts other than *Regiella*, whether it can be altered by co-infection with another symbiont species in the same hosts, and whether it can be expressed in other species beyond the pea aphid. We also ask whether the provision of resistance to entomopathogens may be more common among facultative endosymbionts of aphids. In the first experiment reported in this chapter, we separated the facultative endosymbionts from two naturally double-infected pea aphid clones, harbouring *Spiroplasma* and *Hamiltonella* in the first case, and *Rickettsia* and *Hamiltonella* in the second. These symbionts were introduced, both in co-infections and separately, into five clonal genotypes of two aphid species. The newly developed host-symbiont associations were tested for their susceptibility to

Pandora and had their fecundity measured. In the second experiment, we introduced seven additional strains of pea aphid endosymbionts representing five species, *Spiroplasma*, *Rickettsia*, *Hamiltonella*, *Regiella* and *Rickettsiella*, into one of the novel host genotypes, and tested these novel lineages for susceptibility to *Pandora*.

Material and methods

Study organisms

The aphid clones used in this study were collected in South England between 2003 and 2010, and maintained in laboratory culture since then. Most of the time, they were cultured at 14±1°C in 90-mm non-vented Petri dishes with leaves of broad beans (*Vicia faba*, v. the Sutton) or wheat, which were kept fresh by inserting their stems in 2% agar. Culturing newly injected aphids, building up populations of experimental lineages, fecundity experiments and some of the post-fungal exposure culturing were all conducted in identical dishes at 20±2°C. All clones were assessed for infection with the known facultative symbionts of aphids: *Hamiltonella defensa*, *Regiella insecticola*, *Serratia symbiotica*, X-type, *Rickettsia*, *Spiroplasma* and *Rickettsiella*, following DNA extraction with DNeasy Blood & Tissue Kit (Qiagen), using diagnostic PCRs with specific primers (McLean et al., 2011; Tsuchida et al., 2010). The names, symbiont complements and original host plants of all the experimental clones are listed in Table 1. Sequences of 16S rRNA genes of *Rickettsia* and *Rickettsiella* strains were obtained using Big Dye Terminator v. 3.1 (Applied Biosystems Ltd., USA) and will be deposited in Genbank prior to publication of this chapter. Sequences of 16S rRNA genes of *Spiroplasma* were of high quality and at least 98% identical to the sequence of the pea aphid *Spiroplasma* deposited in Genbank, but chromatograms revealed polymorphisms at several

nucleotides, suggesting the presence of distinct *Spiroplasma* genotypes within the experimental clones. We have not attempted to distinguish between them.

The *Pandora neoaphidis* strain X4 (= isolate NW 327) used in the study was provided by Rothamsted Resarch. This isolate was originally obtained from infected pea aphids on Rothamsted Farm, Hertfordshire, UK, in 1997 (Shah et al., 2004), and used in previous studies on the role of defensive endosymbionts in aphids (Ferrari et al., 2004; Scarborough et al., 2005; Chapter 7 of this thesis). Before this study, the isolate was maintained *in vivo* by regular passage through aphids of a susceptible pea aphid clone 145 for several generations. Cadavers of recently fungus-killed aphids were dried and stored at 4°C in low humidity conditions, and used within four weeks of preparation.

Establishment of experimental lineages

The first experiment presented in this chapter aimed at identifying the roles of the facultative endosymbionts originally infecting two pea aphid clones, *Hamiltonella* and *Spiroplasma* co-infecting clone 161 and *Hamiltonella* and *Rickettsia* co-infecting clone 208. McLean et al. (2011) removed *Hamiltonella* from both clones by orally administering a mixture of antibiotics, but without affecting the infections with *Rickettsia* or *Spiroplasma*. These lineages of clones 161 and 208 cured from *Hamiltonella* infection (with only the second symbiont remaining), as well as the lineages originally double-infected, were used as donors of haemolymph for the injections. The injection procedure is described in detail in Chapter 2 of this thesis. The different complements of symbionts were introduced into five recipient clones free from infection with any of the known secondary symbionts. The recipients included three originally symbiont-free clones of the pea aphid (code numbers 145, J68 and J102) and two clones of the grain aphid cured from the original infection

with *Hamiltonella* (Co23X and Co26X; Chapter 4 of this thesis) (Table 1). That way, in each of the five recipient clonal genotypes we developed five distinct lineages, which harboured different complements of secondary symbionts: 1) symbiont-free; 2) infected with *Spiroplasma* and *Hamiltonella* from 161; 3) infected with *Spiroplasma* from 161 only; 4) infected with *Rickettsia* and *Hamiltonella* from 208; and 5) infected with *Rickettsia* from 208 only. Additionally, in some recipient clones, injection with haemolymph from a doubly infected donor resulted in only *Hamiltonella*, but not the second symbiont establishing. These *Hamiltonella*-only-infected lineages were also included in the study (Table 2).

For the second experiment, we introduced seven additional strains of five symbiont species (Table 1) into the pea aphid clone 145. Three of these additional strains originated from donor clones which were originally doubly infected, but with one of the symbiont species removed by antibiotic treatment (clones 141 and 333 - McLean et al., 2011), or during a passage through another host clone (clone 185 - Chapter 7 of this thesis) well in advance of the present study.

In all cases, successful infections were confirmed with diagnostic PCRs in the second generation after injection, and at least twice more by the end of the study. The identity of the experimental clones was also confirmed at least once using microsatellites (Chapter 5 of this thesis).

Experimental protocols

Fecundity assays were conducted following a protocol described in Chapter 4 of this thesis. The experimental aphids were produced in Petri dishes within 8 hours by synchronised high-quality wingless mothers, and were kept in groups of 6 on plants exchanged every three days. 7 days after birth, before they started reproducing,

young wingless adults or 4th instar juveniles with no wing buds were randomly selected from across the dishes, and isolated in separate Petri dishes. Due to large variation in frequency of morphs across lineages, initial culturing at higher densities was necessary so that sufficient numbers of wingless females could simultaneously be obtained in all lineages in a block, and the fact that the experimental aphids originated from not less than six uniform and high-quality dishes per lineage should have excluded any systematic effects. Following isolation, reproducing aphids were transferred to fresh dishes with new plants every three days, and offspring produced during each three-day interval by each individual aphid were counted. The total number of offspring produced by females surviving until the end of the experiment, that is over 19 days by pea aphids or over 16 days by grain aphids, was used as a measure of fecundity. Lineages of clone 145 were tested in two blocks, with 18-20 females per lineage per block, and lineages of other clones were tested in a separate block for each clone, with 12-22 females per lineage.

The protocol for the fungal susceptibility assay was based on a protocol developed by Ferrari et al. (2001). Briefly, groups of 20 young wingless adult aphids were exposed for 90 minutes to sporulating aphid cadavers in cylinders approx 25mm high and of 15mm diameter. Afterwards, the aphids were kept in Petri dishes (clones Co23X, Co26X, and 145 in Experiment 1) or on bean plants (clones J68, J102 in Experiment 1, and 145 in Experiment 2) at humidity approaching 100% for 24 hours, and then at reduced humidity for another week. Aphid sporulation rate and survival were assessed six days following the exposure, but all surviving aphids were kept for an additional two days, allowing us to measure the rate of late sporulation. In Experiment 1, the lineages were tested in five blocks, separately for each of the clones. We exposed four to ten groups of 20 aphids from each of 29 experimental lineages, or 228 groups in total. In Experiment 2, 12 experimental lineages of clone

145, including the newly developed ones and the ones previously developed for Experiment 1, were tested. Six to ten groups of 20 aphids from each lineage, or 110 groups in total, were tested in a single large block.

Statistical analysis

The proportion of aphids exposed to *Pandora* spores which turned into sporulating cadavers within 8 days was compared across clones and symbiont complements using generalized linear modelling techniques. A quasibinomial distribution was assumed in order to account for overdispersion in the data. In Experiment 1, in order to assess the relative importance of *Hamiltonella*, we compared the full model, with seven levels of symbiont infection status, against a model with three levels of symbiont infection status, either "*Rickettsia*-infected", "*Spiroplasma*-infected" or "*Rickettsia* and *Spiroplasma*-free", thus with information on *Hamiltonella* infection status discarded. The survival of the exposed aphids for 6 days after the exposure was analyzed the same way.

The effects of infection on aphid fecundity were analyzed separately for each recipient clone and for each set of lineages infected with symbionts from a particular donor (161 or 208). In each case, the data was checked for normality and then analyzed with ANOVA, with symbiont complement as a factor. In case of significant differences between lineages, means were compared with Tukey's HSD test. Prior to the main analysis, data for lineages of clone 145, collected in two blocks, was analyzed with ANOVA with block and symbiont complement as factors, and pooled as there was no significant block effect or block × symbiont interaction.

All data were analyzed using the statistical package R v. 2.13.0 (R Development Core Team, 2011).

Results

Experiment 1. Identifying the roles of individual symbionts in multiple infections

The five experimental clonal genotypes of two aphid species, when not harbouring any facultative endosymbionts, were highly susceptibile to the pathogenic fungus *Pandora neoaphidis*. Introduction of the symbionts from the two pea aphid donors strongly affected aphid responses to the pathogen. Across recipient clones, we observed consistent differences between different symbiont complements in their effects on the proportions of aphids turning into sporulating cadavers within eight days from the exposure to fungal spores ($F_{6,223} = 298.30$, $p < 0.001$). Although there were significant differences between aphid clones in the effects of infection with particular symbiont complements (clone × symbiont interaction: $F_{18,217} = 6.29$, $p < 0.001$), the patterns were broadly similar across host clones and species (Figure 1). The differences between symbiont complements in susceptibility to the fungus were associated with the presence of *Rickettsia* and *Spiroplasma*, but not *Hamiltonella*. Discarding the information about *Hamiltonella* infection status, and classifying the experimental lineages as "*Rickettsia*-infected", "*Spiroplasma*-infected" or "*Rickettsia* and *Spiroplasma*-free", and thus reducing the number of levels of symbiont infection status from seven to three, decreased the explanatory power of the model ($F_{4,221} = 2.48$, $p = 0.045$), but 98.6% of the deviance explained by symbiont complement was associated with the presence/absence of *Rickettsia* and *Spiroplasma*.

By reducing their susceptibility to the pathogen, *Rickettsia* and *Spiroplasma* significantly increased the survival of the exposed aphids. Across experimental clones, we detected significant differences between symbiont complements in their effects on aphid survival for six days after the exposure ($F_{6,223} = 97.75$, $p < 0.001$).

While discarding information on *Hamiltonella* infection decreased the explanatory power of the model ($F_{4,221} = 2.56$, $p = 0.040$), 97.2% of the deviance explained by the symbiont complement was associated with the presence/absence of *Rickettsia* and *Spiroplasma*. Parallel with differences in the susceptibility to fungus, we also observed significant differences between the recipient clones in the effect of symbiont complements on aphid survival ($F_{18,217} = 7.79$, $p < 0.001$)(data not shown). However, in all clones except Co26X *Rickettsia*-infected and *Spiroplasma*-infected lineages had a significantly higher survival than lineages free from infection with either of these two symbiont species ($p < 0.001$ in all cases). In Co26X, only *Rickettsia* infection significantly increased the survival of the exposed aphids.

The effects of endosymbiont infection on fecundity varied between recipient clones, and particularly between aphid species (Figure 2). The fecundity data were compared separately for each clone and for symbionts originating from each donor. Below, we separately summarize the effects on fungal susceptibility and fecundity of single and double infections with *Rickettsia* or *Spiroplasma*, and single infections with *Hamiltonella*.

Infection with *Rickettsia* made all recipient clones of the two aphid species immune to *Pandora*, with only 0.15% of the total number of *Rickettsia*-infected aphids sporulating following the exposure. Single infection with *Rickettsia* from clone 208 significantly decreased fecundity of the three pea aphid clones. In all pea aphid clones, the fecundity of the lineages double infected with *Rickettsia* and *Hamiltonella* was intermediate between the fecundities of lineages non-infected and infected with *Rickettsia* only. In contrast, in the two grain aphid clones *Rickettsia* infection did not affect fecundity, regardless of *Hamiltonella* co-infection.

In all pea aphid clones, infections with *Spiroplasma* from 161 conferred partial resistance to *Pandora*, and significantly delayed aphid deaths and fungal sporulation. While among the pea aphid lineages which harboured neither *Spiroplasma* nor *Rickettsia* 99% of all sporulation occurred within 6 days from the exposure, among *Spiroplasma*-infected lineages 37% of all sporulation occurred after the 6th day; this difference was highly significant ($F_{8,902} = 17.98$, $p < 0.001$). Furthermore, in at least 30% of cases, *Spiroplasma* caused the dying pea aphids to drop off the plants before they sporulated, further reducing *Pandora* transmission efficiency. This was not observed in the susceptible symbiont-free or *Hamiltonella*-infected aphids. Single infections with *Spiroplasma* did not significantly affect the fecundity of pea aphids, but in clone J68 double infection with *Spiroplasma* and *Hamiltonella* significantly increased fecundity.

In both grain aphid clones, *Spiroplasma* infection reduced fungal sporulation to zero, and in one of them, Co23X, survival over the six days after exposure was significantly higher in infected lineages compared to susceptible lineages of the same clone. Low survival of *Spiroplasma*-infected aphids of clone Co26X following the fungal exposure was mirrored by low survival in non-exposed control replicates of the same lineages, suggesting that the high mortality was caused by their susceptibility to the high-humidity and high-density conditions rather than by the pathogen. Also, in the two grain aphid clones co-infections with *Spiroplasma* and *Hamiltonella* significantly decreased fecundity, even though single infections with either symbiont had no significant effect on that trait (Figure 2). The negative effects of double infection with *Spiroplasma* and *Hamiltonella* were particularly dramatic in clone Co26X: females surviving until the end of the study had the lowest fecundity of all studied lineages (Figure 3), and 60% of them died by the end of the

experiment. In no other lineage of Co26X or any other clone did mortality exceed 14%.

We recorded no effect of single infection with *Hamiltonella* from clones 161 or 208 on fungal susceptibility of the experimental clones. Also, infection with *Hamiltonella* alone did not have a significant effect on the fecundity of any of the four recipient clones of the two aphid species.

Experiment 2. Survey of pathogen resistance conferred by different symbionts

In the second experiment, in which the pea aphid clone 145 was infected with a wider collection of symbiont strains, the lineages varied greatly in the sporulation rate following the exposure to *Pandora* ($F_{11,109} = 81.8$, $p < 0.001$) (Figure 3). The non-infected lineage and lineages infected with *Hamiltonella* only or with *Spiroplasma* from clones 185 or 333 were highly susceptible to the fungus, with not less than 90% of the exposed aphids turning into sporulating cadavers within 8 days from the exposure. *Spiroplasma* from clone 161 and *Regiella* decreased susceptibility and slowed the development of *Pandora*. The *Rickettsia* and *Rickettsiella* strains conferred immunity to the fungus. During the first six days after the exposure, only 5.4% of the total number of the exposed aphids died for reasons other than *Pandora* infection leading to sporulation, indicating that the protection conferred by the symbionts directly benefitted the exposed aphids.

Discussion

We have demonstrated that strains of four unrelated species of facultative endosymbionts naturally infecting pea aphids can confer protection against the same strain of an entomopathogen in a single pea aphid host genotype. While *Regiella insecticola* (Gammaproteobacteria: Enterobacteriales) has been known to protect pea

aphids against *Pandora* (Ferrari et al., 2004; Scarborough et al., 2005), this is the first report that symbionts representing three other orders of three phyla - *Spiroplasma* (Mollicutes: Entomoplasmatales), *Rickettsia* (Alphaproteobacteria: Rickettsiales), and *Rickettsiella* (Gammaproteobacteria: Legionellales) (Chen et al., 1996; Fukatsu et al., 2001; Tsuchida et al., 2010), can confer the same resistant phenotype to the same host. The resistance provided by two of these symbiont strains, *Rickettsia* from clone 208 and *Spiroplasma* from clone 161, is expressed across a range of host genotypes of at least two aphid species. While previous studies have shown or suggested that different species of endosymbiotic bacteria may be affecting fitness of their hosts in similar ways (Chen et al., 2000; Engelstadter & Hurst, 2009; Ferrari et al., 2004; Nyabuga et al., 2010; Oliver et al., 2006; Oliver et al., 2003; Russell & Moran, 2006), the very similar fitness benefits of infection with such a wide range of unrelated symbionts in a controlled genetic background have not been reported. Our data, together with the results of previous studies, indicate that strains of all of the seven known species of facultative endosymbionts of pea aphids are involved in protecting their hosts against natural enemies, either against pathogens as for *Regiella*, *Rickettsia*, *Spiroplasma* and *Rickettsiella* (Scarborough et al., 2005; this study), or against parasitoids as for *Hamiltonella*, *Serratia* or X-type, which appears to extend *Hamiltonella*-induced protection (Guay et al., 2009; Oliver et al., 2005; Oliver et al., 2003). Correlative studies and results from other species indicate that some symbionts, particularly *Regiella*, or symbiont complements in multiply infected clones, may be capable of protecting their pea aphid hosts from different categories of natural enemies (Ferrari et al., 2004; Ferrari et al., 2001; Nyabuga et al., 2010; Vorburger et al., 2010; Chapter 7 of this thesis). Defensive properties of endosymbionts have also been demonstrated in several other arthropod species (Brownlie & Johnson, 2009; Haine, 2008; Jaenike et al., 2010; Teixeira et al.,

2008), indicating that provision of resistance to natural enemies is a common trait of facultative endosymbionts, and possibly one of the primary reasons for the independent establishment of the numerous and diverse associations between hosts and symbionts.

There is no information on the mechanisms underlying symbiont-mediated protection against pathogenic fungi. It can be expected that aphid symbionts produce antifungal compounds which disrupt growth of fungal hyphae following sprouting on aphid cuticle (Gil-Turnes & Fenical, 1992; Gil-Turnes et al., 1989). No data exist on the nature of such compounds produced by either endosymbiont strain, or on endosymbiont genes or pathways potentially involved in fungal resistance. Given the diversity of chemical compounds known to be synthesized by various bacteria, as well as their modes of action (Piel, 2004), these protection mechanisms in unrelated endosymbiont species may well have evolved independently and converged to provide the same fitness benefit to aphid hosts. Alternatively, they may have resulted from horizontal gene transfer between the symbiont species (Moran et al., 2008; Ochman et al., 2000; Wiedenbeck & Cohan, 2011). Indeed, the bacteriophage-mediated gene transfer between *Hamiltonella* strains appears to be responsible for the patchy distribution of parasitoid resistant phenotype across the symbiont phylogeny (Degnan & Moran, 2008b; Chapter 8 of this thesis), and phage transfers between symbionts strains have been reported in other systems (Chafee et al., 2010; Darby et al., 2010; Kent et al., 2011). However, there is limited information on the gene transfer between species of aphid endosymbionts (Degnan et al., 2010). Analysis of the lineages infected with different bacterial strains with a genomics, transcriptomics or metabolomics approach could provide very useful information on the nature and evolution of resistance to natural enemies in insects. Also, analysis of the specificity of compounds produced by those symbionts against a range of isolates

of different fungal pathogens could further explain their ecological significance, and maybe suggest their commercial applications.

The fitness effects of infection with different strains of the same species of endosymbiotic bacteria can be highly variable. For example, the effects of infection with a symbiont *Hamiltonella defensa* on aphid resistance to parasitoids range from none to complete immunity (Nyabuga et al., 2010; Oliver et al., 2009; Oliver et al., 2003; Vorburger et al., 2010; this thesis), and vary between parasitoid species and genotypes (Dion et al., 2011; Ferrari et al., 2004; Vorburger et al., 2009), environmental conditions (Bensadia et al., 2006) and may be influenced by co-infections with other endosymbionts (Guay et al., 2009). Resistant phenotypes conferred by particular symbiont strains could also vary across host genotypes (Bordenstein et al., 2003; this study). Similarly, isolates of fungal entomopathogens differ in virulence against aphid species and clones. Aphid biotypes immune to some isolates of *Pandora* may be susceptible to others (Milner, 1982; Shah et al., 2004), and *Regiella* strains differ in the amount of protection against a single pathogen isolate that they confer (Chapter 7 of this thesis). It is not known whether the protection conferred by the experimental symbiont strains is a more generalized and systemic response to pathogens, or has arisen as a result of frequent interactions with a particular pathogen type. Potentially, specialisation against different fungal genotypes could explain the differences in protective effects between *Spiroplasma* strains tested in the present study. On the other hand, the donor clones 185 and 333 co-infected with *Regiella* and *Spiroplasma* had been collected in 2003, and only in 2011 were their *Spiroplasma* strains found not to confer resistance. It is plausible that the ability to confer protection against fungus have been lost by the symbiont populations during the eight years of culturing in the absence of selective pressure for maintaining the resistance.

We have shown that single infections with *Rickettsia* strain from clone 208 had significant detrimental effects on the fecundity of its novel pea aphid hosts (Figure 2). These results are in accordance with findings of other authors, who detected fecundity costs of infection with *Rickettsia* under benign conditions both in original and in novel aphid hosts (Chen et al., 2000; Montllor et al., 2002; Sakurai et al., 2005; Simon et al., 2007). Interestingly, we found that in pea aphids co-infection with *Hamiltonella* did not further decrease aphid fecundity, and at least in one case compensated for the costs of infection with *Rickettsia*. In our study, single infections with *Spiroplasma* never significantly decreased the fecundity of the pea aphids, in contrast to what has been previously reported (Fukatsu et al., 2001). However, the fecundity of lineages co-infected with *Hamiltonella* was again not lower, and in one case significantly higher than that of lineages infected with *Spiroplasma* only. These data suggest that in some cases co-infection with *Hamiltonella* may compensate for the costs of infection with other symbionts, without affecting the fitness benefits they confer. This suggests the presence of complex interactions between bacterial species within hosts, with potentially important effect on host fitness (Engelstadter et al., 2007; Vautrin & Vavre, 2009). Another example of such an interaction was presented by Oliver and colleagues (2006): severe fecundity costs reported in aphids infected with *Serratia symbiotica* following superinfection with *Hamiltonella* were likely associated with a 20-fold increase in *Serratia* density, and concurrent decrease of a population of the primary endosymbiont *Buchnera* (Koga et al., 2003). We do not have data on symbiont densities in singly and doubly infected clones used in the present study. However, *Rickettsia* infection has been shown to negatively affect *Buchnera* populations (Sakurai et al., 2005), and it is possible that *Hamiltonella* co-infection reduces *Rickettsia* densities to the level where negative effects of infection on the hosts are minimized, but the pathogen-resistant phenotype is fully expressed.

Interestingly, *Rickettsia* in grain aphid genotypes does not incur fitness costs, regardless of *Hamiltonella* co-infection. In contrast, double infections with *Spiroplasma* and *Hamiltonella* significantly reduced grain aphid fecundity. In clone Co26X, the fitness costs of harbouring *Spiroplasma* appeared to exceed the benefits. This has been demonstrated during the *Pandora* resistance assay, in which the mortality of symbiont-infected aphids, either exposed to the fungus or kept as control, was not significantly less than the mortality caused by the pathogen in susceptible lineages. These interactions between strains and species of facultative endosymbionts, affected by the host genotype (Kondo et al., 2005; Chapter 7 of this thesis) could have an important effect on the evolution of symbioses, and affect the dynamics of symbiont infections in aphid species and communities. Assessing densities and comparing distributions of endosymbionts within aphid hosts infected with different complements of bacteria, and correlating them with the magnitude of fitness effects in infected hosts, would considerably increase our understanding of the ecology of symbioses.

Facultative endosymbionts, with their ability to confer important fitness effects on their hosts, to transmit maternally with high fidelity, but also to transfer horizontally between unrelated hosts, are increasingly regarded as an important force affecting the evolution of insects (Moran et al., 2008; Oliver et al., 2010; Werren et al., 2008). We have previously shown that symbionts, once successfully established in novel grain aphid hosts, do not typically incur fecundity costs, but also do not often provide fitness benefits such as resistance to natural enemies (Chapter 7 of this thesis). Thus, our previous results did not indicate that these symbiont strains could spread in populations of their hosts. In contrast, the results of the present study demonstrate important fitness benefits of infection, which can be expressed in novel hosts, including hosts of different species, following a successful horizontal

transmission. While the fecundity measure we used here cannot be regarded as a reliable estimate of fitness under highly variable natural conditions, and the degree of symbiont-conferred protection against natural enemies is bound to differ between genotypes of natural enemies and between environments (Ferrari et al., 2004; Ferrari et al., 2001; Milner, 1982; Oliver et al., 2008; Vorburger et al., 2009), it is plausible that some of the highly pathogen-resistant novel host-symbiont associations would be able to successfully compete and spread in natural host populations (Feng et al., 1991; Pickering et al., 1989). The fact that unrelated bacterial species can confer the same beneficial resistant phenotype in a range of host genotypes, and can often establish in novel hosts without incurring major fitness costs, suggest that the facultative endosymbionts may act as a horizontal gene pool in communities of aphids, and likely also in other insects. However, further work on interactions between symbionts within hosts, their effects on host fitness, and on the incidence of horizontal transmission of symbionts in natural host populations are essential for developing understanding of the effects of endosymbiotic bacteria on insect ecology and evolution.

Tables and figures

Clone	Species	Collection plant	Original symbionts	Role in the study
145	AP	*Lathyrus pratensis*	none	recipient
J68	AP	*Lotus corniculatus*	none	recipient
J102	AP	*Lotus corniculatus*	none	recipient
Co23X	SA	*Triticum sp.*	Ha*	recipient
Co26X	SA	*Triticum sp.*	Ha*	recipient
161	AP	*Medicago sativa*	**Ha, Sp**	donor
208	AP	*Lotus pedunculatus*	**Ha, Ri**	donor
101	AP	*Ononis spinosa*	**Ha**	donor for 145
132	AP	*Lotus pedunculatus*	**Ha**	donor for 145
J240	AP	*Medicago lupulina*	**Rcl**	donor for 145
126	AP	*Trifolium pratense*	**Reg**	donor for 145
141	AP	*Lotus pedunculatus*	Ha*, **Ri**	donor for 145
185	AP	*Trifolium pratense*	Reg*, **Sp**	donor for 145
333	AP	*Medicago sativa*	Reg*, **Sp**	donor for 145

Table 1. Aphid clones used in the present study. The clones belonged to one of two species, the pea aphid Acyrthosiphon pisum (AP) or the grain aphid *Sitobion avenae* (SA), and were all collected in South England between 2003 and 2010. The clones were originally infected with different combinations of *Hamiltonella defensa* (Ha), *Spiroplasma* (Sp), *Rickettsia* (Ri), *Regiella insecticola* (Reg) and/or *Rickettsiella* (Rcl), but the symbionts marked with asterisk (*) were removed from the experimental lineages well in advance of the present study.

Donor / Recipient	none	*A. pisum* 161			*A. pisum* 208		
		original		cured	original		cured
		Ha + Sp	Ha	Sp	Ha + Ri	Ha	Ri
A. pisum 145	✓	✓		✓	✓		✓
A. pisum J68	✓	✓	✓	✓	✓		✓
A. pisum J102	✓	✓	✓	✓	✓		✓
S. avenae Co23X	✓	✓		✓	✓	✓	✓
S. avenae Co26X	✓	✓		✓	✓	✓	✓

Table 2. Host-endosymbiont combinations used in Experiment 1 of the present study. Five recipient genotypes of two aphid species were injected with haemolymph from two lineages of each of the two donor clones. Clone 161 "original" was originally infected with *Spiroplasma* and *Hamiltonella*, and clone 208 - with *Rickettsia* and *Hamiltonella*, but both were cured from *Hamiltonella* infection in advance of the present study ("cured" donors). In some cases, injection of haemolymph containing two symbionts resulted in only *Hamiltonella* establishing in a particular lineage. All lineages of clone 145 were also used for Experiment 2, together with seven additional lineages infected with other strains of endosymbionts, detailed in Table 1.

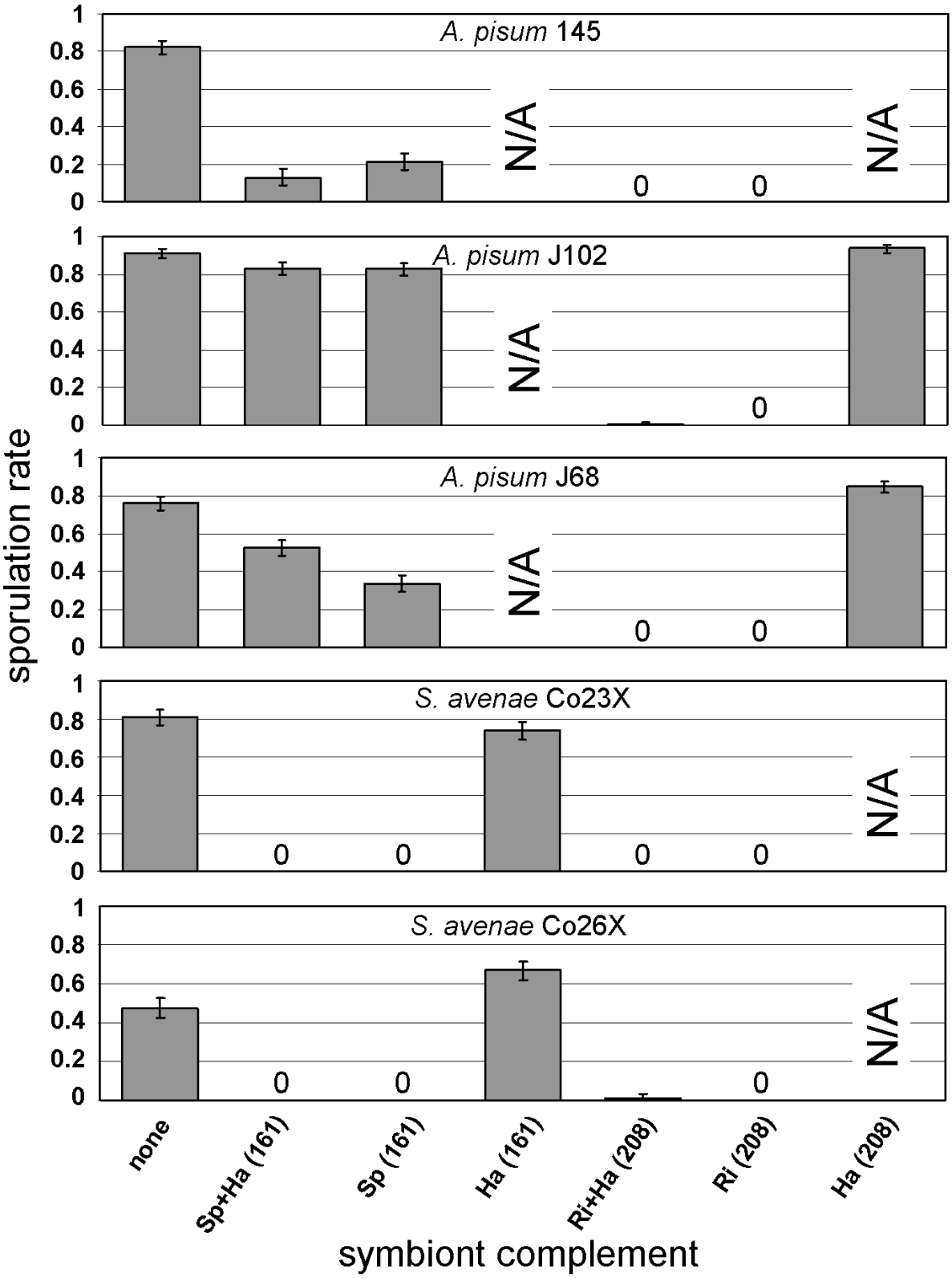

Figure 1. Susceptibility (mean ± S.E.) to *Pandora neoaphidis* of five aphid clonal genotypes, symbiont-free or infected with *Spiroplasma* (Sp), *Rickettsia* (Ri) and/or *Hamiltonella* (Ha) originating from pea aphid clones 161 and 208. Clone-symbiont combinations marked "N/A" were not available.

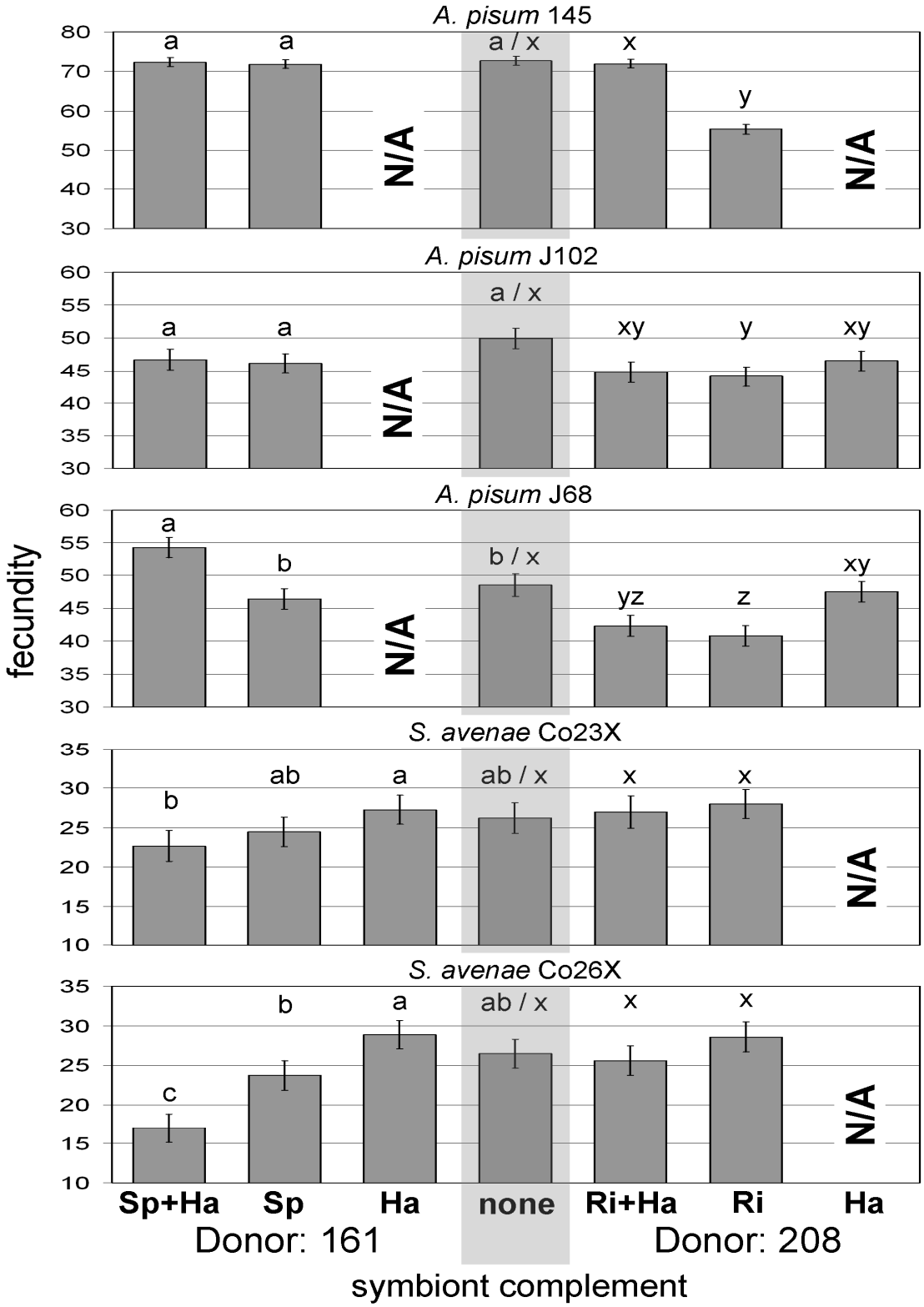

Figure 2. Fecundity (mean ± S.E.) of five aphid genotypes, either symbiont-free or infected with *Spiroplasma* (Sp), *Rickettsia* (Ri) and/or *Hamiltonella* (Ha) originating from one of the two donor clones. Clone-symbiont combinations marked "N/A" were not available. In each clone, the lineages infected with symbionts from a particular donor were compared with each other and with a non-infected lineage, but not with lineages infected with symbionts from another donor. Letters identify lineages not significantly different from others within the same comparison according to Tukey's HSD test with 95% confidence interval. The non-infected lineages were used for two comparisons each, and therefore have two sets of letters.

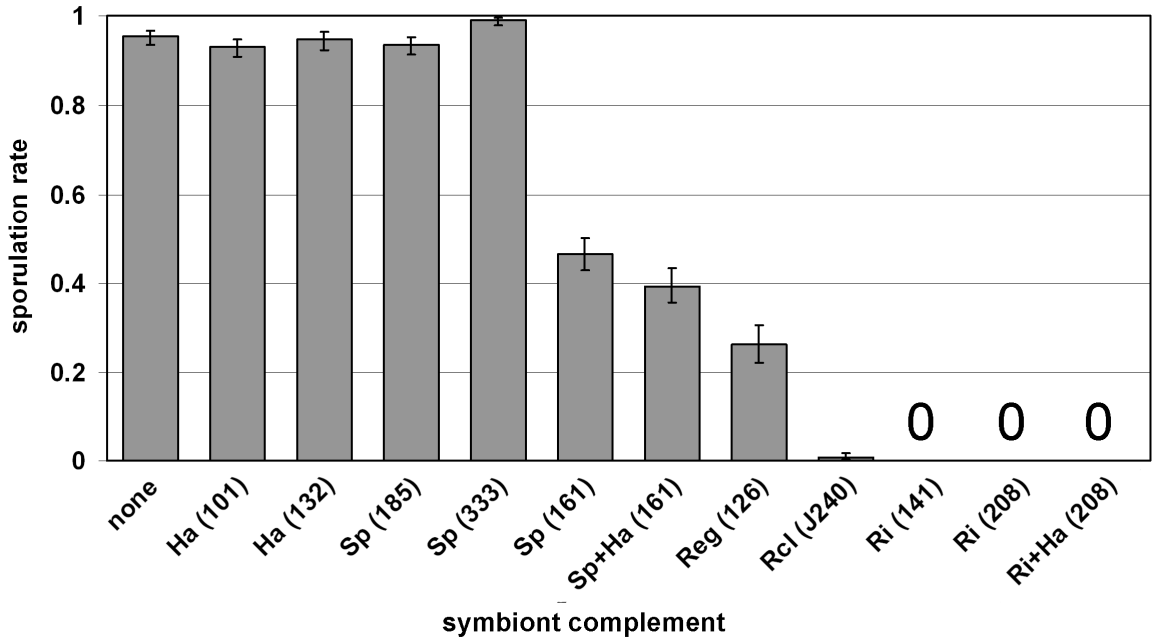

Figure 3. Susceptibility (mean ± S.E.) to *Pandora neoaphidis* of pea aphid clone 145, symbiont-free or infected with the following symbionts: *Hamiltonella* (Ha), *Spiroplasma* (Sp), *Regiella* (Reg), *Rickettsiella* (Rcl) or *Rickettsia* (Ri).

Chapter 10

General discussion

Overview

Since Buchner's (1965) first report on the incidence and diversity of microorganisms associated with insects, the understanding of their roles and importance has improved enormously. The pea aphid, *Acyrthosiphon pisum*, has emerged as one of the model organisms for the study of interactions between insects and their symbionts. In April 2008, when I was starting my D.Phil. research, many aspects of aphid symbioses were well understood. For more than a decade aphid reliance on nutrients provided by the primary endosymbiont *Buchnera*, and the extreme stability of the association between the aphids and the primary symbionts had been known (Douglas, 1998). There were also several reports on the diversity of facultative endosymbionts in pea aphid populations (Ferrari et al., 2004; Leonardo & Muiru, 2003; Simon et al., 2003; Tsuchida et al., 2002), as well as on their fitness effects of infection (Montllor et al., 2002; Oliver et al., 2005; Oliver et al., 2003; Russell & Moran, 2006; Scarborough et al., 2005; Tsuchida et al., 2004). It had been demonstrated that the symbionts can be manipulated within aphid clones; this, as well as the incongruence of the host and symbiont phylogenies (Russell et al., 2003; Sandstrom et al., 2001) had revealed the potential for transmission of these bacteria not only within host matrilines, but also between unrelated hosts. However, many aspects of pea aphid symbioses were not well understood, including the differences between aphid clones in responses to infection, or the interactions between multiple symbiont species infecting common hosts (Oliver et al., 2006). Also, the information on the distributions of facultative endosymbionts within other aphid species was scarce (Haynes et al., 2003), and data on their roles outside of the pea aphid model system virtually non-existent (Oliver et al., 2005). Thus, the conclusions which could

have been made on the importance of the symbionts in ecology and evolution of aphids were necessarily limited.

My research aimed at filling these gaps. I attempted to unravel the role of facultative endosymbiotic bacteria in the ecology and evolution of the grain aphid, *Sitobion avenae*. I anticipated that my results, in addition to providing insights into the biology of this important pest, could be compared against existing information on the pea aphid symbionts, helping to understand the role of symbioses in aphid communities. In this chapter, which summarizes my work, I draw together the results from the different experimental chapters, discuss how they complement the existing knowledge of symbiont biology, and place this work into a broader context within the study of symbiosis. I also identify directions for future exploration.

Discussion of experimental work

In a population of the grain aphid I detected a diversity of facultative endosymbionts rivalling, and likely surpassing, symbiont diversity in pea aphids. The variety of symbiont infection statuses within widespread asexual aphid multilocus genotypes collected in replicates from different host plant species in different locations indicated the ongoing horizontal transmission of secondary symbionts. It also indicated the possibility of local adaptation in obligatorily asexual clones mediated by acquisition of facultative endosymbionts (Chapter 3 of this thesis). The high incidence of infections and the number of symbiont genotypes detected in only a small sample of insects suggested the importance of secondary symbionts in the biology of grain aphids, justifying further research on this system. The rest of my work has been devoted to understanding these fitness effects of endosymbiont infection in the grain aphid ecology, but also their importance to the evolution of the multi-species community mediated by the horizontal transmission of the bacteria. By

adapting techniques previously used for work on pea aphids and their symbionts, and designing some additional methods which are briefly characterized in Chapter 2 of this thesis, I established a powerful study system which allowed me to test a range of novel hypotheses regarding the ecology and evolution of the associations between grain aphids and their facultative endosymbionts.

Infection with facultative endosymbiotic bacteria in grain aphids does not typically carry major fitness costs, despite the symbionts' nutritional dependence on their hosts. As I demonstrated in Chapters 4 and 6, antibiotic treatment resulting in elimination of secondary symbionts from their original host clones did not have a significant effect on aphid fecundity. Also, subsequent re-introduction of the same bacterial strains, or other strains of grain aphid symbionts into their original carriers did not affect aphid fecundity (Chapter 7). Furthermore, the *Hamiltonella* strains originating from four aphid species, once established in two of these naturally symbiotic grain aphids clones, did not have an effect on their reproduction. However, negative effects of infection with some other symbiont strains originating from pea aphids were detected (Chapter 7). Interestingly, the costs of infection with one of the pea aphid *Regiella* strains in two originally infected grain aphid genotypes appeared morph-specific, as the infection caused severe deformations of wings, an effect obviously not seen in the wingless morph (Chapter 7). I am not aware of such morph-specific fitness costs of infection having been reported previously.

Interestingly, in two out of four grain aphid clones which were originally free from infection with secondary symbionts, artificial introduction of *Hamiltonella* affected fecundity (Chapter 6). These effects were not consistent across clones, as in one of the clones *Hamiltonella* decreased fecundity, and in another it positively affected the number of offspring produced. A follow-up experiment revealed

variation among clonal genotypes of grain aphids in their tolerance to infection, and among symbiont strains in the costs which they impose on their novel hosts. However, the intrinsic differences in tolerance to infection between clonal genotypes of grain aphids do not appear to be a major barrier to the horizontal transmission of facultative endosymbionts in aphid populations so limiting the spread of these bacteria: even aphids representing the microsatellite genotype which I found the least tolerant to infection, Co50, may naturally harbour *Hamiltonella* (Chapter 3). Naturally symbiont-bearing clones of the pea aphid also usually suffer only limited, if any, costs of infection with gammaproteobacterial symbionts (Leonardo, 2004; McLean et al., 2011). However, such costs have been reported in naturally secondary symbiont-free pea aphid clones (Chen et al., 2000; Koga et al., 2003; Montllor et al., 2002; Russell & Moran, 2005). It is important to note that the fitness consequences of infection with novel symbionts clearly depend on the environmental conditions, including experimental temperatures, host plant species and aphid density (Chen et al., 2000; Oliver et al., 2008; Tsuchida et al., 2004), as well as on symbiont genotypes. However, never before were the consequences of infection explicitly compared between original, naturally symbiotic hosts and hosts which were not naturally infected with secondary symbionts. Also, such a large collection of secondary symbiont strains introduced into common host genotypes as that described in Chapter 7 has not been previously developed or tested. Therefore, I feel that my data provide novel insights into the fitness consequences of symbioses in aphid populations, and the potential of facultative symbionts for horizontal transmission in aphid communities.

However, data on aphid fecundity under benign conditions provide limited information on their performance under fluctuating natural conditions, when insects are subjected to a range of stressors. As Oliver and colleagues (2008) have

demonstrated, the fitness costs associated with infection may not be apparent under benign low-density conditions, but stress associated with competition for limited resources in high-density population cages can reveal the competitive inferiority of the infected lineages. It is not unusual to detect such trade-offs only under stressful conditions in other model systems (Fellowes et al., 1998). However, the protection against factors such as parasitoid-induced mortality, which the symbionts often confer, can lead to a spread of infection in a population where such stressors play an important role. It appears that this balance between costs and benefits under different conditions may be responsible for intermediate frequencies of symbiont infections in natural populations of arthropods.

I proposed that the high incidence of symbiont infections among grain aphids collected in South England, where populations of *S. avenae* are largely composed of genotypes incapable of sexual reproduction and overwintering as active stages on vegetation (Dewar & Carter, 1984; Llewellyn et al., 2003; Newton & Dixon, 1988b; Sunnucks et al., 1997), may be associated with the symbionts' influence on aphid susceptibility to freezing (Chapter 4 of this thesis). However, the comparison of frost hardiness of aphid lineages indicated that the important variation across clones in survival following a cold shock and in fecundity of survivors, previously demonstrated in *S. avenae* (Griffiths & Wratten, 1979), was not influenced by infection with facultative endosymbionts. This is the first assessment of the effects of heritable symbionts on the cold hardiness of their hosts, and it suggested that the grain aphid susceptibility to freezing is not primarily influenced by these bacteria. Therefore, the prevalence and the diversity of infections in grain aphid populations need to be explained by their other effects on aphid fitness.

The most spectacular of the effects of infection with facultative mutualists reported from pea aphids, but also from other arthropods, may be their ability to confer protection against natural enemies of their hosts, including predators, parasitoids, pathogens and viruses (Brownlie & Johnson, 2009; Haine, 2008; Oliver et al., 2010). Examples of such defensive phenotypes have been reported from several different systems, with many of the recent reports referring to observations made on *Drosophila* species (Hedges et al., 2004; Jaenike et al., 2010; Teixeira et al., 2008; Xie et al., 2010). Despite this, the parasitoid-resistant phenotype conferred on pea aphids by the gammaproteobacterium *Hamiltonella defensa* may remain the best understood of these defensive effects. A series of studies in Nancy Moran's lab at the University of Arizona has demonstrated that symbiont genotypes vary in their defensive properties (Oliver et al., 2005; Oliver et al., 2003), and that this variation may be associated with the diversity of toxins encoded by variants of the bacteriophage APSE, which infects many *Hamiltonella* strains (Degnan & Moran, 2008a, b; Moran et al., 2005a; Oliver et al., 2009). Similarly, a strong positive correlation has been reported between *Hamiltonella* infection and the resistance of the black bean aphid, *Aphis fabae*, to parasitoids (Vorburger et al., 2009). Contrary to expectations, in grain aphids the four APSE-infected *Hamiltonella* strains representing three divergent bacterial genotypes did not negatively affect the development of two species of parasitoids, as reflected by the wasps' pupation and emergence rates, or size at emergence (Chapter 5 of this thesis). A comparison of the effects of infection with the same symbiont strains on susceptibility to parasitoids of the original and novel hosts revealed that the previously observed susceptibility was not a result of a disruption of co-adapted associations between host and symbiont genotypes (Chapter 6 of this thesis). Later, I gathered additional evidence that protection against parasitoids is not a universal feature of *Hamiltonella* strains

infecting *S. avenae*, but also other aphid species in England. Only two of the 11 APSE-infected *Hamiltonella* strains originating from four aphid species and tested in the main experiment described in Chapter 7 of this thesis decreased the parasitoid emergence rate, but no strain increased survival of the exposed aphids. However, the co-infection with *Hamiltonella* and X-type originating from a parasitoid-resistant pea aphid clone reduced the mummification rate in the exposed grain aphids to zero, indicating that the susceptibility of the lineages infected with various *Hamiltonella* strains was not due to some inherent properties of the host clones or the parasitoid lines, or flaws in the testing protocol (Chapter 7 of this thesis). However, despite the absence of direct negative effects of the experimental *Hamiltonella* strains on the life history traits of parasitoid developing within infected aphids, experienced parasitoid females preferentially oviposited into aphids free from infection (Chapter 5 of this thesis). This suggests a certain degree of indirect protection conferred by the symbionts, but the importance of this phenomenon under natural conditions remains to be investigated.

However, the presence of facultative endosymbionts in the grain aphid population can at least partly be explained by their defensive properties. Resistance to a fungus *Pandora neoaphidis* of grain aphid lineages infected with *Regiella* originating from the grain aphid clone Co34 was the first demonstration of the protection against pathogens conferred by an endosymbiont outside of the pea aphid model system (Ferrari et al., 2004; Scarborough et al., 2005). In the same experiment, I demonstrated that a single symbiont strain can express the same resistant phenotype in different host species, but also that distinct strains of the same symbiont species from a single population can vary in their phenotypic effects on the hosts (Chapter 7). All these conclusions were confirmed, and further expanded, by the results of experiments presented in Chapter 9. After separating symbiont species from natural

double infections by introducing them into novel hosts, I demonstrated that the same fungal-resistant phenotype can be expressed by two facultative symbionts other than *Regiella* in five clones of two aphid species. These bacteria, *Rickettsia* and *Spiroplasma*, while common and widespread in insects (Duron et al., 2008; Harris et al., 2010; Perlman et al., 2006; Weinert et al., 2009), have traditionally been regarded as pathogens and reproductive manipulators, even though fitness benefits of infection with some strains have recently been reported (Himler et al., 2011; Jaenike et al., 2010). *Rickettsiella* has also been known as a pathogen of arthropods (Cordaux et al., 2007; Leclerque, 2008), although the fitness effects of colour change it can induce in pea aphids are likely to depend on the environment (Tsuchida et al., 2010). Like *Rickettsia* and *Spiroplasma*, *Rickettsiella* can also protect aphids against a pathogen (Chapter 9 of this thesis). These three species and *Regiella* are the only endosymbiotic bacteria which have so far been shown to protect their hosts from fungal pathogens. Although compounds produced by other bacteria, particularly strains of *Streptomyces*, may affect interactions of other insects with fungi which are important in their ecology (Currie et al., 1999; Gil-Turnes & Fenical, 1992; Gil-Turnes et al., 1989; Kaltenpoth et al., 2005; Scott et al., 2008), these other bacteria are all carried on the cuticle of their hosts rather than in their haemocoel, which suggests more labile associations. Therefore, the fact that four unrelated endosymbiotic bacteria can confer the same phenotype, expressed as resistance to a particular isolate of an entomopathogenic fungus, to a single clonal genotype of host, indicates that defensive properties of facultative endosymbionts can be very common. Three remaining species of the known pea aphid secondary symbionts, *Hamiltonella*, *Serratia* and X-type, as well as *Regiella*, have all been implicated in aphid resistance to parasitoids (Ferrari et al., 2004; Guay et al., 2009; Nyabuga et al., 2010; Oliver et al., 2003; Vorburger et al., 2010). Such widespread ability to confer

protection against natural enemies among distinct facultative endosymbionts infecting a single host species strongly suggests that this may have been one of the key traits which enabled bacteria to engage in such intimate associations with their insect hosts. By becoming infected with heritable endosymbiotic bacteria, with their often complex metabolic pathways for synthesis of defensive chemical compounds (Gil-Turnes & Fenical, 1992; Gil-Turnes et al., 1989; Kellner, 2002), insects can instantaneously acquire an ecologically important trait, whose rapid evolution *de novo* in a metazoan genome would be unlikely.

Phylogenetic data indicate that at least some of the aphid facultative endosymbionts have independently invaded insects and evolved the ability to lead an intracellular lifestyle and transmit maternally between host generations (Burke et al., 2009; Moran et al., 2008; Oliver et al., 2010). Little information is available on how these unrelated bacteria acquired the ability to confer similar phenotypic effects on their hosts. The fact that toxins classified in not less than three protein families and implicated in protecting aphids against parasitoids are all encoded by variants of bacteriophage APSE infecting different *Hamiltonella* strains (Degnan & Moran, 2008a; Moran et al., 2005a), suggests that a similar phenotype can evolve independently more than once. At the same time, defensive effects of infection are distributed across the phylogeny of *Hamiltonella* and its toxin-encoding bacteriophage APSE (Degnan & Moran, 2008a; Chapter 8), and it is clear that the phage can be exchanged between distant symbiont genotypes (Chafee et al., 2010; Degnan & Moran, 2008b; Kent et al., 2011). Thus, the horizontal gene transfer between strains, and maybe also species of bacteria also plays a role in the evolution of defensive phenotypes. It is possible that a combination of these mechanisms – horizontal transfer of some independently evolved genetic pathways - is responsible for the distribution of defensive properties among other symbionts of the pea aphid.

A comparative study of these pathways between aphid symbionts and other bacteria would provide invaluable information on the role of horizontal gene transfer in the evolution of interactions between species.

Horizontal gene transfer among endosymbiont species is the most likely to occur between strains of bacteria co-infecting the same host, and residing within the same cells (Kent et al., 2011). Recent research has revealed that multiple infections with different symbiont species or strains are common in aphids, and also in other insects (Burke et al., 2009; Ferrari et al., accepted; Frantz et al., 2009; McLean et al., 2011; Skaljac et al., 2010). However, the ecological and evolutionary implications of these co-infections are not well understood. Symbionts can interact within hosts, and these interactions can dramatically affect the dynamics of their populations (Engelstadter et al., 2007), as well as the fitness effects of infections for the hosts. For example, fecundity costs reported in pea aphids artificially infected with two gammaproteobacterial secondary symbionts appeared to be associated with a dramatic increase in the density of one of them (Oliver et al., 2006), which likely negatively affected the population of the primary endosymbiont *Buchnera* (Koga et al., 2003). Competition between different facultative symbionts for limited host resources can lead to the loss of one or more of them within a few host generations (Chen & Purcell, 1997; Moran & Dunbar, 2006; Chapter 3 of this thesis). However, in multiple infections which are stable for many host generations there is much potential for bacteria residing together within a single host to co-evolve, most likely with benefits to the host (Vautrin & Vavre, 2009). Indeed, transferring symbionts from natural double infected clones into novel host genotypes, both singly and in co-infections (Chapter 9) indicated the presence of complex interactions between symbiont species. Different symbiont species within common hosts may confer protection against different natural enemies, and one strain may ameliorate the

fitness costs of infection with another symbiont. These effects can be influenced by the host genotype. In some cases, though, multiple infections with distinct symbiont species can incur significant fitness penalties, apparently exceeding the benefits resulting from symbiont-conferred resistance (Chapters 7 and 9). Understanding the interactions between different facultative endosymbionts within common hosts, and their effects on host fitness, remains one of the most pressing problems in symbiosis research. As I have demonstrated, separating symbionts from natural multiple infections in originally symbiont-free aphid clonal genotypes can be a powerful technique, which enables exploring these effects and extending our understanding of the evolution of symbiotic bacteria.

One of the most interesting aspects of symbiont biology is the bacteria's ability to be transmitted horizontally between unrelated hosts, both within and between species (Ferrari & Vavre, 2011; Oliver et al., 2010). This ability has previously been demonstrated by phylogenetic studies (Burke et al., 2009; Russell et al., 2003; Sandstrom et al., 2001), as well as by artificial transfers of the symbionts under laboratory conditions (e.g., Russell & Moran, 2005). During my course, I combined different approaches to understanding the potential for horizontal transmission of symbionts in an aphid community, including assessing the symbiont diversity and distribution in a natural population, developing and testing 74 novel host-symbiont associations, and phylogenetic analyses of bacteria and their phages. I have shown that symbiont complements can vary within widespread aphid asexual genotypes in the field (Chapter 3), indicating horizontal transmission of the bacteria in grain aphid populations over ecological timescales. I showed that symbiont origin influenced their establishment rate in novel hosts: while bacteria established easily following a transfer between clones of *S. avenae*, the establishment success was lower and infections tended to be less stable following an artificial transfer between aphid

species. However, once successfully established, symbionts did not typically incur significant costs to their carriers, which suggests that the infections could persist in a population (Chapter 7). The differences in the effects of infections with some symbiont strains between originally symbiont-free and originally infected clones, as well as apparent differences in aphid tolerance to infection, did not appear to constitute a major barrier to symbiont spread (Chapter 6). Moreover, the fact that the same important fitness benefit of infection – resistance to an entomopathogen – can be expressed both in the original and in novel host clones and species indicates that under certain environmental conditions the successfully established symbiont may well spread (Chapters 7 and 9). Finally, a multi-locus phylogeny of several *Hamiltonella* strains has shown no relationship between their original host species and their position on a phylogenetic tree, and the same *Hamiltonella*-APSE multilocus haplotype was found to infect two distantly related host species, albeit both feeding on the same plants (Chapter 8). Although phage exchange between strains of bacteria, including facultative endosymbionts, is regarded as common (Chafee et al., 2010; Degnan & Moran, 2008b; Kent et al., 2011), I found no evidence that it happens more frequently than successful horizontal transfers of symbionts between aphid species. Thus, while barriers in the horizontal transmission of symbionts exist, the typically limited costs associated with infection and the important benefits which can be conferred by the symbionts to the novel hosts clearly make successful transfers between unrelated hosts plausible.

We are beginning to understand the distribution and roles of facultative endosymbionts within species and communities of aphids and other insects. Data on the fitness effects of infections with symbiotic bacteria in grain aphids which are presented in this thesis reveal similarities, but also differences, between the symbioses of grain aphids and pea aphids. Such comparisons help us to comprehend

the role and importance of symbioses in aphids and insects as a whole. Furthermore, the data regarding the potential for horizontal transmission of symbionts to new hosts broadens our understanding of the role of facultative endosymbionts as units of lateral transfer of genes encoding ecologically important traits within aphid and insect communities. This phenomenon is well known in prokaryotes, which can adapt to environmental challenges not only by chromosomal sequence evolution, but also by recruiting sets of new beneficial genes from outside the species, often in the form of plasmids. Spread of important traits such as resistance to antibiotics or the ability to utilize new carbon sources across unrelated bacterial species within communities has largely been determined by such lateral gene transfer. The genetic material exchanged within communities has often been described as a horizontal gene pool (Dionisio et al., 2002; Ochman et al., 2000; Thomas & Nielsen, 2005; Wiedenbeck & Cohan, 2011). It is now becoming clear that insects can also recruit genes from a horizontal pool; in their case, this pool consists of the sum of the facultative symbionts in multi-species insect communities (Ferrari & Vavre, 2011; Oliver et al., 2010). The possibility of rapid adaptation of insects to environmental challenges through acquisition of symbiotic bacteria, which, once acquired, can transfer within host matrilines with high fidelity and thus extend the heritable genetic variance in insects, and which can dramatically alter traits critical to insect fitness, challenges the traditional view of evolution of novel traits as a slow process determined by a series of gradual changes to insect genome (Boto, 2010). While further research on different aspects of insect interactions with symbionts is necessary for understanding their ubiquity and importance, it is becoming apparent that unravelling the roles of facultative endosymbiotic bacteria may change the way we think about the ecology and evolution of insects.

Future directions

When discussing the results presented in the individual chapters of this thesis, I pointed out their limitations, and suggested additional work which could expand our understanding of the reported phenomena. Here I present some lines of future research which could further our knowledge of the distribution, ecology and evolution of aphid symbioses.

As discussed in Chapter 3, there is limited information on the incidence of infections and diversity of facultative endosymbionts within aphid populations, species and communities, with the existing data largely limited to a single species, the pea aphid. Also, there have been few comprehensive surveys of symbiont diversity in other groups of arthropods, with most published projects either having scanned for a limited set of symbionts, or looked at only few individuals per host species (Duron et al., 2008). However, as I have demonstrated, a relatively small sample of insects from a single population can harbour a surprising diversity of symbionts. Describing the incidence of symbiont infections, and the bacterial diversity and distribution within and between sympatric host species, is crucial for understanding the large-scale dynamics of symbionts in multi-species communities, and their role in insect ecology and evolution.

My data have indicated variation within multilocus microsatellite genotypes of the grain aphid in the complements of secondary symbionts they carry, and suggested that acquisition of facultative symbionts may have played a role in aphid adaptation to local conditions. Many populations of *S. avenae*, but also other aphid species including numerous important pests, are largely composed of a few anholocyclic clones that are widespread across geographical areas and host plants and stable over time (e.g., Carletto et al., 2009; Figueroa et al., 2005; Haack et al., 2000; Harrison &

Mondor, 2011; Llewellyn et al., 2003). A survey of the diversity of facultative symbionts within insects representing the same genotypes, but collected in different habitats and areas, potentially followed by experimental studies, would provide information on whether symbionts can be acquired by such clones and aid their adaptations to novel environmental challenges. Study of such effects could considerably alter our understanding of the ecology and evolution of asexually reproducing organisms.

Much of our current knowledge on the fitness effects of infection with endosymbionts has been gathered in a long series of laboratory-based experiments, generally conducted under carefully controlled and favourable conditions, with the possible exception of the application of a single stress factor. However, the effects of symbionts on the relative fitness of their hosts can vary considerably between experimental conditions (Chen et al., 2000; Oliver et al., 2008). Despite this, our understanding of the effects the symbionts have on their hosts under highly fluctuating natural conditions are limited (Darby et al., 2003). Studying the consequences of symbiont manipulations in the field could provide valuable information on the real consequences of carrying symbionts. The grain aphid may be a convenient model organism for such studies, as during spring and early summer clonal colonies are typically restricted to single inflorescences of grasses or cereals. This may considerably facilitate assessing the fates of individual colonies.

In the field, aphids are attacked by a wide range of natural enemies whose relative importance changes across habitats, seasons and years (Feng et al., 1991; Kröber & Carl, 1991). Aphid clonal genotypes can vary considerably in their susceptibility to different species or genotypes of natural enemies (Ferrari et al., 2001; Milner, 1982). It can be expected that in the field the fitness benefits of

infection with a particular strain of a defensive symbiont would be related to the degree of protection it confers. However, there are no published studies reporting the effects of symbiont manipulations on susceptibility of their clonal hosts to more than one type of natural enemy (Ferrari et al., 2004; Vorburger et al., 2009). Understanding the variation in the effects of symbionts on aphid resistance to various parasitoids and pathogens would improve our understanding of the ecological roles of these bacteria. Also, studying the range of conditions under which resistance can be conferred (Bensadia et al., 2006; Guay et al., 2009) is important for understanding the dynamics of interactions between symbionts, hosts and natural enemies. Finally, learning about the nature, properties and specificity of the defensive compounds which the symbionts produce may suggest their commercial applications.

Another interesting problem concerning the biology of facultative endosymbionts is the evolution of the ability to make their aphid hosts resistant to natural enemies, present in strains of most species of secondary symbionts known to infect aphids. Analyzing the genetic pathways associated with resistance and the chemical compounds which participate in defense can reveal whether the similar phenotypic effects of infection with different bacteria are a consequence of convergent evolution in unrelated symbiont strains or whether they have resulted from horizontal gene transfer between species. Also, experimental approaches to detect horizontal gene transmission between symbiont strains and species could provide useful data on the evolution of defensive phenotypes. For example, bacteriophages infecting some *Hamiltonella* strains could potentially infect other strains of the same symbiont species if both temporarily co-occur in the same host following haemolymph microinjection.

The incidence of multiple infections with different secondary symbionts in the same hosts provides several additional exciting questions. Investigations of the dynamics of strains sharing common hosts would aid our understanding of how multiple symbionts are stably vertically transferred alongside one another, how they interact within hosts, and how this interaction is affected by environmental conditions, including the host genotype. I have shown that separating bacteria from multiple infections by introducing them into new host clones can be a powerful technique for understanding interactions between symbionts. Studies on the densities and distributions of the same secondary symbiont strains in single and multiple infections, on their effects on the primary symbiont *Buchnera*, and on the degree and evolution of mutual adaptation between particular strains should be carried out. Finding out whether multiple infections typically result in additive effects of individual strains on their hosts, synergistic mutualisms, costs through conflicts or amelioration of negative effects is essential for understanding symbioses in insects.

Conclusions

Studies conducted over the last decade have demonstrated that there is a wide diversity of facultative endosymbiotic bacteria in arthropods, and revealed many exciting aspects of their biology, as well as their importance for arthropod evolution. While research on the microbial associates of the pea aphid, *Acyrthosiphon pisum*, has provided a wealth of information on the roles of these symbionts in their hosts' ecology, several aspects of interactions between aphids and bacteria have not yet been understood. Furthermore, limited data on the diversity, distribution and the ecological role of facultative endosymbiotic bacteria in other aphid species compromise our understanding of these microbes as components of the horizontal gene pool accessible to multi-species communities, which may aid aphid adaptation

to novel environmental challenges (Ferrari & Vavre, 2011; Oliver et al., 2010). This thesis explores the roles of symbiotic microorganisms in a commercially important pest, but also explains some of the insufficiently understood aspects of symbioses, with implications for understanding the horizontal transmission of symbionts within aphid and insect communities.

I have shown that the grain aphid, *Sitobion avenae*, an important pest of cereals, frequently harbours diverse facultative symbionts, which under natural conditions are capable of moving between aphid asexual genotypes within the species, but also between species. As in pea aphids, these bacteria can be manipulated within aphid asexual populations through antibiotic treatment or haemolymph microinjection. Secondary symbionts typically establish more easily and form more stable associations following transfer between clones of the same species compared to transfers between heterospecific hosts, which indicates the presence of some barriers to horizontal transmission within communities. Facultative symbionts of grain aphids, but also symbionts originating from other aphid species, do not typically incur significant fecundity costs to their carriers under benign conditions, although there are differences among host and symbiont genotypes, and some multiple infections with heterospecific symbionts can dramatically decrease aphid fitness. Strikingly, unlike strains of *Hamiltonella* infecting North American pea aphids (Oliver et al., 2009; Oliver et al., 2005; Oliver et al., 2003), strains of the same bacterium originating from four species of British aphids do not generally protect grain aphids against parasitoids, despite all harbouring the bacteriophage APSE which is associated with resistance (Degnan & Moran, 2008a, b). There are no clear patterns in the distribution of the ability to confer parasitoid-resistant phenotypes across phylogenetic trees of *Hamiltonella* and APSE, and more detailed studies of phage- or symbiont-encoded toxins are essential for understanding the evolution of

this important trait. However, the ability to confer protection against fungal pathogens is common among pea aphid symbionts, with strains of at least four unrelated species conferring the same pathogen-resistant phenotype in a single pea aphid host clone. At least three of these symbionts can also protect grain aphids from the same fungal pathogen. Finally, while infection with some of these resistance-enhancing bacteria may be costly to their aphid hosts, in some host genotypes co-infections with other symbionts may reduce that cost.

The ability of prokaryotes to adapt to changes in their environment by recruiting new beneficial genes in the form of plasmids from outside the species, from what has been called the horizontal gene pool, has received much attention. It is now becoming clear that aphids, but also other insects, can recruit genes from a horizontal pool consisting of the sum of the facultative symbionts in a community, mirroring some of the effects known from bacteria. My research has added to the growing body of evidence for the ongoing horizontal transmission of the ecologically important traits conferred by facultative symbiotic bacteria in insects. While barriers to the horizontal transmission of symbionts exist, the typically low costs associated with infection and the important benefits which can be conferred by the symbionts in their novel hosts clearly make successful transfers between unrelated hosts plausible. These findings open a whole new chapter in our understanding of how insects interact with their environment, and how they have achieved their tremendous evolutionary success.

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