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Defensive symbiosis in the wild – patterns and dynamics of symbiont-conferred resistance in natural host-parasitoid communities

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Summary

Defensive symbiosis describes the interaction between two species where one species protects the other from dangers, in exchange for another benefit. Protagonist of this thesis is *Hamiltonella defensa,* a vertically transmitted bacterial endosymbiont of aphids. *H. defensa* can defend its aphid host against parasitoid wasps and in return profits from nutrients and shelter inside the aphid body. In wild aphid populations, *H. defensa* often occurs at intermediate prevalence, that is, some aphid individuals carry the bacteria, but others do not. This might be explained by balancing selection, as carrying *H. defensa* has not only benefits but also costs for the aphid. The tripartite interaction between aphids, *H. defensa* and aphid parasitoids is considered a model system for symbiont-driven host-parasite coevolution and has been studied from various angles during the past twenty years. However, there is still a lack of data on the role of defensive symbiosis in the ecology and evolution of natural communities. With my PhD work, I sought to improve on that by studying patterns and dynamics of *H. defensa*-conferred resistance in the field.

The core of this thesis is a large two-year field survey on the seasonal dynamics of *H. defensa* and other aphid endosymbionts in natural populations of the black bean aphid, *Aphis fabae fabae*. By estimating parasitoid abundance in parallel, I could test the hypothesis that *H. defensa* prevalence varies over the course of the season in response to parasitoid-mediated selection. *H. defensa* prevalence indeed varied over time, but despite a strong seasonal peak in parasitoid abundance, the relationship between parasitoid abundance and *H. defensa* prevalence was relatively weak. *H. defensa* frequency was, in fact, more strongly correlated with temperature. This opens the door for speculations on yet unknown characteristics of *H. defensa* that might influence its prevalence in addition to parasitoid-mediated selection (Chapter II).

Further, I showed experimentally that in the field community under study, *H. defensa*-conferred resistance is effective only against *A. f. fabae*'s most abundant parasitoid, *Lysiphlebus fabarum*, but not against other wasps parasitizing this aphid (Chapter III). Laboratory studies have shown that *H. defensa*-conferred resistance against *L. fabarum* depends on the precise combination of parasitoid genotype and symbiont strain. My work shows that such genotype-by-genotype (G × G) interactions between the aphid's symbiont

and the parasitoid can indeed drive aphid-parasitoid coevolution in the field, as they occur at the scale of a local field community (Chapter III), and because they are stable across different biotic environments (Chapter I): the genotype-specificity of the *H. defensa-L. fabarum* interaction was not altered when the aphids fed on different host plants (no G × G × E interaction).

Finally, this thesis includes a population genetic study of the diversity within the *A. fabae* species complex, to which my focal subspecies *A. f. fabae* belongs (Chapter IV). *A. fabae* is host-alternating, with different, morphologically cryptic subspecies using different summer host plants, but returning to a common winter host plant to mate. I show that despite this regular opportunity for gene flow, *A. fabae* subspecies are genetically distinct and differing in the endosymbiont complements they harbor. Hybrids between subspecies can be found in natural populations, however, these are rare and seem to be under negative selection.

Altogether, this thesis provides evidence for the importance of *H. defensa* in mediating aphid-parasitoid co-evolution in natural communities, while motivating further research on the role of defensive symbiosis in the wild.

Zusammenfassung

Symbiose beschreibt das enge Zusammenleben zweier unterschiedlicher Organismen. In sogenannten defensiven Symbiosen bietet eine Art der anderen Schutz oder Resistenz gegen Feinde oder Gefahren. Protagonist der vorliegenden Arbeit ist *Hamiltonella defensa*, ein endosymbiotisches Bakterium, das im Körper von Blattläusen lebt und von einer zur nächsten Blattlausgeneration vererbt wird. *H. defensa* kann Blattläusen Resistenz gegen parasitische Schlupfwespen verleihen, welche wichtige natürliche Feinde der Läuse sind. *H. defensa* ist also ein defensiver Symbiont von Blattläusen. Im Gegenzug profitiert *H. defensa* von 'Kost und Logis' im Innern der Blattlaus. In natürlichen Blattlauspopulationen leben meist einige Individuen mit, andere ohne symbiotische *H. defensa*. Das wird damit erklärt, dass *H. defensa* für die Läuse nicht nur Vorteile bringt, sondern auch Kosten. Diese Kosten kommen besonders dann zur Geltung, wenn keine Schlupfwespen die Blattläuse bedrohen, weil die Resistenz gegen solche dann nichts bringt, *H. defensa* aber weiterhin von den Blattlausressourcen zehrt. Je nach Situation sind also Blattläuse mit oder solche ohne *H. defensa* fitter.

Die 'Dreiecks-Beziehung' zwischen Blattläusen, *H. defensa* und Schlupfwespen gilt als Modellsystem für die von defensiven Symbionten beeinflusste Wirt-Parasit-Koevolution, und sie wurde in den vergangenen zwanzig Jahren von verschiedensten Blickwinkeln aus untersucht. Die heutigen Kenntnisse über *H. defensa* basieren allerdings weitgehend auf im Labor durchgeführten Experimenten. Wir wissen tatsächlich noch sehr wenig darüber, welche Bedeutung defensive Symbiosen für die Ökologie und Evolution von natürlichen Insektengemeinschaften haben. Ziel meiner Doktorarbeit war es daher, Muster und saisonale Dynamiken der von *H. defensa* vermittelten Resistenz in natürlichen Blattlauspopulationen zu untersuchen.

Herzstück meiner Arbeit ist eine Feldstudie über zwei Jahre, im Rahmen derer ich monatlich die Häufigkeit von *H. defensa* und anderen Blattlaus-Symbionten in Populationen der Schwarzen Bohnenblattlaus, *Aphis fabae fabae*, ermittelt habe. Parallel dazu habe ich die Häufigkeit parasitischer Schlupfwespen bestimmt. So konnte ich testen, ob sich die Häufigkeit von *H. defensa* in den Blattlauspopulationen als Reaktion auf die saisonal variable Häufigkeit parasitischer Schlupfwespen verändert. Die Häufigkeit von

H. defensa variierte im Jahresverlauf, aber die Korrelation mit der Häufigkeit von Schlupfwespen war schwächer als erwartet. Dies, obwohl wir im Frühsommer beider Jahre einen klaren Peak in der Häufigkeit von Schlupfwespen beobachtet haben. Tatsächlich korrelierte die Häufigkeit von *H. defensa* stärker mit Temperaturwerten. Diese Resultate geben Anlass zu Spekulationen über noch unbekannte Eigenschaften von *H. defensa*, welche die Häufigkeit des Symbionten möglicherweise mitbeeinflussen (Kapitel II).

Weiter konnte ich experimentell zeigen, dass *H. defensa* in der untersuchten Blattlauspopulation ausschliesslich gegen die häufigste Schlupfwespen-Art schützt, *Lysiphlebus fabarum*, nicht aber gegen andere Schlupfwespen-Arten (Kapitel III). Labor-Experimente haben gezeigt, dass die von *H. defensa* vermittelte Resistenz davon abhängt welche Kombinationen von Wespen-Genotyp und Symbionten-Genotyp in der Blattlaus aufeinandertreffen. Meine Arbeit zeigt, dass solche genotypischen (G × G) Interaktionen treibende Kraft der natürlichen Koevolution von Blattläusen und Schlupfwespen darstellen können, da sie auch in lokalen Insektengemeinschaften vorkommen (Kapitel III), und in verschiedenen Umgebungen stabil sind: die genotypische Spezifität der Interaktion zwischen *H. defensa* und *L. fabarum* ist unabhängig von der Blattlaus-Wirtspflanze (Kapitel I).

Schliesslich beinhaltet diese Arbeit auch eine populationsgenetische Studie zur Vielfalt innerhalb des *A. fabae*-Artenkomplexes, zu dem auch die für die restlichen Kapitel meiner Arbeit relevante Unterart *A. f. fabae* gehört (Kapitel IV). *A. fabae* ist wirtswechselnd, wobei morphologisch nicht unterscheidbare Unterarten verschiedene Sommer-Wirtspflanzen bevorzugen, aber Ende Sommer auf eine gemeinsame Winter-Wirtspflanze zurückkehren und sich dort paaren. Meine Daten bestätigen, dass sich die Unterarten von *A. fabae* trotz dieser wiederkehrenden Paarungsgelegenheit genetisch und in Bezug auf die Häufigkeit ihrer Endosymbionten klar unterscheiden. Ferner zeige ich, dass Hybride im Feld vorkommen, dass diese aber selten sind und vermutlich unter negativer Selektion stehen.

Insgesamt unterstützt meine Arbeit die Relevanz von *H. defensa* für die natürliche Koevolution von Blattläusen und parasitischen Schlupfwespen, gleichzeitig motiviert sie weitergehende Forschung zur Bedeutung defensiver Symbiose in natürlichen Gemeinschaften.

General Introduction

Defensive symbiosis

Virtually all organisms are involved in host-parasite interactions at some point of their life, be it as host or as parasite (Lafferty, 2008; Sorci & Garnier, 2008). In such interactions, a gain in fitness for one player implies a loss in fitness for the other. The resulting reciprocal selection acting on host and parasite can fuel antagonistic coevolutionary processes: both host and parasite populations continuously need to acquire new or different adaptations, in order not to lose out to their opponents (Combes, 2005). To keep up with their parasites in potentially endless 'arms races', hosts have evolved a vast array of physical, chemical, or behavioral mechanisms of resistance. On top of these endogenous lines of defense, host immunity and resistance may also be influenced by beneficial microbes living in close contact with the host (Ford & King, 2016; Kaltenpoth & Engl, 2014). The type of species interaction where symbionts provide their host with protection against an enemy, in exchange for nutrients and shelter, is referred to as defensive symbiosis (Haine, 2008). Defensive microbial symbionts have been found in association with hosts as different as plants, mollusks, or vertebrates, though particularly many intriguing examples come from insect systems (Florez *et al*., 2015). For instance, females of various species of solitary beewolves host symbiotic *Streptomyces* bacteria in specialized reservoirs of their antennas (Goettler *et al*., 2007). The beewolves inoculate their larval brood cells with these bacteria, which will produce a cocktail of different antibiotics that protect the beewolf pupae against infestation by other microbes (Kaltenpoth *et al*., 2005; Koehler *et al*., 2013). Another famous example of defensive symbiosis concerns *Wolbachia*, a multifaceted bacterial endosymbiont likely occurring in more than half of all insect species (Landmann, 2019). While *Wolbachia* is frequently identified as a reproductive parasite (Stouthamer *et al*., 1999), certain strains of the endosymbiont are able to protect their hosts against RNA viruses (Hedges *et al*., 2008; Teixeira *et al*., 2008). This defensive characteristic is currently being exploited for the control of mosquito-borne viral diseases such as Dengue or Chikungunya: mosquitoes carrying *Wolbachia* are released in disease-affected countries, with the aim of reducing virus transmission from mosquito vectors to humans by increasing the virus resistance in native mosquito populations (Powell, 2022). *Wolbachia* and the beewolves' *Streptomyces* have in common that their fate is tightly linked to that of their host: *Wolbachia* is vertically transmitted from one to the next host generation via the mosquito germ line and therefore requires successful mosquito reproduction for its transmission (Landmann, 2019), and also *Streptomyces* gets vertically transmitted from the beewolf mothers to their offspring and profits from highly selective application to the beewolf brood cells (Kaltenpoth *et al*., 2014). Such strong dependence of microbe

fitness on host fitness and reproduction is thought to favor the evolution of defensive symbiosis (Ewald, 1987; Jones *et al*., 2011).

At first sight, microbial defensive symbiosis seems to provide equal benefits for host and symbiont. However, while the symbiont tends to profit from food or shelter unconditionally, the symbiont's presence may be costly for the host. Costs could for example arise from the symbiont's resource consumption, or from toxic effects of protective compounds produced by the symbiont (Clay, 2014; Oliver *et al*., 2014). The mutualistic character of defensive symbioses therefore hinges on the presence of an enemy against which the symbiont can defend (Ford & King, 2016; Lively *et al*., 2005). Whether the host experiences net costs or benefits will also depend on the environment more generally (e.g. Sochard *et al*., 2019; Wolinska & King, 2009). For instance, temperature can influence the ease of parasites or pathogens to infect hosts (e.g. Blanford *et al*., 2003; Vale *et al*., 2008), and therefore also the host's necessity of being protected. In summary, defensive symbionts are mutualists if they can actually provide protection, but if they cannot, they may become parasites themselves (Ford & King, 2016). The interaction between hosts and defensive symbionts is thus moving along a continuum between parasitism and mutualism, depending on biotic and abiotic factors of the environment (e.g. Rogalski *et al*., 2021).

Aphids as model organisms for defensive symbiosis

Aphids (Hemiptera: Aphidoidea) are small, hemimetabolous insects that feed on plant phloem sap by piercing through the plant surface into the sieve elements with their specialized piercing mouthpart, the stylet. There are more than 5000 aphid species worldwide, of which just about a hundred are considered as serious agricultural pests (Blackman & Eastop, 2017). These few pest species, however, can lead to serious harvest losses. If occurring in high numbers, aphids may weaken their host plant by branching off plant sap, or by hindering photosynthetic activity when large patches of the plant get covered by honeydew, the aphid's sugar-rich excretion . However, the most harmful effects arise from many aphid's ability to act as vectors of viral plant diseases (Dedryver *et al*., 2010). For example, after the recent ban on neonicotinoid insecticides, Swiss sugar beet farmers are currently worried about a re-emergence of the aphid-transmitted Beet Yellow Virus (BYV), which decreases sugar yield by reducing the photosynthetic area of leaves and thus size and sugar content of the beets (Mahillon *et al*., 2022). The present work is focusing on the black bean aphid *Aphis fabae fabae* as a model (Figure 1). This is the nominal subspecies of the *Aphis fabae* complex comprising multiple taxa that vary in their preference for different host plants (see Chapter IV of this thesis for more details). *A. f. fabae* can feed on agricultural crops like broad beans, chards, and sugar beet, and also on a range of non-cultivated plants and weeds. Notably, *A. fabae* belongs to the small proportion of aphid species that show a host alternating

(heteroecious) life cycle (Blackman & Eastop, 2000): summer generations of the aphid feed on herbaceous plants, while autumn generations migrate to woody shrubs for overwintering.

The various traits that render aphids such successful species and sometimes redoubtable pests include (cyclical) parthenogenesis, high phenotypic plasticity, and – most important for this thesis – their common symbiotic associations (Le Ralec *et al*., 2010). Most aphid species produce multiple generations per year, and they alternate between one sexual generation with males and females and a succession of parthenogenetic, only-female generations. This is referred to as cyclical parthenogenesis (Blackman & Eastop, 2000). While the sexual generation generates genetic variation and yields winter-resistant, diapausing eggs, the multiple viviparous parthenogenetic generations allow the rapid built-up of large aphid populations during the growing season. Possible negative aspects of clonal reproduction, such as a lack of adaptability, are counteracted by a high degree of phenotypic plasticity. For instance, winged morphs that can migrate to new host plants are induced among the clonal offspring of unwinged aphids when these grow under crowded conditions, when they experience decreasing host plant quality, or when they perceive high enemy pressure (Müller *et al*., 2001).

Plant sap is rich in sugars but low in amino acids. Aphid nutrition is therefore highly unbalanced. Most aphids have solved this problem by living in a stable symbiosis with the bacterium *Buchnera aphidicola*. The strictly vertically inherited endosymbiont lives in specialized bacteriocytes in the aphid's body and has the ability to produce amino acids that lack in the aphid's diet (Douglas, 1998). The mutually obligate interaction – neither partner can live without the other – was established around 160-280 million years ago in a common aphid ancestor, and *B. aphidicola* has subsequently diversified in parallel with its aphid hosts (Douglas, 1998; Moran *et al*., 1993). *B. aphidicola* as an obligate and so-called primary endosymbiont stands in contrast to several facultative, secondary endosymbionts that on their part are characteristic companions of many aphid species. The attribute 'facultative' expresses that these endosymbionts are not necessary for aphid survival under benign conditions. They may, however, confer various benefits that are of decisive ecological relevance to their host. The most common facultative aphid symbionts are the bacterial taxa *Hamiltonella defensa*, *Serratia symbiotica*, *Regiella insecticola*, *Fukatsuia symbiotica*, *Rickettsia*, *Rickettsiella*, *Spiroplasma*, *Wolbachia* and *Arsenophonus* (Guo *et al*., 2017; Zytynska & Weisser, 2016). They are all vertically transmitted and can provide host plant-specific adaptive traits (Hosokawa *et al*., 2007; Wagner *et al*., 2015) or nutritional benefits (De Clerck *et al*., 2015; Koga *et al*., 2003), but most of them have defensive functions: they confer protection against abiotic stresses like heat, or against natural enemies like entomopathogenic fungi, parasitoids, or predatory insects (reviewed in Oliver *et al*., 2010). Different facultative symbionts occur in different aphid species, and, very typically, only some intermediate proportion of the

aphids in a specific population carry these symbionts. These intermediate frequencies are assumed to reflect the balance between costs of infection and the ecological benefits the symbionts provide (Oliver *et al*., 2014).

The relatively simple and heritable aphid microbiome facilitates the assessment of the effects that single bacterial taxa have on their host. Aphids can be selectively cured of their secondary endosymbionts by the application of specific antibiotics, and symbionts can be transferred between individuals using micro-injection techniques (Sochard *et al*., 2020). In combination with the ease of rearing clonal aphid lines via parthenogenesis, it is thus possible to study genetically identical aphids that differ uniquely in their facultative symbiont complement. These characteristics together have made aphids rewarding model organisms for studying defensive symbiosis.

Figure 1: young black bean aphids, *Aphis fabae fabae*, feeding on broad bean (picture by C. Hudson)

The defensive symbiont *Hamiltonella defensa*

Protagonist of this thesis is the γ-proteobacterium *Hamiltonella defensa*. It lives as an endosymbiont inside the bodies of hemipteran insects such as whiteflies, psyllids, spittlebugs, and aphids (Darby *et al*., 2001; Kapantaidaki *et al*., 2021; Moran *et al*., 2005b; Russell *et al*., 2003). In aphids, *H. defensa* can be found both intracellularly in specialized bacteriocytes, and extracellularly in the hemolymph (Moran *et al*., 2005b). The facultative symbiont is best known for its protective effect against parasitoids wasps, a characteristic that was first observed in the pea aphid *Acyrthosiphon pisum* (Oliver *et al*., 2003), and subsequently in a variety of additional aphid species

(e.g. Asplen *et al*., 2014; Leybourne *et al*., 2020; Postic *et al*., 2020; Vorburger *et al*., 2009). Parasitoid wasps exploit aphids as cradle and food source for their offspring: they oviposit their eggs inside the body of living aphids, such that upon hatching the wasp larva can feed on the aphid body. The aphid initially stays alive and continues feeding and growing. Only late in the development of the parasitoid larva the aphid dies, and the larva pupates inside or below the emptied aphid husk before emerging as an adult parasitoid (Figure 2).

H. defensa-conferred protection leads to a failure of wasp development and thus aphid survival. This is connected to a bacteriophage present in the endosymbiont genome (Brandt *et al*., 2017; Lynn-Bell *et al*., 2019; Moran *et al*., 2005a; Oliver *et al*., 2009), called APSE, for '*Ac. pisum* secondary endosymbiont', since such phages were first detected in an endosymbiont of pea aphids (van der Wilk *et al*., 1999). APSE bacteriophages contain putative toxin genes which are likely involved in conferring *H. defensa*'s protective phenotype (Boyd *et al*., 2021; Brandt *et al*., 2017; Degnan & Moran, 2008; Rouïl *et al*., 2020). Different APSE variants vary in their protective effect, which results in strain-specificity of *H. defensa*-conferred resistance: a specific *H. defensa* strain may confer resistance against some but not other parasitoid species (e.g. Asplen *et al*., 2014; Cayetano & Vorburger, 2015; McLean & Godfray, 2015), and even within parasitoid species different genotypes vary in their susceptibility to different *H. defensa* strains (e.g. Cayetano & Vorburger, 2013; Schmid *et al*., 2012).

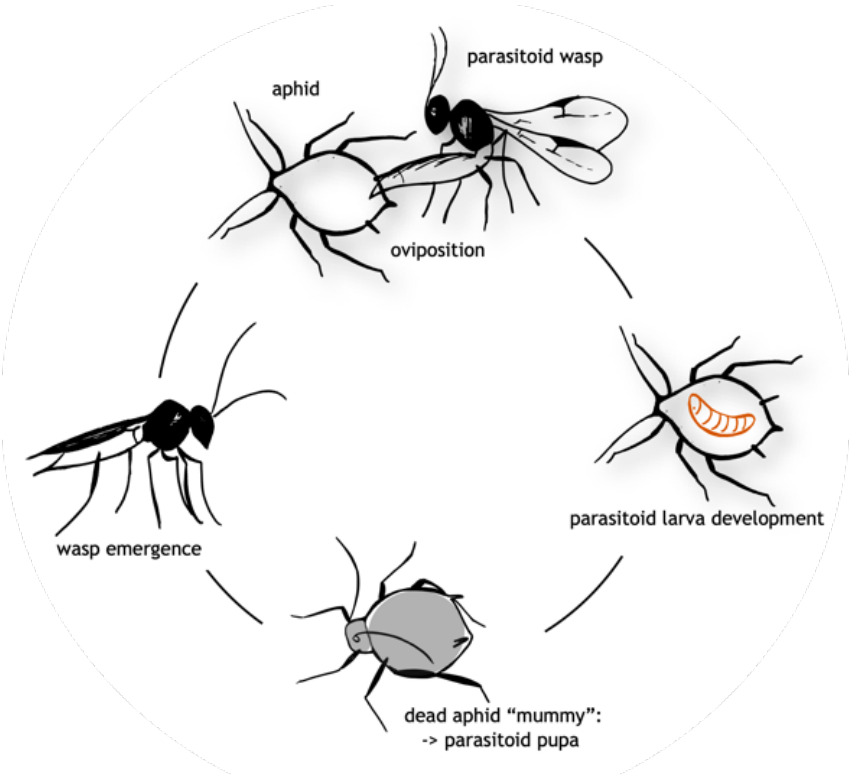

Figure 2: life cycle of a parasitoid wasp

H. defensa's fate is tightly linked to successful reproduction of its host, since its main transmission route is vertical from aphid mothers to their offspring. The success rate of transmission from one to the next aphid generation is thought to be very high (Darby & Douglas, 2003; Vorburger *et al*., 2017), though not perfect (Dykstra *et al*., 2014; Rock *et al*., 2018). Horizontal transmission of *H. defensa* is possible and has certainly occurred on an evolutionary time scale (Henry *et al*., 2013; Russell *et al*., 2003), while the frequency of it happening on an ecological time scale is unknown. Horizontal transmission might occur, for example, via contaminated parasitoid ovipositors (Gehrer & Vorburger, 2012), via shared host plants (Caspi-Fluger *et al*., 2012; Li *et al*., 2018), or during mating (Moran & Dunbar, 2006; Vorburger *et al*., 2017).

While *H. defensa* obligately needs a host to reproduce, *H. defensa* becomes unnecessary or even costly for the aphid if there is no parasitism pressure rewarding resistance: costs of *H. defensa*-infection may include negative effects on life history traits (Leybourne *et al*., 2020; Vorburger & Gouskov, 2011), generally on intraspecific competitivity (Dykstra *et al*., 2014; Hafer-Hahmann & Vorburger, 2020; Oliver *et al*., 2008), or on interaction with predators (Dion *et al*., 2011a; Polin *et al*., 2014). Whether aphids are selected for or against maintaining symbiotic interactions with *H. defensa* is therefore context-dependent. In particular, it depends on the presence and abundance of parasitoid wasps selecting for aphid resistance (Hafer-Hahmann & Vorburger, 2020; Oliver *et al*., 2008).

H. defensa and symbiont-driven host-parasite coevolution

In host-parasite interactions, costs and specificity of resistance can result in negative frequency-dependent selection and an advantage of rare genotypes (Agrawal & Lively, 2002; Hamilton, 1980). For instance, if there are multiple host genotypes, parasites should be under selection to overcome the resistance of the most frequent host genotype, but once this point is reached, rare host genotypes will have a selective advantage. As a consequence of their increased fitness, the frequency of the rare host genotypes may raise, up to a point when they will be sufficiently abundant to exert strong selection on parasites to overcome their resistance. In this way, negative frequency-dependent selection can lead to cyclical changes in the frequencies of different resistance and susceptibility traits over evolutionary time (Woolhouse *et al*., 2002). It may explain the maintenance of genetic diversity within host and parasite populations, because multiple rare genotypes are more fit on average than are few frequent ones (Clarke, 1976). Negative frequency-dependent selection has also been proposed to contribute to the evolution or maintenance of sexual reproduction, as a means to provide novel and thus rare genotypes via recombination (Bell, 1982; Hamilton, 1980; Hamilton *et al*., 1990; Jaenike, 1978). While the rounds of selection and adaptive responses may be separated

by large time periods, the average fitness of coevolving populations should remain similar over time, an idea summarized as the Red Queen hypothesis (Bell, 1982; Van Valen, 1973).

In aphid populations harboring *H. defensa*, aphid resistance to parasitoids is largely determined by infection with *H. defensa* and the genetic identity of the infecting *H. defensa* strains. *H. defensa*-conferred resistance is heritable, costly, and genotype-specific, and it entails counteradaptation by parasitoids (Dennis *et al.*, 2017; Dion *et al.*, 2011b; Rouchet & Vorburger, 2014). Taken together, these traits set the stage for intense and dynamic, symbiont-driven coevolution between aphids and parasitoids (Kwiatkowski *et al.*, 2012; Vorburger & Perlman, 2018), and they imply a major role of defensive symbiosis in shaping aphid-parasitoid interactions.

Defensive symbiosis and biological control of aphid pests

There is certainly an inherent fascination with the phenomenon of defensive symbiosis and its role in host-parasite coevolution. However, gaining an improved understanding of the role of defensive symbionts in aphid ecology is also relevant in regard of some species' status as agricultural pests. The devastating effects aphid can have on crop production are prompting the massive application of chemical insecticides with well-known negative side effects for biodiversity and human health (Stokstad, 2013). An alternative or complement to the use of insecticides is the biological control of aphids, that is, their suppression with the help of natural enemies. Classically, biocontrol intended the deliberate introduction of a host-specific natural enemy to a new region or continent, to target an alien pest species after this has spread to the new place without its natural enemies (van Lenteren, 2012). For example, the parasitoid *Lysiphlebus testaceipes* has been introduced from Cuba to the Mediterranean Europe in the 1970s to control two exotic citrus aphid species (Starý *et al.*, 1988). In the more recent past, augmentative biological control of aphids through the mass-release of parasitoids has been adopted in particular for greenhouse cultures, where the confined spaces allow to obtain high enemy densities and thus successful aphid suppression (van Lenteren, 2000). In open fields, rather than releasing separately mass-bred natural enemies, today's focus lies on conserving and promoting naturally occurring beneficial insects through habitat management, for example via the implementation of flowering strips and intercropping (Gurr *et al.*, 2017).

Defensive symbionts have the potential to interfere with biological control, and in particular, *H. defensa* might compromise the effectiveness of biocontrol with parasitoid wasps (Vorburger, 2018). Knowledge on mechanisms and costs of symbiont-conferred resistance as well as aphid-parasitoid co-evolution are therefore necessary to avoid the emergence of resistance in aphid populations (Käch *et al.*, 2018; Rossbacher & Vorburger, 2020). An understanding of the complexity of the interactions between insect pests, their natural enemies, and their bacterial

symbionts, is crucial to improve and support measures of biological control as a sustainable alternative for crop protection.

Research gap and thesis outline

Research on defensive symbiosis in aphids has advanced in big steps over the last years, and it is hinting at an influential role of *H. defensa* in shaping natural communities: by driving parasitoid evolution and adaptation (Vorburger, 2022), by mediating antagonistic coevolution (Ford & King, 2016; Kwiatkowski *et al*., 2012; Vorburger & Perlman, 2018), or by affecting the structure and equilibrium of natural food webs (Hrcek *et al*., 2016; Rothacher *et al*., 2016). However, most of these far-reaching predictions are derived from studies implemented under standardized conditions in laboratories. They have rarely been tested in more complex environments, and even less so in natural populations. As a consequence, there is substantial need for field data to further advance our understanding of defensive symbiosis and aphid-parasitoid coevolution. With my PhD project, I aimed to address this demand for more realistic data by testing some of the exciting predictions on *H. defensa*-conferred resistance under more natural conditions, and by collecting data on defensive symbiosis 'in the wild'.

In **Chapter I**, I took a step towards increasing environmental realism while studying *H. defensa*-conferred resistance still under laboratory conditions. Taking advantage of asexual lines of the parasitoid *Lysiphlebus fabarum*, it has been shown multiple times that aphid resistance depends on genotype-by-genotype (G × G) interactions between *H. defensa* and *L. fabarum*. Because in other study systems, such G × G interactions have shown to be susceptible to the environment in which they were assessed (G × G × E interactions), we were wondering whether the importance we currently attribute to G × G interactions including *H. defensa* would hold in a more complex and variable natural environment. I thus re-assessed infection patterns of known combinations of *H. defensa* and parasitoid genotypes, but rather than using a single standardized environment, I varied the aphids' biotic environment by rearing and exposing them to parasitoids on three different host plants. There are two main conclusions from Chapter I: (i) G × G interactions between *H. defensa* and the parasitoid *L. fabarum* remained stable independently of the biotic environment, that is, we did not find any G × G × E interaction. (ii) We found that both aphid and parasitoid fitness varied with the aphid's host plant, suggesting that net selection acting on both species differs between biotic environments.

Chapter II is the centerpiece of my thesis. It summarizes the principal findings of an extensive field study that we conducted over two seasons at three different sites around Zurich, Switzerland. On a monthly basis, we collected data on the frequency of the nine most common facultative

bacterial aphid endosymbionts in *Aphis fabae fabae,* with a particular focus on *H. defensa*. In parallel, we estimated the risk imposed on aphids by parasitoids using sentinel hosts. I used this data to test the prediction that *H. defensa* frequencies in natural aphid populations should respond to the seasonally variable strength of selection imposed by parasitoids. While the major achievement of this work may be the broad overview it gave us on a complex and seasonally dynamic aphid-endosymbiont-parasitoid community, the principal conclusions are (i) *H. defensa* frequencies indeed varied over time and (ii) the correlation between parasitism risk and *H. defensa* frequency dynamics was – though not absent – weaker than expected. Instead, *H. defensa* frequency showed stronger temporal correlation with warm temperatures, suggesting that multifarious selection rather than parasitism risk alone shapes *H. defensa* prevalence in the wild.

Chapter III is a follow-up on the field survey: I designed a classical infection matrix experiment to study infection patterns among the different combinations of parasitoid wasp species or genotypes and *H. defensa* haplotypes that actually co-occur in the field. The latter is what distinguishes my experiment from previous, similar work: the choice of parasitoids and *H. defensa* haplotypes exposed to each other was informed directly by the field data collected for Chapter II, and the parasitoids used in the experiment were collected on that occasion. The results thus illustrate resistance patterns one can observe in a locally co-adapted, natural field community. I found that in the studied field community (i) *H. defensa* protects *A. f. fabae* uniquely against the most frequent parasitoid species, *L. fabarum*, but not against more rare parasitoid species, and (ii) protection against *L. fabarum* depends on G × G interactions between *L. fabarum* and *H. defensa*. These results indicate that species- and genotype-specificity of *H. defensa*-conferred resistance are relevant for aphid-parasitoid coevolution in natural communities, and they suggest *H. defensa*-mediated adaptation of aphids to their parasitoids.

Chapter IV is also based on data collected for Chapter II, but it is somewhat detached from the other parts of my thesis in that it focuses on the problem of the different, morphologically cryptic subspecies within the *A. fabae* complex and their genetic differentiation. To follow the dynamics of *H. defensa* in *A. f. fabae* over the full growing season for Chapter II, we had collected black bean aphids from summer host plants as well as from the winter host *Euonymus europaeus*. Because *E. europaeus* is known to host multiple, morphologically cryptic *A. fabae* subspecies, I genotyped these samples using microsatellite markers in order to genetically distinguish *A. f. fabae*, the subspecies I was mainly interested in, from any other taxa potentially present on this host plant. I then took advantage of the extensive dataset of microsatellite genotypes of *A. fabae* I had produced, to explore and describe the little-known genetic structure of the black bean aphid species complex. Complementing the dataset with samples from a second winter host and fourteen different summer host plants of *A. fabae*, I could delineate six distinct genetic clusters that are largely consistent with

the ecologically defined subspecies as described in the taxonomic literature. I confirmed that they have distinct host plant preferences and I found that they also differ in the frequencies of infection with the facultative endosymbionts *H. defensa* and *R. insecticola*, further supporting their status as distinct taxa. Finally, I show that even though they are rare, hybrids between subspecies can be found in natural populations, but these are mainly found in spring and do not appear to return to the winter host plants in autumn. This is indicative of a reduced fitness of hybrid genotypes during the clonal phase of the aphid life-cycle, which could reinforce the genetic separation of the different black bean aphid subspecies.

My thesis ends with a general discussion where I briefly synthesize the main conclusions that derive from my work, and the directions for further research it suggests.

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Chapter I

Strong genotype-by-genotype interactions between aphid-defensive symbionts and parasitoids persist across different biotic environments

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Abstract

The dynamics of coevolution between hosts and parasites are influenced by their genetic interactions. Highly specific interactions, where the outcome of an infection depends on the precise combination of host and parasite genotypes (G × G interactions), have the potential to maintain genetic variation by inducing negative frequency-dependent selection. The importance of this effect also rests on whether such interactions are consistent across different environments or modified by environmental variation (G × G × E interaction). In the black bean aphid, *Aphis fabae*, resistance to its parasitoid *Lysiphlebus fabarum* is largely determined by the possession of a heritable bacterial endosymbiont, *Hamiltonella defensa*, with strong G × G interactions between *H. defensa* and *L. fabarum*. A key environmental factor in this system is the host plant on which the aphid feeds. Here, we exposed genetically identical aphids harbouring three different strains of *H. defensa* to three asexual genotypes of *L. fabarum* and measured parasitism success on three common host plants of *A. fabae*, namely *Vicia faba*, *Chenopodium album* and *Beta vulgaris*. As expected, we observed the pervasive G × G interaction between *H. defensa* and *L. fabarum*, but despite strong main effects of the host plants on average rates of parasitism, this interaction was not altered significantly by the host plant environment (no G × G × E interaction). The symbiont-conferred specificity of resistance is thus likely to mediate the coevolution of *A. fabae* and *L. fabarum*, even when played out across diverse host plants of the aphid.

Keywords: aphids, defensive symbiosis, genotype-by-genotype interactions, host-parasite coevolution, parasitoids, resistance

1 | Introduction

Whether the encounter between a host and a parasite leads to infection or not is often determined by the distinct combination of their respective genotypes. In classical infection matrix experiments, where multiple host and parasite genotypes are exposed to each other, such genotype specificity manifests itself in genotype-by-genotype (G × G) interactions on the probability of infection (e.g. Carius *et al*., 2001; Salvaudon *et al*., 2007; Schulenburg & Ewbank, 2004). G × G interactions may contribute to the maintenance of genetic diversity within species through negative frequency-dependent selection: a frequent host or parasite genotype exerts selection on its antagonist to counter-adapt and is soon going to be at a disadvantage compared to rare genotypes. Many rare genotypes are therefore favoured over few frequent ones in the long term (Clarke, 1976; Judson, 1995). G × G interactions may also contribute to the evolutionary maintenance of sexual reproduction and recombination, which promote diversity by combining existing genes and producing new, rare offspring genotypes (The Red Queen Hypothesis: Bell, 1982; Hamilton, 1980; Hamilton *et al*., 1990; Jaenike, 1978).

The potentially far-reaching influence of G × G interactions on host-parasite coevolution also hinges on their sensitivity to environmental variation. With the environment varying across the geographic distribution of interacting species, also the conditions for successful host infection or defence can change, potentially altering the strength and direction of selection acting on host and parasite (Nuismer *et al*., 2000; Tétard-Jones *et al*., 2007; Thompson, 2005; Wolinska & King, 2009). Different genotypes of a species may respond unequally to changing environmental conditions, resulting in genotype-by-environment (G × E) interactions that influence the outcome of host-parasite encounters. Moreover, the strength and specificity of G × G interactions may be dependent on the environment, which is referred to as a genotype-by-genotype-by-environment interaction (G × G × E). Such three-way interactions may contribute to the maintenance of genetic diversity at the species level as well, and they make the outcome of genotype-specific selection of hosts and parasites less predictable (Mostowy & Engelstadter, 2011; Wolinska & King, 2009). Experimental evidence for G × G interactions that are sensitive to environmental variation, that is for G × G × E interactions, is not abundant but exists for diverse systems. Examples include G × G × E interactions with the rhizosphere environment influencing the outcome

of specific interactions between plants and herbivore genotypes (Tétard-Jones *et al*., 2007), or the food environment influencing the G × G interactions that determine parasite infection in bumblebees (Sadd, 2011). Similarly, Bryner and Rigling (2011) found that temperature interacts with the genotypes of tree pathogenic fungi and their hyperparasitic viruses when predicting fungal virulence, and a three-way interaction between temperature, host genotype and parasite genotype may determine the transmission potential of viral diseases by mosquito vectors (Zouache *et al*., 2014). Apart from experimental evidence, modelling approaches support the potential influence of environmental changes on host-parasite coevolution through three-way interactions (Mostowy & Engelstadter, 2011). Taken together, these references underline the importance of incorporating environmental variability into classical G × G interaction studies, prior to generalizing conclusions to more complex natural systems.

Aphids have become popular model organisms for studying host-parasite interactions, not least because of the fascinating way by which certain species resist natural enemies: they carry defensive symbionts protecting them, for example, against pathogenic fungi (Lukasik *et al*., 2013; Scarborough *et al*., 2005) or parasitoid wasps (Asplen *et al*., 2014; Oliver *et al*., 2003; Vorburger *et al*., 2009). The best-studied example for the latter is the gammaproteobacterium *Hamiltonella defensa* (Moran *et al*., 2005b; Oliver *et al*., 2003), a maternally transmitted endosymbiont. There are different strains of the endosymbiont *H. defensa* which may confer different levels of resistance against parasitoid wasps, depending on the species or also the genotype of the attacking parasitoids (Asplen, Bano et al. 2014, Cayetano and Vorburger 2015). Because maternal transmission of *H. defensa* is very reliable (Darby & Douglas, 2003; Peccoud *et al*., 2014; Vorburger *et al*., 2017), *H. defensa* may be regarded as a form of a selectable resistance trait of its aphid host, with different strains acting as different genotypes determining the characteristics of this trait (Jaenike, 2012). Since *H. defensa*-conferred resistance tends to be much stronger than the basal resistance of the aphid (Oliver *et al*., 2005; Vorburger *et al*., 2009), coevolution between aphid hosts and parasitoids is likely mediated by defensive symbionts (Vorburger & Perlman, 2018).

The present study is concerned with the interaction between the black bean aphid, *Aphis fabae* (Hemiptera: Aphididae), and its main parasitoid *Lysiphlebus fabarum* (Hymenoptera: Braconidae, Aphidiinae). Different experiments have shown that the rate of successful

parasitism in this system is determined by strong G × G interactions between *L. fabarum* and the aphids' symbiont *H. defensa* (Rouchet & Vorburger, 2012; Schmid *et al.*, 2012). This suggests a decisive role of *H. defensa* in governing natural coevolutionary dynamics between *A. fabae* and *L. fabarum* (Kwiatkowski *et al.*, 2012). However, many of the experiments yielding the current knowledge were justifiably done under constant ambient conditions in the laboratory, which leaves open the question of how consistent – and thus relevant – such G × G interactions may be in heterogeneous natural environments. The only environmental variable that has been explicitly manipulated in this system is temperature: on the one hand, Cayetano and Vorburger (2013b) showed that G × G interactions between *L. fabarum* and *H. defensa* remain qualitatively the same at different ambient temperatures, even though the level of resistance conferred by *H. defensa* drops with increasing temperature, as also seen in pea aphids (Bensadia *et al.*, 2006; Doremus *et al.*, 2018). On the other hand, the same authors found that heat shocks experienced by the aphids could affect G × G interactions, albeit only at very high temperatures that are rarely experienced in nature (Cayetano & Vorburger, 2013a). We aimed to complement these studies by manipulating a different, yet crucially important environmental variable of the same host-endosymbiont-parasitoid system, namely the aphid's host plant.

Plants are both the food source and habitat of an aphid, and their availability and quality are an important determinant of seasonal and spatial environmental variation. There is ample evidence for plants having a direct influence on the host-parasite ecology of their insect inhabitants. For instance, Sochard *et al.* (2019) recently found that costs imposed on pea aphids (*Acyrthosiphon pisum*) by both parasitoids and endosymbionts depend on the aphid's host plant. And while Sochard *et al.* (2019) did not find any evidence for an influence of the host plant on parasitism rates, Goldson and Tomasetto (2016) found that parasitism rates in a weevil species are different depending on the grass species on which the weevils feed. Correlations between host plant, resistance to parasitoids and natural *H. defensa* infections of pea aphids led McLean *et al.* (2011) to suggest that selection pressure by parasitoids varies across different host plants. As shown for the same aphid species, also predation rates can be influenced by the host plant (Aquilino *et al.*, 2005). Generally, host plant choice may affect the fitness of herbivorous insects and consequently the fitness of their predators or parasitoids (Pan *et al.*, 2020).

While the influence of the host plant on insect interactions is striking in many systems, actual studies of G × G × E interactions with host plant as the environmental variable are rare. Investigating such interactions requires a study system where specific genotype combinations can be replicated, as is the case for the *A. fabae*/*H. defensa*/*L. fabarum* system. *A. fabae* can reproduce clonally and its infection with *H. defensa* can readily be manipulated by microinjection (Oliver *et al*., 2003; Sochard *et al*., 2020), while the occurrence of asexual reproduction in *L. fabarum* (thelytoky, see Sandrock *et al*. 2011) enables the use of distinct, genetically homogeneous lines also for the parasitoid. Environmental variability due to host plants is of high relevance for the multivoltine *Aphis fabae*, where opportunistic switches between crops and weeds from one aphid generation to the other are important to allow continuous feeding and reproduction over a whole growing season. We thus investigated the influence of three different, common host plants of *A. fabae* on G × G interactions between the aphid-defensive symbiont *H. defensa* and the parasitoid *L. fabarum*, using a full factorial design. The host plant had a strong effect on overall parasitism rates and thus on wasp reproductive success, and it also affected aphid fitness independent of parasitism rates. The host plant did, however, not alter the strong G × G specificity between *L. fabarum* and *H. defensa*. Hence, our results support earlier studies suggesting that in our model system, coevolution between aphids and parasitoids is largely symbiont-mediated and governed by genotype-specific interactions, which remain remarkably stable across different environments.

2 | Methods

Organisms

As host plants we used broad bean (*Vicia faba*, var. Fuego, UFA Samen, Winterthur, Switzerland), the common goosefoot (*Chenopodium album*) and green chard (*Beta vulgaris*, var. Grüner Schnitt, Samen Mauser AG, Winterthur, Switzerland), hereafter referred to as *Vicia*, *Chenopodium* and *Beta*. Seeds of *Vicia* and *Beta* were purchased, while the *Chenopodium* seeds were collected in the field in Zurich, Switzerland in autumn 2019. Within each host plant treatment, plants had the same age (*Vicia* 7 days, *Beta* and *Chenopodium* 27 days) and were chosen for most similar height and habitus. All plants are

important summer hosts of *A. fabae fabae*, the nominal subspecies of *Aphis fabae* and a notorious agricultural pest (Blackman & Eastop, 2000).

We chose four aphid lines from our laboratory collection showing a broad spectrum of resistance to different lines of the parasitoid *L. fabarum*, as seen in previous studies (Cayetano & Vorburger, 2013a; Cayetano & Vorburger, 2013b; Cayetano & Vorburger, 2015). The four lines originate from a single clone of *A. fabae fabae* (clone ID: 407), which was collected in Switzerland in 2006 from *Chenopodium*. One of these lines was free of any facultative symbionts (407), and three lines were uniquely infected with one of three genetically different strains of *H. defensa*: H15, H76 and H402. To obtain these lines, the *H. defensa*-free aphid clone 407 had been infected by microinjection of haemolymph from *H. defensa*-carrying aphid clones (Cayetano & Vorburger, 2015), resulting in stable, heritable infections. The infected lines are referred to as 407^{H15}, 407^{H76} and 407^{H402} and have been maintained parthenogenetically since the infection. Their identity was reconfirmed immediately before the experiment by microsatellite genotyping of the aphid (Coeur d'acier *et al.*, 2004) and sequencing of the *H. defensa* gene murE (Degnan & Moran, 2008b). Using a single aphid clone should exclude genetic variation beyond the endosymbiont strain in the aphid lines. We included the *H. defensa*-free aphid line in order to relate levels of *H. defensa*-conferred resistance to the aphid's basal resistance.

As parasitoids, we used three asexual, isofemale lines of *L. fabarum* (06-242, 07-64 and 09-369), the most frequent parasitoid species of *A. fabae* in Switzerland (Rothacher *et al.*, 2016). The lines had been started from single asexual females collected between 2006 and 2009. Parasitoid wasps oviposit single eggs into aphids. After hatching, the wasp larva feeds on the aphid's body, eventually kills the aphid and pupates within the emptied aphid exoskeleton. At this stage, a parasitized aphid is clearly recognizable and referred to as a mummy.

Experimental setup

We combined three host plants (*Vicia*, *Chenopodium*, *Beta*) with four aphid lines (407, 407^{H15}, 407^{H76} and 407^{H402}) and three parasitoid lines (06-242, 07-64 and 09-369) in a full factorial design with 36 treatment combinations and eight replicates, thus 288 experimental units. We performed the experiment in eight randomized complete blocks containing one replicate of each treatment. Four blocks were processed on the same day over two

consecutive days. An experimental unit consisted of one plant populated by one aphid line, exposed to one parasitoid line. The plants were grown in pots of 5 cm diameter and covered with a ventilated plastic cup. The experiment was conducted in a climate chamber at constant 22 °C with a 16 h photoperiod.

We split up the four aphid lines to the 288 experimental units and reared them on the respective plants for two generations prior to the experiment, with each new generation being transferred to a new set of plants. These two generations of prior rearing for each replicate helped to level out potential environmental effects carried over from the stock cultures and allowed for physiological adaptation to the different plant species. To initiate the experimental generation, we put four adult female aphids on a plant on day 1. We let them reproduce for up to two days in order to have similar numbers of nymphs on each plant, before removing the adults and counting the nymphs on day 3. On day 4, we added two female parasitoids to each plant and removed them again after six hours. We then waited until the aphid mummies (= parasitized aphids) were clearly visible on day 13 and counted the parasitized as well as the adult non-parasitized aphids. As response variable we used the number of mummies divided by the number of nymphs initially exposed to the wasps (parasitism rate). A considerable number of aphids died in the time between the counting of the aphid nymphs and the counting of the mummies. To ensure that this would not falsify our conclusions, we did a second analysis with parasitism rate calculated as the number of mummies divided by the number of aphids still present (alive or parasitized) on the plant when counting mummies.

We further measured the fresh weight of the mothers of the experimental aphid generation on a precision balance (MX5, Mettler Toledo, Greifensee, Switzerland) to assess potential effects of host plant and aphid line on aphid size. As a measure connected to parasitoid fitness, we determined the proportion of wasps hatching from the mummies we collected during the experiment (emergence rate). For this, we cut the whole plant after having counted the mummies and kept it in an air permeable bag until the adult wasps emerged from the mummies.

Analysis

All statistical analyses were done in RStudio v1.2.5001 (RStudio Team, 2020) with R v3.6.1 (R Core Team, 2019) and using the package ggplot2 v3.3.2 (Wickham, 2016) for

producing figures. To analyse parasitism rates, we used a generalized linear model with a logit link function and quasibinomial errors to account for overdispersion. We performed a three-way factorial analysis of deviance testing for the effects of aphid line, parasitoid line and host plant as well as the two- and three-way interactions, and for the main effect of experimental block. We used the function *Anova* from the R package *car* v3.0.7 (Fox & Weisberg, 2019) with F-tests as recommended by Crawley (2014) for quasilikelihood fits. We treated experimental block as fixed since quasibinomial errors are not implemented for generalized mixed models in R. For consistency, we treated block as a fixed effect in all further analyses. While the initial number of nymphs exposed to the wasps differed between plants (*Beta* 15.6 ± 7.2 sd, *Chenopodium* 13.8 ± 6.0, *Vicia*: 17.3 ± 6.7), we did not include this value in the final model, since it had no significant effect on parasitism rates when aphid line and host plant were included (Table S1), suggesting that parasitoids were host limited (and not time limited) in our assays. We did the analysis once for the full dataset (all aphid lines) and once with the dataset restricted to the *H. defensa*-infected aphid lines. Only in the latter case does the aphid line × parasitoid line interaction strictly reflect the G × G interactions between symbionts and parasitoids. Certain treatment combinations resulted in zero mummies in all replicates, and thus a group variance of zero, which led to problems with model convergence. To avoid this, we edited our data such that we manually added one mummy to one replicate of each 'zero parasitism' treatment combination. This minor intervention should have reduced treatment differences and hence made comparisons more conservative.

The parasitoid emergence rate was analysed with generalized linear models with logit link function and binomial errors. Since we could analyse only samples where at least one mummy had formed, we performed separate analyses on data subsets from the aphid lines 407 and 407^{H15}, where we had enough replicates, and tested only for the main effects of parasitoid line, host plant, and block. Likewise, we tested for the main effects of aphid line, parasitoid line, and block on a subset of the data including mummies collected from *Vicia* plants only. We calculated pairwise differences (Tukey HSD) between categories of significant predictors using the package *multcomp* v1.4.10 (Hothorn *et al*., 2008).

A model with quasibinomial errors as described above was also applied to analyse the proportion of aphids surviving until the end of the experiment among the non-parasitized aphids (initially exposed aphids minus parasitized aphids). The log-transformed aphid

body weight measures were analysed using a linear model and an analysis of variance. Here we tested for the effects of aphid line, host plant and the aphid × host plant interaction while accounting for the experimental block. We calculated pairwise differences (Tukey HSD) between aphid lines conditional on host plant using the R package *lsmeans* v2.30.0 (Lenth, 2016).

3 | Results

Parasitism rate and parasitoid emergence

There were highly significant main effects of aphid line, parasitoid line and host plant on parasitism rates, as well as a significant block effect, both in the complete dataset and the dataset restricted to *H. defensa*-infected aphid lines (Table 1). The *H. defensa*-free aphids and aphids carrying H15 were on average more susceptible to parasitoids than the aphids with the other two strains of *H. defensa*, and the observed rates of parasitism were mostly higher on *Vicia* than on the other two plants (Figure 1).

The main effect of parasitoid line largely reflects the low parasitism success of line 09-369, even on aphids without *H. defensa*. In both analyses, parasitism rates were also strongly dependent on the specific combinations of host and parasitoid lines, i.e. there was a highly significant aphid line × parasitoid line interaction, which is uniquely determined by the G × G interaction between *L. fabarum* and *H. defensa* in the restricted dataset (Table 1). There were no significant effects of the other interaction terms in the full dataset, while the parasitoid × plant interaction was marginally significant in the restricted dataset. Most notably with regard to the study question, the three-way interaction between aphid line, parasitoid line and host plant was non-significant in both analyses (Table 1). Hence, we observed no evidence for a G × G × E interaction on parasitism rates, indicating that the genetic specificity of the interaction between *H. defensa* and *L. fabarum* is not significantly altered by the different host plant environments. These conclusions remain unchanged when parasitism rates are calculated as the proportion of parasitized aphids among parasitized and surviving adult aphids at the end of the experiment, i.e. excluding aphids that died of reasons other than parasitism (Table S2).

Table 1: Analysis of deviance table for the proportion of aphids parasitized (parasitism rate). A generalized linear model with logit link and quasibinomial fit was applied. Column A: results using the full dataset including all four aphid lines (288 samples), the dispersion parameter is 3.468. Column B: results for including only the three *H. defensa*-infected aphid lines (216 samples), the dispersion parameter is 2.446.

	A. All aphid lines				**B. *H. defensa*-infected lines**			
Effect	df	Sum Sq	*F*	*P*	df	Sum Sq	*F*	*P*
Block	7	112.86	4.649	<0.001	7	99.57	5.816	<0.001
Aphid	3	308.42	29.644	<0.001	2	224.31	45.858	<0.001
Parasitoid	2	213.55	30.79	<0.001	2	110.82	22.656	<0.001
Plant	2	287.4	41.437	<0.001	2	125.69	25.696	<0.001
Aphid × parasitoid	6	208.15	10.003	<0.001	4	190.38	19.461	<0.001
Aphid × plant	6	10.78	0.518	0.794	4	8.77	0.8961	0.467
Parasitoid × plant	4	21.84	1.574	0.182	4	24.54	2.509	0.044
Aphid × parasitoid × plant	12	22.51	0.541	0.887	8	15.25	0.78	0.621
Residual	245	849.65			182	445.11		

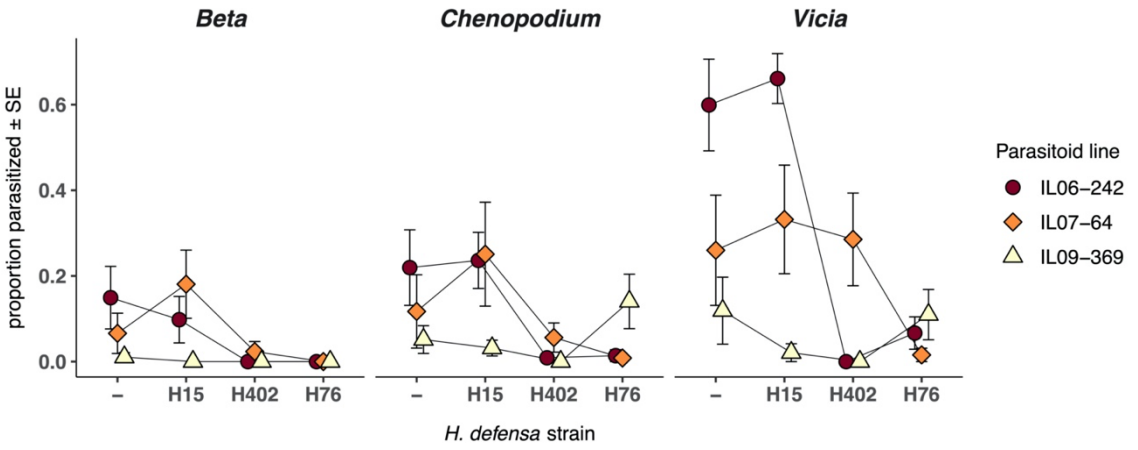

Figure 1: Parasitism rates calculated as number of mummies divided by number of exposed aphids. We used four lines of a single aphid clone: a line without *H. defensa* (407), and three infected with a different *H. defensa* strain each (lines 407^{H15}, 407^{H402} and 407^{H76}). The bars indicate standard errors.

Table 2: Analysis of deviance table for the parasitoid emergence rate. Generalized linear models with logit link and binomial errors were applied on three data subsets covering sufficient replicates.

Data subset	Effect	df	LR $\chi 2$	P
Aphid line	Block	7	13.84	0.054
407	Parasitoid	2	1.76	0.416
	Plant	2	70.95	<0.001
Aphid line	Block	7	8.76	0.27
407-H15	Parasitoid	2	8.4	0.015
	Plant	2	62.92	<0.001
Plant	Block	7	24.96	<0.001
Vicia	Aphid	3	13.25	0.004
	Parasitoid	2	1.36	0.508

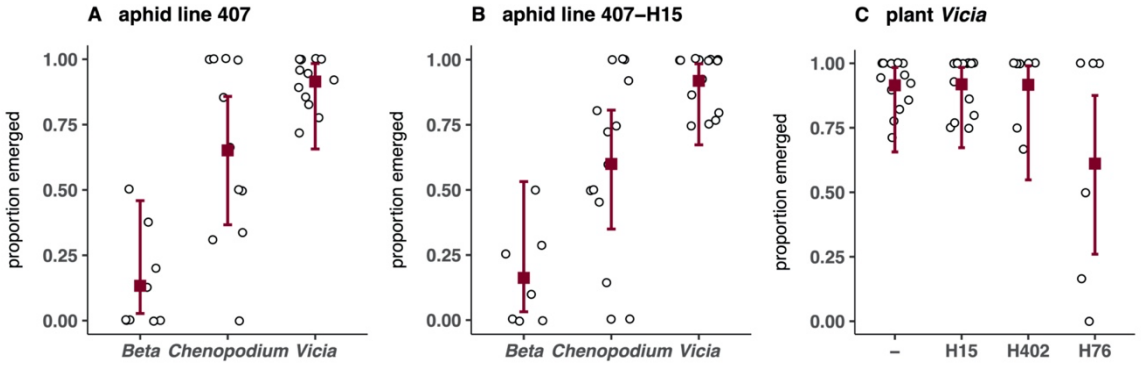

Figure 2: Proportion of parasitoids that emerged from the collected mummies, averaged over all parasitoid lines, for three data subsets: aphid lines 407 and 407^{H15}, and plant *Vicia*. Single datapoints are shown as white dots, the dark squares show the mean per host plant (A, B) or *H. defensa* strain associated with the aphid clone 407 (C), respectively. The bars indicate 95% confidence intervals.

Parasitoid emergence from parasitized aphids differed among treatments. Considering data from aphid lines 407 and 407^{H15} separately, parasitoid emergence was in both cases significantly influenced by the host plant, and to a lower extent also by the parasitoid line in aphid line 407^{H15} (Figure 2A & B, Table 2, S3). Mean parasitoid emergence for 407 and 407^{H15}, respectively, was highest on *Vicia* (91% and 92%), intermediate on *Chenopodium*

(65% and 60%) and lowest on *Beta* (13% and 16%). Considering data from *Vicia* plants only, there was a significant main effect of block and aphid line on emergence rate, and no effect of parasitoid line (Table 2). The effect of aphid line reflects a lower emergence from mummies of the aphid line 407^{H76} (61 % emerged on *Vicia*) compared to all other aphid lines (91 - 92% emerged on *Vicia*, Figure 2C, Table S3).

Table 3: Analysis of deviance table for the proportion of aphids surviving until the end of the experiment among the non-parasitized aphids. A generalized linear model with logit link and quasibinomial errors was applied, the dispersion parameter was 3.880.

Effect	df	Sum Sq	*F*	*P*
Block	7	88.45	3.257	0.003
Aphid	3	9.59	0.824	0.481
Parasitoid	2	5.04	0.649	0.523
Plant	2	426.03	54.901	<0.001
Aphid × parasitoid	6	13.23	0.568	0.756
Aphid × plant	6	12.48	0.536	0.781
Parasitoid × plant	4	37.11	2.391	0.051
Aphid × parasitoid × plant	12	56.83	1.221	0.269
Residual	244	946.71		

Aphid survival and body weight

From all initially exposed aphid nymphs one part got mummified, one part survived, and one part died before the end of the experiment for reasons other than visible mummification (Figure S1). Among the non-parasitized aphids, the proportion of surviving aphids varied significantly, explained by a significant main effect of host plant and experimental block (Table 3). Averaged over all parasitoid and aphid lines, 59% of all aphids survived on *Vicia*, 57% on *Chenopodium* and 36% on *Beta*.

The aphids also varied in body weight, with significant main effects of aphid line ($F_{3,259}$=15.37, p<0.001), host plant ($F_{2,259}$=109.97, p<0.001) and experimental block ($F_{7,259}$=4.50, p<0.001), as well as a significant aphid line × host plant interaction ($F_{6,259}$=4.08, p<0.001). The effect of aphid line and the interaction effect manifest in lower weight of the *H. defensa*-infected aphid lines compared to the *H. defensa*-free aphid line

on *Vicia* and *Beta*, but not on *Chenopodium* (Figure 3, Table S4). On average, aphid weight was highest on *Beta* (0.704mg ± 0.233mg SD), followed by *Vicia* (0.555mg ±0.161mg SD) and lowest on *Chenopodium* (0.400mg ± 0.110mg SD).

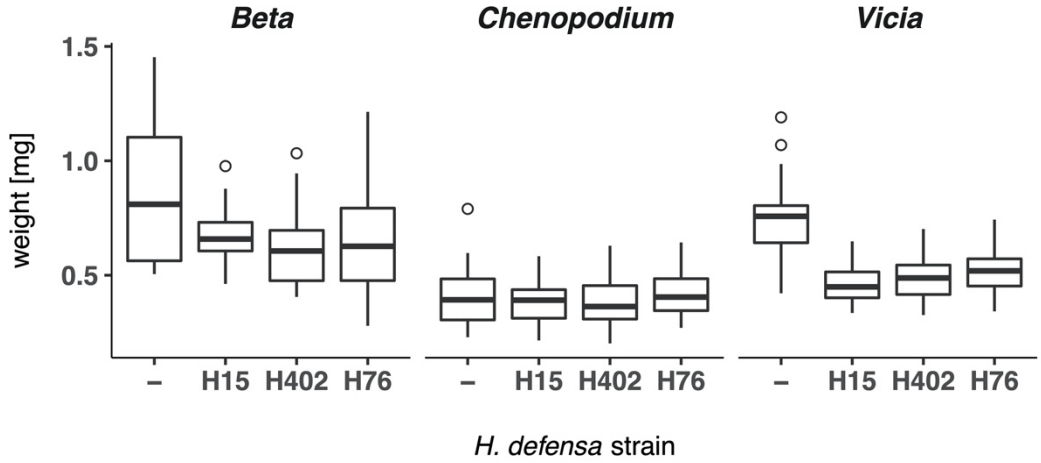

Figure 3: Adult weight of the mothers of the experimental aphid generation, in milligrams. Aphids on *Beta* had the highest weight on average, aphids on *Chenopodium* the lowest. Boxplot hinges correspond to the 1st and 3rd quartiles, the whiskers extend to a length of 1.5 times the inter-quartile range. x-axis: endosymbiotic *H. defensa* strain associated with the aphid clone 407.

4 | Discussion

Genotype-by-genotype interactions between the parasitoid *L. fabarum* and the aphid-protective endosymbiont *H. defensa* have been observed in multiple laboratory experiments (e.g. Cayetano and Vorburger (2015); Schmid *et al*. (2012)), and are therefore assumed to be an important driver of the coevolutionary dynamics in this host-parasitoid system (Hafer & Vorburger, 2019; Hafer-Hahmann & Vorburger, 2020; Kwiatkowski *et al*., 2012). Here we investigated such G × G interactions on different host plants, a key environmental variable for aphids and their parasitoids in natural populations. We observed the expected G × G interactions on all host plants, but also a strong main effect of the host plants on overall parasitism rates. In contrast, we saw no significant G × G × E interaction, suggesting that environmental heterogeneity generated by the availability of different host plants in the field does not reduce the hierarchy of G × G interactions between *L. fabarum*

and *H. defensa*. Taken together, these findings imply that host plant variation across space and time can indeed create a mosaic of varying selection strength (Thompson, 2005), but without changing the specificity of reciprocal selection between symbiont-protected hosts and parasitoids. Coevolutionary dynamics may proceed at different pace in different host plant environments, but the environmental variation is unlikely to change the direction of selection (Wolinska & King, 2009). Our findings thus resemble those of Cayetano and Vorburger (2013b), who assessed G × G interactions in the same system, but at different ambient temperatures. They found a clear effect of temperature on overall parasitism rates, but no evidence for a G × G × E interaction. While investigating two completely different aspects of the environment, the two studies reinforce each other in that the G × G interactions between *H. defensa* and *L. fabarum* are very robust to environmental perturbation.

While parasitism rates were affected by the plant they were measured on, the protective effect conferred by the different *H. defensa* strains remained similar on all plants (no aphid × plant interaction, Table 1, Figure 1). This may not be surprising considering the mechanistic basis underlying *H. defensa*-conferred resistance, which is related to the presence of a bacteriophage, called APSE, within the bacterial genome (Oliver *et al.*, 2009). Different APSE types carry specific toxin cassettes, which encode for different putative toxins, likely responsible for variation in the protective phenotype among *H. defensa* strains (Degnan & Moran, 2008a; Moran *et al.*, 2005a; Oliver *et al.*, 2009; Oliver & Higashi, 2019). The three strains used here also represent clearly distinct genotypes (Kaech *et al.*, 2021). The phage toxins are assumed to kill susceptible parasitoids at an early stage, that is as eggs or early larvae, but they may also have later-acting effects when parasitoids manage to complete development despite the presence of *H. defensa*, such as reduced adult weight or delayed emergence of parasitoids (Dennis *et al.*, 2017; Schmid *et al.*, 2012). Such a late-acting detrimental effect may explain the low parasitoid emergence rate from mummies of the aphid line 407^{H76} (Figure 2, Table S2), which was already observed in an earlier experiment (Schmid *et al.*, 2012).

Infection with a toxin-producing symbiont can also be associated with costs to the host, and indeed the frequency of *H. defensa*-carrying aphids tends to decline within aphid populations that are not under selection by parasitoids (Dykstra *et al.*, 2014; Hafer-Hahmann & Vorburger, 2020; Oliver *et al.*, 2008). Here we measured adult weight as a

rough proxy for aphid performance and found that *H. defensa*-free aphids were clearly larger than *H. defensa*-carrying ones on *Vicia* and *Beta*, but not on *Chenopodium*, where aphids were generally smaller. This was reflected in a significant aphid line × host plant interaction, indicating that the cost of infection with *H. defensa* may depend on the host plant an aphid is feeding on. Host plant-dependent costs of symbiont-conferred resistance would also have the potential to affect host-parasitoid coevolution, in that the net cost or benefit of possessing a resistance-conferring symbiont would vary across a geographic mosaic of host plant availability.

That the stability of the observed G × G interaction between *H. defensa* and *L. fabarum* is not simply a result of weak environmental differences is indicated by more than the aphid body weight varying between host plants. Despite the low parasitism rate on *Beta*, aphid mortality was clearly elevated there compared to *Chenopodium* or *Vicia* (Table 3, Figure S1). Moreover, differences in the reproductive success of parasitoids due to lower parasitism rates on *Beta* and *Chenopodium* were amplified further by variation in emergence rates, which were also lowest on *Beta* and intermediate on *Chenopodium* (Figure 2A & B, Table S2). In summary, *Vicia* was the most favourable host plant for aphids as well as parasitoids, while *Beta* represented a comparatively adverse environment for both antagonists. We could thus expect that the strength of reciprocal selection between hosts and parasitoids is higher on relatively benign hosts such as *Vicia*.

How do differences in host plant quality for both antagonists come about mechanistically? For the parasitoids, differences in parasitism success may be related to variation in plant structure affecting how efficiently aphids are attacked (e.g. Grevstad & Klepetka, 1992; Kareiva & Sahakian, 1990), or to variation in host quality (Pan *et al*., 2020). The entire parasitoid development takes places within the aphid's body, hence the more vital and well-fed the aphids are, the more resources may be available for the parasitoid. A resource deficit compared to *Vicia*-feeding aphids is a possible explanation for the low parasitism success on low-weight aphids from *Chenopodium,* but another explanation must apply to the frequent parasitoid failure on aphids from *Beta*, which were even heavier on average than the aphids from *Vicia*. *Beta* leaves may contain high concentrations of oxalates (Baker & Eden, 1954), which can have negative effects on aphids (Massonié, 1980) and could thus be responsible for the lower survival in our experiment, but of course many other reasons are conceivable. If the lower vitality of *Beta*-feeding aphids came from the uptake

of some toxic plant compound, this may also have hampered the parasitoids' development (Turlings & Benrey, 1998). The low aphid survival on *Beta* in our experiment was surprising, though, since *A. fabae* is known as a severe pest in sugar beet cultures (Blackman & Eastop, 2000), and we indeed regularly observe heavy infestations of sugar beet during field work. However, the species *Beta vulgaris* unites a multitude of crop varieties which may differ in their quality as host plants for *A. fabae*. The green chard variety we used in this experiment may be a less favourable host plant than the commonly grown varieties of sugar beet.

Whatever the precise reasons, *Vicia*, *Chenopodium* and *Beta* represented very different environments for the aphids and the parasitoids, and still, the genetic interactions tested within remained virtually unaffected. This is not self-evident, as several studies examining host-parasite interactions under different environmental conditions have reported significant G × G × E effects (Bryner & Rigling, 2011; Piculell *et al*., 2008; Sadd, 2011; Tétard-Jones *et al*., 2007; Wendling *et al*., 2017; Zouache *et al*., 2014). Explicit reports of the lack of a G × G × E interaction in host-parasite systems are scarce to our knowledge (but see Cisarovsky *et al*. (2012) and Cayetano and Vorburger (2013b)). To some extent, this may reflect a publication bias against reporting negative results (Csada *et al*., 1996). We argue that the absence of a significant G × G × E interaction is relevant here because it corroborates the importance we may attribute to the G × G effects. The more robust G × G interactions are to environmental variability, the more pervasive they will be in natural populations, and thus the more likely they explain fundamental evolutionary phenomena, such as the maintenance of genotypic diversity (Hafer & Vorburger, 2019; Judson, 1995). G × G interactions between *H. defensa* and *L. fabarum* have been shown to persist over a range of average temperatures and, as we newly report here, aphid host plants. This consolidates the role of the defensive symbiont *H. defensa* as a key mediator of coevolution between aphids and parasitoids, also in a heterogeneous environment.

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Author contributions

EG and CV designed the study. EG conducted the experiment. Both authors contributed to data analysis and interpretation. EG wrote the first draft of the manuscript, which was edited and revised by both authors.

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Supplementary Material

Table S1: Analysis of deviance table for the proportion of aphids parasitized (parasitism rate), applying a generalized linear model with logit link and quasibinomial fit. In contrast to the analysis presented in Table 1, we included the number of nymphs exposed to the parasitoids ("nymphs") here. Since "nymphs" is not significantly contributing to the model as long as "plant" and "aphid" are included, we did not keep the variable in the final model presented in Table 1. Column A: results using the full dataset including all four aphid lines (288 samples), the dispersion parameter is 3.666. Column B: results for including only the three *H. defensa*-infected aphid lines (216 samples), the dispersion parameter is 2.436.

	A. all aphid lines				**B. *H. defensa*-infected lines**			
Effect	df	Sum Sq	*F*	*P*	df	Sum Sq	*F*	*P*
Block	7	109.31	4.26	<0.001	7	101.86	5.974	<0.001
Nymphs	1	8.09	2.207	0.139	1	2.29	0.941	0.333
Aphid	3	275.04	25.011	<0.001	2	225.74	46.339	<0.001
Parasitoid	2	218.64	29.823	<0.001	2	110.95	22.774	<0.001
Plant	2	270.08	36.84	<0.001	2	128.56	26.39	<0.001
Aphid × parasitoid	6	208.32	9.472	<0.001	4	190.24	19.526	<0.001
Aphid × plant	6	9.6	0.437	0.854	4	9.32	0.956	0.433
Parasitoid × plant	4	22.49	1.534	0.193	4	23.47	2.409	0.051
Aphid × parasitoid × plant	12	24.21	0.551	0.88	8	14.98	0.769	0.631
Residual	244	894.41			181	440.87		

Table S2: Analysis of deviance table for the proportion of aphids parasitized (parasitism rate). Here, the proportion is calculated as mummies / (mummies + surviving_aphids), while for Table 1 the proportion is calculated as mummies / exposed_aphids. A generalized linear model with logit link and quasibinomial fit was applied. Column A shows the results for analyzing the full dataset including all four aphid lines (279 samples, 9 samples were excluded compared to the analysis in Table 1 since (mummies + surviving_aphids) = 0); the dispersion parameter was 2.931. Column B shows the results for analyzing only the *H. defensa*-infected aphid lines (208 samples), the dispersion parameter was 2.136.

	A. all aphid lines				**B. *H. defensa*-infected lines**			
Effect	df	Sum Sq	F	P	df	Sum Sq	F	P
Block	7	156.4	7.623	<0.001	7	136.38	9.119	<0.001
Aphid	3	270.96	30.813	<0.001	2	204.37	47.828	<0.001
Parasitoid	2	177.13	30.214	<0.001	2	86.72	20.296	<0.001
Plant	2	99.99	17.056	<0.001	2	33.13	7.754	<0.001
Aphid × parasitoid	6	199.01	11.315	<0.001	4	183.24	21.442	<0.001
Aphid × plant	6	18.25	1.038	0.401	4	15.9	1.861	0.119
Parasitoid × plant	4	14.63	1.248	0.292	4	17.19	2.011	0.095
Aphid × parasitoid × plant	12	21.5	0.611	0.832	8	16.36	0.957	0.471
Residual	236	691.77			174	371.75		

Table S3: Pairwise post hoc Tukey HSD of the parasitoid emergence rate between pairs of significant predictors as resulting from the analysis of deviance presented in Table 2. A generalized linear fit with logit link and binomial errors was used, the estimates are given at the logit (not response) scale.

Data subset	Contrast	Estimate	SE	*Z*	*P*
Aphid line	Cheno – Beta	1.54	0.714	2.158	0.078
407	Vicia – Beta	4.075	0.651	6.259	<0.001
	Vicia – Cheno	2.535	0.572	4.434	<0.001
Aphid line	Cheno – Beta	2.337	0.609	3.838	<0.001
407-H15	Vicia – Beta	4.148	0.662	6.263	<0.001
	Vicia – Cheno	1.811	0.438	4.132	<0.001
	IL07-64 – IL06-242	0.848	0.434	1.955	0.099
	IL09-369 – IL06-242	18.493	2751.987	0.007	1
	IL09-369 – IL07-64	17.644	2751.987	0.006	1
Plant	407H15 – 407	0.192	0.411	0.467	0.963
Vicia	407H402 – 407	0.632	0.746	0.847	0.818
	407H76 – 407	-2.555	0.761	-3.358	0.004
	407H402 – 407H15	0.44	0.724	0.607	0.923
	407H76 – 407H15	-2.748	0.821	-3.348	0.004
	407H76 – 407H402	-3.187	1.054	-3.023	0.012

Table S4: Pairwise post hoc Tukey HSD of aphid fresh weight between aphid lines, per host plant. We used the command lsmeans(wmodel, pairwise ~ a_clone | plant) from the R package *lsmeans* v2.30.0. We used a linear fit on log-transformed weight measures, thus the estimates are given on the log (not response) scale.

Plant	Contrast	Estimate	SE	df	t ratio	*P*
Beta	407 - 407H15	0.194	0.077	259	2.537	0.057
	407 - 407H402	0.257	0.077	259	3.358	0.005
	407 - 407H76	0.258	0.076	259	3.406	0.004
	407H15 - 407H402	0.063	0.078	259	0.811	0.849
	407H15 - 407H76	0.064	0.077	259	0.831	0.84
	407H402 - 407H76	0.001	0.077	259	0.01	1
Chenopodium	407 - 407H15	0.053	0.072	259	0.732	0.884
	407 - 407H402	0.073	0.072	259	1.002	0.748
	407 - 407H76	-0.017	0.072	259	-0.237	0.995
	407H15 - 407H402	0.02	0.072	259	0.27	0.993
	407H15 - 407H76	-0.07	0.072	259	-0.969	0.767
	407H402 - 407H76	-0.09	0.072	259	-1.239	0.603
Vicia	407 - 407H15	0.474	0.072	259	6.544	<0.001
	407 - 407H402	0.423	0.072	259	5.84	<0.001
	407 - 407H76	0.369	0.072	259	5.101	<0.001
	407H15 - 407H402	-0.051	0.072	259	-0.704	0.896
	407H15 - 407H76	-0.104	0.072	259	-1.443	0.473
	407H402 - 407H76	0.054	0.072	259	0.74	0.881

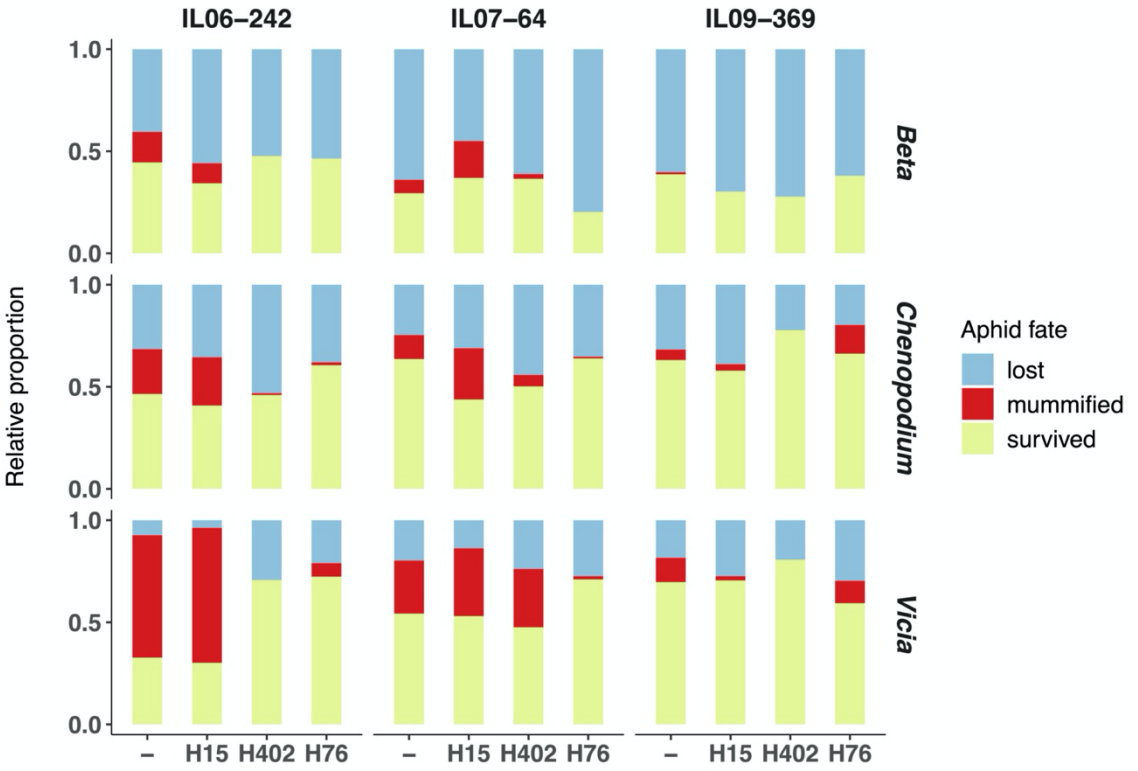

Figure S1: Proportion of surviving aphids (green, bottom color), parasitized aphids (red, middle color) and aphids dying for reasons other than visible mummification (blue, top color), out of all initially exposed aphids. Rows represent host plants; main columns represent parasitoid lines (IL06-242, IL07-64 and IL09-369); and single bars correspond to the endosymbiotic *H. defensa* strain associated with the aphid clone 407 (none, H15, H402 or H76).

Chapter II

Defensive symbiosis in the wild: seasonal dynamics of parasitism risk and symbiont-conferred resistance

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Abstract

Parasite-mediated selection can rapidly drive up the resistance levels in host populations, but fixation of resistance traits may be prevented by costs of resistance. Black bean aphids (*Aphis fabae*) benefit from increased resistance to parasitoids when carrying the defensive bacterial endosymbiont *Hamiltonella defensa*. However, due to fitness costs that come with *H. defensa*-infection, *H. defensa*-conferred resistance may result in either a net benefit or a net cost to the aphid host, depending on parasitoid presence as well as on the general ecological context. Balancing selection is therefore a likely explanation for why in natural aphid populations, *H. defensa* is often found at intermediate frequencies: periods when infection with *H. defensa* provides a net fitness advantage may alternate with periods when infection represents a liability. Here we present a two-year field study where we set out to look for signatures of balancing selection in three natural aphid populations. We collected temporally well-resolved data on the prevalence of *H. defensa* in *A. f. fabae* and estimated the risk imposed by aphid parasitoids using sentinel hosts. Despite a marked and spatially consistent early summer peak in parasitism risk in both years, and significant changes in the prevalence of *H. defensa* over time, we found just a weak correlation between parasitism risk and *H. defensa* frequency dynamics. *H. defensa* prevalence in the populations under study was, in fact, better explained by the number of heat days that previous aphid generations were exposed to. Our study grants an unprecedentedly well-resolved insight into the dynamics of endosymbiont and parasitoid communities of *A. f. fabae* populations, and it adds to a growing body of field observations suggesting that not only parasitism risk, but rather multifarious selection is shaping *H. defensa* prevalence in the wild.

Keywords: balancing selection; defensive symbiosis; *Hamiltonella defensa;* host-parasite interactions; symbiont-conferred resistance; trade-offs

1 | Introduction

Parasites can exert strong selection on host populations by favoring resistant over susceptible individuals (e.g. Briese & Mende, 1983; Duncan & Little, 2007). Yet, the selective advantage of resistance may be short-lived, when parasites coevolve with their hosts and develop counteradaptations for overcoming host resistance. If host resistance is specific to parasite genotypes, negative-frequency dependent selection can fuel the continuous turnover of a diversity of resistance types (Clarke, 1976; Judson, 1995; Woolhouse *et al*., 2002). Both the evolution and maintenance of host resistance may also be counteracted by life-history costs (Kraaijeveld *et al*., 2002; Schmid-Hempel, 2003) or trade-offs with other ecologically relevant traits (Cotter *et al*., 2004; Polin *et al*., 2014), which will be contingent on environmental conditions (Kraaijeveld & Godfray, 1997; Wolinska & King, 2009). We therefore expect temporal and spatial variation in host resistance to parasites, created and maintained by a range of evolutionary and ecological mechanisms (Duffy & Forde, 2009; Schmid-Hempel & Ebert, 2003).

For parasite-mediated selection to act on resistance traits, these need to be heritable, but not necessarily encoded in the host's own genes. Many organisms house heritable microbial endosymbionts, which live in their host's body and have evolved the ability to protect it against parasites (Ewald, 1987; Florez *et al*., 2015; Jones *et al*., 2007). As a consequence, the so called defensive endosymbionts can be regarded as a resistance trait of the host (Jaenike, 2012). The mutualistic character of defensive symbiosis is conditional on the balance between the costs of sharing resources and the benefits of protection experienced by the host. Whether symbiont-conferred resistance, and thereby defensive symbionts, are selected for or against is therefore a matter of ecological context (Bronstein, 1994; Duffy & Forde, 2009; White & Torres, 2009).

Defensive symbiosis is particularly well studied in insects (e.g. Brownlie & Johnson, 2009; Kaltenpoth, 2009); for example in aphids. In this insect group, at least nine repeatedly occurring facultative defensive endosymbionts are known (Guo *et al*., 2017). They can have protective effects against heat (Chen *et al*., 2000; Montllor *et al*., 2002), fungal infections (Lukasik *et al*., 2013) or other adversities (reviewed e.g. in Oliver *et al*., 2010), but as the attribute 'facultative' implies, they are not necessary for aphid survival, at least

under benign conditions. Facultative symbionts stand in contrast to obligate symbionts such as *Buchnera aphidicola*, a nutritional symbiont without which most aphids cannot survive (Douglas, 1998). The best-studied facultative defensive symbiont in aphids is *Hamiltonella defensa*, a gammaproteobacterium noted for its ability to protect against parasitoid wasps (Asplen *et al*., 2014; Oliver *et al*., 2003; Schmid *et al*., 2012). Parasitoid wasps oviposit eggs into living aphids, where the developing wasp larva eventually causes the aphid's death. The wasp development may be stopped by endosymbiotic *H. defensa* or, more precisely, by toxins produced by so-called APSE bacteriophages present in the symbiont genome (Oliver & Higashi, 2019). As a result, the presence or absence of endosymbiotic *H. defensa* may decide about life or death of a parasitized aphid.

H. defensa-conferred resistance behaves like a classical resistance trait in many aspects, first of all, in that the defensive symbiont is costly to its host: in the absence of parasitoids, *H. defensa*-infected aphids tend to lose out in competition with *H. defensa*-free individuals (Dykstra *et al*., 2014; Hafer-Hahmann & Vorburger, 2020; Oliver *et al*., 2008). This suggests a trade-off between resistance and parasitoid-independent fitness of aphids, and indeed the presence of *H. defensa* can for example shorten aphid lifespan and lifetime reproduction (Vorburger *et al*., 2013; Vorburger & Gouskov, 2011) or reduce defensive behavior, leading to higher predation risk in natural environments (Polin *et al*., 2014). Different *H. defensa* strains provide resistance to different parasitoid species or even genotypes (Asplen *et al*., 2014; Cayetano & Vorburger, 2015), suggesting that *H. defensa*-conferred resistance is determined by specific host-parasite combinations, which is a pre-requisite for symbiont-mediated negative frequency-dependent selection (Kwiatkowski *et al*., 2012). *H. defensa*-conferred resistance can also evoke rapid parasitoid counteradaptation (Dennis *et al*., 2017; Dion *et al*., 2011). Finally, vertical transmission of *H. defensa* from one to the next aphid generation may not be perfect (Dykstra *et al*., 2014; Rock *et al*., 2018), but likely represents the most reliable way of transmission for the symbiont (Darby & Douglas, 2003; Vorburger *et al*., 2017). This heritability allows for natural selection to act upon the presence and genetic composition of *H. defensa* at the host level (Hafer & Vorburger, 2019; Hafer-Hahmann & Vorburger, 2020).

Strong and directional parasitoid-mediated selection for *H. defensa*-conferred resistance can be observed within weeks in finite, experimental aphid populations (Hafer-Hahmann & Vorburger, 2020; Oliver *et al*., 2008). Nevertheless, endosymbiont-infected and

endosymbiont-free aphids often occur side-by-side in natural populations, a fact attributed to balancing selection (Oliver *et al*., 2014). Intermediate symbiont frequencies are typical for real aphid populations and may not represent stable equilibria, but rather snapshots of dynamic processes driven by trade-offs that change over space and time, and the resulting variation in selection pressure (Oliver *et al*., 2014). The present study was motivated by the fact that – considering the clear costs and benefits of *H. defensa* and the short generation times of aphids and parasitoids – resistance levels in natural aphid populations may be modulated on an ecological and thus traceable time scale, in response to variation in selection pressures. In temperate latitudes, we expect pronounced seasonal variation in parasitism risk, as a response to strong seasonal dynamics in aphid population sizes: initiating in early spring from individuals that overwintered as eggs, aphid populations grow rapidly until early summer, when usually a dramatic mid-season population crash is observed, with subsequent recovery towards autumn. High abundance of natural enemies certainly contributes to the mid-season population crash (Karley *et al*., 2004), and indeed the proportion of parasitized aphids increases towards summer, with a certain time lag relative to the aphid population density (e.g.Kavallieratos *et al*., 2004; Leclair *et al*., 2021; Yang *et al*., 2017). These patterns suggest that there is a period in early spring when aphids are selected primarily for fast reproduction, and thereby against *H. defensa*. In contrast, *H. defensa*-infection should be selected for in summer, when parasitism risk is high. We would therefore expect the frequency of *H. defensa*-infected aphids to cycle over the year, with a decline early in the season, and an increase during summer.

Smith *et al*. (2015) found large and consistent shifts in defensive symbiont frequencies in US populations of pea aphids (*Acyrthosiphon pisum*) within short time intervals. In one of these populations, parasitoid-induced mortality was indeed positively correlated with an increase in defensive symbiont frequencies. However, in an extensive follow-up study, there were still large symbiont frequency shifts recorded, but rather than by parasitism, *H. defensa* frequency was best explained by temperature (Smith *et al*., 2021). Contrary to the large shifts observed in both these studies, Leclair *et al*. (2021) described infection frequencies of various endosymbionts to be surprisingly stable when monitoring French *Ac. pisum* populations over a whole growing season. They did not find parasitoid abundance to correlate with *H. defensa* presence in general, but with the co-infection of *H. defensa* and *Fukatsuia symbiotica*, another facultative endosymbiont. The different

results of these field studies suggest that there might be additional selective forces acting on defensive symbionts in natural systems, or that hitchhiking and non-additive effects of co-infecting symbionts might mask the expected effects of selection by parasitoids (Carpenter *et al*., 2021; Smee *et al*., 2021; Smith *et al*., 2015). While challenging the straight-forward hypothesis presented above, this emerging complexity emphasizes the need for extensive field studies to scrutinize laboratory observations under natural conditions and to refine our knowledge on the heritable aphid microbiome (Oliver *et al*., 2014).

For the present study, we worked with the black bean aphid *Aphis fabae fabae* (Hemiptera: Aphididae), a widespread and notorious pest feeding on various agricultural crops and weeds (Blackman & Eastop, 2000). Black bean aphids are commonly infected with *H. defensa* (Vorburger & Rouchet, 2016; Vorburger *et al*., 2009). We carried out a two-year field study collecting monthly data on the prevalence of facultative endosymbionts and the risk imposed by parasitoids of *A. f. fabae* at three field sites. We asked whether there were significant patterns in the frequency of facultative endosymbionts in natural *A. f. fabae* populations on ecologically relevant time scales, and in particular, whether the frequency of *H. defensa* correlated with preceding parasitism risk or temperature.

We found that despite a marked early-summer peak in parasitism risk in both study years, the patterns in *H. defensa* frequency are not well explained by parasitism risk. Instead, *H. defensa* frequencies are best described by a model where *H. defensa* frequency positively correlates with the number of heat days that previous aphid generations were exposed to. Overall, our study gives a well-resolved picture of the dynamics of endosymbionts and parasitoids in natural *A. f. fabae* populations, and it supports earlier studies in that the effects of *H. defensa* may extend beyond protection from parasitoids.

2 | Methods

Sampling organization

Over two entire growth seasons (2019 and 2020), we regularly estimated the prevalence of up to nine facultative endosymbionts in *A. f. fabae*, and the risk for these aphids of getting parasitized by parasitoid wasps, at three different sites near Zurich, Switzerland. We generally estimated endosymbiont frequencies and parasitism risk at 4-week intervals, and on two extra time points in May and June 2020 to improve temporal resolution during the period for which the 2019 data indicated a high abundance of parasitoids. Only endosymbiont infection frequencies were additionally estimated in late October 2019 and 2020, and in April 2021, in order to have data closely spanning two overwintering periods. The exact sampling dates and sample sizes are provided in Table S1. The three sites *Faellanden*, *Gossau* and *Steinmaur* (map in Figure S1) were between 10 and 30 km distant from each other and covered an area of roughly 2 km^2 each. Located in agricultural areas, they were structurally very similar, comprising fields of various crops interspersed with woody hedges growing along small streams. The European spindle tree (*Euonymus europaeus*), the main winter host of *A. f. fabae*, was abundant in these hedges, as well as the guelder rose (*Viburnum opulus*), which can be used as an alternative winter host (Blackman & Eastop, 2000). All sites included fields of sugar beet (*Beta vulgaris*), an important summer host of *A. f. fabae*, and a second important summer host, the goosefoot *Chenopodium album*, was growing in high numbers as a weed across fields of various crops.

Estimation of symbiont frequencies in the aphid populations

A. f. fabae is a cyclical parthenogen with a host-alternating life cycle. In late autumn, sexual morphs mate and lay overwintering eggs on the woody winter host plants. Parthenogenetic and live-bearing females hatch from these eggs in spring. After a few asexual generations on the winter hosts, the aphids migrate to their herbaceous summer host plants in late spring, where they continue to reproduce asexually for multiple generations throughout the summer, until returning to their winter hosts in autumn. To estimate symbiont frequencies in the *A. f. fabae* populations, we collected approximately 60 aphids per timepoint and site

within 2-3 days from the reference sampling date (Table S1). We sampled *A. f. fabae* from the summer hosts *B. vulgaris* and *C. album* between May and early October and from its winter hosts (*E. europaeus* and to a small extent *V. opulus*) at the other timepoints (Table S1). At each timepoint and site, we sampled aphids from multiple fields and within fields from host plants that were at least 5 m apart from each other, in order to reduce the likelihood of collecting clones. Each aphid was picked up using a fresh pipette tip and placed in a separate Eppendorf tube, which then was stored at -20°C until further processing. We extracted aphid DNA using a salting out protocol as in Sunnucks and Hales (1996). We then set up separate PCR reactions using specific primers for each endosymbiont and determined the presence or absence of amplified endosymbiont DNA in each sample using a QIAxcel capillary electrophoresis device. Apart from *H. defensa*, we also tested for the presence of the facultative endosymbionts *Regiella insecticola, Serratia symbiotica, Rickettsia, Wolbachia, Fukatsuia symbiotica, Arsenophonus, Rickettsiella* and *Spiroplasma*. As a control for successful DNA extraction, we confirmed the presence of the obligate symbiont *Buchnera aphidicola* (Douglas, 1998) in each sample using the same diagnostic PCR approach. The PCR protocol and primer informations are provided in Table S2. *Wolbachia, F. symbiotica, Arsenophonus, Rickettsiella* and *Spiroplasma* occurred at very low frequency or not at all in the samples from 2019 (Table S5), thus we no longer screened for them in the 2020 samples and did not analyze their frequency dynamics. The frequency of each symbiont was calculated as the number of aphids infected with the symbiont divided by the total number of aphids sampled, for each timepoint and site.

The nominal subspecies of the black bean aphid, *A. f. fabae*, is part of the *A. fabae* complex (Blackman & Eastop, 2000; Heie, 1986) which comprises several subspecies that are morphologically cryptic but may differ genetically and in their facultative symbiont composition (Jörg & Lampel, 1996; C. Vorburger *et al.*, 2017). While the summer host plants *B. vulgaris* and *C. album* are used almost exclusively by *A. f. fabae*, the winter hosts *E. europaeus* and *V. opulus* are also used by other subspecies of the *A. fabae* complex (Blackman & Eastop, 2000; Müller, 1982). To distinguish the individuals belonging to *A. f. fabae* among the samples from winter host plants, we genotyped all aphids collected from these at eight microsatellite loci as described in Coeur d'acier *et al.* (2004). After PCR amplification (see Table S3 for PCR protocol and primer information), the

microsatellite fragments were run on an ABI 3730 automated sequencer and analyzed with Genemarker 3.0.1. Among the 1713 successfully genotyped samples, we found only 9 genotypes that were collected twice, and one genotype that was collected three times. Samples of *A. f. fabae* were separated from genetically distinct samples using an expectation-maximization clustering method implemented in the function *snapclust* from the R package *adegenet* 2.1.5 (Beugin *et al*., 2018), using genotypes from Vorburger *et al*. (2017) as references (Chapter IV). With this approach, 942 of the 1713 aphids sampled from the winter host plants were classified as *A. f. fabae* and used for the further analysis.

Estimation of parasitoid frequency

Aphid parasitoids oviposit single eggs into aphids. The hatched wasp larva feeds on the aphid's body, eventually killing it and pupating within the empty aphid exoskeleton. At this state, parasitized aphids are recognizable as so-called "mummies". To estimate the risk of parasitism by various parasitoids in the field, we exposed laboratory-reared, symbiont-free aphids on sentinel plants as baits to the natural parasitoid community. The bait aphids were then returned to the laboratory and kept until those that had been parasitized in the field turned into mummies. This method allowed us to estimate parasitoid abundance and species composition at each site independently of the levels of *H. defensa*-conferred resistance in the local aphid population (low numbers of mummies at a field site could be the result of low parasitoid abundance, or of a high resistance level in the aphid population at this site). To prepare the sentinel plants, we inoculated three weeks old broad bean plants (*Vicia faba*, var. Fuego, height circa 20 cm, pot size 10x10x15 cm) with 15 adults of one single *A. f. fabae* clone from our laboratory collection (A08-28H$^-$). This clone is free of facultative endosymbionts and therefore susceptible to parasitoids. In a climate chamber (22°C, 16h light), we let the adult aphids reproduce on the plants for two days before removing them, leaving behind approx. 120 aphid nymphs on the plant. The day after, we placed 25 aphid-infested sentinel plants at each of our three field sites, digging them into the soil together with their pot so they would fit into the landscape (Figure S2). After an exposure time of four days, the plants were brought back to the laboratory, where we immediately counted the number of aphids remaining on the plant (70 ± 51 SD) and removed any visible animals other than our aphids (especially aphid predators and their eggs, ants, aphid parasitoids and slugs). The plant was then covered with a cellophane bag

and returned to the climate chamber. From the initially 1350 plants that we set out in the field, 963 were safely returned to the laboratory with bait aphids on them that could be analyzed. The other plants could not be recovered from the field because they were destroyed inadvertently by people, consumed by slugs, or the plants were recovered but without remaining bait aphids on them. In August 2019, we could analyze particularly few plants from the sites *Gossau* and *Faellanden* (two and six), since the aphids from all other plants had been predated by hoverfly larvae, but on average, we recovered 18 ± 6 SD plants per timepoint and site. Nine days after bringing the plants back to the laboratory, we counted all mummies that had formed in the aphid colonies and collected them in ventilated plastic dishes. Once hatched, we determined the parasitoids to species level. Parasitism risk by each parasitoid species was then calculated as the number of hatched parasitoids divided by the number of recovered bait aphids per plant. For statistical analysis, we used the joint risk of parasitism by any parasitoid species. Even though probably not all parasitoid species are equally susceptible to *H. defensa* (Asplen *et al*., 2014; Cayetano & Vorburger, 2015; Vorburger *et al*., 2009), we assume this to be the most robust estimate for selection pressure on *H. defensa*-conferred resistance, considering the restricted number of bait plants and the short bait aphid exposure times.

H. defensa strain diversity

Different strains of *H. defensa* may occur in different individuals of the same aphid species and vary in the level and specificity of protection they confer (e. g. Cayetano *et al*., 2015; Oliver & Higashi, 2019). Selection by parasitoids could thus also act on the relative frequencies of different *H. defensa* strains, rather than just on the overall prevalence of *H. defensa* (Hafer-Hahmann & Vorburger, 2020; Rossbacher & Vorburger, 2020). In order to investigate the strain diversity of *H. defensa* and potential seasonal variation in haplotype frequencies, we sequence-typed *H. defensa* of 9 or 10 infected aphid samples per site of one timepoint each in spring, summer and autumn of both years. From these 175 samples, we amplified fragments of the bacterial housekeeping gene *murE* and the *P41* gene of the APSE bacteriophage associated with *H. defensa*, using the primers of Degnan and Moran (2008) and a PCR protocol as for symbiont diagnosis (Table S2). Both sequences allow clear distinction of the *H. defensa* haplotypes 1, 2 and 3 described from *A. f. fabae* by Cayetano *et al*. (2015), and they are also informative for distinguishing *H. defensa* strains

from other species of the genus *Aphis* (Henry *et al*., 2022). PCR products were sent for Sanger sequencing to Microsynth AG (Balgach, Switzerland). We manually checked the sequences with MEGA 11.0.11 (Stecher *et al*., 2020) and aligned them in R using *seqinr* 4.2.8 (Charif & Lobry, 2007) and *msa* 1.26.0 (Bodenhofer *et al*., 2015). Using a neighbor joining method and Tamura-Nei distances, we built separate phylogenetic trees for the *murE* and *P41* sequences using *ape* 5.6.2 (Paradis & Schliep, 2019).

Data Analysis

All analyses were done in R 4.1.2 (R Core Team, 2019) using R studio 2022.02.3 (RStudio Team, 2020) and *ggplot2* 3.3.5 (Wickham, 2016) for plotting. After calculating the individual frequency of every aphid symbiont in the full dataset, we used Fisher's exact tests to assess whether aphids co-infected with different combinations of two symbionts occurred more or less frequently than expected by chance. We tested only for combinations where the expected number of double infected aphids in our dataset was > 5, thus for co-infections of *H. defensa* with each *R. insecticola*, *Rickettsia*, *S. symbiotica* and *Wolbachia* and for co-infections of *R. insecticola* and *Rickettsia*.

To generally test for the presence of a seasonal pattern in the frequency of *H. defensa*, *R. insecticola*, *Rickettsia* or *S. symbiotica*, we used generalized additive models (GAMs) using the R package *mgcv* 1.8-40 (Wood, 2017) with default settings. We modelled symbiont frequency separately for each year as a flexible function of time (day of year) while correcting for differences between sites. We used binomial errors and logit links, except for the *Rickettsia* 2020 data where we switched to quasi-binomial errors to account for overdispersion. We then proceeded with the analysis for *H. defensa* and *Rickettsia*, the two symbionts whose frequencies showed significant patterns in time.

Visual inspection of the seasonal pattern of *Rickettsia* frequency revealed its close similarity to the pattern of parasitism risk. We confirmed this unexpected result using a model with logit link and quasibinomial errors (to account for overdispersion) with site, year and (contemporaneous) parasitism risk as explanatory variables. *Rickettsia* endosymbionts are known to occur in *A. fabae* and other aphid species (Chen *et al*., 1996; Zytynska *et al*., 2016; Zytynska & Weisser, 2016) but also in many other arthropods, including parasitoid wasps (Pilgrim *et al*., 2021). We therefore suspected that the *Rickettsia* we found in certain aphid extractions might not be aphid symbionts, but rather symbionts

of parasitoids that had been present as eggs or early larvae in the aphids we collected. This could explain the high *Rickettsia* prevalence in our dataset just during the period of peak parasitism, while *Rickettsia* prevalence was zero or near zero at most other timepoints. To explore this hypothesis, we did a small follow-up analysis which is described in the Appendix (Analysis S1).

We expected to see a seasonal pattern in *H. defensa* frequency as the result of seasonally variable strength of selection by parasitism. However, in the field study of Smith *et al.* (2021), *H. defensa* frequency positively correlated with temperature, and experimental evidence suggests that in particular extreme heat could lead to a fitness advantage of *H. defensa* infected aphids independently of parasitism (Russell & Moran, 2006). We therefore considered also heat as a factor potentially influencing *H. defensa* frequencies. To incorporate this factor, we used data from a weather station close to our field sites (Duebendorf, Zurich, 47°24'N, 8°37'E) provided by the Federal Office of Meteorology and Climatology, MeteoSwiss (a single value per timepoint for all three sites). If there was a cause-effect relationship between either parasitism or temperature and *H. defensa* frequency, the maximum of *H. defensa* frequency should follow upon the maximum of positive selection pressure with a certain time lag, depending on the generation time of the involved species but also on how long the selection on *H. defensa* presence remains positive after having reached its maximum. The clearest possible linear correlation between selection strength and *H. defensa* frequency should then be observed under consideration of this time lag. Because the time lag which results in the highest correlation is system-specific (e.g. Blanquart *et al.*, 2017; Dybdahl & Lively, 1998; Nee, 1989) and might not be determined a priori, we compared the explanatory power of parasitism and temperature using a lag of either one or two sampling timepoints. This means that we tested for a correlation between *H. defensa* frequency and parasitism risk 4 or 8 weeks earlier ("lag 1" or "lag 2"), and for a correlation between *H. defensa* frequency and the number of heat days (max. temperature >30°C) summed up either within the 4 weeks preceding a sampling timepoint ("lag 1") or within the 8 and 4 weeks preceding a sampling timepoint ("lag 2"). We used generalized linear models with binomial errors and logit link, with *H. defensa* frequency as the response variable. We pooled the data of the two sampling years and used year and site as fixed covariates in addition to the effects of parasitism and/or temperature with lag 1 or lag 2. We compared AIC values to determine which model

describes our data best, and we used *Anova* from the R package *car* 3.0-12 (Fox & Weisberg, 2019) with default parameters for deviance analysis. Using parasitism estimated 8 weeks before *H. defensa* frequency as an explanatory variable had the consequence that the *H. defensa* frequency estimates of the first two timepoints of each year, as well as the estimates of the two in-between sampling timepoints in 2020 (June 4 and July 6) were excluded from this analysis, because we have no parasitism estimates from 8 weeks before these timepoints (as indicated in Table S1). Similarly, the last two estimates of parasitism risk of each year did not enter this analysis, since we have no *H. defensa* frequency estimate 8 weeks after these timepoints.

To get an estimate of how many aphid generations the time lags we tested may include, we used the day-degree method from Campbell *et al.* (1974) exactly as used in Smith *et al.* (2021) for pea aphids. This should be a good approximation also for *A. fabae* (Tsitsipis & Mittler, 1976). Accordingly, one aphid generation may have taken between 21 days (in spring and autumn) and 8 days (in mid-summer), and time lags of 4 or 8 weeks may roughly span up to three and six aphid generations (Figure S3).

Finally, we tested whether *H. defensa* frequency changed during the two overwintering periods using Pearson's $\chi 2$ to test for differences in *H. defensa* frequency between the last sampling timepoint in autumn and the first sampling timepoint in spring.

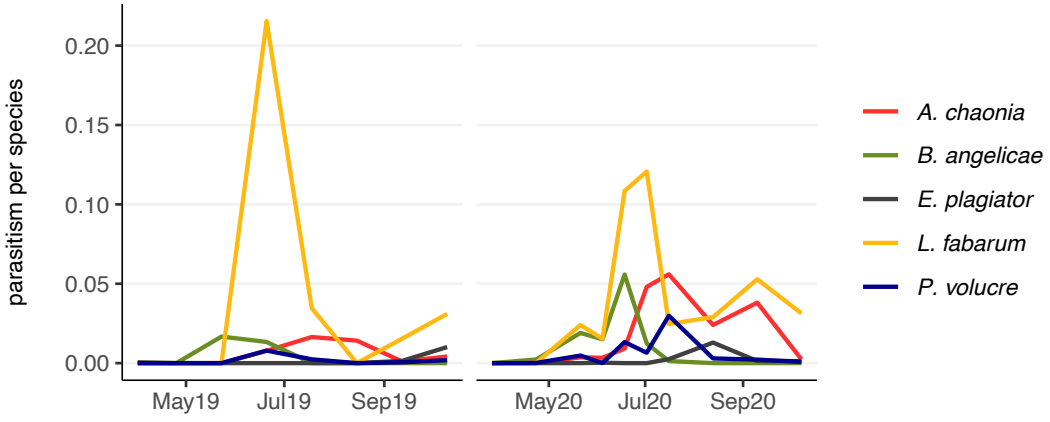

Figure 1: Proportion of exposed bait aphids that got parasitized (parasitism rate) as functions of date, separated by parasitoid species and averaged over the three sampling sites.

3 | Results

Parasitoid species and dynamics

We found parasitized aphids on 258 of 963 analyzed sentinel plants. A total of 5029 parasitoids hatched from the collected mummies, of which 70% belonged to *Lysiphlebus fabarum*. We further found *Aphelinus chaonia* (13%), *Binodoxys angelicae* (8%), *Praon volucre* (4%), *Ephedrus plagiator* (2%) and four other primary parasitoid species, as well as two secondary parasitoid species (all <1%, Table S4). The low number of secondary parasitoids was expected considering the short exposure time of our bait aphids. In 2019, there was a marked peak in parasitism rates on June 20, mainly driven by *L. fabarum*. In 2020, parasitism risk peaked on June 18 and July 2, again driven by *L. fabarum* but less exclusively so (Figure 1). *A. chaonia*, the second most frequent primary parasitoid, reached its peak frequency four weeks after *L. fabarum* in both years. The overall parasitism risk was zero or near zero at the first two sampling timepoints in both years (late March and April) and remained low (2019) to intermediate (2020) after the parasitism peak, that is from mid to late summer (Figures 1 and 2).

Facultative symbionts of *Aphis fabae fabae*

In total, 3449 samples of *A. f. fabae* were successfully analyzed and contained amplifiable bacterial DNA, as indicated by the detection of the obligate aphid symbiont *B. aphidicola*. *H. defensa* was the most frequent facultative symbiont, with 35 % of all aphid individuals infected. *R. insecticola* was the second most frequent symbiont and was detected in 8 % of the samples. *Rickettsia, Wolbachia* and *Serratia* were detected in 3, 2 and 1 % of the analyzed samples. In the 1465 samples from 2019 we did not detect infections with either *Arsenophonus*, *F. symbiotica, Rickettsiella* or *Spiroplasma* (Table S5). Co-infections between *H. defensa* and *R. insecticola* occurred less frequently than expected by chance (expected: 91, observed: 18, p<2.2e-16 in Fisher's exact test). The numbers of co-infections between *H. defensa* and *Rickettsia*, *Wolbachia* or *S. symbiotica* or between *R. insecticola* and *Rickettsia* did not significantly deviate from the expectations based on the individual symbiont frequencies (Table S6). *R. insecticola* and *S. symbiotica* frequencies did not significantly differ between sites and remained stable over time in both

years (Table 1, Figure S5). There were significant patterns in the frequencies of *H. defensa* and *Rickettsia* (Table 1, Figure S4, Figure S5), which are described in the following paragraphs.

Table 1: Results from Wald tests for the significance of site (parametric) and time (day of year, smooth term) in generalized additive models for symbiont frequencies, per year.

symbiont	year	family	scale est.	site			s(day of year)			
				df	Chisq	p	edf	ref.df	Chisq	appr. p
H. defensa	2019	bin.	1	2	11.54	**0.003**	2.19	2.72	6.00	0.112
	2020	bin.	1	2	0.98	0.614	7.30	7.85	31.12	**<0.001**
R. insecticola	2019	bin.	1	2	3.52	0.172	1	1	1.31	0.251
	2020	bin.	1	2	2.68	0.261	3.84	4.73	5.50	0.387
Rickettsia sp.	2019	bin.	1	2	2.65	0.266	5.32	6.09	39.35	**<0.001**
	2020	quasib.	2.12	2	F=3.09	0.062	2.64	3.40	F=3.29	**0.032**
S. symbiotica	2019	bin.	1	2	2.89	0.236	1.29	1.52	4.58	0.088
	2020	bin.	1	2	1.58	0.453	6.41	7.01	12.79	0.072

Notes: we used binomial errors and logit links (family = bin.) except in the model for *Rickettsia* frequency in 2020, where we used quasibinomial errors to account for overdispersion (family = quasib.); F statistics rather than Chi-square statistics are given in this case and the scale estimator is 2.12. The p-value of the smooth term is only approximate. Significance of the smooth term (day of year) indicates a consistent pattern in symbiont frequency over time.

Exploring *Rickettsia* frequency dynamics

Rickettsia frequency showed a significant pattern in time, peaking in mid-June and July of both years, but did not differ between sites (Table 1, Figure S4, Figure S5). *Rickettsia* frequencies are strongly correlated to (contemporaneous) parasitism risk ($\chi^2 = 39.18$, df = 1, $p<0.001$), which reflects the coincidence of the peaks of *Rickettsia* frequency and parasitism frequency in June or July of both years (Figure 1, Figure S5). This result might be caused by aphids collected during the period of peak parasitism being more likely to (invisibly) carry parasitoid eggs or larvae, which themselves might be hosts to endosymbionts such as *Rickettsia* (Pilgrim *et al.*, 2021). We found support for this hypothesis in a small follow-up analysis (Analysis S1): first, we confirmed that *Rickettsia* occurs in some of the parasitoid individuals that we collected in the field; they were most

frequent in samples of *B. angelicae*. Second, we found a strong correlation between the presence of *Rickettsia* and the presence of wasp DNA in our aphid extractions ($\chi^2 = 77.57$, df = 1, p-value < 0.001). Although not all *Rickettsia*-positive aphids contained amplifiable parasitoid DNA, this represents strong evidence that the marked and brief surges of *Rickettsia* prevalence described in June and July of both years are driven by the detection of this endosymbiont from parasitoid eggs or larvae present in part of the sampled aphids.

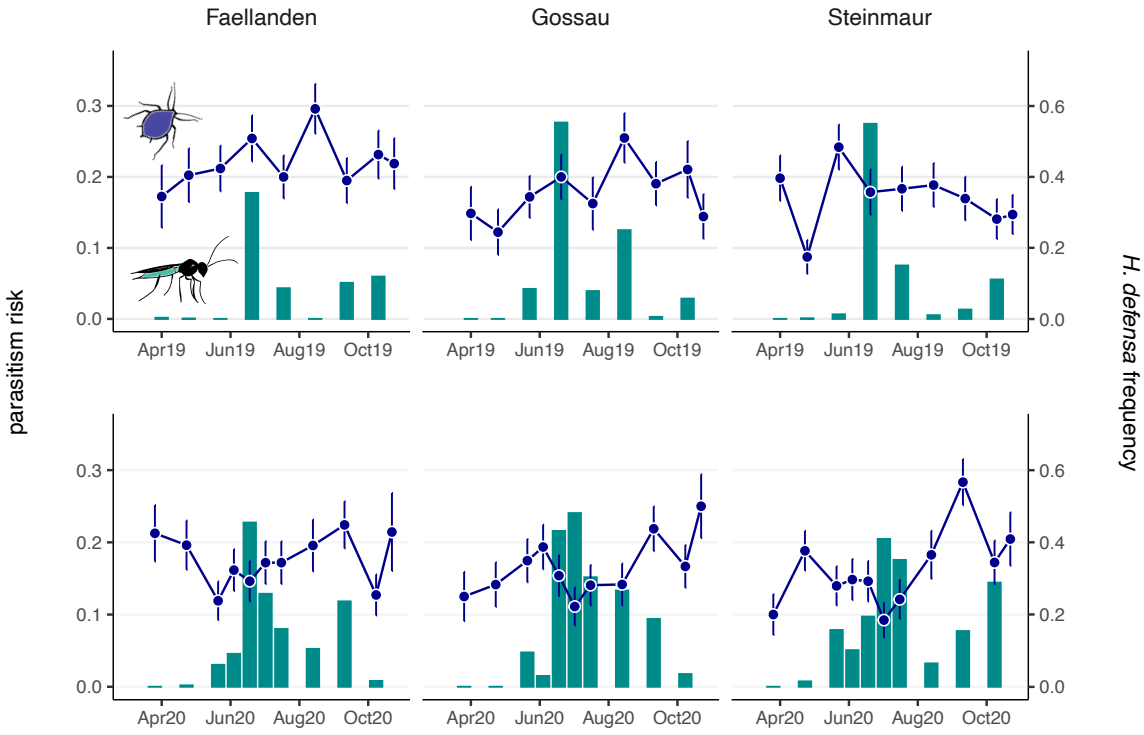

Figure 2: *H. defensa* frequency and parasitoid frequency at the three sampling sites *Faellanden*, *Gossau* and *Steinmaur* as functions of date. Right y-axis, dark blue points: *H. defensa* frequency, i.e. the proportion of aphids infected with *H. defensa*, with standard errors. Left y-axis, bars: parasitoid frequency, i.e. the proportion of exposed bait aphids that got parasitized.

Table 2: Results from analyses of deviance (using type II sums of squares) to test for correlations between *H. defensa* frequency and parasitism and/or the number of heat days: a) only temperature at lag 2; b) only parasitism at lag 2; c) both temperature and parasitism at lag 2.

	effect	Chisq	Df	*P*
a	site	2.09	2	0.352
	year	10.21	1	**0.001**
	heatdays lag 2	16.07	1	**<0.001**
	AIC 205.97			
b	site	3.33	2	0.189
	year	13.83	1	**<0.001**
	parasitism lag 2	9.83	1	**0.002**
	AIC 212.20			
c	site	2.38	2	0.305
	year	10.65	1	**0.001**
	heatdays lag 2	6.70	1	**0.010**
	parasitism lag 2	0.47	1	0.495
	AIC 207.50			

Notes: generalized linear models with logit link and binomial errors were used. The base model (only site and year as explanatory variables) has an AIC of 220.04. Lag 2 means that we test for a correlation between *H. defensa* and the number of heat days within the 8 to 4 preceding weeks, or the parasitism risk 8 weeks before, respectively.

Exploring *H. defensa* frequency patterns

H. defensa frequency differed between sites but showed no consistent pattern over time in the first sampling year (Table 1, Figure S4). The lack of consistency is due to site *Steinmaur* showing a very different trajectory compared to *Faellanden* and *Gossau* (Figure 2, Figure S5). In *Steinmaur* we recorded a steep drop and unexpected minimum at the end of April and hardly any frequency changes for the rest of the year, while in *Faellanden* and *Gossau, H. defensa* frequencies were lowest in early spring, highest in August (Figure 2, Figure S5) and low again in October. In the second sampling year, *H. defensa* frequency did not differ between sites and showed a more consistent pattern in time, in particular an increase at all sites between July and September followed by a drop in October (Table 1, Figure 2, Figure S4, Figure S5).

H. defensa frequency is best described by a model using the number of heat days in the preceding 8-4 weeks (time lag 2) as only explanatory variable apart from site and year; the model indicates a significant positive association (Table 2a, Figure 4). This effect is driven

by the overall peaks in *H. defensa* frequency observed in August 2019 and September 2020, thus in both years at the end of the summer period (Figure 3). Heat days were almost exclusively recorded in in June, July and August of both years (Figure 3), and it is thus unfortunate but not surprising that we see a strong positive correlation between temperature and parasitism risk, which peaked in June and July (Figure S6). This correlation between our two main potential explanatory variables makes it impossible to fully disentangle their effects. Indeed, also parasitism with time lag 2 (8 weeks before estimating symbiont frequency) is significantly correlated to *H. defensa* frequency when ignoring temperature (Table 2b, Figure 2, Figure 4), but it loses significance when the temperature term is added to the model (Table 2c). The models including temperature and/or parasitism with time lag 1 are clearly inferior to any of the lag 2-models, as suggested by the higher AIC values (Table S7).

H. defensa frequency slightly decreased during both winters and at all sites, but the difference between the last sample in autumn and the first in spring was significant only for winter 2020/21 at site *Gossau* ($X^2 = 6.26$, df = 1, $p = 0.012$ without correction for multiple testing, Table S8).

Low *H. defensa* strain diversity

Of 175 *H. defensa* samples from all three sites and 6 sampling timepoints, 171 samples shared the same sequences for both loci we investigated. These sequences corresponded to the reference sequence of *H. defensa* haplotype 2 (Cayetano *et al.*, 2015). Two samples clustered with *H. defensa* haplotype 1 considering both the bacterial housekeeping gene *murE* and the phage gene *P41*, and two samples clustered with haplotype 2 for their *murE* sequences but contained *P41* sequences that we would have expected for haplotype 3 (Figures S7 and S8). While it is possible that the *H. defensa* samples we analyzed show some variation outside the *murE* and *P41* sequences, our results suggest that the *H. defensa* strain referred to as haplotype 2, which was the prevalent haplotype also in the dataset of Henry *et al.* (2022), is by far the dominant strain in our sample collection. Hence, the overall *H. defensa* frequency in both years and at all three sites may be virtually tantamount to the frequency of the *H. defensa* haplotype 2. For this reason, we did not analyze *H. defensa* strain diversity any further.

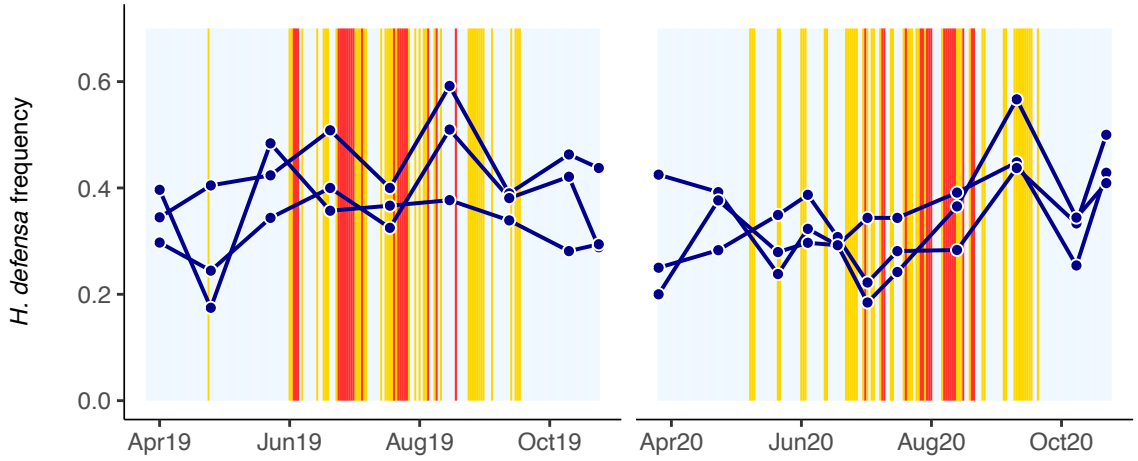

Figure 3: *H. defensa* frequency (dark blue points and lines) and temperature as functions of date. Yellow bars indicate days where the maximal temperature was > 25 °C (summer days), red bars indicate days where the maximal temperature was > 30 °C (heat days). *H. defensa* frequency is significantly correlated to the number of heat days within the 8 to 4 weeks preceding a sampling timepoint.

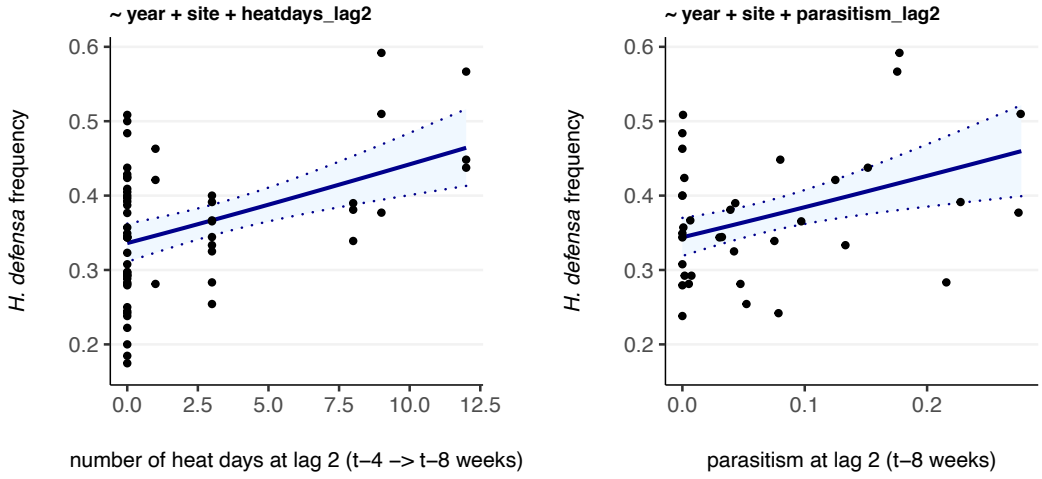

Figure 4: Partial effect plots for the model including only the number of heat days at lag 2 (left plot, corresponding values in Table 2a) and the model including only parasitism risk at lag 2 (right plot, corresponding values in Table 2b) to explain *H. defensa* frequency. The dotted lines delimit 95% confidence intervals of the regression line.

4 | Discussion

Temporal and spatial variation in the balance between costs and benefits may influence resistance levels in natural aphid populations. With our field survey, we explored the hypothesis that seasonally variable parasitism risk provokes fluctuating frequencies of the costly but resistance-conferring symbiont *H. defensa* in populations of *A. f. fabae*. We observed significant changes in the prevalence of *H. defensa* over time, but the temporal dynamics are not well explained by parasitism risk alone, and they just partly correspond to our predictions. On the one hand, there was no decrease in *H. defensa* frequency despite zero or near-zero parasitism risk in the spring period; the expected net costs of *H. defensa* during the period of rapid aphid population growth are thus not evident. On the other hand, we recorded changes in *H. defensa* frequency that were apparently unrelated to parasitoid presence or absence, for example a sudden, concerted drop at the end of the second sampling season. We find that rather than by parasitism risk, *H. defensa* frequency is best explained by the number of heat days that previous aphid generations were exposed to (Table 2).

Discrepancies between expectations based on laboratory experiments and field observations have also been reported from the observational studies on *H. defensa* frequencies in *Ac. pisum*: either a link between parasitism risk and *H. defensa* frequency was not observed at all (Smith *et al.*, 2021), or in some but not all studied populations (Smith *et al.*, 2015), or only when considering *H. defensa* in co-infection with another symbiont (Leclair *et al.*, 2021). In the field cages set up by Smith *et al.* (2021), high parasitism risk slowed down but could not prevent a decrease in *H. defensa* frequency. In other field experiments again, the protective effect of *H. defensa* against natural parasitoid communities was not significant at all (Narayan *et al.*, 2022), or it manifested in a significantly reduced proportion of parasitized aphids, but without resulting in increased population growth of *H. defensa*-infected compared to uninfected aphids (Hrcek *et al.*, 2016; Rothacher *et al.*, 2016).

Even though not unprecedented, the weakness or lack of a relationship between parasitism and *H. defensa*-conferred resistance in the field is surprising. Parasitoid-mediated selection has been documented numerous times from laboratory experiments (e.g. Herzog *et al.*, 2007; Käch *et al.*, 2018; Oliver *et al.*, 2008; Xie *et al.*, 2015), but also from a large-scale

field experiment under semi-natural conditions (Ives *et al*., 2020). Why could it be, in contrast, so difficult to see a relationship between parasitism and *H. defensa*-conferred resistance in natural systems? Rather than whether parasitoid-mediated selection exists, one question might be whether symbiont-conferred resistance is, by itself, strong enough to compensate for the costs of *H. defensa* under field conditions. The most frequently mentioned constitutive costs of *H. defensa* are reduced survival and lowered lifetime reproduction (Zytynska *et al*., 2021), but the actual extent of the fitness reduction caused by *H. defensa* likely varies in space or time and with biological context: costs depend for instance on environmental factors like the host plant (Sochard *et al*., 2019) and on the combination of aphid genotypes and *H. defensa* strains (Martinez *et al*., 2018; Vorburger & Gouskov, 2011). Costs can also be of ecological nature and, for example, vary with predator abundance, if resistance to parasitism traded off with resilience to predators as shown in Polin *et al*. (2014). In our study we tried to estimate a source of positive selection – parasitism – but not negative selection: we thus ignored that the *net* positive selection acting on *H. defensa* might not be fully proportional to parasitism due to costs which also vary in time. This is one possible reason for the apparently weak relationship between parasitism risk and symbiont frequency we found.

It is well known that the extent of *H. defensa*-conferred resistance depends on the parasitoid species and genotype, in combination with the *H. defensa* strain present in the attacked aphid (Asplen *et al*., 2014; Cayetano & Vorburger, 2015; McLean & Godfray, 2015; Vorburger *et al*., 2009). Our results from sequence-typing *H. defensa* in a subset of aphid samples suggest that there was a single dominant strain of *H. defensa* at all our field sites and during both sampling years. We are therefore unlikely to miss relevant changes in the relative frequencies of *H. defensa* strains when looking at overall *H. defensa* frequency. Regarding parasitism risk, however, it is possible that not all parasitoid species and genotypes present at our field sites (Figure 1, Table S4) were equally affected by the presence of *H. defensa*, such that only part of the parasitoids might have selected for *H. defensa*-conferred resistance. The seasonal dynamics of those species or genotypes that are affected by the resistance conferred by the dominant *H. defensa* strain in our data might therefore look different from the dynamics of all parasitoids taken together. The dominance of a single *H. defensa* strain could even facilitate counteradaptation of the parasitoid

community to *H. defensa*-conferred protection (Käch *et al*., 2018; Rossbacher & Vorburger, 2020), such that the mean protective effect might decrease in time.

That *H. defensa* prevalence responded less to parasitism risk than we expected could also be due to an unanticipated characteristic of *H. defensa* offering an additional target for positive or negative selection. Considering the correlation between *H. defensa* frequency and heat days apparent in our data, and the similar findings of Smith *et al*. (2021) for pea aphids, such a characteristic might be conditional on temperature. Heat can have strong negative effects on aphids (e. g. Asin & Pons, 2001; Ma *et al*., 2004), and a role in resistance to heat damage is known for other facultative aphid symbionts (e. g. Chen *et al*., 2000; Montllor *et al*., 2002), but has also been put up for discussion for *H. defensa* (Russell & Moran, 2006). Endosymbiont-conferred heat tolerance could for instance operate through mitigation of negative effects of heat on the obligate symbiont *B. aphidicola* (Burke *et al*., 2010; Heyworth *et al*., 2020), or through preparing the host for thermal stress by provoking general stress responses (Brumin *et al*., 2011). However, heat might also have a negative impact on the ability of *H. defensa* to protect its aphid host against parasitoids (Bensadia *et al*., 2006; Cayetano & Vorburger, 2013; Doremus *et al*., 2018; Guay *et al*., 2009; Higashi *et al*., 2020). Therefore, the relationship between heat and *H. defensa* could be double-edged: *H. defensa*-infected aphids might benefit from better heat tolerance on the one hand – this should be tested more specifically – but on the other hand, the effect of parasitoid-mediated selection for *H. defensa*-infection could be reduced during heat periods, which would weaken the link between parasitism risk and symbiont prevalence. Generally, the interaction between heat and parasitism, reaching their maximum in the same time period in our survey, might have a different impact on the costs and benefits conferred by *H. defensa* than one would expect based on their individual effects (Heyworth & Ferrari, 2016).

Smith *et al*. (2021) propose that not only heat, but also cold might have an influence on *H. defensa* frequencies. This suggestion is based on observing lower symbiont prevalence during colder periods, and more specifically on the drop in *H. defensa* prevalence observed between autumn of one year and spring of the next year. The same trend is seen in Ives *et al*. (2020) and – although subtly – in our data. This is unlikely due to symbiont losses at the overwintering egg stage, as virtually no losses were observed in hatchlings from eggs of *H. defensa*-infected mothers that were overwintered under artificial as well as natural

conditions in a field experiment (Vorburger *et al*., 2017). In contrast to this experiment, we did not sample the very first aphid generation, thus the observed decrease in *H. defensa* frequency between autumn and spring might not be a direct result from overwintering but rather reflect reduced fitness of *H. defensa*-infected aphids on the primary host plant early in the year, before there starts to be any kind of positive selection for *H. defensa*.

With an overall frequency of 35%, *H. defensa* was by far the most frequent facultative symbiont in the aphid populations we studied, followed by *R. insecticola* with only 8% prevalence (Table S5). The dominance of *H. defensa* and the resulting scarcity of symbiont co-infections in the same aphid reduces the probability that some of the patterns we observe are artefacts of so-called hitchhiking effects, that is, selection for or against a co-infecting symbiont resulting in unexpected frequency shifts of the focal symbiont (Carpenter *et al*., 2021). We noted that co-infections between *H. defensa* and *R. insecticola* occurred significantly less than expected by chance, and the same observation has been mentioned multiple times in the past for *A. fabae* (Vorburger & Rouchet, 2016) and *Ac. pisum* (Ferrari *et al*., 2012; Henry *et al*., 2013; Mathé-Hubert *et al*., 2019; Russell *et al*., 2013; but note the variable results in Rock et al., 2018). Mechanistically, such an effect might come for instance from competition between the symbionts or from increased costs experienced by double-infected aphids (e.g. Leclair *et al*., 2017; but no such effects were found by McLean *et al*., 2018). Anyway, since the frequency of *R. insecticola* was low and showed no significant temporal dynamics (Table 1, Figure S5), it is unlikely that selection acting on *R. insecticola* had a significant influence on the observed *H. defensa* frequencies. Hitchhiking effects may also be observed if *H. defensa* occurred by chance in a particularly fit or unfit aphid clone, that may have been selected for or against in the course of the season. However, in the genotyped aphid samples from spring and autumn hardly any aphid clone occurred more than once, suggesting that at least on the clone level there were no important frequency shifts that could have interfered with selection for *H. defensa*.

In conclusion, we confirm past studies on *H. defensa* dynamics in stating that short-time parasitoid-mediated selection is more difficult to trace in natural aphid populations than one would expect from experimental evidence. While the repeatedly observed intermediate *H. defensa* frequencies are strongly suggestive of balancing selection, it might be overly simplified to imagine that they are maintained by a simple two-way trade-off between resistance and reproduction. The correlation between *H. defensa* frequencies and heat in

our data asks for a more thorough investigation of the role of *H. defensa* under stressful temperatures and adds to the growing evidence that *H. defensa*-conferred benefits may reach beyond protection from parasitoids. Our results underpin how important it is to scrutinize laboratory observations in the field, in order to gain understanding of the multifarious selection that is acting on defensive symbiosis in the wild.

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Author Contributions

CV and EG designed the study. EG, JW and CV carried out the field work, JW and EG the laboratory work. EG and CV analyzed the data. EG wrote the first draft of the manuscript with inputs from CV and JW, the manuscript was edited and revised by EG and CV. All authors approved the final version for publication.

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Supplementary Material

Table S1: Summary of the field data. Columns 2-4 show the number of *A. f. fabae* infected with *H. defensa* (Ham+) and the total number of *A. f. fabae* individuals collected at each timepoint, per site. Winter host plants are *Euonymus europaeus* and to a small extent *Viburnum opulus*; summer host plants are *Beta vulgaris* and *Chenopodium album*. The timepoints for which we have no data on parasitism risk at a lag of eight weeks (2 timepoints) are not used in the GLMs testing for potential explanatory variables of *H. defensa* frequency.

sampling timepoint	Ham+ / total *Faellanden*	Ham+ / total *Gossau*	Ham+ / total *Steinmaur*	host plant	parasitoids sampled?	used for GLMs?
01-04-2019	10 / 29	11 / 37	23 / 58	winter	yes	no
25-04-2019	17 / 42	11 / 45	11 / 63	winter	yes	no
23-05-2019	25 / 59	22 / 64	30 / 62	summer	yes	yes
20-06-2019	30 / 59	24 / 60	20 / 56	summer	yes	yes
18-07-2019	26 / 65	13 / 40	22 / 60	summer	yes	yes
15-08-2019	29 / 49	26 / 51	23 / 61	summer	yes	yes
12-09-2019	23 / 59	24 / 63	20 / 59	summer	yes	yes
10-10-2019	25 / 54	16 / 38	18 / 64	summer	yes	yes
24-10-2019	21 / 48	15 / 52	20 / 68	winter	no	no
26-03-2020	17 / 40	10 / 40	10 / 50	winter	yes	no
23-04-2020	20 / 51	15 / 53	29 /77	winter	yes	no
21-05-2020	15 / 63	22 / 63	19 / 68	summer	yes	yes
04-06-2020	21 / 65	24 / 62	19 / 64	summer	yes	no
18-06-2020	19 / 65	20 / 65	19 / 65	summer	yes	yes
02-07-2020	22 / 64	14 / 63	12 / 65	summer	yes	no
16-07-2020	22 / 64	18 / 64	15 / 62	summer	yes	yes
13-08-2020	18 / 46	17 / 60	19 / 52	summer	yes	yes
10-09-2020	26 / 58	28 / 64	34 / 60	summer	yes	yes
08-10-2020	15 / 59	21 / 63	21 / 61	summer	yes	yes
22-10-2020	9 / 21	16 / 32	18 / 44	winter	no	yes
09-04-2021	3 / 10	5 / 30	19 / 51	winter	no	no

Table S2: Cycling conditions and primers for symbiont-diagnostic PCR and *H. defensa* haplotyping

PRIMERS SYMBIONT DIAGNOSIS

symbiont	product size [bp]	primer F	sequence Primer F	primer R	sequence Primer R	reference
Buchnera aphidicola	196	16SA1	AGAGTTTGATCMTGGCTCAG	Buch_R_CV2	CCCCCACTTTRGTTTTTCAAC	Hafer-Hahmann and Vorburger (2020)
Hamiltonella defensa	471	10F	AGTTTGATCATGGCTCAGATTG	T419R	AAATGGTATTCGCATTTATCG	Ferrari *et al*. (2012)
Regiella insecticola	480	10F	AGTTTGATCATGGCTCAGATTG	U443R	GGTAACGTCAATCGATAAGCA	Ferrari *et al*. (2012)
Serratia symbiotica	350	murES6F	CTGTTCGCTGGGCATGATGTGG	murES6R	GCCCGGTGCGTTAAACACTTCC	Henry *et al*. (2013)
Spiroplasma sp.	234	Sp16S_618F	GTGGCAAGCGTTATCCGGAT	Sp16S_834R	CCCACGCTTTCGTGCCACAA	Cariou *et al*. (2018)
Fukatsuia insecticola	468	10F	AGTTTGATCATGGCTCAGATTG	X420	GCAACACTCTTTGCATTGCT	Ferrari *et al*. (2012)
Rickettsia sp.	205	16SA1	AGAGTTTGATCMTGGCTCAG	Rick16SR	CATCCATCAGCGATAAATCTTTC	Fukatsu *et al*. (2001)
Arsenophonus sp.	456	16SA1	AGAGTTTGATCMTGGCTCAG	Ars16S_R2	CCTTAACACCTTCCTCACGAC	Henry *et al*. (2022)
Rickettsiella viridis	281	RCL16S-211F	GGGCCTTGCGCTCTAGGT	RCL16S-470R	TGGGTACCGTCACAGTAATCGA	Henry *et al*. (2013)
Wolbachia sp.	438	wspecF	CATACCTATTCGAAGGGATAG	wspecR	AGCTTCGAGTGAAACCAATTC	Werren and Windsor (2000)

PRIMERS HAPLOTYPING

gene	product size [bp]	primer F	sequence Primer F	primer R	sequence Primer R	reference
murE	885	murE16F	ACTAACGGGAAAACCACTAATAC	murE936R	TTGAGAATGTCAGCGGTAATC	Degnan & Moran (2008)
P41	774	APSE25.0F	ATCCTGTATTGCCCGTTTTG	APSE26.0R	ATCATTCCGGTTACGCAAAG	Degnan & Moran (2008)

PCR REACTION MIX (per sample)

reagent	volume [μl]
ddH20	2.3
Promega GoTaq® G2 Colorless Master Mix	5.5
Primer F	1.1
Primer R	1.1
Reagent mix per reaction	10
DNA solution per reaction	1
Final vol. per reaction	11

PCR PROTOCOL

temp [°C]	time [min]	cycles
95	3	
95	0.5	
65-56	0.5	10x
72	1	
95	0.5	
55	0.5	25x
72	1	
72	6	

Table S3: Primers for microsatellite PCR as published by Coeur d'acier *et al*. (2004) and PCR protocol

marker	size range [bp]	primer name	sequence
AfF	113 - 204	AfF forward	GCGTTGCAGCAGCATATACT
		AfF reverse	CCTATATCGTGTGCGTGCAT
Af82	159 - 236	Af82 forward	GCGTAATGCAAGTAACGACC
		Af82 reverse	CGTCGTTCCAGCGAATTCTC
Af86	207 - 221	Af86 forward	CGCGTTCTCTCCAATAACTC
		Af86 reverse	TAATGTTGCGGATTGTTTGC
Af85	208 - 228	Af85 forward	CGCGTGCAGTGTAGGTCCAT
		Af85 reverse	CAAGGTGCGATTGACGACGA
Af50	255 - 276	Af50 forward	TGGTGAGTGCAGGCTAGTAT
		Af50 reverse	AAGGCACTTAGTCGACGTGT
Afbeta	260 - 377	Afbeta forward	GAGGACGCGGCTAAGAAGAA
		Afbeta reverse	CGAAAAGGGACGTCTACGAG
Af48	303 - 355	Af48 forward	TTAAACCTTTGAGCGTAGCG
		Af48 reverse	CCGAAGCAGCAGTAACATTG
Af181	299 - 362	Af181 forward	GGCATGTGCACGACGAATAC
		Af181 reverse	CGTTTCTTCGTGTGCGATTT

PCR PROTOCOL

temp [°C]	time [min]	cycles
95	15	
94	0.5	30 x
60	1.5	
72	1	
60	30	

PCR REACTION MIX (per sample)

Reagent	volume [μl]
ddH20 + primers (conc. see table on the right)	4.5
QIAGEN Multiplex PCR Master Mix	5.5
DNA solution	1
Final vol. per reaction	11

Primer and label	conc. in PCR [µM]
AfF forward + PET	0.1
AfF reverse	0.1
Af82 forward + NED	0.4
Af82 reverse	0.4
Af86 forward + VIC	0.2
Af86 reverse	0.2
Af85 forward + FAM	0.2
Af85 reverse	0.2
Af50 forward + PET	0.2
Af50 reverse	0.2
Afbeta forward + NED	0.4
Afbeta reverse	0.4
Af48 forward + VIC	0.4
Af48 reverse	0.4
Af181 forward + FAM	0.2
Af181 reverse	0.2

Table S4: Numbers of parasitoid individuals collected, per species. Ordered by number of collected individuals.

parasitoid species	nr. collected	prop. of total
Lysiphlebus fabarum	3504	69.7
Aphelinus chaonia	648	12.9
Binodoxys angelicae	397	7.9
Praon volucre	219	4.4
Ephedrus plagiator	121	2.4
Aphidius colemani	47	0.9
Lipolexis gracilis	28	0.6
Aphelinus flaviventris	21	0.4
Alloxysta sp.	18	0.4
Binodoxys acalephae	8	0.2
Syrphophagus aphidivorus	5	0.1
Species not determined	13	0.3

Table S5: Number of aphid samples analyzed for each of nine secondary symbionts, number of aphids tested positive for the respective symbiont infection and individual frequency of each secondary symbiont.

symbiont	nr. aphids analyzed	nr. aphids positive	symbiont frequency
Hamiltonella defensa	3449	1206	0.350
Regiella insecticola	3448	261	0.076
Rickettsia sp.	3448	92	0.027
Serratia symbiotica	3449	33	0.010
Wolbachia sp.	1452	22	0.015
Fukatsuia symbiotica	1464	0	0
Arsenophonus sp.	1465	0	0
Rickettsiella viridis	1464	0	0
Spiroplasma sp.	1465	0	0

Table S6: Observed and expected frequencies of co-infections with two secondary symbionts for all combinations where > 5 co-infected aphid individuals are expected in our dataset, based on the individual symbiont frequencies (see Table S5). The p-values stem from Fisher's exact tests. Co-infections between *H. defensa* & *R. insecticola* occur significantly less often than expected by chance, also at a Bonferroni-corrected significance level α of 0.05/5 = 0.01.

symbiont combination	nr. aphids analyzed	observed	expected	p
H. defensa & *R. insecticola*	3448	18	91	**< 0.001**
H. defensa & *Rickettsia*	3448	34	32	0.740
H. defensa & *S. symbiotica*	3449	16	12	0.141
H. defensa & *Wolbachia*	1452	8	8	1
R. insecticola & *Rickettsia*	3447	10	7	0.228

Table S7: AIC values of the GLMs describing *H. defensa* frequency as a function of parasitism and/or heat days, at two different time lags (lag 1: parasitism 4 weeks prior to the *H. defensa* frequency estimate, heat days within the 4 weeks preceding the *H. defensa* frequency estimate; lag 2: parasitism 8 weeks prior, heat days within the 8 and 4 weeks preceding the *H. defensa* frequency estimate). Sampling site and year are used as covariates in all models.

time lag cause-effect	effects included in the model	AIC
lag 2	site + year + heatdays	205.97
lag 2	site + year + heatdays + parasitism	207.50
lag 2	site + year + parasitism	212.20
lag 1	site + year + heatdays + parasitism	217.44
-	site + year	220.04
lag 1	site + year + heatdays	220.10
lag 1	site + year + parasitism	221.15

Table S8: Comparison of *H. defensa* frequency before and after overwintering, that is between the last sampling in autumn and the first sampling in spring of the next year. We performed two-sided Chi-square tests. Although *H. defensa* frequency is lower in spring for all comparisons, the difference is significant only for winter 20/21 at site Gossau, and yet only at the significance level of 0.05 but not at a Bonferroni-corrected level of 0.05/9=0.006.

data subset	prop. fall	prop. spring	Chisq	df	p
all sites & both years	0.374	0.290	3.45	1	0.063
all sites 2019/20	0.333	0.285	0.60	1	0.439
all sites 2020/21	0.443	0.297	3.71	1	0.054
Faellanden 2019/20	0.438	0.425	0	1	1
Gossau 2019/20	0.288	0.250	0.03	1	0.861
Steinmaur 2019/20	0.294	0.200	0.90	1	0.344
Faellanden 2020/21	0.429	0.300	0.09	1	0.770
Gossau 2020/21	0.500	0.167	6.26	1	0.012
Steinmaur 2020/21	0.409	0.373	0.02	1	0.878

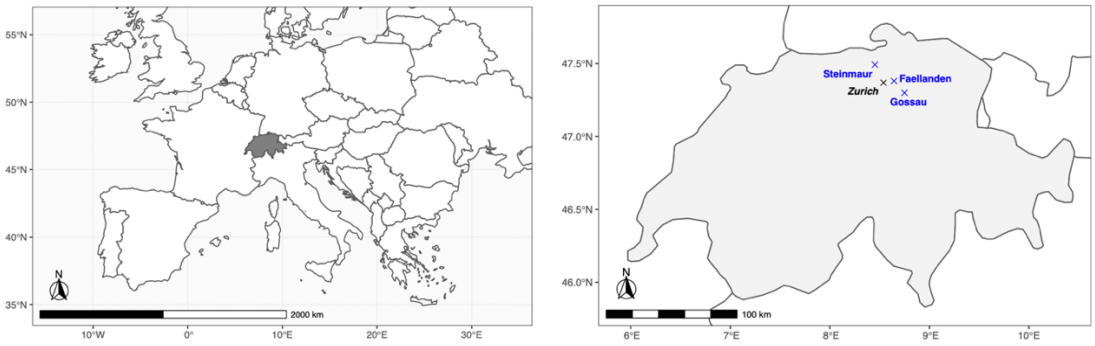

Figure S1: Location of our three sampling sites *Faellanden* (47° 22′ N 8° 38′ E), *Gossau* (47° 19′ N 8° 45′ E) and *Steinmaur* (47° 30′ N 8° 27′ E) near Zurich in Switzerland.

Figure S2: Examples of our sentinel plants: potted *Vicia faba* plantlets infested with bait aphids that were exposed to the parasitoid field community for four days. (Pictures: E. Gimmi)

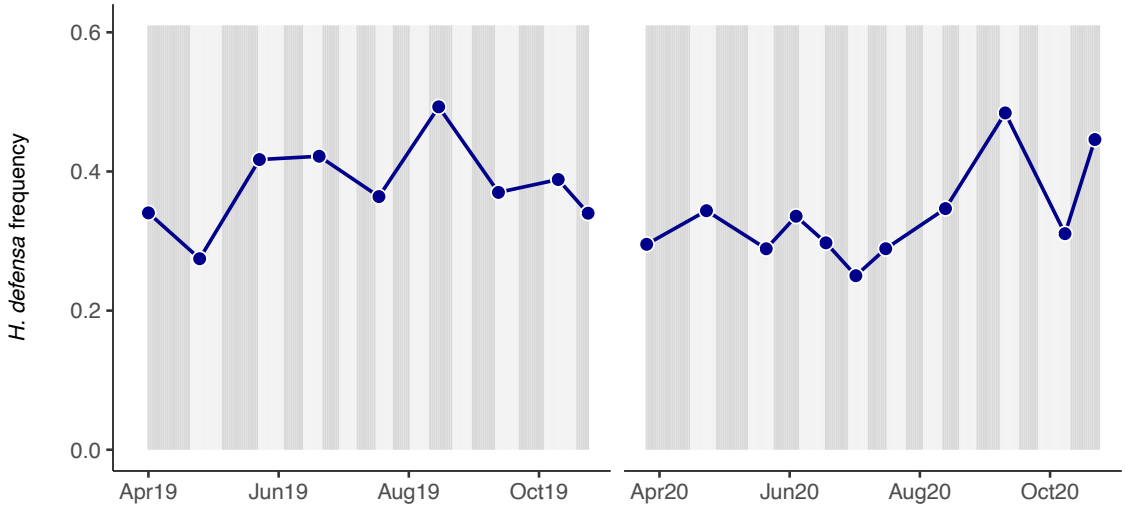

Figure S3: Aphid generation times are temperature-dependent and therefore different over the year. We calculated generation times using the day-degree method of Campbell *et al*. (1974) as described in Smith *et al*. (2021). Here, generation time is defined as 4*d/3, with d the age at first reproduction equal to age at adulthood + 2 days. Age at adulthood is calculated as the number of days at which the sum of mean day temperatures (starting at day of birth) passes 109.5 °C. The dark blue lines and points show *H. defensa* frequency averaged over all three sites.

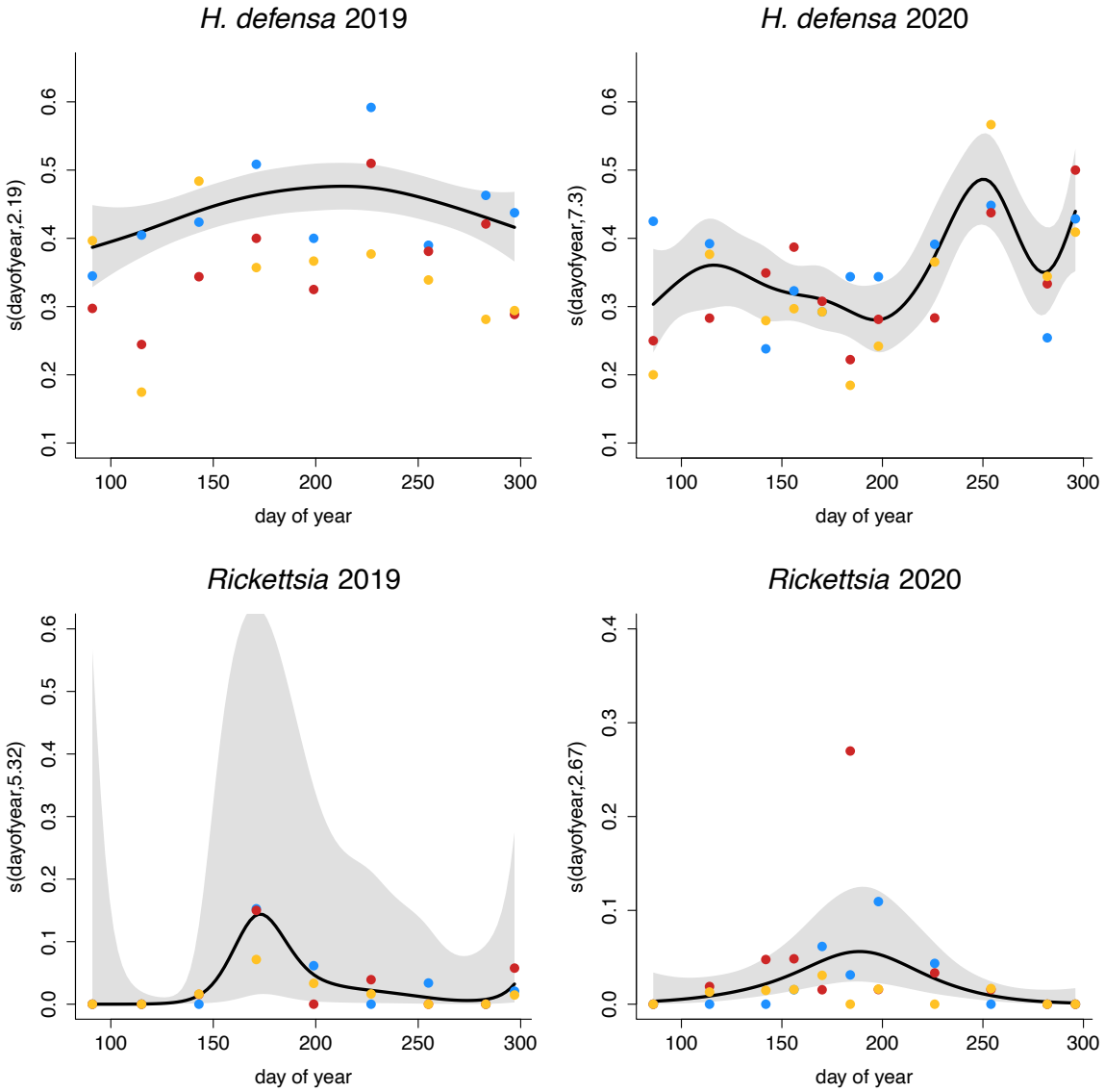

Figure S4: Component effect plots for the smooth term in GAMs testing whether the relationship between time (x-axis) and symbiont frequency (y-axis) is different from a straight line with slope zero. The corresponding test statistics are presented in Table 1 in the main manuscript. The black line is the function proposed by the model, shaded areas show the 95% confidence interval for the mean shape of the effect. Points show the actual data and are colored by site (blue: *Faellanden*, red: *Gossau*, yellow: *Steinmaur*).

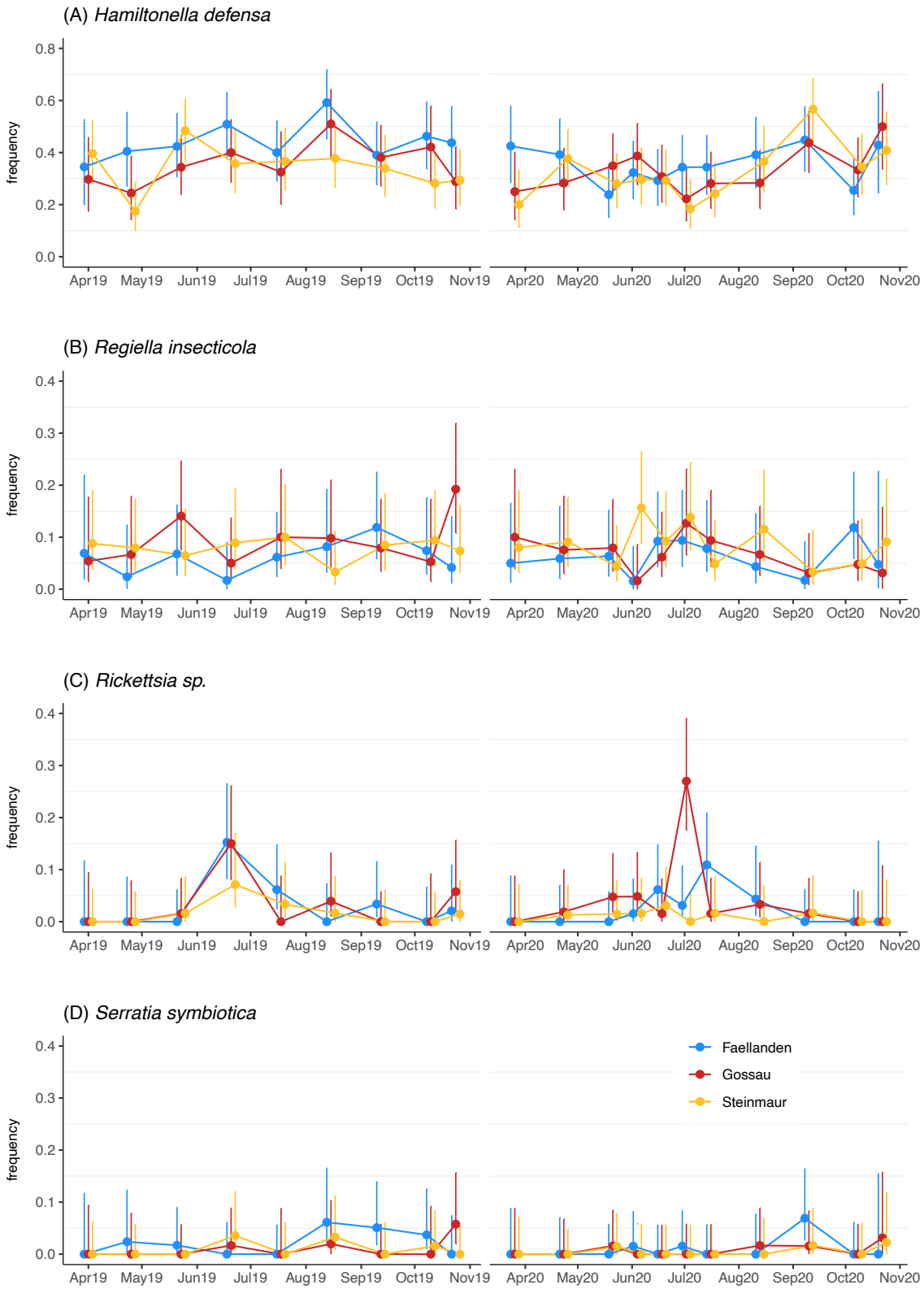

Figure S5: Symbiont frequencies per sampling timepoint, colored by site. Note the different scales of the y-axis. Error bars show 95% binomial confidence intervals.

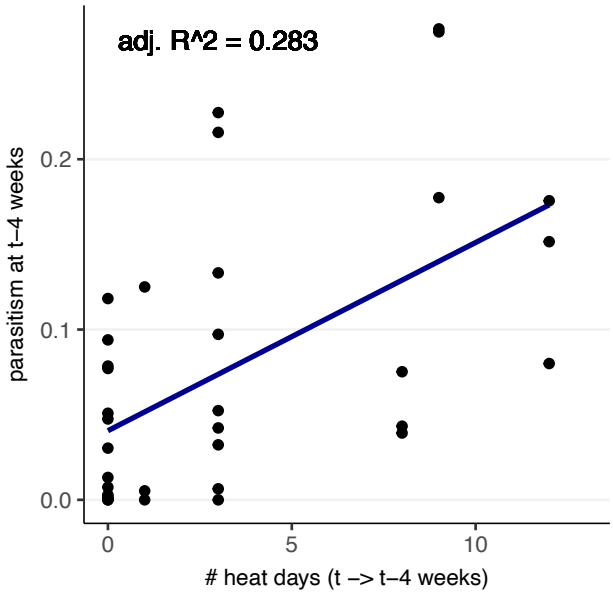

Figure S6: Correlation between number of heat days (summed up over a 4-week period, i.e. from t -> t-4 weeks) and parasitism risk (at timepoint t-4 weeks). Both variables are used as explanatory variables for *H. defensa* frequencies. The dark blue line describes a linear relationship between the two variables with an adjusted R^2 of 0.283.

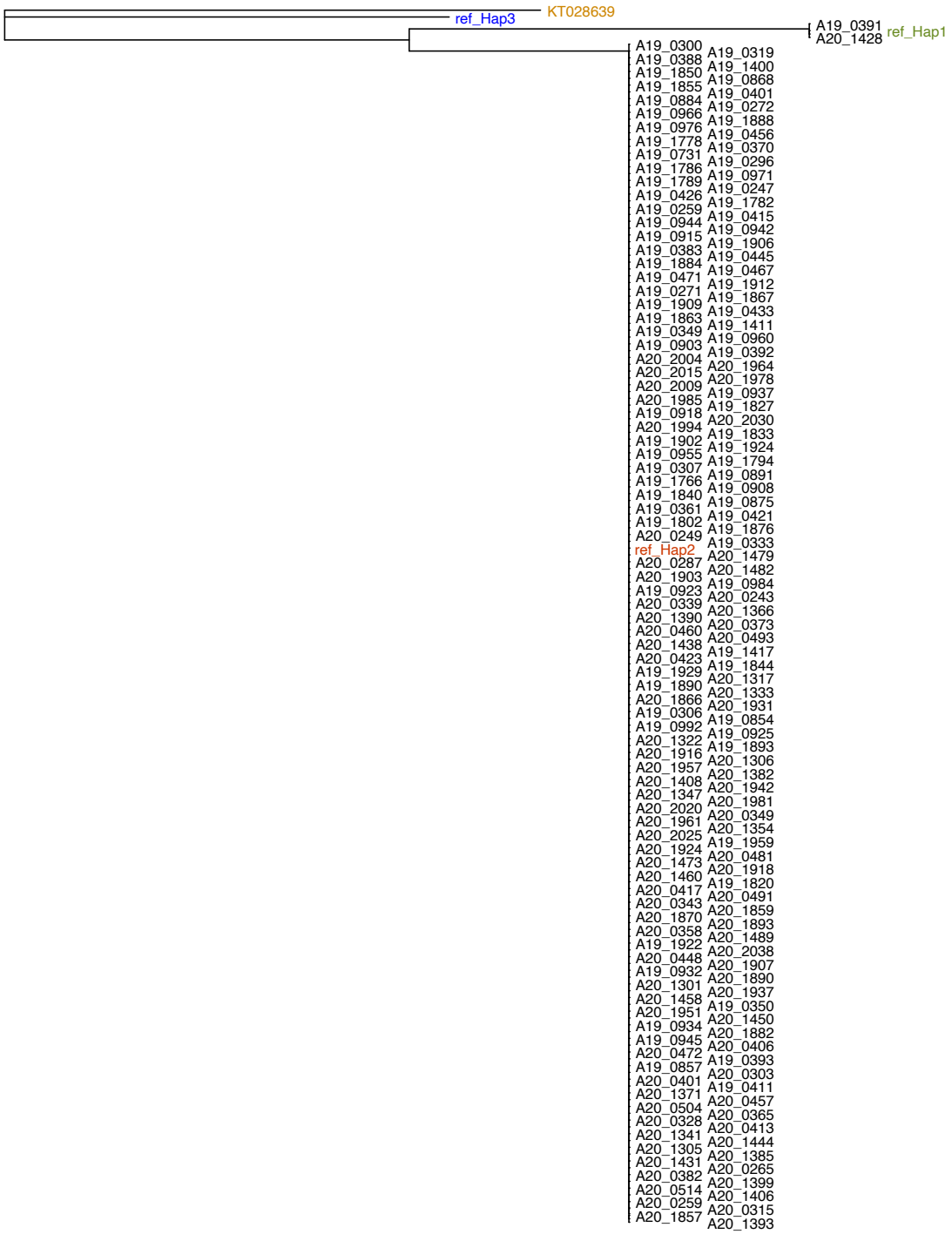

Figure S7: Phylogenetic tree built from partial sequences of the *H. defensa* gene *murE*. We used a distance matrix following Tamura and Nei (1993) and a neighbor join method (Saitou & Nei, 1987). Ref_Hap1, ref_Hap2 and ref_ Hap3 (in green, red and blue) are the reference sequences found in *H. defensa* haplotypes 1, 2 and 3, respectively. The outgroup (KT028634, orange) is a sequence from a *H. defensa* in a pea aphid (*Acyrthosiphon pisum*) from GeneBank.

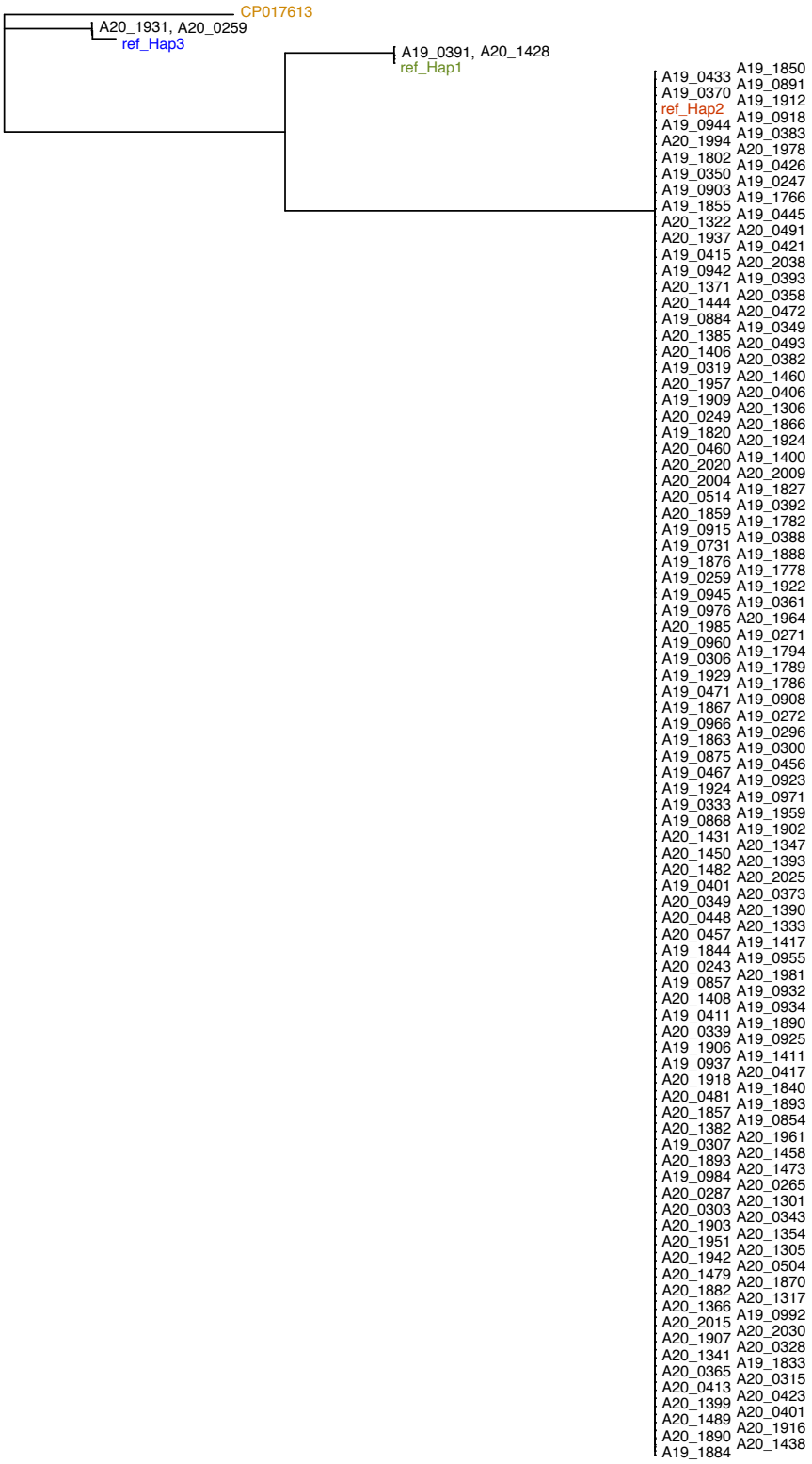

Figure S8: Phylogenetic tree built from partial sequences of the *P41* gene of the APSE phage within the *H. defensa* genome. We used a distance matrix following Tamura and Nei (1993) and a neighbor join method (Saitou & Nei, 1987). Ref_Hap1, ref_Hap2 and ref_ Hap3 (in green, red and blue) are the reference sequences found in *H. defensa* haplotypes 1, 2 and 3, respectively. The outgroup (CP017613, orange) is a sequence from a *H. defensa* in a pea aphid (*Acyrthosiphon pisum*) from GeneBank

Supplementary Analysis S1

Rationale

In our field data, overall prevalence of the endosymbiont *Rickettsia* was low (3%), yet there was a significant increase in *Rickettsia* frequency that strongly correlated with parasitoid frequency (see main manuscript). *Rickettsia* endosymbionts are known to occur in *A. fabae* and other aphid species (Chen et al., 1996; Zytynska et al., 2016; Zytynska & Weisser, 2016) but also in many other arthropods, including parasitoid wasps (Pilgrim et al., 2021). We therefore reasoned that the *Rickettsia* we found in certain aphid extractions might not be aphid symbionts, but rather symbionts of recently injected parasitoid eggs or young parasitoid larvae. This could explain the high *Rickettsia* prevalence in our aphid samples just during the period of peak parasitism. Here we describe how we explored this hypothesis.

Methods

1. Does Rickettsia occur in parasitoid wasps from the field?

To answer this question, we obtained DNA from 48 individuals of the parasitoids collected during the field study (parasitoids that hatched from the parasitized bait aphids). We used the same DNA extraction protocol as for the aphid samples (Sunnucks & Hales, 1996). We then tested the samples for *Rickettsia* presence using diagnostic PCR with *Rickettsia*-specific primers, as described in Table S1. The product of this PCR is a fragment of ~210bp that is found in *Rickettsia* from a multitude of hosts, and which is thus not sufficient to determine whether it stems from a parasitoid host or an aphid host.

2. Are Rickettsia-positive aphid samples parasitized by parasitoid wasps more often than Rickettsia-negative aphid samples? This question was addressed using a subset of 204 aphid DNA extracts from the main field study. Nine samples were from the first sampling timepoint in 2019 where no *Rickettsia*-positive aphid was detected and estimated parasitoid frequency was zero. The other samples came from the timepoints in mid-June 2019 (85 samples), mid-July 2019 (15 samples) and begin of July 2020 (95 samples), thus from the "high parasitism" period. 20% (N=40) of the samples in the subset contained DNA from *Rickettsia*, thus *Rickettsia*-positive extractions were overrepresented compared to their frequency in the full dataset (3% *Rickettsia*). To test whether the aphid extractions contained parasitoid DNA, we used the primers that were developped by Derocles et al. (2012) to detect parasitoid larvae or eggs in extractions of parasitized aphid hosts by PCR. These primers (16S-F: 5' CGC CGT TTT ATC AAA AAC ATG T 3', 16S-Rspe: 5' TCT AWA GGG TCT TCT CGT CT 3') target a mitochondrial 16S sequence of Aphidiinae wasps. By sequencing the amplified fragment and comparing it to a reference database, one can identify a broad range of Aphidiinae species, including the most frequent wasp species that we recovered in the field, except A. chaonia, which belongs to the Aphelinidae. We carried out PCR amplification in two steps, starting with a 11 μl reaction volume containing 2μl extracted DNA, 2.4 μl ddH2O, 5.5 μl Promega GoTaq® G2 Colorless Master Mix, and 0.55 μl of each primer. Amplification conditions were as following: 180s at 94°C; then 40 cycles of 30s at 94°C, 60s at 58°C, 90s at 72°C; and a final elongation step of 10min at 72°C. We performed a second PCR step taking 5 μl of the product from the first step , 5.5 μl Promega GoTaq® G2 Colorless Master Mix, and 0.55 μl of each primer per reaction and amplification conditions as in step one but for 30 cycles. We added the second step because this part of the analysis was done >2 years after the original DNA extraction, and sample quality appeared reduced so that after one round of PCR product concentration was yet too low for sequencing. With fresh aphid extracts, performing only step 1 showed to be sufficient. Following PCR we screened the samples for presence or absence of amplified parasitoid DNA using a QIAxcel capillary electrophoresis device. PCR products that contained parasitoid DNA were sent for Sanger sequencing to Microsynth AG (Balgach, Switzerland), and the resulting sequences were compared to GenBank (Sayers et al., 2022) using BLAST (Altschul et al., 1990) to determine the parasitoid species from which it originated. To test whether *Rickettsia* was detected more frequently in parasitized aphids than in unparasitized aphids we used Pearson's $\chi 2$ tests.

Results and Discussion

We found *Rickettsia* in 13 of the 48 analysed parasitoid extractions, confirming that *Rickettsia* commonly infects parasitoid wasps. Interestingly, none of the analysed *L. fabarum* (0/32) but all analysed *B. angelicae* (10/10) carried *Rickettsia*. Further, we found *Rickettsia* in 0/1 *B. acalephae*, 1/2 *L. gracilis* and 2/3 *P. volucre*. While the samples numbers are too low to infer any frequency estimates for *Rickettsia* in these species, we can confirm that the symbiont occurs in different parasitoid species present in the field.

We detected parasitoid DNA in 37/204 (18%) of the analyzed aphid extractions. The occurrence of parasitoid DNA and *Rickettsia* in the aphid extractions was highly correlated. That is, in extractions from aphids that had apparently been attacked by a parasitoid before being sampled, *Rickettsia* was found more frequently than in extractions from unparasitized aphids ($\chi2 = 77.57$, df = 1, p-value < 2.2e-16). This effect remains highly significant also when removing the nine *Rickettsia*-free samples from the first timepoint in 2019, where we a priori did not expect to find parasitoid DNA because parasitism risk independently estimated with the bait plants was low ($\chi2 = 73.16$, df = 1, p-value < 2.2e-16). This supports our hypothesis that at least some of the *Rickettsia* we detected in aphid extractions were in fact symbionts of parasitoids, rather than aphid symbionts. However, not all *Rickettsia*-positive aphid extractions contained parasitoid DNA. Hence, we do not exclude that some of the detected *Rickettsia* were true symbionts of *A. f. fabae*. Nevertheless, the marked peak in *Rickettsia* prevalence that we see in June and July of both years in our seasonal data is likely driven by the detection of this endosymbiont in parasitoid eggs or larvae present in part of the sampled aphids.

We successfully sequenced the parasitoid DNA fragment of 35 samples. One sequence corresponded to *Aphidius* sp, 26 to *B. angelicae*, six to *L. fabarum* and two to *P. volucre*. Corroborating the results from above (especially that *Rickettsia* is frequent in *B. angelicae* but not in *L. fabarum*), all but one of the extractions containing DNA from *B. angelicae* (25/26) or *P. volucre* (2/2) were *Rickettsia*-positive, while all the extractions containing DNA from *Aphidius* sp or *L. fabarum* were *Rickettsia*-negative.

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Chapter III

High specificity of symbiont-conferred resistance in an aphid-parasitoid field community

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Abstract

Host-parasite coevolution is mediated by genetic interactions between the antagonists and may lead to reciprocal adaptation. In the black bean aphid, *Aphis fabae fabae*, resistance to parasitoids can be conferred by the heritable bacterial endosymbiont *Hamiltonella defensa*. *H. defensa* has been shown to be variably protective against different parasitoid species, and different genotypes of the black bean aphid's main parasitoid *Lysiphlebus fabarum*. However, these results were obtained using haphazard combinations of laboratory-reared insect lines with different origins, making it unclear how representative they are of natural, locally (co)adapted communities. We therefore sampled the parasitoids of a natural *A. f. fabae* population comprehensively and measured the ability of the five most abundant species to parasitize aphids carrying the locally prevalent *H. defensa* haplotypes. *H. defensa* provided resistance only against the dominant parasitoid *L. fabarum* (70% of all parasitoids), but not against less abundant parasitoids, and resistance to *L. fabarum* acted in a genotype-specific manner (G × G interactions between *H. defensa* and *L. fabarum*). These results confirm that strong species- and genotype-specificity of symbiont-conferred resistance is indeed a hallmark of wild *A. f. fabae* populations, and they are consistent with symbiont-mediated local adaptation of aphids to parasitoids.

Keywords: specificity of resistance, genotype-by-genotype interactions, aphids, parasitoids, *Hamiltonella defensa*, local adaptation, defensive symbiosis, coevolution

1 | Introduction

Host-parasite relationships are characterized by continuous adaptation and counteradaptation between the interacting species, a process referred to as antagonistic coevolution (Inouye, 2012). It requires heritable genetic variation in host resistance and parasite infectivity, which among other can be maintained by negative frequency-dependent selection when genotype-by-genotype interactions between hosts and parasites determine infection success (Agrawal & Lively, 2002; Hamilton, 1980), or when host resistance and parasite virulence are costly (Agrawal & Lively, 2002; Nuismer, 2006). Host-parasite coevolution may result in local adaptation, that is, adaptation of hosts to the local parasites, or of parasites to the local hosts. On average, the direction of the effect will depend on whether the host or the parasite has the upper hand in the coevolutionary arms race. Local adaptation may be promoted in the antagonist that shows comparatively high migration or mutation rates, as this increases the genetic variability upon which selection can act (Gandon *et al*., 1996; Gandon & Michalakis, 2002). Adaptation may be sped up and thus facilitated by short generation times and high reproductive rates (Gandon *et al*., 1996; Kaltz & Shykoff, 1998). Both are typical of many parasites, and indeed, local adaptation of parasites to their hosts is frequently observed (e.g. Ebert, 1994; Lively *et al*., 2004). However, also local adaptation of hosts to parasites, sometimes equated with parasite local maladaptation, can occur in natural systems (e.g. Kaltz *et al*., 1999; Lemoine *et al*., 2012; Oppliger *et al*., 1999). In either case, observed levels of resistance in a host population could originate from past and present selection imposed by the local environment, and in particular the local parasite community (Decaestecker *et al*., 2007; Kerfoot & Weider, 2004; Sadd & Schmid-Hempel, 2009; Schmid-Hempel & Ebert, 2003). Selection for resistance acts not only on the host genome but may also affect resistance conferred by host-associated symbiotic organisms. So-called defensive symbionts bear the potential for rapid evolution of host resistance (e.g. Hedges *et al*., 2008; Jaenike *et al*., 2010; Teixeira *et al*., 2008) and may enable symbiont-driven host-parasite coevolution (Vorburger & Perlman, 2018). Many examples of defensive symbioses concern insects: they can harbor various endosymbiotic bacteria known to confer ecological benefits, including defense against pathogens, parasites, and predators (Florez *et al*., 2015; Oliver & Moran, 2009; Oliver *et al*., 2014). One of the most extensively studied insect defensive

symbioses is that between aphids (Aphidoidea) and the gammaproteobacterium *Hamiltonella defensa* (Moran et al., 2005), a heritable endosymbiont known to provide several aphid species with resistance against parasitoid wasps (Asplen *et al*., 2014; Oliver *et al*., 2003; Vorburger *et al*., 2009). *H. defensa* is a facultative endosymbiont in that it is not necessary for aphid survival under benign conditions. Its presence even entails fitness costs for the aphid host in the absence of parasitoids (Dykstra *et al*., 2014; Oliver *et al*., 2008; Vorburger & Gouskov, 2011), which likely contributes to the fact that *H. defensa* is rarely fixed in natural aphid populations (e.g. Brady *et al*., 2014; Clarke *et al*., 2018; Sepúlveda *et al*., 2017). Individual aphids do usually not carry more than one *H. defensa* strain (Russell *et al*., 2013), but multiple strains can occur within a single aphid species (e.g. Henry *et al*., 2022; Leclair *et al*., 2016; Wu *et al*., 2022). Different *H. defensa* strains carry different variants of a bacteriophage residing in the bacterial genome (Degnan & Moran, 2008; Rouïl *et al*., 2020). These phage variants may encode distinct toxins that are likely involved in parasitoid resistance by inhibiting the development of the parasitoid egg or larva within the aphid (Brandt *et al*., 2017; Lynn-Bell *et al*., 2019; Oliver *et al*., 2009; Oliver & Higashi, 2019). *H. defensa*-conferred resistance against parasitoid wasps is therefore a variable and heritable trait that can be subject to selection by a local parasitoid community. Parasitoids, for their part, possess genetic variation for overcoming *H. defensa*-conferred resistance and can evolve counteradaptations to different *H. defensa* strains (Dennis *et al*., 2017; Dion *et al*., 2011; Rouchet & Vorburger, 2014).

In accordance with this, multiple studies have demonstrated strong variation and a high specificity of *H. defensa*-conferred resistance to parasitism. A given strain of *H. defensa* can confer resistance against some parasitoid species but not against others (e.g. Asplen *et al*., 2014; Hopper *et al*., 2018; Łukasik *et al*., 2013; Martinez *et al*., 2016; McLean & Godfray, 2015), and it can also provide different resistance against different genotypes of the same parasitoid species (e.g. Cayetano & Vorburger, 2013; Cayetano & Vorburger, 2015; Schmid *et al*., 2012). This suggests that local parasitoid communities can influence patterns and types of symbiont-conferred resistance in their hosts. However, much of the available evidence is based on experiments in which *H. defensa* strains and parasitoids were combined haphazardly, using lines collected from different sites or time points (e.g. Cayetano & Vorburger, 2013; Hopper *et al*., 2018; Schmid *et al*., 2012), or parasitoids obtained from commercial breeders of biocontrol agents (e.g. Asplen *et al*., 2014; Cayetano

& Vorburger, 2015; Łukasik *et al*., 2013). The antagonists confronted in these experiments thus did not have a common evolutionary history, such that the experimentally observed specificities and apparent trade-offs for resistance are not necessarily representative of resistance patterns that occur in natural, locally (co)adapted insect communities.

In an attempt to improve on this, Wu *et al*. (2022) shuffled *H. defensa* strains among three aphid species found in the UK and then exposed each aphid species to its dominant parasitoid. They found that in the majority of cases, the aphids' native *H. defensa* strains were protective against the species' dominant parasitoids, consistent with the hypothesis of symbiont-mediated aphid adaptation to the local parasitoid community. However, the focus on a single parasitoid species captures only part of the risk experienced by each host species, and we therefore took a different approach for the present study. We took advantage of a two-year field study on the black bean aphid, *Aphis fabae fabae*, where we had collected very detailed information on i) the relative abundances of different parasitoid species exploiting the local aphid populations near Zurich, Switzerland and ii) the prevalence of different *H. defensa* haplotypes in the same aphid populations (Chapter II). This data was used to design a full-factorial experiment in which we tested the ability of the five most frequent parasitoid species of *A. f. fabae* – using locally collected insects – to parasitize aphids carrying the two most frequent *H. defensa* haplotypes present in the local aphid populations. The results provide a comprehensive picture of the strength and specificity of symbiont-conferred resistance in a locally assembled, natural insect community.

2 | Methods

Organisms

In the field study preceding the present experiment (Chapter II), we collected black bean aphids (*A. f. fabae*) and estimated their risk of infection by parasitoids on a monthly basis for two full growing seasons (2019 and 2020) at three sites close to Zurich, Switzerland. Parasitoids were collected using sentinel hosts, i.e. susceptible, *H. defensa*-free black bean aphids that were brought to the field and exposed to the local parasitoid community. Parasitized aphids were returned to the laboratory to count and identify the hatching

parasitoids. In total, we identified 5029 individuals belonging to eleven parasitoid species (Chapter II). For the present experiment we considered the five most frequent parasitoid species, which together made up 97% of the collected samples: *Lysiphlebus fabarum* (70% of the collected samples), *Aphelinus chaonia* (13%), *Binodoxys angelicae* (8%), *Praon volucre* (4%) and *Ephedrus plagiator* (2%). *A. chaonia* belongs to the family Aphelinidae, the other four species are braconid wasps (Braconidae) from the subfamily Aphidiinae. We established laboratory populations of these species with individuals collected during the second year of the field study. *A. chaonia*, *B. angelicae*, *P. volucre* and *E. plagiator* are sexual species (arrhenotokous reproduction) and were bred as large cage populations on a *H. defensa*-free clone of *A. f. fabae* that was different from the clone used in the experiment (see below). *L. fabarum* is predominantly asexual (thelytokous reproduction), hence we initiated 11 asexual lines from single females and reared them on the same aphid clone as the other parasitoid species. We genotyped these lines at 10 microsatellite loci (Sandrock *et al*., 2007; Sandrock *et al*., 2011b), showing that they belonged to six different genotypes. For the experiment we used four genetically different lines, including the two genotypes that had been collected multiple times (*L.fab* 5, collected five times and *L.fab* 1, collected twice), likely representing abundant genotypes of this species in Zurich, as well as two of the genotypes collected only once (*L.fab* 2 & 3).

35% of the 3449 aphids collected in the field study carried *H. defensa*. Sequence typing of the symbiont in a subset of samples (n = 175) determined that 98% of the infected aphids carried the same known *H. defensa* haplotype (haplotype 2, Cayetano *et al*., 2015), while another 1% each carried the known haplotype 1 (Cayetano *et al*., 2015) and a previously unknown haplotype (Chapter II). For the present experiment, we assessed the resistance conferred by *H. defensa* haplotypes 1 and 2 in a common aphid genetic background, that is, we worked with three different lines of a single clone of *A. f. fabae* from our laboratory collection (clone ID: 407): one line carried no facultative endosymbiont (407), one line carried a *H. defensa* strain with haplotype 1 (strain 76, aphid line 407-H76), and one line carried a *H. defensa* strain with haplotype 2 (strain 42, aphid line 407-H42). The *H. defensa*-infected aphid lines had been created by microinjection of hemolymph from aphids carrying the desired *H. defensa* strains into the *H. defensa*-free 407 clone (Cayetano & Vorburger, 2015, Y. Henry & C. Vorburger, unpublished). The common genetic background allowed us to exclude variation in resistance due to the aphid genotype, even

though the endogenous resistance of *A. f. fabae* is likely low compared to *H. defensa*-conferred resistance (Vorburger *et al.*, 2009). All aphid lines have been maintained parthenogenetically in the lab for multiple generations prior to the experiment. Aphid and parasitoid rearing and the experiment took place in a climate chamber at constant 19°C and with a 16/8 h light/dark cycle.

Experimental Setup

We tested the parasitism success of 8 parasitoid types (4 sexual species, 4 different isofemale lines of the asexual *L. fabarum*) on three different aphid lines (407, 407-H42, 407-H76) using a full-factorial design with 10 replicates per aphid line-parasitoid type combination (240 experimental units). The experiment was performed in 10 randomized complete blocks containing each one replicate of the 24 treatment combinations. 5 blocks were processed on each of two consecutive days. An experimental unit consisted of a seedling of *Vicia faba* – the plant on which we routinely rear *A. f. fabae* – in a pot of ca. 5cm diameter, covered by a ventilated plastic cage of ca. 15cm height. The aphid lines were split up onto 240 separate plants and reared for two generations prior to the start of the experiment, to avoid carrying over (grand)maternal environmental effects from the stock populations (Kindlmann & Dixon, 1989). On day 1 of the experiment, we transferred four adult aphids per experimental unit onto fresh *V. faba* seedlings. On day 2, the adult aphids were removed from the plants, leaving behind a cohort of aphid nymphs. We counted the number of nymphs on the plants on day 3 (average $n = 18 \pm 6$ SD). On day 4, we added two female wasps to each of the plants and allowed them to parasitize the aphids for 8 hours. 14 days later (on day 18), the successfully parasitized aphids were clearly recognizable as so-called mummies (parasitoid cocoons within the emptied aphid husk). We counted the mummies and calculated parasitism rate per experimental unit as the number of mummies divided by the number of nymphs that were initially exposed to the parasitoids.

Analysis

Data analysis was carried out with R version 4.1.2 (R Core Team, 2019) in Rstudio 2022.02.3 (RStudio Team, 2020) and we used ggplot2 3.3.5 (Wickham, 2016) for plotting. To test for overall patterns of *H. defensa* resistance, we first considered two models

analyzing either the parasitism rates of the sexual parasitoid species (*A. chaonia*, *B. angelicae*, *P. volucre* and *E. plagiator*) or those of the four asexual *L. fabarum* lines. For both data subsets we applied generalized linear models (GLMs) to the proportion of aphids that got parasitized, using logit links and a quasibinomial error distribution to account for overdispersion. *Anova* from the R library car 3.0.7 (Fox & Weisberg, 2019) was used for analysis of deviance with F-tests as recommended for quasilikelihood fits (Crawley 2014). For the four sexual parasitoid species we tested for the effects of experimental block, aphid line, parasitoid species, and the aphid line × parasitoid species interaction. For the four *L. fabarum* lines, we tested for the effects of block, aphid line, *L. fabarum* line, and the aphid line × *L. fabarum* line interaction. Furthermore, we specifically tested for the protective effect of each *H. defensa* strain against each parasitoid species and *L. fabarum* line. Because of high overdispersion due to zero-inflation in some data subsets, we did not use GLMs for this but rather applied Kruskal-Wallis tests for overall differences among aphid lines, for each parasitoid species or line separately. We then tested for a difference in parasitism rate between the *H. defensa*-free aphid line and either of the two *H. defensa*-infected aphid lines using two pairwise Wilcoxon tests with Bonferroni correction.

3 | Results

Parasitoid species was the only significant effect in the analysis of parasitism rates achieved by the four sexual species *A. chaonia*, *B. angelicae*, *P. volucre* and *E. plagiator* (Table 1a, Figure 1a). The effect is mainly driven by the low parasitism rates of *B. angelicae* (around 10% on all three aphid lines, Figure 1a) in contrast to the other species. The aphid line × parasitoid species interaction was marginally non-significant in this model (Table 1a), the relatively low p-value resulting mostly from the slight difference in parasitism by *E. plagiator* between the *H. defensa*-infected aphid lines 407-H42 and 407-H76 (Figure 1a). The main effect of aphid line was not significant for the parasitism rates of the sexual species (Table 1a). This is in accordance with the fact that neither of the two *H. defensa*-infected aphid lines showed significantly changed parasitism rates compared to the *H. defensa*-free line when exposed to any of these parasitoid species (Figure 1a,

Table S1). The two tested *H. defensa* strains are therefore not protective against these parasitoid species in the studied field community.

In contrast, parasitism rates of *L. fabarum* were significantly dependent on aphid line (Table 1b), with strongly reduced parasitism on the *H. defensa*-infected aphid lines 407-H42 and 407-H76 compared to the uninfected line 407 when averaging over all four *L. fabarum* lines (Figure 1b, Table S1). There was also a highly significant interaction between aphid line and *L. fabarum* line (Table 1b), indicating a genotype-specific protection by *H. defensa*: all four *L. fabarum* lines could parasitize the *H. defensa*-free aphids, the *L. fabarum* lines 1 and 2 could parasitize 407-H42 but not 407-H76, line 3 could parasitize 407-H76 but not 407-H42, and line 5 had very poor parasitism success on both *H. defensa*-infected aphid lines (Figure 1c, Table S1). Both tested *H. defensa* strains thus protect against parasitism by *L. fabarum*, but the protection depends on the genotype of the attacking parasitoid.

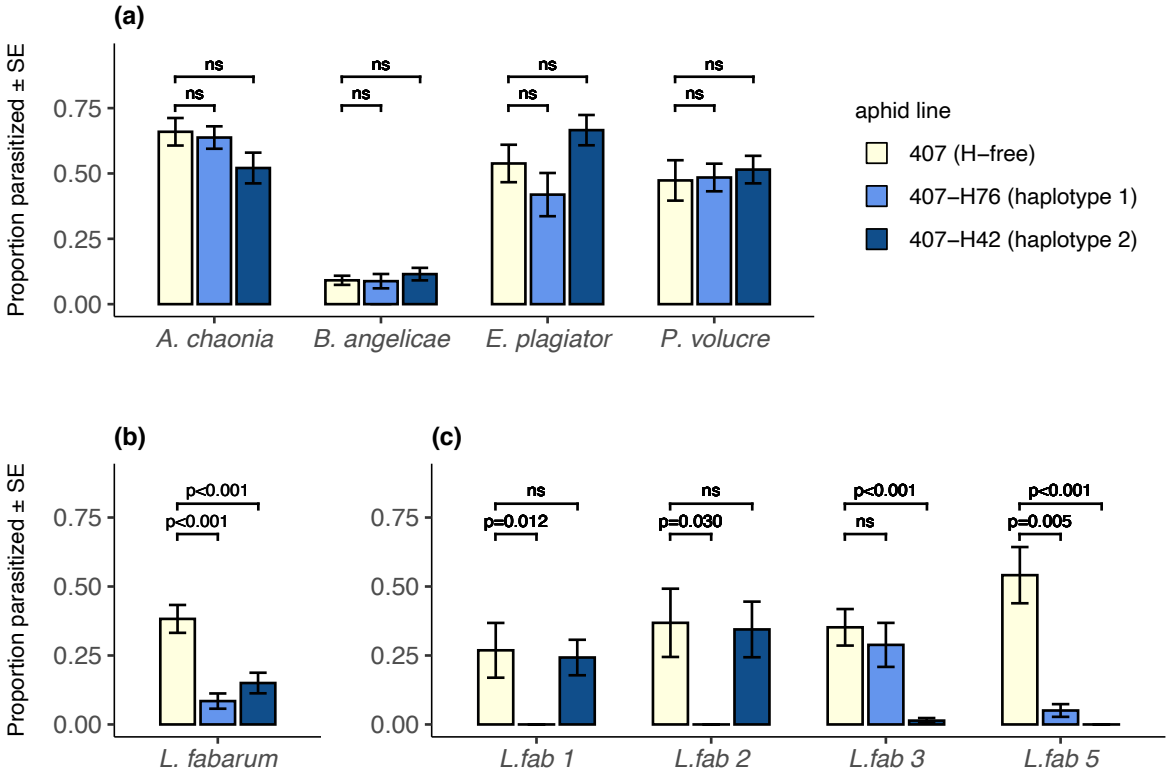

Figure 1: Mean parasitism rates calculated as number of mummies divided by number of exposed aphids. (a) shows the four sexual parasitoid species on the x-axis, (b) shows the results for *L. fabarum* when averaging over the four different lines, and (c) shows each of the four different lines of *L. fabarum* separately. The p-values above the bars stem from pairwise Wilcoxon tests; values below 0.05 indicate a significant protective effect of the respective *H. defensa* strain against the wasp species or line after Bonferroni correction. Bar colors correspond to the three different aphid lines: 407 (*H. defensa*-free), 407-H76 (*H. defensa* haplotype 1) and 407-H42 (*H. defensa* haplotype 2).

Table 1: Analysis of deviance table for the proportion of aphids parasitized (parasitism rate). Two generalized linear models with logit link and quasibinomial fit are shown: (a) on the data set containing only the sexual wasp species (dispersion parameter Φ = 2.38) and (b) on the data of *L. fabarum* lines only (Φ = 4.49).

	Df	**Sum Sq**	***F***	***P***
(a) sexual parasitoid species (*A. chaonia, B. angelicae, E. plagiator, P. volucre*)				
block	9	29.1	1.36	0.2176
aphid line	2	7.3	1.54	0.2203
parasitoid species	3	391.7	54.94	<0.0001
aphid line x parasitoid species	6	29.8	2.09	0.0611
Residuals	99	235.3		
(b) *Lysiphlebus fabarum*				
block	9	51.62	1.28	0.2585
aphid line	2	186.62	20.79	<0.0001
L. fabarum line	3	10.58	0.79	0.5047
aphid line x *L. fabarum* line	6	320.03	11.88	<0.0001
Residuals	99	444.41		

4 | Discussion

In a Swiss population of *A. f. fabae*, we tested the ability of two *H. defensa* haplotypes, the dominant haplotype (98%) and one of the rare haplotypes (1%), to protect aphids against the local parasitoid community. *H. defensa* conferred high levels of resistance only to the most abundant parasitoid species, *L. fabarum*, and this resistance acted in a genotype-specific manner (Figure 1, Table 1). That *H. defensa*-conferred resistance is differently effective against different parasitoid species has been demonstrated before (e.g. Asplen *et al*., 2014; Cayetano & Vorburger, 2015; Kraft *et al*., 2017; Martinez *et al*., 2016; McLean & Godfray, 2015), as have strong genotype-by-genotype interactions between *L. fabarum* and *H. defensa* (Cayetano & Vorburger, 2013; Gimmi & Vorburger, 2021; Schmid *et al*., 2012; Vorburger & Rouchet, 2016). However, previous experiments sometimes used commercially bred parasitoid stocks or somewhat arbitrary combinations of host and

parasitoid lines from different origins, making it unclear how representative the results are of interactions in natural populations. Our experiment shows that species- and genotype-level specificity of symbiont-conferred protection indeed prevails in a field population of black bean aphids and thus has the potential to affect the evolution of host resistance.

The fact that *H. defensa* provides aphids with protection against the most frequent parasitoid species is similar to the finding of Wu *et al.* (2022), who described that other aphid species' native *H. defensa* strains provided protection against those species' dominant parasitoids. It is indicative of host adaptation in response to parasite-mediated selection as also seen, for instance, in experimental populations of *Daphnia* waterfleas (Capaul & Ebert, 2003; Haag & Ebert, 2004) – with the difference that in our example, host adaptation is realized via defensive symbiosis (Hafer-Hahmann & Vorburger, 2020; Oliver *et al.*, 2008; Rossbacher & Vorburger, 2020). In this context, it is interesting that both tested *H. defensa* strains protected strongly against *L. fabarum* line 5, which appeared to be an abundant genotype in the field populations of *L. fabarum* (see Methods).

Even though our experiment was comprehensive in using all parasitoid species representing a significant risk for the studied aphid population, it remains a snapshot in space and time. As suggested by Thompson (2005), environmental variability and corresponding changes in community composition could result in mosaic-like variation of selection forces. It is possible, therefore, that we would have observed different resistance patterns if the same experiment had been carried out with insects and symbionts from another geographic area or year (Kaltz & Shykoff, 1998). For instance, Lenhart and White (2017) found no protection of *H. defensa* against a local parasitoid community in *Aphis craccivora*. Nevertheless, there are conditions under which we expect local adaptation of hosts to parasites, rather than parasite local adaptation, to prevail. One such condition is when the host is more mobile than the parasite (Gandon *et al.*, 1996; Gandon & Michalakis, 2002; Greischar & Koskella, 2007). Many aphids, including *A. f. fabae*, are highly migratory and show wind-assisted dispersal (Loxdale *et al.*, 1993). The frequently reported weakness of genetic differentiation between aphid populations even from very distant sites is consistent with such large-scale dispersion abilities (e.g. Llewellyn *et al.*, 2003; Rattanawannee *et al.*, 2019; Sandrock *et al.*, 2011a). In contrast, aphid parasitoids generally seem to be poor dispersers (e.g. Nyabuga *et al.*, 2010; Rauch & Weisser, 2007). The comparatively high dispersal abilities of hosts may distinguish the aphid-parasitoid

system from other host-parasite systems with higher mobility of parasites, where local adaptation of parasites is the predominantly observed pattern (Greischar & Koskella, 2007; Hoeksema & Forde, 2008; Lively *et al*., 2004). Generation time might be another important factor: as long as genetic variability is not limiting, adaptation of parasites to hosts may predominate in those interactions where parasites have much shorter generation times than their hosts (Gandon & Michalakis, 2002; Kaltz & Shykoff, 1998; Nee, 1989; Price, 1980). This clearly does not apply for parasitoids, which have similar or slightly longer generation times than aphids, making host local adaptation a likely alternative outcome.

The strong genotype-specificity of symbiont-conferred resistance makes the host's benefit of harboring *H. defensa* contingent on the genotypic composition of its main parasitoid. This could set the stage for intense and dynamic coevolution (Kwiatkowski *et al*., 2012). Specificity promotes negative frequency dependent selection, which can account for the maintenance of genetic variation in both hosts and parasites due to the selective advantage of rare over common genotypes (Clarke, 1976; Judson, 1995). Laboratory studies have demonstrated that parasitoid (genotypic) diversity indeed bears the potential to maintain strain diversity among protective endosymbionts (Hafer & Vorburger, 2019; Hafer-Hahmann & Vorburger, 2020). The high degree of specificity we observed here among lines of a natural community lends credibility to the relevance of these previous studies and supports the importance of *H. defensa* as a driver of coevolutionary dynamics between aphids and parasitoids (Kwiatkowski *et al*., 2012; Vorburger, 2014). It is therefore surprising that the field survey preceding our experiment (Chapter II) revealed that *H. defensa* haplotype 2 was totally dominant in the *A. f. fabae* population (98% of the infected aphids carried this haplotype). This does not really fit with the picture of a highly dynamic turnover of symbiont strains, especially since the same haplotype was already found at high prevalence in collections of Central European *A. f. fabae* that preceded ours by more than a decade (Cayetano *et al*., 2015). We do not currently know why *H. defensa* haplotype 2 remains so dominant in our study area. This is particularly puzzling since we have evidence that haplotype 2 (here represented by strain H42) is somewhat less protective than haplotype 1 (H76) when averaged over multiple *L. fabarum* genotypes, and also more costly to the aphid hosts in the absence of parasitoids (Cayetano *et al*., 2015; Vorburger & Rouchet, 2016). Possible explanations include differences in the vertical transmission reliabilities between haplotypes, or other ecological benefits provided by

H. defensa that may obscure parasitoid-mediated selection (e.g. Chapter II, Smith *et al*., 2015; Smith *et al*., 2021), but these hypotheses remain to be tested.

All parasitoid species other than *L. fabarum* remained virtually unaffected by *H. defensa* and mostly showed parasitism rates as high or higher than *L. fabarum*. Low rates of parasitism were recorded only for *B. angelicae* (Figure 1a). This could suggest some endogenous resistance of *A. f. fabae* to these wasps, yet we observed that only few aphids survived the exposure to *B. angelicae* during our experiment (E. Gimmi: pers. observation), suggesting that the low realized parasitism rates arose from increased aphid mortality after attack by *B. angelicae*, rather than from aphid resistance (see also Cayetano & Vorburger, 2015). There is no *a priori* reason why parasitoids of the genera *Aphelinus*, *Binodoxys*, *Ephedrus* or *Praon* should not be susceptible to *H. defensa*-mediated defenses: other studies have reported *H. defensa*-conferred protection against *Aphelinus abdominalis* in pea aphids (McLean & Godfray, 2015) or against two different species of *Binodoxys* in cowpea aphids (Asplen *et al*., 2014). To our knowledge, no protection by *H. defensa* against *Praon* or *Ephedrus* species has been reported yet, but the number of pertinent studies is low so far (Łukasik *et al*., 2013; Martinez *et al*., 2016). What shapes the effectiveness of (or susceptibility to) symbiont-conferred resistance in the context of an entire community of natural enemies is an interesting problem for further study.

Finally, it should be noted that by working with a single aphid clone, we completely ignored potential variation in endogenous aphid resistance to parasitoids for our experiment. Aphid populations do exhibit genotypic variation in their susceptibility to parasitoids independently of carrying *H. defensa* (Martinez *et al*., 2014; Sandrock *et al*., 2010), which may add another layer of complexity to the coevolutionary interactions between aphids, endosymbionts, and parasitoids, though without denying the adaptive value of *H. defensa*-conferred resistance that we here observed in black bean aphids.

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Author Contributions

EG and CV designed the study, conducted the experiment, and analyzed and interpreted the data. EG wrote the first draft of the manuscript, which was edited and revised by both authors.

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Supplementary Material

Table S1: Results from pairwise Wilcoxon tests comparing mean parasitism success between the *H. defensa*-free aphid line (407) and either of the two *H. defensa*-infected lines in data subsets containing data from only one wasp species or *L. fabarum* line, respectively (*A. chaonia*, *B. angelicae*, *P. volucre*, *E. plagiator* and *L. fabarum; L.fab 1, L.fab 2, L.fab 3* and *L.fab 5*). Values in brackets indicate the results from Kruskal-Wallis tests for differences between any aphid lines. Bonferroni-adjusted p-values below the significance level of 0.05 are printed in bold.

Comparison	**W**	***P***	**adj. *P***
Aphelinus chaonia ($\chi^2 = 2.72$, df = 2, p = 0.257)			
407-H76 - 407 == 0	55.5	0.705	1
407-H42 - 407 == 0	70	0.140	0.280
Binodoxys angelicae ($\chi^2 = 0.76$, df = 2, p = 0.683)			
407-H76 - 407 == 0	52	0.909	1
407-H42 - 407 == 0	41	0.520	1
Ephedrus plagiator ($\chi^2 = 4.941$, df = 2, p = 0.085)			
407-H76 - 407 == 0	62.5	0.364	0.728
407-H42 - 407 == 0	30	0.143	0.286
Praon volucre ($\chi^2 = 0.158$, df = 2, p = 0.924)			
407-H76 - 407 == 0	49	0.970	1
407-H42 - 407 == 0	47	0.850	1
Lysiphlebus fabarum (all genotypes, $\chi^2 = 24.07$, df = 2, p < 0.001)			
407-H76 - 407 == 0	1248	<0.001	**<0.001**
407-H42 - 407 == 0	1150	<0.001	**<0.001**
L.fab 1 ($\chi^2 = 10.01$, df = 2, p = 0.007)			
407-H76 - 407 == 0	80	0.006	**0.012**
407-H42 - 407 == 0	50	1	1
L.fab 2 ($\chi^2 = 10.34$, df = 2, p = 0.006)			
407-H76 - 407 == 0	75	0.015	**0.030**
407-H42 - 407 == 0	47	0.847	1
L.fab 3 ($\chi^2 = 14.22$, df = 2, p < 0.001)			
407-H76 - 407 == 0	55	0.733	1
407-H42 - 407 == 0	100	<0.001	**<0.001**
L.fab 5 ($\chi^2 = 18.78$, df = 2, p < 0.001)			
407-H76 - 407 == 0	90	0.002	**0.005**
407-H42 - 407 == 0	95	<0.001	**<0.001**

Chapter IV

Genotypes and symbiotypes shed light on cryptic diversity in the black bean aphid species complex

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A selection of summer host plants of *Aphis fabae*, from left to right: *Anthriscus sylvestris, Chenopodium album, Tropaeolum majus, Cirsium arvense*.

Abstract

Different host plants represent ecologically dissimilar environments for phytophagous insects. The resulting divergent selection can promote the evolution of specialized host races, provided that gene flow is reduced between insect populations feeding on different plants. In black bean aphids belonging to the *Aphis fabae* complex, several morphologically cryptic subspecies were already described prior to the advent of genetic markers, based on their distinct preferences for different summer host plants. This is astounding, because host choice and mate choice are largely decoupled in these aphids: they have a host-alternating life cycle and migrate between specific summer host plants and shared winter hosts, where they mate and lay overwintering eggs. This provides a yearly opportunity for gene flow among aphids returning from different summer hosts, and raises the question of whether the ecologically defined subspecies are also genetically differentiated. Here, we analyzed an extensive, geographically and temporally structured dataset of microsatellite genotypes from *A. fabae* samples that were collected from their main winter host *Euonymus europaeus*. We complemented these with additional samples from a second winter host and fourteen summer host plants. Our data confirms the presence of multiple, strongly differentiated genetic clusters within the *A. fabae* complex, which largely match previously described subspecies in number and host association. These subspecies also differ in the frequency of infection with the facultative endosymbionts *Hamiltonella defensa* and *Regiella insecticola*, which is additional evidence for divergent ecological selection between host plants. Furthermore, we found evidence for hybridization among subspecies, but putative hybrids were rare and collected more frequently in spring than in autumn. This suggests that prezygotic barriers as well as postzygotic selection against hybrids maintain genetic differentiation among *A. fabae* subspecies, despite their using a common mating habitat.

Keywords: genetic structure, host races, biotypes, species complex, cryptic species, ecological speciation, divergent selection, facultative symbionts, aphids, microsatellites

1 | Introduction

Contrasting environments can impose differential selection on separate populations of a species, thereby causing ecologically based adaptive divergence. In this process of specialization, reduced gene flow and assortative mating may represent both drivers and effects of increasing population differentiation, and could eventually lay the ground for ecological speciation (e.g. Dobzhansky, 1940; Rice, 1987; Rundle & Nosil, 2005; Schluter, 2001). The evolution of separate, specialized species thereby represents the endpoint of a wide continuum of divergence, ranging from weak genetic differentiation to complete reproductive isolation between populations (Dobzhansky, 1940; Nosil, 2012; Schluter, 2000). Among model organisms studied to investigate ecologically based population divergence and the potential of ecological speciation, phytophagous insects assume a prominent position (e.g. Berlocher & Feder, 2002; Funk *et al*., 2002; Matsubayashi *et al*., 2010; Via, 2001). Their host plants often represent habitat, food source, and mating site all in one, and the variable chemical and physical properties of different plant species may impose very specific selection pressures on the insects exploiting them. Examples of polyphagous insect species that appear structured into host-specialized lineages, often referred to as biotypes or host races, are abundant (Jaenike, 1990), and novel examples are frequently discovered (e.g. Mlynarek & Heard, 2018; Villacis-Perez *et al*., 2021). Specialization may be associated with variable amounts of genetic differentiation and reproductive compatibility between host races (Drès & Mallet, 2002; Ehrlich & Murphy, 1988; Harrison *et al*., 2022; Mitchell, 1981), which makes them attractive models for exploring how the interplay of ecology and evolution shapes genetic structure within and among species (Berlocher & Feder, 2002).

At least two non-exclusive starting points might lead to a realized reduction of gene flow and the genetic divergence between insect populations feeding on different host plants. On the one hand, specialization may be initiated by the physical separation of individuals, for example following the acquisition of a new host species. This might facilitate subsequent adaptation to either host due to the reduced likelihood of encounters among individuals feeding on different hosts, under the condition that the full insect life cycle, including mating, occurs on the same host plant species (Guldemond & Mackenzie, 1994; Rice & Salt, 1988). On the other hand, specialization can originate from polymorphisms for

performance on specific plants in a polyphagous insect population. Such polymorphisms would favor those individuals that can choose their optimal host plant species when dispersing (Jaenike, 1978), and might therefore promote the linkage of performance and preference traits (Felsenstein, 1982; Fry, 2003; Futuyma & Peterson, 1985; Sandoval & Nosil, 2005; Soudi *et al*., 2015). If different genes or combinations of genes are responsible for adaptation to different plant species, individuals with intermediate phenotypes could be less fit than individuals with pure phenotypes, and in particular, hybrids could be less fit than either of their parents (Egan & Funk, 2009; Thompson *et al*., 2019). This might promote the evolution of mechanisms allowing assortative mating to escape the costs of producing unfit hybrids (Howard, 1993; Mackenzie & Guldemond, 1994), thereby reinforcing reproductive isolation between populations.

A prime example for host plant associated ecological specialization is the species complex formed by the pea aphid, *Acyrthosiphon pisum* (Hemiptera: Aphididae), a sap-sucking insect that feeds on a range of legume plants (Fabaceae). *Ac. pisum* comprises multiple genetically distinct populations, that differ in their preference for, and performance on, different legume genera (Frantz *et al*., 2006; Peccoud *et al*., 2009; Simon *et al*., 2003; Via, 1991) and also in the communities of facultative bacterial endosymbionts they harbor (Ferrari *et al*., 2012; Smith *et al*., 2015). In the pea aphid complex, host preference and host performance are heritable (Via, 1991; Via, 1999), the responsible loci seem to be linked (Hawthorne & Via, 2001), and there is evidence for selection against both migrants and hybrids (Via *et al*., 2000). It appears that strong host fidelity, with individuals feeding and mating on the same plant species throughout their life cycle, provides a significant barrier to gene flow among pea aphid host races, facilitating divergence. Furthermore, aphids reproduce by cyclical parthenogenesis: the numerous clonal generations between the annual sexual generations may potentiate fitness differences among genotypes, allowing selection to choose the fittest clones for each host plant over multiple generations, without the homogenizing effect of recombination (Neiman & Linksvayer, 2006; Vanoverbeke & De Meester, 2010; Vorburger, 2006). These fittest genotypes on each host plant mate among each other in autumn, thereby likely sustaining host race differentiation even independently of reproductive compatibility.

The evolution of host races or host-specialized species is more difficult to explain when host choice and mate choice are unlinked, providing more opportunity for gene flow

between ecologically specialized populations. In contrast to *Ac. pisum*, a minority of aphid species are host alternating (dioecious): they undergo the sexual generation on a woody primary host plant species and most of the parthenogenetic generations on herbaceous secondary host plant species. A well-studied example for this dioecious lifestyle is the black bean aphid, *Aphis fabae*: females called fundatrices hatch in spring from overwintering eggs on the primary host (predominantly the European spindle tree, *Euonymus europaeus*), from where their clonal offspring migrate to secondary hosts and reproduce parthenogenetically during summer. In autumn, sexual males and females are produced and migrate back to the primary hosts, where they mate and lay overwintering eggs. Intriguingly, *A. fabae* also forms a complex of morphologically cryptic lineages, generally treated as subspecies, that show a high degree of specialization to certain secondary host plant species, even though they meet and mate on common primary hosts (Blackman & Eastop, 2000; Iglisch, 1968; Müller, 1982; Thieme, 1987). The use of the same mating habitat implies the potential for homogenizing gene flow among subspecies, which may be counteracted by trade-offs in secondary host plant utilization (Mackenzie, 1996), reduced hybrid fitness (Müller, 1982; Tosh *et al*., 2004b), or behavioral mechanisms (Raymond *et al*., 2001; Thieme & Dixon, 1996). The genetic structure of the *A. fabae* complex is still insufficiently understood. While mitochondrial COI/II and CytB sequences appear noninformative for distinguishing *A. fabae* subspecies (Béji *et al*., 2015; Coeur d'acier *et al*., 2007; Coeur d'acier *et al*., 2014; Zhang *et al*., 2010), genetic differences have been found among multiple subspecies using enzyme electrophoresis (Jörg & Lampel, 1996). Furthermore, microsatellite markers revealed strong genetic differentiation between *A. fabae cirsiiacanthoides*, the subspecies feeding on thistle (*Cirsium vulgare*) and *A. fabae fabae*, the nominal subspecies feeding on goosefoot (*Chenopodium album*) during summer (Coeur d'acier *et al*., 2004; Vorburger *et al*., 2017). In summary, there is clear evidence for host specialization and reduced gene flow between different members of the *A. fabae* complex, but the fact that this specialization is maintained without an obvious ecological barrier to gene flow remains puzzling. These circumstances raise questions about the actual diversity present in the *A. fabae* complex, and the extent of reproductive isolation among secondary host-associated populations.

The present work is based on an extensive, temporally and geographically structured dataset of *A. fabae* samples collected from their primary host plant *E. europaeus*. The

samples were collected as part of a different study (Chapter II), which required identifying those individuals belonging to the nominal subspecies *A. f. fabae* by microsatellite genotyping. Here we analyzed this dataset with the goal of describing the genetic structure of *A. fabae* on *E. europaeus*. To do this we complemented the original dataset with a collection of *A. fabae* individuals from one alternative primary host plant and from 14 different secondary host plants (Table S1). We asked how many and which genetic clusters (presumed subspecies) can be detected among the collected black bean aphids, and whether individuals belonging to different genetic clusters indeed disperse to distinct summer host plants. We also looked for evidence of hybridization between presumed subspecies from *E. europaeus*. In addition to the genetic analyses, we tested for the presence of two known facultative bacterial endosymbionts in all collected aphids. Since heritable endosymbionts, which are commonly associated with aphids and other herbivorous insects, may provide different ecological functions (Feldhaar, 2011; Oliver *et al.*, 2010), differing symbiont complements can be considered as independent indication for population divergence and ecological specialization (Ferrari *et al.*, 2012; Hosokawa *et al.*, 2007; Tsuchida *et al.*, 2004).

2 | Methods

The *Aphis fabae* complex

According to Blackman and Eastop (2017), *Aphis fabae* s. str. comprises five taxa, *A. f. cirsiiacanthoides*, *A. f. euonymi*, *A. f. fabae*, *A. f. mordwilkoi*, and *A. f. solanella*, of which all but *A. f. mordwilkoi* use the European spindle tree, *Euonymus europaeus*, as winter host. *A. f. mordwilkoi* and *A. f. cirsiiacanthoides* use the guelder rose *Viburnum opulus* as winter host, and *A. f. cirsiiacanthoides* can also be found on the mock orange *Philadelphus coronarius*. A wide range of cultivated and wild plants can be used as summer hosts (Blackman & Eastop, 2000). Among these, *Cirsium* species (thistles) are typical summer hosts of *A. f. cirsiiacanthoides*, *Vicia faba* (broad bean), *Beta vulgaris* (beet varieties) and *Chenopodium album* (goosefoot) are typical for *A. f. fabae*, *Arctium* (burdock) and *Tropaeolum* (nasturtium) species are typical for *A. f. mordwilkoi*, and *Solanum nigrum* (nightshade) is typical for *A. f. solanella* (Blackman & Eastop, 2000). These "diagnostic"

plant species are also used to identify *A. fabae* subspecies based on their acceptance of these as hosts (Müller, 1982). *A. f. euonymi* does not host alternate but remains on *E. europaeus* throughout the year (Blackman & Eastop, 2000; Lampel & Meier, 2007). Although slight morphological differences might exist between some *A. fabae* subspecies (e.g. Müller & Steiner, 1986; Müller & Steiner, 1990), it is widely accepted that biological information on host plant preference or genetic methods should be considered to identify 'black bean aphids' beyond the general term *A. fabae* (Blackman & Eastop, 2000; Heie, 1986; Iglisch, 1968; Jörg & Lampel, 1996; Lampel & Meier, 2007; Müller, 1982; Thieme, 1987).

Aphid samples

The dataset used in this study consists of two parts: the first part comprises black bean aphids collected exclusively from their predominant primary host *E. europaeus*. These samples were originally collected for a different study (Chapter II) with sampling dates in March, April, and October of the years 2019 and 2020, and in April 2021. For each time point, approximately 80 aphids were sampled at each of three rural sites near Zurich, Switzerland, situated 10 to 30 km apart from each other: *Faellanden* (47° 22′ N 8° 38′ E), *Gossau* (47° 19′ N 8° 45′ E) and *Steinmaur* (47° 30′ N 8° 27′ E). The three sampling sites included cultivated fields of various crops interspersed with weeds serving as summer hosts of *A. fabae*, and they were structured by woody hedges containing *E. europaeus* and *V. opulus*. The exact sample sizes and dates are provided in Table S1. The second sample set was collected in 2020 and 2021 from various host plants at various sites in the Zurich area, including *Faellanden*, *Gossau*, and *Steinmaur*. We first collected individuals from the alternative winter host *V. opulus* in April 2020 and April 2021 (the third possible winter host, *P. coronarius*, is not native to our study area). In spring and summer 2021 we collected individuals from various summer hosts: *Achillea millefolium*, *Aegopodium podagraria*, *Anthriscus sylvestris, Arctium lappa, Beta vulgaris*, *Capsella bursa-pastoris*, *Chenopodium album, Cirsium vulgare, Cirsium arvense*, *Galium aparine*, *Galium mollugo*, *Matricaria chamomilla*, *Papaver rhoeas*, *Rumex obtusifolius* and *Tropaeolum majus*. Sample sizes ranged from 14 to 37 per summer host plant species (Table S1).

DNA extraction and genotyping

Aphid DNA was extracted using a salting out protocol as in Sunnucks and Hales (1996). Each sample was genotyped at eight microsatellite loci (Af85, Af181, Af86, Af48, Af82, Afbeta, AfF, and Af50) using the primers of Coeur d'acier *et al*. (2004), which proved to be reliable and successful in separating *A. f. fabae* and *A. f. cirsiiacanthoides* in the study of Vorburger *et al*. (2017). Primer sequences and the PCR protocol are provided in Table S2. After PCR amplification, the microsatellite fragments were run on an ABI 3730 automated sequencer. GeneMarker 3.0.1 (SoftGenetics) was used to visualize the electropherograms and to score the alleles. Samples were used for further analysis if the alleles of at least seven of the eight markers were successfully scored (1.4% missing data in the final dataset). In the original dataset, 16 aphid genotypes occurred twice and one genotype three times, and we kept only one sample of each genotype for further analysis. To help identify genetic clusters within our sample collection, we complemented our dataset with the genotypes of 30 samples that were clearly identified as either *A. f. fabae* or *A. f. cirsiiacanthoides* in Vorburger *et al*. (2017). The final dataset comprised 1619 aphid microsatellite genotypes from *E. europaeus* and 480 collected from other host plants, i.e. 2099 genotypes in total (Table S1). Allele numbers per locus varied from seven (Af86 and Af50) to 58 (Afbeta, Table S4).

Analysis of genetic structure

All analyses using R were performed in Rstudio 2022.02.3 (RStudio Team, 2020) with R 4.1.2 (R Core Team, 2019) and using *ggplot2* 3.3.5 (Wickham, 2016) for plotting. To assess the genetic structure present in our data and to assign samples to genetic clusters, we compared the results of three different clustering methods: *snapclust* (Beugin *et al*., 2018) implemented in the R package *adegenet* 2.1.5 (Jombart, 2008), STRUCTURE 2.3.4 (Falush *et al*., 2003; Pritchard *et al*., 2000), and DAPC (Jombart *et al*., 2010) implemented in *adegenet* as well. *Snapclust* applies a combination of geometric and model-based steps and the Expectation-Maximization algorithm to cluster genotypes (Beugin *et al*., 2018). The latter makes *snapclust* much faster than STRUCTURE which uses a Bayesian MCMC approach; both rely on population genetic models assuming Hardy-Weinberg equilibrium (HWE) and linkage equilibrium within real clusters to calculate the likelihood of specific clustering solutions (Beugin *et al*., 2018; Pritchard *et al*., 2000). DAPC is a model-free

approach where the genotype data is first transformed using PCA, then the principal components (PCs) are used as input for linear discriminant analysis (DA). We used DAPC to assign individuals to groups and especially for a visual assessment of between-population differentiation.

We applied *snapclust* with default settings for numbers of genetic clusters (K) ranging from 1 to 20. To decide on the most probable K, we consulted the three information criteria AIC, BIC, and KIC in combination with visual assessment of the solutions for different values of K. The *snapclust* analysis suggested using a K value of 6 (see Results). However, the number of individuals assigned to the smallest cluster in this solution was more than 10× smaller than the number of individuals assigned to the largest cluster (Table S5), and uneven sample sizes can hamper the 'correct' identification of clusters (Kalinowski, 2011; Neophytou, 2014; Puechmaille, 2016; Wang, 2017). We therefore checked the robustness of the K=6 solution by extending the analysis in two ways: first, we ran *snapclust* as above on each of six data subsets containing only those samples with the highest group membership probabilities for the same cluster. Second, we ran *snapclust* on a more balanced subset of our data containing all samples from clusters 2-6 but only 222 samples from the largest cluster 1 (222 = mean number of samples in clusters 2-6). These samples consisted of the 15 *A. f. fabae* reference samples plus 207 samples selected randomly from those assigned to cluster 1 under K=6. Both approaches would help to detect substructure within the primarily inferred clusters that could remain hidden when considering the full, unbalanced dataset.

We ran STRUCTURE with the admixture model and without prior information on sample origin. Considering the large variation in cluster sizes detected with *snapclust*, we used the settings suggested by Wang (2017) to improve detection of clusters in unbalanced datasets. These include uncorrelated allele frequencies among populations (FREQSCORR=0) and separate alpha values per population (POPALPHAS=1, UNIFPRIORALPHA=0), with an initial alpha of 0.17 (= 1/6, six being the number of clusters inferred with *snapclust*). The other settings were left to their default. We ran ten independent simulations for each K between 1 and 10, doing 200 000 iterations after discarding the first 25 000 iterations as burn-in. We also ran STRUCTURE with the same settings on the more balanced data subset as described above. To infer the most probable number of genetic clusters we considered mean LnP(K) as suggested by Pritchard *et al*. (2000) and Evanno's DeltaK

(Evanno *et al*., 2005) as implemented in STRUCTURE HARVESTER (Earl & vonHoldt, 2011). To combine and summarize the output of the replicate STRUCTURE runs we used CLUMPAK (Kopelman *et al*., 2015).

For DAPC, initial groups used as input need to be defined in a preceding step, for which we used the k-means algorithm implemented in the *adegenet* function *find.clusters*, retaining all PCs. The function provides BIC values that can be used to assess goodness-of-fit for different clustering solutions (Jombart *et al*., 2010). For the subsequent DAPC analyses we generally retained all eigenvalues as recommended by the authors for small numbers of clusters (Jombart *et al*., 2010), and we used cross validation with *adegenet::xvalDapc* and default settings to decide on the number of principal components to retain. We applied DAPC first for K=2 retaining 10 PCs. The resulting split corresponded to a separation of the largest cluster inferred by *snapclust* and STRUCTURE from all other samples, and no substructure was suggested within this large cluster when we considered it separately from the other samples (see Results). Since also DAPC showed to be sensitive to unequal sample sizes, we thus focused the further DAPC analysis on the more balanced dataset as described above. We divided this data into 6 or 7 clusters retaining 20 PCs in both cases.

For all clustering approaches, we arbitrarily assigned samples to a group if they showed a group membership probability >0.8.

Genetic diversity and differentiation

We calculated the number of alleles, observed (H_o) and expected (H_e) heterozygosity for all microsatellite loci for the full dataset and for each of the six genetic groups inferred by STRUCTURE with *adegenet* 2.1.5 (Jombart, 2008). We also tested for deviations from HWE overall and within the six groups using *pegas* 1.1 (Paradis, 2010). We calculated pairwise F_{ST} values (Weir & Cockerham, 1984) between groups with the function *pairwise.WCfst* and 95% confidence intervals with *boot.ppfst* (nboots = 1000) using *hierfstat* 0.5-10 (Goudet, 2005). To compare the differentiation among the inferred groups to potential spatial and temporal differentiation within, we further calculated pairwise F_{ST} values between the three sampling sites and the different sampling time points within each of the four dominant groups found in our large collection of individuals from the winter host *E. europaeus*.

Hybrid detection

We specifically sought for potential hybrids among aphid individuals collected from *E. europaeus* and belonging to one of the four dominant genetic clusters we found on this winter host (clusters 1, 2, 3, and 5, see Results). We considered two different approaches: (i) *snapclust* with settings allowing for the detection of F1 hybrids (hybrids = TRUE, hybrid.coef = c(0.5)), and (ii) the software *NewHybrids* 2.0 which applies a Bayesian clustering method (Anderson & Thompson, 2002). Both methods expect the input data to consist of just two parental populations and their offspring. We therefore looked for hybrids separately in all pairwise combinations of the four genetic groups. Each of the input datasets consisted of the genotypes that were assigned to one of the two considered clusters with a probability >0.8, plus genotypes whose assignment probabilities were highest to one and second highest to the other considered cluster, based on the STRUCTURE analysis of the full dataset (K=6). We ran *NewHybrids* with a burn-in of 100 000 followed by 400 000 sweeps, using uniform priors for both π and θ, and looking for F1 hybrids only. The hybrids detected by *NewHybrids* represented a subset of those detected with *snapclust*, and we conservatively considered only those samples as hybrids that showed higher membership probability to the hybrid category than to either parental category in both the *snapclust* and *NewHybrids* results. To assess how well *snapclust* and *NewHybrids* are able to detect hybrids, we applied both to datasets containing simulated hybrids that we obtained with the function *adegenet*::*hybridize*. As parental genotypes we used those individuals with an assignment probability >0.8 to the clusters under consideration in the STRUCTURE analysis of the full dataset and for which we had data for all eight markers. For each of the six combinations of parental clusters, we simulated 100 times 20 hybrids. On each dataset, we ran *snapclust* and *NewHybrids* as above but with a burn-in of just 1000 followed by 4000 sweeps for the latter. The simulated hybrids were reliably detected in five combinations of parental groups (16-20 of 20 hybrids detected on average), while in the datasets with clusters 2 and 3 as parents of the simulated hybrids, just 16 (*snapclust*) and 11 (*NewHybrids*) of the 20 hybrids were detected on average (Table S10). This suggests that real hybrids should be detectable in our datasets with a high probability for all combinations of parental populations except for hybrids between clusters 2 and 3.

Finally, we compared the frequency of inferred hybrid individuals on *E. europaeus* between spring and autumn samples. If individuals with hybrid genotypes were less fit than parental genotypes during the asexual summer generations on secondary host plants, they might be more frequent in spring, i.e. in newly hatched genotypes resulting from sexual reproduction, than in autumn when returning to *E. europaeus* after a full growth season of clonal selection. We thus used Pearson's Chi-square tests to determine whether putative hybrids arising from crossings between individuals belonging to the four major genetic groups found on *E. europaeus* were significantly more frequent in spring (March and April) than in autumn (late October), considering all putative hybrids of all combinations of parents and of all three sampling years together.

Because of the notable number of samples showing admixture between cluster 4 (green) and cluster 1 (yellow) in the STRUCTURE K=6 results (see Results), we further applied both *snapclust* and *NewHybrids* as above to assess these samples more closely. To account for the large difference in samples size between the two clusters in the full dataset, we chose the input data from among the more balanced data subset, i.e. we included those samples that showed >0.8 membership probability to either the yellow cluster 1 (N=221) or the green cluster 4 (N=38) plus those that showed highest and second highest assignment probability to the yellow and green cluster (N=37) in the STRUCTURE solution for the more balanced data subset under K=6.

Endosymbiont detection

We determined the presence of the obligate symbiont *Buchnera aphidicola* (as a positive control) and of the facultative symbionts *Hamiltonella defensa* and *Regiella insecticola* in each aphid sample using diagnostic PCR. Other known facultative endosymbionts of aphids are very rare in *A. fabae* (see Chapter II) and were therefore not considered here. We did separate PCR reactions using specific primers for each endosymbiont and determined the presence or absence of amplified endosymbiont DNA using a QIAxcel capillary electrophoresis device. The PCR protocol and primer sequences are provided in Table S3. For the analysis, we filtered out samples with missing data (*A. f. fabae* and *A. f. cirsiiacanthoides* reference samples) or negative results for *B. aphidicola*, remaining with N = 2047 samples. We tested for differences in the frequency of symbiotypes ($Ham^{\cdot}Reg^{-}$,

Ham^{-} Reg^{+}, Ham^{+}Reg^{-} or Ham^{+} Reg^{+}) among genetic groups with pairwise Fisher's exact tests and a Bonferroni-adjusted significance level of 0.05/14 = 0.00357.

3 | Results

Genetic structure within the black bean aphid complex

Combining the evidence from the three clustering approaches with *snapclust*, STRUCTURE, and DAPC, we conclude that the optimal number of genetic clusters in our dataset is six. The vast majority of individuals can be assigned to these six clusters with high confidence. Applying *snapclust* to the full dataset and comparing solutions for various number of clusters (K), BIC values clearly suggest dividing our genotypes into six clusters (Figure S1), while AIC and KIC values are more ambiguous and show little difference between K=6 and somewhat higher numbers of K (Figure S1). Applying *snapclust* to the more balanced data subset (containing all samples assigned to clusters 2-6 but only a random subset of samples from the largest cluster 1), all information criteria support K=6 as the optimal number of clusters (Figure S2). Under K=6, only 23 out of 2099 genotypes from the full dataset show a membership probability of less than 0.8 to any cluster (Figure 1, Table S5a), whereas applying *snapclust* with K=7 or K=8 leads to further subdivision of the largest cluster 1 (yellow), including the 15 reference samples known to represent *A. f. fabae*, but with much lower certainty of assignment for most individuals (Figure 1). Both are hints for these splits not being biologically meaningful. Indeed, re-applying *snapclust* separately to the genotypes from each of the six primarily inferred genetic clusters does not suggest any further subdivision (Figure S3). Six genetic clusters are also supported by the STRUCTURE analysis applied to the balanced data subset: while there is some uncertainty when applying Evanno's DeltaK due to inconsistent runs at K=5, the posterior probabilities clearly plateau at K=6 (Figure S4), supporting this as the optimal subdivision. Applying K=6 to the full dataset, the assignment of genotypes to clusters in the STRUCTURE analysis is largely consistent with the assignment resulting from *snapclust* under K=6 (Figure 1, S5, S6). The major difference is that membership probabilities are generally lower in the STRUCTURE results, resulting in more samples being categorized

as admixed than with *snapclust* when using the same assignment threshold of p>0.8 (Table S5, Figure S6). This is most evident for cluster 4 (green), to which many samples are assigned with p>0.8 by *snapclust* but not by STRUCTURE. Rather than being clearly assigned to the green cluster 4 as in the *snapclust* results, most of these samples result as admixed between the yellow cluster 1 and the green cluster 4 in the STRUCTURE analysis (Figure 1). Using STRUCTURE with K=7 or K=8, cluster 2 (orange, including the *A. f. cirsiiacanthoides* reference samples) rather than cluster 1 (as in *snapclust*) gets subdivided further (Figure 1, S6), but the additional clusters comprise only few individuals (Table S5b) and with low assignment probabilities, arguing against this further subdivision.

The DAPC analysis was strongly influenced by unequal group sizes in the data. When applying k-means to the full dataset to obtain input clusters for DAPC, BIC values hint at K=2 as the optimal number of clusters (Figure S7a), which is clearly an insufficient subdivision. Hence, we considered only the more balanced dataset, for which BIC values also indicate K=6 as the optimal number of clusters (Figure S7b,c). The group assignments resulting from DAPC are very similar to the ones obtained with *snapclust* or STRUCTURE under K=6 (Figure S8). Under K=7, a different cluster than with either *snapclust* or STRUCTURE gets subdivided further (cluster 5, blue), albeit with low confidence of assignment, which additionally argues for K=6 as the optimal solution. Assuming six clusters, all but clusters 2 (orange) and 3 (violet) get clearly separated along the first two linear discriminants (Figure 2). Clusters 2 and 3 are separated along the 3rd and 5th linear discriminants, while the 4th discriminant is mainly separating cluster 4 (green) from all other samples (Figure 2).

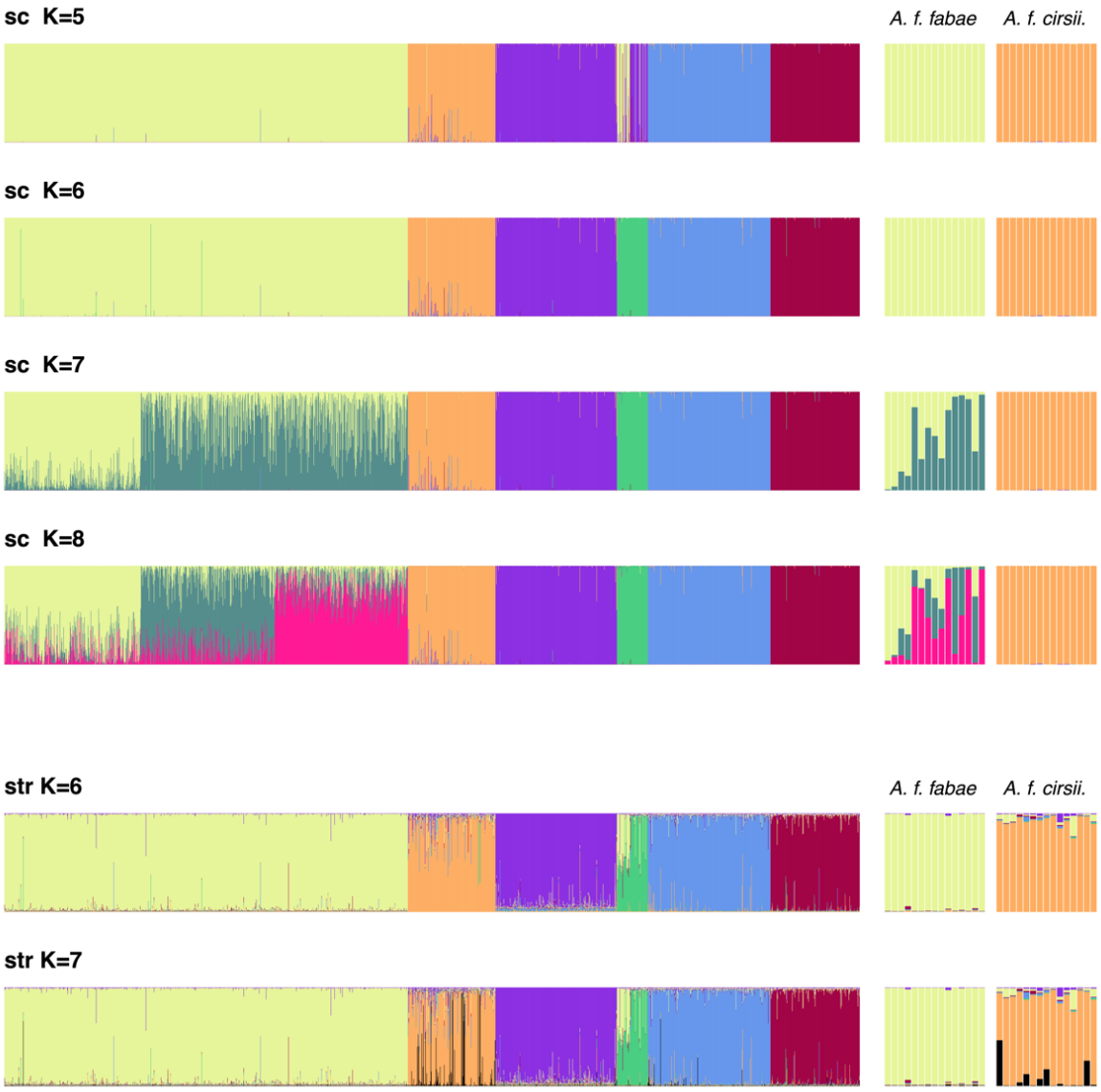

Figure 1: Clustering results from *snapclust* (sc, top rows) and STRUCTURE (str, bottom rows) using different numbers of clusters (K). Each aphid individual is represented by a vertical bar, the proportion of this bar in a specific color represents the likelihood that the sample belongs to the respective cluster (membership probability). For each K, the wide boxes to the left show all 2099 samples used in the analysis. For all solutions the samples are ordered according to the cluster for which they show highest membership probability in the K=8 result. The two narrow boxes to the right zoom in on the reference samples known to represent *A. f. fabae* and *A. f. cirsiiacanthoides*, respectively.

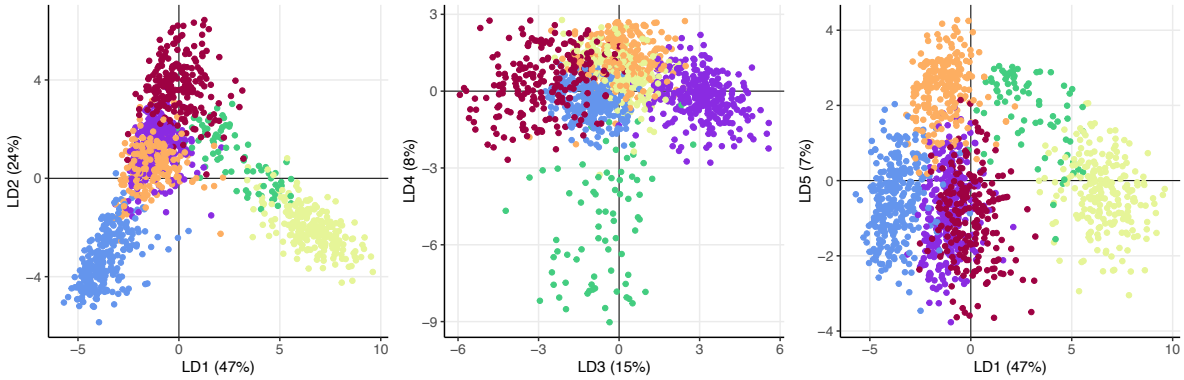

Figure 2: Discriminant analysis of principal components (DAPC) after dividing the more balanced data subset into six groups using the k-means algorithm. The axes represent the 1st and 2nd (left) or the 3rd and 4th (middle) and the 1st and 5th (right) linear discriminants, the number in brackets shows the percentage of variance explained by the discriminant. Each dot represents an individual aphid, its color corresponds to its group assignment as used for DAPC (which is very similar but not identical to the group assignment resulting by the *snapclust* and STRUCTURE analyses).

Host plant associations, taxonomic identity, and genetic differentiation

To illustrate host plant associations, we used the assignment to genetic clusters resulting from the STRUCTURE analysis when requiring a membership likelihood p>0.8, i.e. the most conservative assignment. The genetic composition of aphids was strikingly different on the two primary host plants *E. europaeus* and *V. opulus*, where sexual reproduction takes place (Figure 3a): on *E. europaeus*, cluster 1 (yellow) was dominant, followed by clusters 5 (violet) and 3 (blue), whereas on *V. opulus* two different clusters dominated, namely 4 (green) and 6 (red). Only cluster 2 (orange) was similarly frequent on both *E. europaeus* and *V. opulus*. Both primary hosts also contained some proportion of individuals that were not clearly assigned to any cluster.

Their associations with secondary host plants allow the identification of several of these clusters. All reference samples of *A. f. fabae* as well as all samples collected from *Ch. album* or *B. vulgaris* (known as diagnostic hosts of this subspecies) were assigned to cluster 1 (yellow), clearly indicating that this cluster represents *A. f. fabae*. The reference samples of *A. f. cirsiiacanthoides* were assigned to cluster 2 (orange), which indeed dominates on thistles (*Cirsium* spp.), corroborating that this cluster represents *A. f. cirsiiacanthoides*. This subspecies was found on both primary host plants and also occurs on various secondary host plants other than thistles, especially *Ca. bursa-pastoris* and *M. chamomilla* (Figure 3b). Cluster 6 (red) likely represents *A. f. mordwilkoi*, since it is dominant on

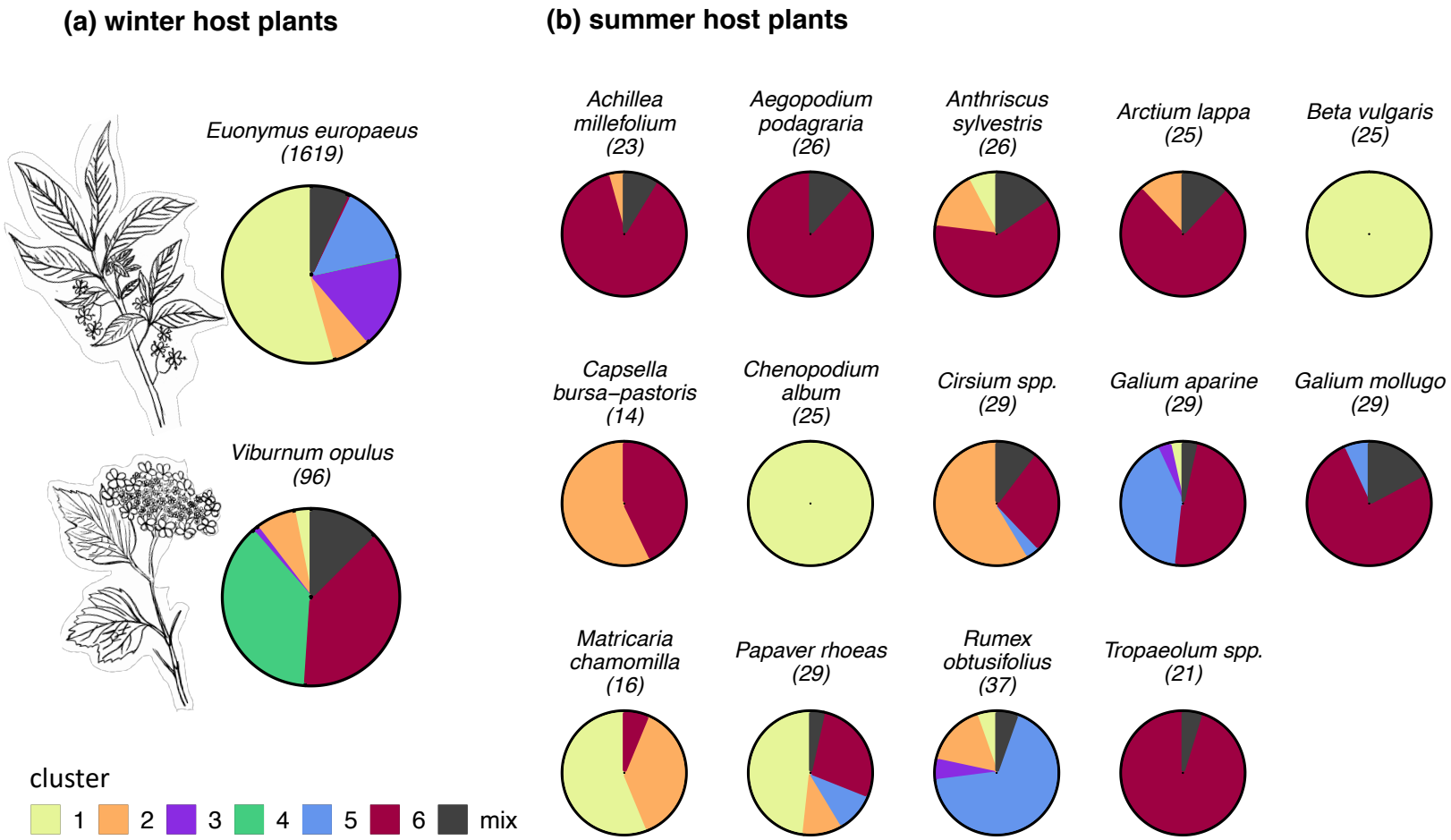

Figure 3: Distribution of aphid individuals assigned to the six genetic groups on two winter host plants (a) and various summer host plants (b). Samples were assigned to a cluster if they showed a membership probability >0.8 in the STRUCTURE analysis under K=6, samples that are not assigned to any cluster are categorized as mixed. The sample size per host plant is given in brackets, note that there are many more samples from *E. euonymus* than from any other host plant. The summer hosts are ordered alphabetically.

Ar. lappa and *T. majus*, known as diagnostic secondary hosts of this subspecies. That aphids belonging to cluster 6 also dominate on *Ac. millefolium*, *Ae. podagraria* and *An. sylvestris* and use *V. opulus* as primary host (40/96 samples) rather than *E. euonymus* (6/1619) is also consistent with this assignment (Jörg & Lampel, 1996). The remaining three genetic clusters are less straightforward to identify. Cluster 5 (blue) overwinters on *E. europaeus* and is found frequently on the summer hosts *R. obtusifolius* and *G. aparine*. Cluster 3 (violet) also overwinters on *E. europaeus* and is virtually absent from the summer hosts we sampled (Figure 3). According to Blackman and Eastop (2017), *A. f solanella* and *A. f. euonymi* are also using *E. europaeus* as primary host, and we refer to the Discussion for the likely assignment of the inferred clusters to these taxa. Cluster 4 (green) uses *V. opulus* as its primary host and was not observed on any of the sampled secondary host plants. As detailed in the Discussion, we suppose this cluster might be *A. viburni*. Finally, some summer hosts such as *P. rhoeas*, *M. chamomilla*, *R. obtusifolius*, or *G. aparine* harbored a high degree of genotypic diversity, thus they appear to attract multiple genetic groups of black bean aphids to a similar extent (Figure 3).

Table 1: Pairwise F_{ST} values (Weir & Cockerham) and 95% confidence intervals (values in brackets) between the six main genetic groups identified in our dataset, considering the clustering solution from STRUCTURE with K=6 and assigning samples to a cluster if they show an assignment probability >0.8.

	2 - orange	**3 - violet**	**4 - green**	**5 - blue**	**6 - red**
1 - yellow *A. f. fabae*	0.094 [0.062, 0.127]	0.104 [0.072, 0.138]	0.128 [0.088, 0.17]	0.128 [0.100, 0.155]	0.105 [0.072, 0.139]
2 - orange *A. f. cirsii.*		0.050 [0.032, 0.069]	0.095 [0.058, 0.133]	0.053 [0.033, 0.077]	0.054 [0.032, 0.078]
3 - violet			0.093 [0.050, 0.141]	0.068 [0.046, 0.087]	0.063 [0.041, 0.085]
4 - green				0.120 [0.082, 0.159]	0.104 [0.071, 0.136]
5 - blue					0.070 [0.048, 0.095]

When comparing the observed heterozygosity (H_o) with the expected heterozygosity (H_e) for the full dataset, we see the heterozygote deficit and significant deviation from Hardy-Weinberg equilibrium (HWE) expected for a dataset containing strong genetic structure ($p<0.0001$ for all loci, Table S4). Within the six genetic clusters inferred by STRUCTURE, H_o and H_e are close to each other, and with four exceptions of individual loci in different groups (one locus in each group 1 (Af86), 2 (AfF), 4 (Afbeta), and 6 (Af86)) there are no significant deviations from HWE after Bonferroni correction (Table S4). The pairwise F_{ST} values between these six groups are all significantly larger than zero, but the extent of genetic differentiation varies (Table 1). Cluster 1 (*A. f. fabae*, yellow) and cluster 4 (supposedly *A. viburni*, green) are most strongly differentiated from all other clusters with pairwise F_{ST} values ranging from and 0.094 to 0.128 and 0.093 to 0.128, respectively. The remaining four groups are more closely related to each other with pairwise F_{ST} values between 0.050 and 0.070 (Table 1). The large samples collected from *E. europaeus* at three distinct sites and at different time points allow us to put these values in relation to genetic differentiation that may result from spatial or temporal separation. The relative proportions of the four genetic clusters dominating on *E. europaeus* showed some variation across space and time (Figure 4), but within these groups, genetic differentiation was very weak, with F_{ST} values between sites or between time points vastly smaller than those between groups, and with confidence intervals that included zero in the majority of comparisons (Table S6, S7).

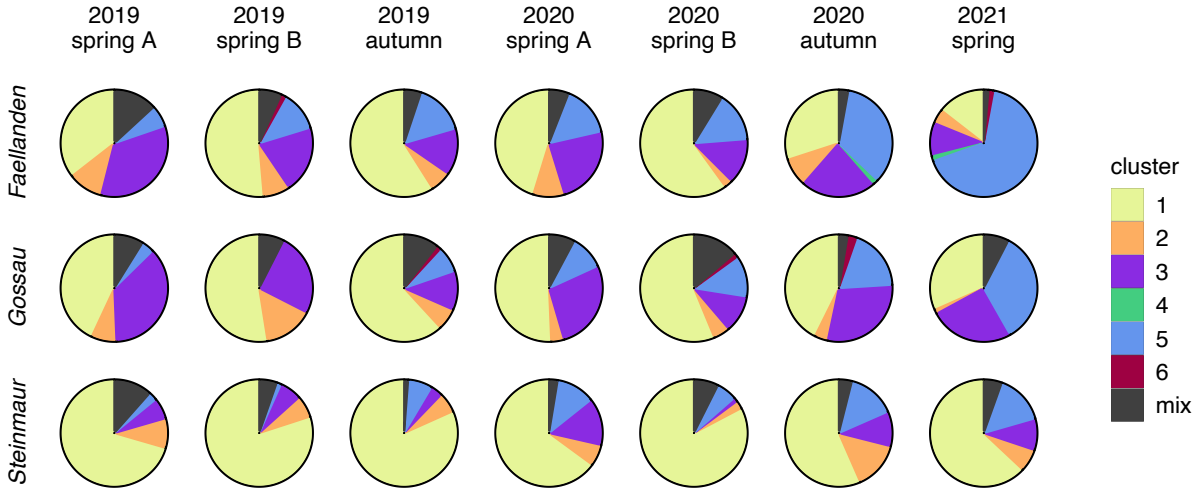

Figure 4: Distribution of individuals belonging to the six genetic clusters defined by STRUCTURE on the winter host *E. euonymus* between sites (*Faellanden*, *Gossau* and *Steinmaur*) and over time. The four main genetic clusters (yellow, orange, violet and blue) are present at all sites at all but one time point.

Endosymbiont prevalence in *Aphis fabae* genetic clusters

The genetic clusters we identified with microsatellite genotypes exhibit significant differences in the prevalence of the two endosymbionts *Hamiltonella defensa* and *Regiella insecticola* (Figure 5, Table S8), also supporting their distinctiveness. In cluster 1 (yellow, *A. f. fabae*) we found *H. defensa* in 34% and *R. insecticola* in 8% of the aphids. Cluster 3 (violet) shows a lower *H. defensa* (14%) and a much higher *R. insecticola* frequency (92%), while in cluster 4 (green), 100% of the aphids carried *H. defensa* but only 3% *R. insecticola*. In the remaining three clusters, both endosymbionts are very rare (Figure 5). Accordingly, the symbiotypes of clusters 2 (*A. f. cirsiiacanthoides*), 5 and 6 do not significantly differ from each other, but they all differ from the three groups with higher endosymbiont prevalences (Table S8).

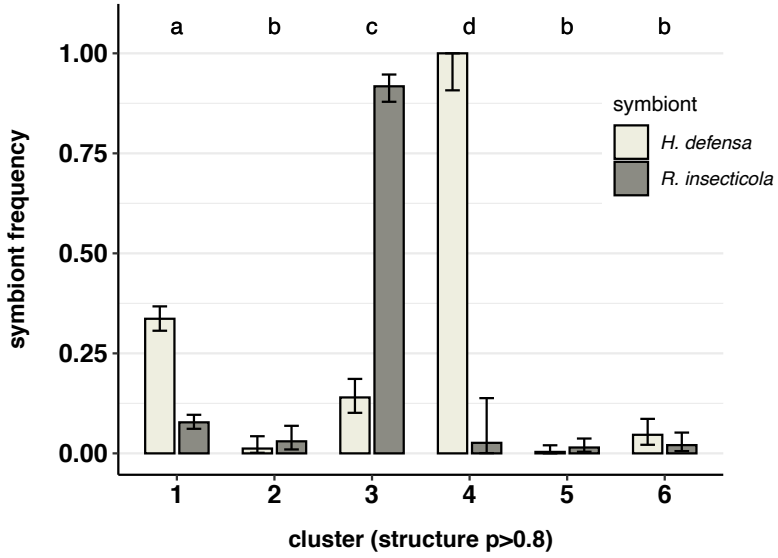

Figure 5: Frequencies of the two endosymbiotic bacteria species *Hamiltonella defensa* and *Regiella insecticola* in each of the six genetic clusters of *A. fabae*, assigning samples based on the STRUCTURE results. Different letters indicate significant differences in symbiotypes in pairwise comparisons using Fisher's exact tests with Bonferroni corrections (see Table S8 for p-values).

Evidence for hybridization between *Aphis fabae* subspecies

Twenty-eight of 1572 samples belonging to the four major genetic clusters found on *E. europaeus* were inferred to be hybrids by both methods we used (Table S9). Two hybrids each were determined as mixtures between cluster 3 and cluster 1 (*A. f. fabae*) or cluster 5,

and three hybrids were determined as crosses between *A. f. fabae* and cluster 5 (Table S9). The remaining 21 hybrids were all determined to be crosses between cluster 2 (*A. f. cirsiiacanthoides*) and cluster 5. All but two of these putative hybrids were collected in spring months, such that hybrid frequency among the samples from *E. europaeus* was higher in spring (26/1094=2.4%) than in autumn (2/450=0.4%, p=0.010 in Fisher's Exact test).

Furthermore, 37 samples were inferred to be hybrids originating from crosses between cluster 1 (*A. f. fabae*) and cluster 4 (supposed *A. viburni*). These are the samples that are identified as admixed between cluster 1 and 4 in the STRUCTURE analysis, while all but one of them are assigned to cluster 4 using *snapclust* on the full dataset. All of these putative hybrids were collected in spring months, spread out over all three sampling years, and from *E. europaeus* and *V. opulus* in similar frequencies (32/1162=3% of *E. europaeus* samples, 5/91=5% of *V. opulus* samples). As expected for hybrid genotypes, H_o (0.80) is distinctly larger than H_e (0.66) within these samples, and allele distributions are intermediate between those of the *A. f. fabae* and the green cluster (Figure S9). A separate argument for these 37 samples actually being hybrids is that their symbiont frequencies (43 % *H. defensa* and 5 % *R. insecticola*) are intermediate between those of cluster 1 and 4 (Figure 5). Statistically, their symbiotype frequency is not significantly different from that of cluster 1 but different from that of cluster 4 ($p=0.587$ and $p<0.0001$ in pairwise Fisher's Exact tests).

4 | Discussion

Different plant species may impose divergent selection on the phytophagous insects exploiting them, but for the evolution and maintenance of genetically differentiated host races reduced gene flow between host-associated populations is necessary. Here we show that despite a shared mating habitat, black bean aphids of the *A. fabae* complex can be assigned to at least six genetically distinct groups, which differ in their host plant preferences and in the frequency of association with facultative endosymbionts.

The existence of multiple *A. fabae* taxa characterized by distinct feeding preferences has been described already 100 years ago (Börner & Janisch, 1922). Also Müller (1982) argued that biological tests of host plant acceptance are the only reliable way to distinguish the morphologically cryptic subspecies. Apart from being tedious, the assignment of individuals to taxa using host plant tests may also hinge on the developmental stage and the condition of both host plants and aphids (Thieme, 1987), as well as on the degree of phenotypic plasticity in aphid performance traits (Gorur *et al*., 2005; Gorur *et al*., 2007). Still, our data shows that there is clear genetic differentiation between black bean aphids that dominate on plants considered 'diagnostic' for certain taxa. This is remarkable and attests to the careful work of the entomologists who studied this complex group with biological assays. In accordance with and extending on previous population genetic studies (Jörg & Lampel, 1996; Vorburger *et al*., 2017), we thus confirm the clear genetic differentiation of ecologically defined subspecies. This is particularly interesting considering that the lack of resolution in mitochondrial COI/II and CytB sequences led some authors to suggest that there might be little genetic distinction among *A. fabae* subspecies (Béji *et al*., 2015; Coeur d'acier *et al*., 2007; Coeur d'acier *et al*., 2014; Zhang *et al*., 2010). This also suggests that the genetic clusters we found represent evolutionarily young taxa.

Most authors agree that the winter host *E. europaeus* is used principally by four *A. fabae* subspecies (summarized by Blackman & Eastop, 2017). This matches well with the four main genetic groups (1, 2, 3, and 5) we found on *E. europaeus*, two of which we can clearly identify as *A. f. fabae* and *A. f. cirsiiacanthoides* thanks to reference genotypes and their summer host plant use. The other two subspecies expected on *E. europaeus* are *A. f. solanella* and *A. f. euonymi*. *A. f. solanella*'s diagnostic summer host is *Solanum nigrum*, from which we could not find any aphid samples, unfortunately. It is also reported to feed on a range of other summer host plants as well, including *R. obtusifolius* (e.g. Thieme, 1988). We found cluster 5 (blue) in large numbers on *R. obtusifolius* and also on *Galium* species and *P. rhoes* (Figure 3) and therefore propose that cluster 5 corresponds to *A. f. solanella*. The remaining cluster 3 (violet) likely represents the monoecious *A. f. euonymi*, which would explain the scarcity of aphids from this cluster on the summer host plants we sampled (Figure 3). *A. f. euonymi* is generally thought to differ visually from other *A. fabae* subspecies due to its brownish body coloration (Blackman & Eastop, 2000), however, no

such divergent body color was recorded during sample collection (E. Gimmi: personal observation). This suggests that body color may not always be reliable for *A. f. euonymi*'s identification, at least not during spring and autumn, when all subspecies are present on *E. europaeus*.

Even though the number of samples we collected from the alternative winter host *V. opulus* is much lower than from *E. europaeus*, we could confirm the presence of two subspecies expected to use *V. opulus* as primary host according to the literature (Blackman & Eastop, 2017), namely *A. f. mordwilkoi* (cluster 6, red – identified by its summer host preferences) and *A. f. cirsiiacanthoides*. We did not expect to find yet another very abundant cluster that appears to be *V. opulus*-specific (cluster 4, green). It could either represent a yet undocumented *A. fabae* host race, or a different but closely related aphid species that we mistook for *A. fabae* when identifying aphids only by the unaided eye in the field. With hindsight, we suspect cluster 4 to represent *A. viburni*, a monoecious taxon that feeds on *V. opulus* throughout the year and is generally regarded as a member of the *A. fabae* complex in the broad sense (s. l.) (Blackman & Eastop, 2000). *A. viburni* would show morphological differences to other black bean aphids under microscopic examination (Lampel & Meier, 2007), but since we extracted DNA destructively for this study, we would need to collect new aphid samples to confirm our hypothesis. According to Coeur d'acier *et al*. (2007; 2014), mitochondrial markers cannot distinguish between *A. viburni* and members of *A. fabae* s. str., which would fit in with our finding that the nuclear genetic differentiation of cluster 4 from other members of *A. fabae* s. str. is comparable to that of the nominal subspecies *A. f. fabae* (Table 1).

The confirmation that black bean aphid populations with different secondary host plant use indeed represent genetically distinct taxa brings back the question of how this differentiation can be maintained despite an apparently regular opportunity for gene flow at shared mating sites. Based on a number of crossing experiments (Iglisch, 1968; Raymond *et al*., 2001; Thieme, 1988; Tosh *et al*., 2004b), we can assume that matings between individuals from different subspecies lead at least partially to viable and fertile offspring. Spatial separation could contribute to a reduction in gene flow between aphids using different primary host plants, though we see no general trend for increased genetic differentiation between the groups overwintering on *E. europaeus* and those overwintering on *V. opulus*, compared to the differentiation among groups sharing the same winter host

(cf. Table 1, Figure 3). An alternative explanation would be temporal separation via a difference in the timing of arrival and the production of sexual morphs on the primary host plant. Such temporal separation plays an important role in the maintenance of genetically divergent lineages in another host alternating aphid species, *Rhopalosiphum padi* (Halkett *et al*., 2006; Halkett *et al*., 2005). While we cannot exclude some variation in the timing of sexual reproduction among subspecies, this mechanism is unlikely to be relevant for separating the four main *A. fabae* groups on *E. europaeus*, since all of them were present simultaneously on *E. europaeus* in the autumn of both sampling years (Figure 4). However, separate temporal niches might be realized at a smaller scale, for example can mating-related activities of *A. f. fabae* and *A. f. solanella* be differently distributed over the day (Thieme & Dixon, 1996). It is generally likely that behavioral mechanisms of assortative mating contribute to reproductive isolation between *A. fabae* subspecies. While this is not well studied yet, there is at least evidence that male black bean aphids are able to differentiate between female pheromones of their own and of different subspecies (Raymond *et al*., 2001; Thieme & Dixon, 1996), and it appears also conceivable that a behavioral preference for chemical signals from the summer host plants of specialized subspecies could promote assortative mating.

Assortative mating is selected for when hybrid offspring show reduced fitness, and our data provided some evidence that this may indeed be the case. In the samples from *E. europaeus*, we identified multiple individuals as putative hybrids among the four main groups that reproduce sexually on this plant (Table S9). The fact that all but two putative hybrids we identified were collected in spring rather than in autumn is suggestive for selection acting against hybrids during the summer months, thereby reinforcing reproductive isolation between genetic groups (Howard, 1993). Postzygotic selection may have an intrinsic (e.g. genetic incompatibility of parental chromosomes) or extrinsic basis, and the latter can directly be related to ecological speciation models: extrinsic postzygotic selection may manifest specifically in the environments that parental individuals are adapted to, if the intermediate gene composition present in hybrids results in a reduced fitness compared to adapted parents ("maladaptive intermediacy", Hatfield & Schluter, 1999; Rundle & Whitlock, 2001). Interestingly, this implies also that the effect of extrinsic selection against hybrids might be reduced on host plants on which both parents feed equally well (Tosh *et al*., 2004a). While it is not possible from our observational data to

distinguish between intrinsic and extrinsic selection against hybrids (Rundle & Whitlock, 2001), clear evidence for extrinsic postzygotic selection has been shown for other phytophagous insect systems (Funk, 2010; Nosil *et al*., 2003).

While hybridization between subspecies co-occurring on *E. europaeus* could be expected, we were surprised by the relatively large number of putative hybrids between *A. f. fabae*, using *E. europaeus* as primary host, and cluster 4 (green, presumed *A. viburni*), using *V. opulus* as primary host. We observed these hybrids only in spring and on both primary host plants. That these taxa are reproductively compatible is consistent with Iglisch (1968), who reported the successful production of hybrids between *A. f. fabae* and *A. viburni* in experimental assays. But how are these hybrids formed when the parental taxa use to mate on different hosts? We hypothesize that male aphids (which we did not sample) occasionally visit the 'wrong' winter hosts when actively searching for females during the period of sexual reproduction. This would be a straightforward explanation for the winged males of *A. f. fabae*, but less so for males of *A. viburni*, which are reported to be unwinged (Heie, 1986). However, *E. europaeus* and *V. opulus* are very common hedgerow plants in our sampling area, often growing right next to each other and with touching branches. It would therefore at least be feasible that stray males of either taxon could mate with egg-laying females (oviparae) that are already settled on the correct plant species, which may explain the presence of hybrids on both winter hosts in spring despite the strict host specificity observed for the female aphids.

The correlation between the use of specific host plants and genetic differentiation in black bean aphids, together with performance trade-offs on these different plants (Douglas, 1997; Mackenzie, 1996; Müller, 1982), recapitulates the situation of host specialized biotypes in the pea aphid (Peccoud *et al*., 2009; Via, 1999). Another parallel is that host-associated biotypes in pea aphids are characterized by distinct endosymbiont communities (Ferrari *et al*., 2012; Simon *et al*., 2003). In *A. fabae*, the two most abundant heritable facultative symbionts are *H. defensa* and *R. insecticola*, but their prevalences differ markedly among the different subspecies (Figure 5). These frequency differences may have arisen due to drift and could thus just be a consequence of the reproductive barriers existing between subspecies. However, *H. defensa* and *R. insecticola* may provide their host with various ecological benefits including protection against parasitoids or pathogens (reviewed in Guo *et al*., 2017) but they also entail fitness costs (Polin *et al*., 2014; Vorburger & Gouskov,

2011; Zytynska *et al*., 2021). Net costs likely vary depending on the aphid's host plant environment (McLean *et al*., 2011; Sochard *et al*., 2019), and some symbionts can even directly affect aphid performance on certain host plants (Tsuchida *et al*., 2004; Wagner *et al*., 2015). It is thus likely that differing costs and benefits of hosting heritable endosymbionts and thus diverging selection account for the large differences in symbiont prevalence between *A. fabae* subspecies specialized on different plant species (Figure 5). As such, the frequency differences represent additional evidence for divergent ecological selection on different host plants.

In conclusion, we illustrate an example of genetic divergence within a species complex of phytophagous insects that correlates with specialization on different host plants. Genetic divergence is also associated with differences in the frequency of infection with facultative endosymbionts. Both is suggestive of divergent selection underlying the observed differentiation. The advantage of ecological specialization seems to be strong enough to promote the maintenance of genetic divergence despite the opportunity for gene flow at shared mating sites, and this is likely achieved via an interplay of prezygotic barriers and postzygotic selection against hybrids.

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Author Contributions

CV and EG designed the study. EG, JW and CV carried out the field work, EG and JW the laboratory work. EG analyzed the data with inputs from CV. EG wrote the first draft of the manuscript which was edited and revised by EG and CV. All authors approved the final version for publication.

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Supplementary Material

Table S1: Sampling data overview: number of black bean aphid samples that were used included in the dataset, per sampling time point, sampling site and host plant species.

Host plant species	sampling time point	site	N
Euonymus europaeus	Mar.19	Faellanden	76
Euonymus europaeus	Mar.19	Gossau	79
Euonymus europaeus	Mar.19	Steinmaur	78
Euonymus europaeus	Apr.19	Faellanden	74
Euonymus europaeus	Apr.19	Gossau	80
Euonymus europaeus	Apr.19	Steinmaur	75
Euonymus europaeus	Oct.19	Faellanden	78
Euonymus europaeus	Oct.19	Gossau	76
Euonymus europaeus	Oct.19	Steinmaur	82
Euonymus europaeus	Mar.20	Faellanden	84
Euonymus europaeus	Mar.20	Gossau	77
Euonymus europaeus	Mar.20	Steinmaur	77
Euonymus europaeus	Apr.20	Faellanden	80
Euonymus europaeus	Apr.20	Gossau	80
Euonymus europaeus	Apr.20	Steinmaur	81
Viburnum opulus	Apr.20	Faellanden	15
Viburnum opulus	Apr.20	Steinmaur	19
Euonymus europaeus	Oct.20	Faellanden	70
Euonymus europaeus	Oct.20	Gossau	75
Euonymus europaeus	Oct.20	Steinmaur	76
Euonymus europaeus	Apr.21	Faellanden	69
Euonymus europaeus	Apr.21	Gossau	79
Euonymus europaeus	Apr.21	Steinmaur	73
Viburnum opulus	Apr.21	Faellanden	19
Viburnum opulus	Apr.21	Gossau	17
Viburnum opulus	Apr.21	Steinmaur	21
Viburnum opulus	summer 21	Zurich region	5
Achillea millefolium	summer 21	Zurich region	23
Aegopodium podagraria	summer 21	Zurich region	26
Anthriscus sylvestris	summer 21	Zurich region	26
Arctium lappa	summer 21	Zurich region	25
Beta vulgaris	summer 21	Zurich region	25
Capsella bursa-pastoris	summer 21	Zurich region	14
Chenopodium album	summer 21	Zurich region	25
Cirsium arvense & C. vulgare	summer 21	Zurich region	29
Galium aparine	summer 21	Zurich region	29
Galium mollugo	summer 21	Zurich region	29
Matricaria chamomilla	summer 21	Zurich region	16
Papaver rhoeas	summer 21	Zurich region	29
Rumex spp	summer 21	Zurich region	37
Tropaeolum spp	summer 21	Zurich region	21
Chenopodium album	samples from Vorburger *et al*. 2017		15
Cirsium arvense & C. vulgare	samples from Vorburger *et al*. 2017		15

Table S2: Cycling conditions and primers for used for microsatellite PCR; as applied for and presented in Chapter II. The primers were published by Coeur d'acier *et al*. (2004).

Marker	size range [bp]	primer name	sequence
AfF	113 – 204	AfF forward	GCGTTGCAGCAGCATATACT
		AfF reverse	CCTATATCGTGTGCGTGCAT
Af82	159 – 236	Af82 forward	GCGTAATGCAAGTAACGACC
		Af82 reverse	CGTCGTTCCAGCGAATTCTC
Af86	207 – 221	Af86 forward	CGCGTTCTCTCCAATAACTC
		Af86 reverse	TAATGTTGCGGATTGTTTGC
Af85	208 – 228	Af85 forward	CGCGTGCAGTGTAGGTCCAT
		Af85 reverse	CAAGGTGCGATTGACGACGA
Af50	255 – 276	Af50 forward	TGGTGAGTGCAGGCTAGTAT
		Af50 reverse	AAGGCACTTAGTCGACGTGT
Afbeta	260 – 377	Afbeta forward	GAGGACGCGGCTAAGAAGAA
		Afbeta reverse	CGAAAAGGGACGTCTACGAG
Af48	303 - 355	Af48 forward	TTAAACCTTTGAGCGTAGCG
		Af48 reverse	CCGAAGCAGCAGTAACATTG
Af181	299 - 362	Af181 forward	GGCATGTGCACGACGAATAC
		Af181 reverse	CGTTTCTTCGTGTGCGATTT

PCR PROTOCOL

temp [°C]	time [min]	cycles
95	15	
94	0.5	
60	1.5	30 x
72	1	
60	30	

PCR REACTION MIX (per sample)

Reagent	volume [μl]
ddH20 + Primers (conc. see table on the right)	4.5
QIAGEN Multiplex PCR Master Mix	5.5
DNA solution	1
Final vol per reaction	11

Primer and label	conc. in PCR [µM]
AfF forward + PET	0.1
AfF reverse	0.1
Af82 forward + NED	0.4
Af82 reverse	0.4
Af86 forward + VIC	0.2
Af86 reverse	0.2
Af85 forward + FAM	0.2
Af85 reverse	0.2
Af50 forward + PET	0.2
Af50 reverse	0.2
Afbeta forward + NED	0.4
Afbeta reverse	0.4
Af48 forward + VIC	0.4
Af48 reverse	0.4
Af181 forward + FAM	0.2
Af181 reverse	0.2

Table S3: Cycling conditions and primers used for symbiont-diagnostic PCR.

PRIMERS SYMBIONT DIAGNOSIS

symbiont	product size [bp]	primer F	sequence Primer F	primer R	sequence Primer R	reference
Buchnera aphidicola	196	16SA1	AGAGTTTGATCMTGGCTCAG	Buch_R_CV2	CCCCCACTTTRGTTTTTCAAC	Hafer-Hahmann and Vorburger (2020)
Hamiltonella defensa	471	10F	AGTTTGATCATGGCTCAGATTG	T419R	AAATGGTATTCGCATTTATCG	Ferrari *et al*. (2012)
Regiella insecticola	480	10F	AGTTTGATCATGGCTCAGATTG	U443R	GGTAACGTCAATCGATAAGCA	Ferrari *et al*. (2012)

PCR REACTION MIX (per sample)

reagent	volume [μl]
ddH20	2.3
Promega GoTaq® G2 Colorless Master Mix	5.5
Primer F	1.1
Primer R	1.1
Reagent mix per reaction	10
DNA solution per reaction	1
Final vol per reaction	11

PCR PROTOCOL

temp [°C]	time [min]	cycles
95	3	
95	0.5	
65-56	0.5	10x
72	1	
95	0.5	
55	0.5	25x
72	1	
72	6	

Table S4: Summary of allele numbers, observed and expected heterozygosity, and p-value of an exact test for HWE per locus; for the full dataset and for each of the six genetic groups inferred with STRUCTURE. P-values from HWE tests <0.05 are printed in bold, p-values below the Bonferroni-corrected significance threshold of 0.05/64=0.0008 are printed in red.

	alleles / locus	Ho	He	exact HWE
full data (N=2099, 1.37% missing data)				
Af85	11	0.56	0.67	**0.0000**
Af181	33	0.7	0.81	**0.0000**
Af86	7	0.25	0.57	**0.0000**
Af48	23	0.69	0.85	**0.0000**
Af82	38	0.66	0.86	**0.0000**
Afbeta	58	0.67	0.83	**0.0000**
AfF	44	0.69	0.84	**0.0000**
Af50	7	0.56	0.76	**0.0000**
mean	27.63	0.6	0.77	
total	221			
group_1 (N=976, 1.29% missing data)				
Af85	6	0.55	0.57	**0.0410**
Af181	6	0.64	0.67	0.3228
Af86	6	0.44	0.5	**0.0000**
Af48	11	0.75	0.77	0.1729
Af82	17	0.49	0.49	0.8159
Afbeta	22	0.57	0.59	0.4306
AfF	13	0.56	0.57	0.3098
Af50	5	0.61	0.6	0.3633
mean	10.75	0.58	0.6	
total	86			
group_2 (N=181, 1.66% missing data)				
Af85	6	0.56	0.61	**0.0013**
Af181	18	0.83	0.87	0.0907
Af86	2	0.01	0.01	1.0000
Af48	12	0.66	0.71	0.1488
Af82	21	0.85	0.89	0.1065
Afbeta	38	0.84	0.92	**0.0046**
AfF	33	0.68	0.89	**0.0000**
Af50	5	0.49	0.61	**0.0056**
mean	16.88	0.62	0.69	
total	135			

Table continues on the next side

Continuation Table S4

	alleles / locus	Ho	He	exact HWE
group_3 (N= 280, 2.19% missing data)				
Af85	5	0.5	0.51	0.3277
Af181	17	0.5	0.53	**0.0061**
Af86	3	0.1	0.09	1.0000
Af48	13	0.76	0.8	0.1385
Af82	15	0.83	0.8	0.6533
Afbeta	46	0.89	0.94	0.0904
AfF	21	0.81	0.83	0.4031
Af50	4	0.4	0.43	0.3337
mean	15.5	0.6	0.62	
total	124			
group_4 (N=38, 0.99% missing data)				
Af85	3	0.24	0.22	1.0000
Af181	7	0.68	0.73	0.1947
Af86	2	0.03	0.03	1.0000
Af48	7	0.69	0.54	0.6671
Af82	3	0.55	0.49	0.6466
Afbeta	10	0.47	0.59	**0.0007**
AfF	13	0.89	0.86	0.1945
Af50	4	0.35	0.3	1.0000
mean	6.13	0.49	0.47	
total	49			
group_5 (N=277, 1.08% missing data)				
Af85	4	0.57	0.54	0.7018
Af181	23	0.84	0.85	0.6443
Af86	3	0.06	0.06	1.0000
Af48	11	0.32	0.3	0.6551
Af82	21	0.79	0.8	0.1870
Afbeta	26	0.7	0.71	0.2987
AfF	25	0.84	0.86	0.1397
Af50	4	0.56	0.54	0.1600
mean	14.63	0.59	0.58	
total	117			
group_6 (N=200, 0.88% missing data)				
Af85	9	0.7	0.72	0.5234
Af181	13	0.79	0.81	0.8583
Af86	3	0.04	0.12	**0.0000**
Af48	13	0.77	0.78	0.3573
Af82	25	0.75	0.83	**0.0166**
Afbeta	22	0.72	0.76	0.4838
AfF	12	0.8	0.84	0.4636
Af50	6	0.54	0.57	**0.0416**
mean	12.88	0.64	0.68	
total	103			

Table S5: Number of samples assigned per cluster with *snapclust* (a) or STRUCTURE (b) under different Ks and using an assignment threshold of p>0.8 (p being the group membership probability). Note that cluster names are different between *snapclust* and STRUCTURE as well as under different Ks, e.g., c1 may not contain the same samples under K=6 and K=7. STRUCTURE results are not shown for K<6 since there the single runs converged to two or more different solutions.

	K=2	**K=3**	**K=4**	**K=5**	**K=6**	**K=7**	**K=8**	**K=9**
a) *snapclust* number of samples / cluster (p>0.8)								
c1	1021	1017	1017	1017	985	292	184	190
c2	1059	332	319	204	203	330	147	130
c3		714	341	334	295	203	203	202
c4			373	298	78	295	295	295
c5				221	297	77	117	43
c6					218	298	76	107
c7						218	298	297
c8							218	217
c9								41
mix	19	36	49	25	23	386	561	577
b) STRUCTURE number of samples / cluster (p>0.8)								
c1					976	973	972	970
c2					181	151	126	127
c3					280	279	276	275
c4					38	38	38	38
c5					277	275	272	271
c6					200	196	195	194
c7						13	21	19
c8							3	3
c9								0
mix					147	174	196	202

Table S6: Pairwise FST values (Weir & Cockerham) and 95% confidence intervals (in brackets) between sampling sites within each of the four main groups found in the winter host data with STRUCTURE under K=6 and assigning samples to a cluster if they show an assignment probability >0.8. F_{ST} values were calculated using *pairwise.WCfst*, 95% CI were estimated using *boot.ppfst* with nboot=1000 from the R package *hierfstat* (Goudet, 2005). Values whose confidence intervals do not include zero are printed in bold and red.

	Gossau	***Steinmaur***
1-yellow		
Faellanden	0.000 [0.000, 0.001]	0.001 [0.000, 0.002]
Gossau		0.001 [0.000, 0.002]
2-orange		
Faellanden	0.003 [0.000, 0.007]	0.004 [0.000, 0.007]
Gossau		**0.006 [0.003, 0.009]**
3-violet		
Faellanden	0.000 [-0.001, 0.000]	0.000 [-0.002, 0.001]
Gossau		-0.001 [-0.002, 0.000]
5-blue		
Faellanden	0.000 [-0.001, 0.001]	0.000 [-0.002, 0.002]
Gossau		0.000 [-0.002, 0.001]

Table S7: Pairwise F_{ST} values (Weir & Cockerham) and 95% confidence intervals (in brackets) between sampling time points within each of the four main groups found in the winter host data with STRUCTURE under K=6 and assigning samples to a cluster if they show an assignment probability >0.8. F_{ST} values were calculated using *pairwise.WCfst*, 95% CI were estimated using *boot.ppfst* with nboot=1000 from the R package *hierfstat* (Goudet, 2005). Values whose confidence intervals do not include zero are printed in bold and red.

	19_springB	**19_fall**	**20_springA**	**20_springB**	**20_fall**	**21_spring**
1-yellow						
19_springA	0.001 [0.000, 0.002]	**0.002 [0.001, 0.004]**	0.002 [0.001, 0.004]	0.001 [0.000, 0.002]	0.002 [0.000, 0.003]	0.000 [-0.001, 0.002]
19_springB		0.002 [0.000, 0.004]	0.002 [0.000, 0.003]	0.001 [0.000, 0.001]	0.002 [0.000, 0.004]	0.000 [-0.001, 0.001]
19_fall			0.000 [-0.001, 0.000]	0.000 [-0.001, 0.001]	0.000 [-0.001, 0.001]	0.002 [0.000, 0.003]
20_springA				0.000 [-0.001, 0.000]	0.000 [-0.001, 0.000]	0.001 [0.000, 0.002]
20_springB					0.000 [-0.001, 0.001]	0.000 [0.000, 0.001]
20_fall						0.001 [-0.001, 0.002]
2-orange						
19_springA	-0.003 [-0.005, 0.000]	-0.002 [-0.004, 0.001]	0.001 [-0.003, 0.006]	0.007 [-0.003, 0.019]	0.000 [-0.002, 0.003]	0.002 [-0.003, 0.009]
19_springB		-0.002 [-0.006, 0.002]	0.000 [-0.004, 0.005]	0.000 [-0.007, 0.008]	-0.001 [-0.004, 0.002]	0.007 [0.000, 0.015]
19_fall			0.000 [-0.005, 0.007]	-0.001 [-0.007, 0.007]	0.001 [-0.005, 0.006]	0.007 [0.000, 0.014]
20_springA				0.002 [-0.007, 0.012]	0.004 [-0.002, 0.011]	0.012 [-0.002, 0.032]
20_springB					0.005 [-0.001, 0.012]	**0.029 [0.015, 0.048]**
20_fall						0.002 [-0.005, 0.01]
3-violet						
19_springA	0.000 [-0.002, 0.003]	0.004 [-0.003, 0.013]	0.003 [0.000, 0.005]	-0.001 [-0.004, 0.001]	0.001 [-0.001, 0.004]	-0.001 [-0.002, 0.001]
19_springB		0.007 [-0.003, 0.021]	0.001 [-0.001, 0.002]	-0.001 [-0.003, 0.002]	0.001 [-0.002, 0.004]	0.000 [-0.002, 0.002]
19_fall			0.007 [0.001, 0.015]	0.001 [-0.006, 0.010]	0.002 [-0.002, 0.007]	0.004 [-0.004, 0.014]
20_springA				0.001 [-0.001, 0.003]	0.000 [-0.001, 0.002]	-0.002 [-0.003, -0.001]
20_springB					0.000 [-0.001, 0.002]	-0.003 [-0.004, -0.001]
20_fall						-0.001 [-0.003, 0.001]
5-blue						
19_springA	0.002 [-0.006, 0.010]	0.007 [0.000, 0.015]	0.007 [-0.002, 0.02]	0.006 [-0.001, 0.016]	**0.011 [0.002, 0.022]**	**0.008 [0.001, 0.017]**
19_springB		0.001 [-0.006, 0.009]	-0.001 [-0.006, 0.005]	-0.002 [-0.005, 0.001]	0.000 [-0.004, 0.004]	-0.001 [-0.004, 0.002]
19_fall			0.000 [-0.003, 0.004]	0.000 [-0.004, 0.003]	0.003 [-0.003, 0.012]	0.001 [-0.002, 0.005]
20_springA				0.002 [-0.002, 0.008]	0.002 [-0.001, 0.006]	0.000 [-0.001, 0.002]
20_springB					0.003 [-0.001, 0.008]	0.001 [-0.002, 0.008]
20_fall						0.001 [-0.001, 0.004]

Table S8: Endosymbiont frequencies, p-values from pairwise Fisher's Exact tests to assess the statistical significance of differences in symbiotypes ($Ham^{-}Reg^{-}$, Ham^{-} Reg^{+}, $Ham^{+}Reg^{-}$ or Ham^{+} Reg^{+}) between genetic groups of *Aphis fabae* (as inferred from the STRUCTURE K=6 solution). The Bonferroni-corrected significance level is 0.05/14 = 0.00357.

comparison	**p-value Fisher's Exact Test**
1-yellow vs 2-orange	<0.000001
1-yellow vs 3-violet	<0.000001
1-yellow vs 4-green	<0.000001
1-yellow vs 5-blue	<0.000001
1-yellow vs 6-red	<0.000001
2-orange vs 3-violet	<0.000001
2-orange vs 4-green	<0.000001
2-orange vs 5-blue	0.281171
2-orange vs 3-red	0.140102
3-violet vs 4-green	<0.000001
3-violet vs 5-blue	<0.000001
3-violet vs 4-red	<0.000001
4-green vs 5-blue	<0.000001
4-green vs 6-red	<0.000001
5-blue vs 6-red	0.004436

Table S9: Results from the search for hybrid genotypes using either *snapclust* or *NewHybrids*. We considered only those samples as hybrids which were designated so by both *snapclust* and *NewHybrids*.

parent A	parent B	nr. samples	hybrids detected		
			snapclust only	***snapclust* & *NewHybrids***	*NewHybrids* only
1-yellow (*A. f. fabae*)	2-orange (*A. f. cirsii*.)	997	15	**0**	0
1-yellow (*A. f. fabae*)	3-violet	1166	15	**2**	0
1-yellow (*A. f. fabae*)	5-blue	1121	6	**3**	0
2-orange (*A. f. cirsii*.)	3-violet	403	33	**0**	0
2-orange (*A. f. cirsii*.)	5-blue	373	16	**21**	0
3-violet	5-blue	516	12	**2**	0

Table S10: Results from the search for hybrid genotypes using either *snapclust* or *NewHybrids* in datasets containing 20 simulated hybrids and their parental populations (N=number of samples). Average values from 100 datasets with identical parents but newly simulated hybrids are shown. The input data consisted of those samples that were assigned to either of the parental cluster with p>0.8 in the STRUCTURE analysis under K=6 and for which data was complete for all eight markers.

parent A	N(B)	parent B	N(B)	***snapclust***		**NewHybrids**	
				hybrids total	of which simulated	hybrids total	of which simulated
1-yellow *(A. f. fabae)*	785	2-orange *(A. f. cirsii.)*	96	24.29	19.19	18.35	18.35
1-yellow *(A. f. fabae)*	785	3-violet	228	23.56	19.57	19.2	19.2
1-yellow *(A. f. fabae)*	785	5-blue	212	21.96	19.96	19.85	19.85
2-orange *(A. f. cirsii.)*	96	3-violet	228	32.48	16.06	11.05	10.7
2-orange *(A. f. cirsii.)*	96	5-blue	212	22.73	17.42	18.1	16.05
3-violet	228	5-blue	212	26.95	19.36	18.85	18.85

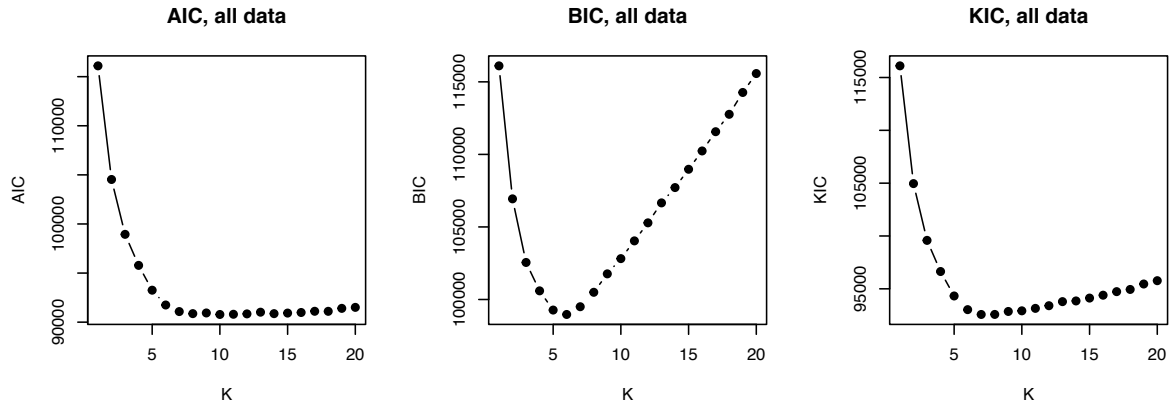


Figure S1: AIC, BIC and KIC values for the clustering results obtained with *snapclust* applied to the full dataset (2099 samples) for different numbers of clusters (K). Minimal values or "elbows" in the curves, i.e. trends that change from decreasing to increasing, may hint at the "optimal" number of clusters in the data, a such is most clearly visible in the BIC plot at K=6.

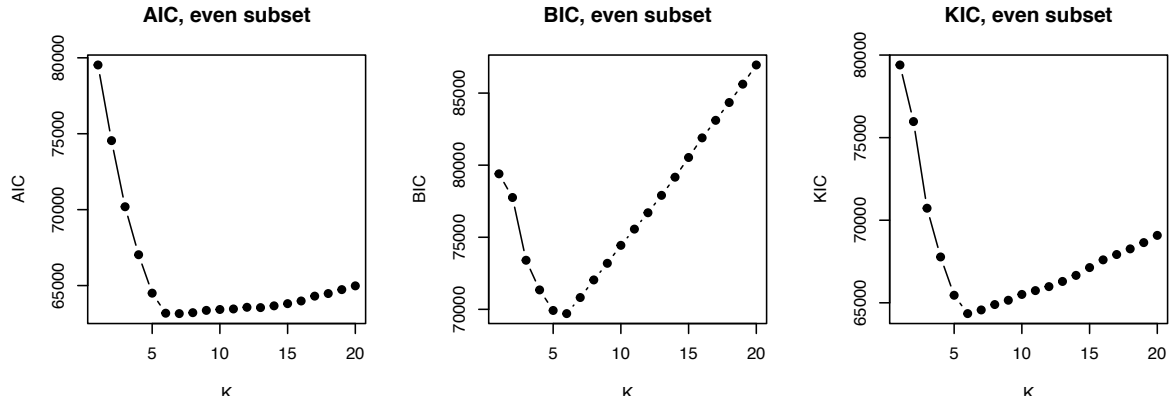


Figure S2: AIC, BIC and KIC values for the clustering results obtained with *snapclust* applied to the more balanced data subsets (containing a subset of data from the largest cluster such as to have more similar numbers of samples belonging to the six clusters initially inferred with *snapclust*), for different numbers of clusters (K).

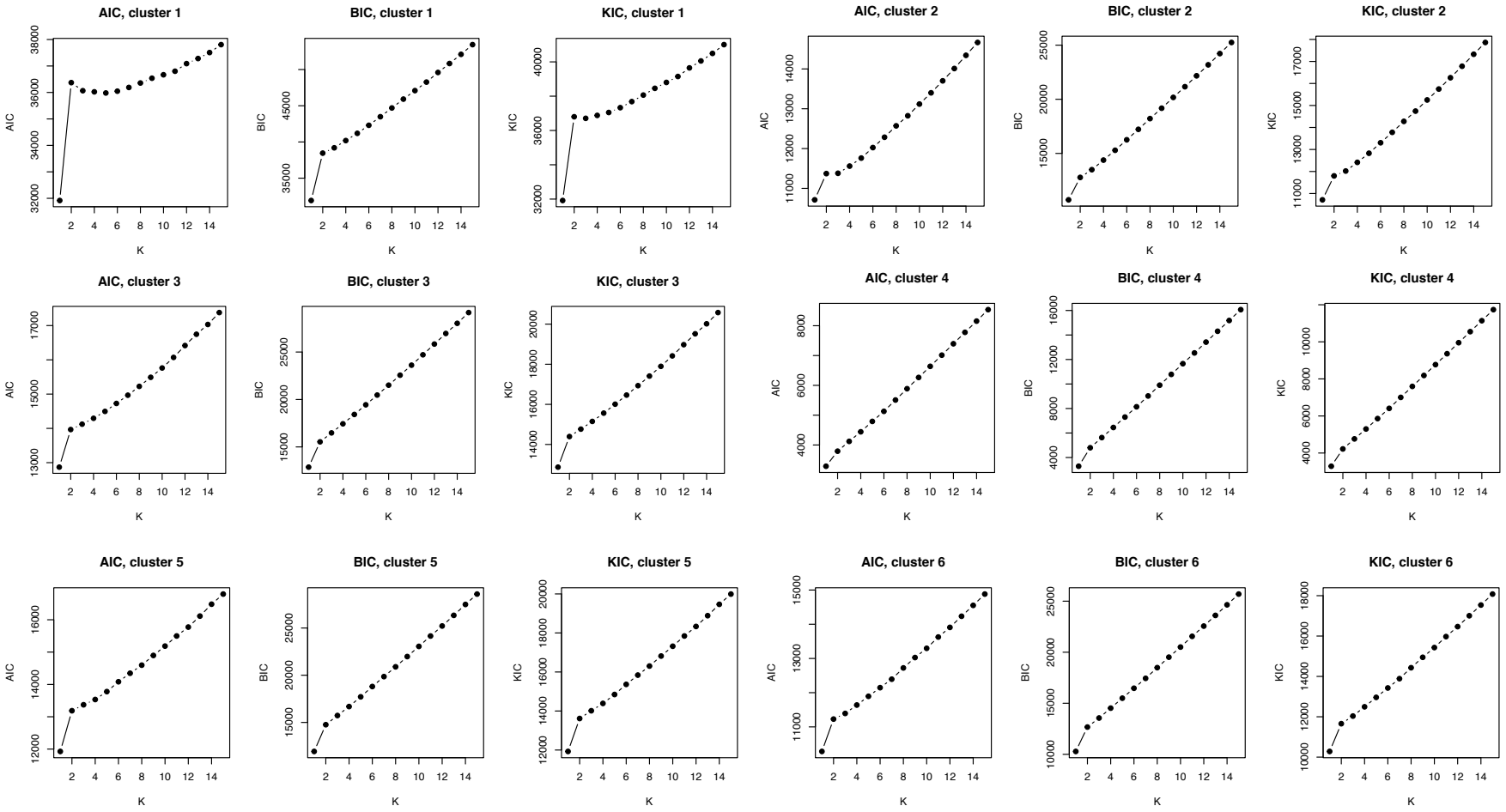
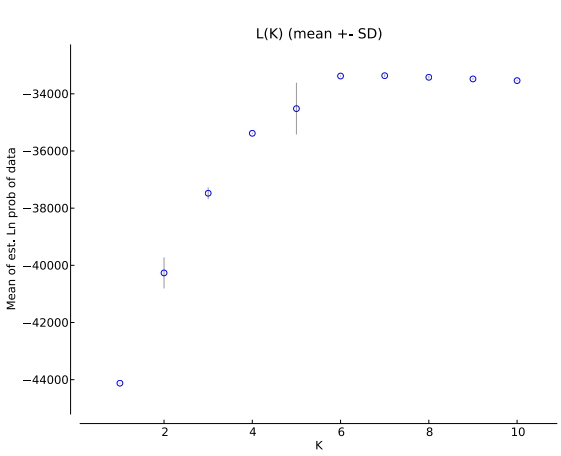
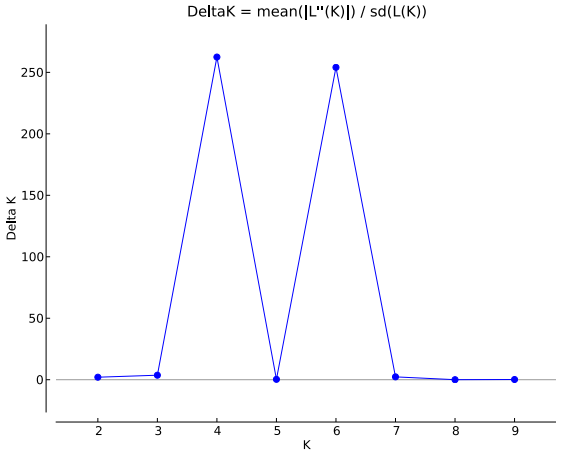


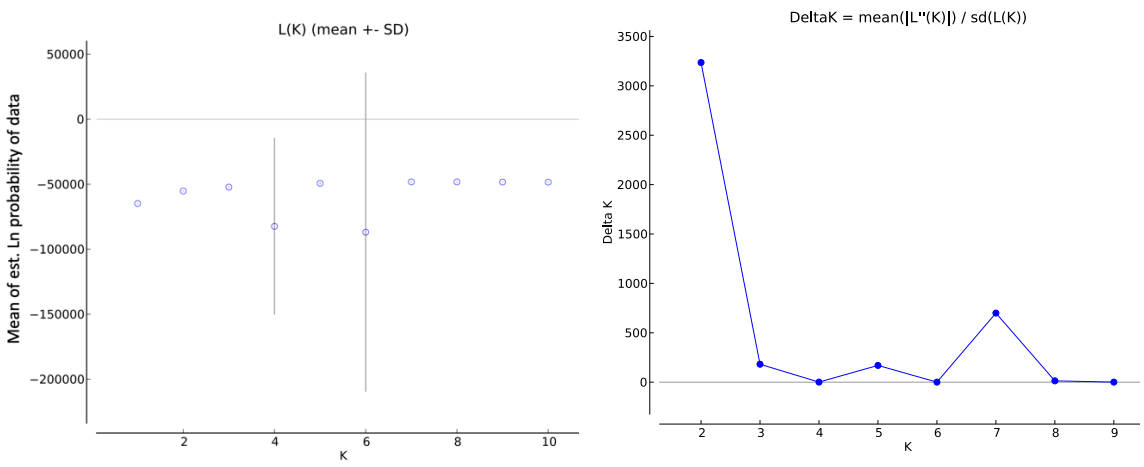
Figure S3: AIC, BIC and KIC values for the clustering results with the number of clusters (K) varying from 1 to 15, obtained with *snapclust* applied to each of six data subsets containing each the samples that showed highest group membership probabilities for the same cluster.





K	Reps	Mean LnP(K)	Stdev LnP(K)	Ln'(K)	|Ln''(K)|	Delta K
1	10	-44123.250000	0.143372	—	—	—
2	10	-40265.540000	526.151973	3857.710000	1070.600000	2.034773
3	10	-37478.430000	188.260570	2787.110000	689.730000	3.663699
4	10	-35381.050000	4.704194	2097.380000	1234.640000	262.455143
5	10	-34518.310000	888.232338	862.740000	277.870000	0.312835
6	10	-33377.700000	4.442722	1140.610000	1128.810000	254.080725
7	10	-33365.900000	29.399131	11.800000	68.070000	2.315375
8	10	-33422.170000	24.820916	-56.270000	0.710000	0.028605
9	10	-33479.150000	12.176685	-56.980000	2.020000	0.165891
10	10	-33538.150000	8.287910	-59.000000	—	—

Figure S4: Output from STRUCTURE HARVESTER (Evanno *et al*., 2005) used to determine the optimal number of clusters (K) in the results from running STRUCTURE on the more **balanced data subset** with the settings suggested by Wang (2017).



K	Reps	Mean LnP(K)	Stdev LnP(K)	Ln'(K)	|Ln''(K)|	Delta K
1	10	-64852.410000	0.202485	—	—	—
2	10	-55207.730000	2.063465	9644.680000	6677.510000	3236.066108
3	10	-52240.560000	182.159059	2967.170000	33101.980000	181.720197
4	10	-82375.370000	67543.718046	-30134.810000	63197.520000	0.935654
5	10	-49312.660000	415.680816	33062.710000	70636.900000	169.930623
6	10	-86886.850000	122394.920388	-37574.190000	76213.340000	0.622684
7	10	-48247.700000	55.195733	38639.150000	38625.440000	699.790332
8	10	-48233.990000	7.946131	13.710000	107.830000	13.570126
9	10	-48328.110000	5.843600	-94.120000	4.670000	0.799165
10	10	-48426.900000	15.261426	-98.790000	—	—

Figure S5: Output from STRUCTURE HARVESTER (Evanno *et al*., 2005) used to determine the optimal number of clusters (K) in the results from running STRUCTURE on the **full dataset** using the settings suggested by Wang (2017). These settings should improve the detection of small clusters in datasets with uneven or unknown samples sizes, but they may also lead to overestimation of the "optimal" number of clusters. The "optimal" number of clusters might be derived from the plot on the left as the K (y-axis) for which the mean Ln of assignment probability (x-axis) is highest, or sometimes also where the curve flattens down (Pritchard *et al*., 2000), no such pattern can be seen here. From the plot on the right, the optimal number of K (y-axis) might be derived as the one where DeltaK is maximal (x-axis, Evanno method, Evanno *et al*., 2005), i.e. K = 2 is determined as the optimal number of clusters here, but there is also a second "bump" at K=7 suggesting to take into consideration also this solution. The summary table shows the values that are visualized in the plot.

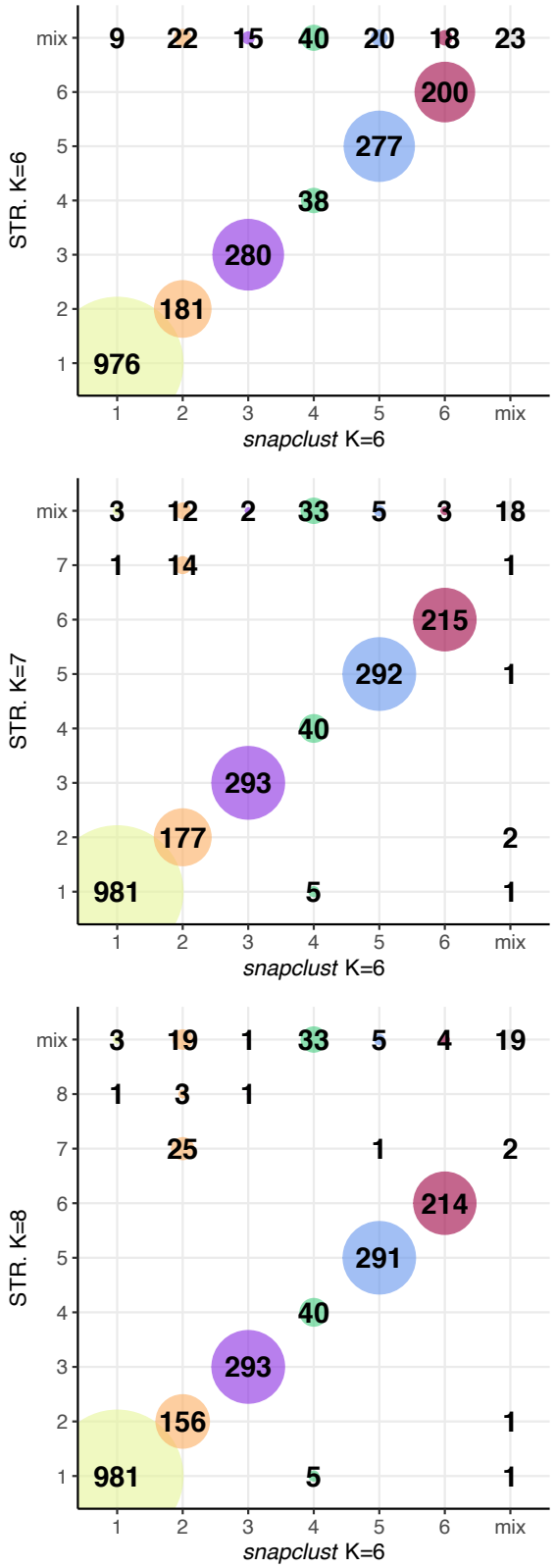


Figure S6: Comparison of the assignment of individuals to clusters using STRUCTURE (top: K=6, middle: K=7, bottom: K=8) or *snapclust* (K=6) on the full dataset. Samples are assigned to the cluster for which they show an assignment probability of at least 0.8, if this is not the case for any cluster the samples are categorized as mixed. The numbering of the levels is arbitrary.

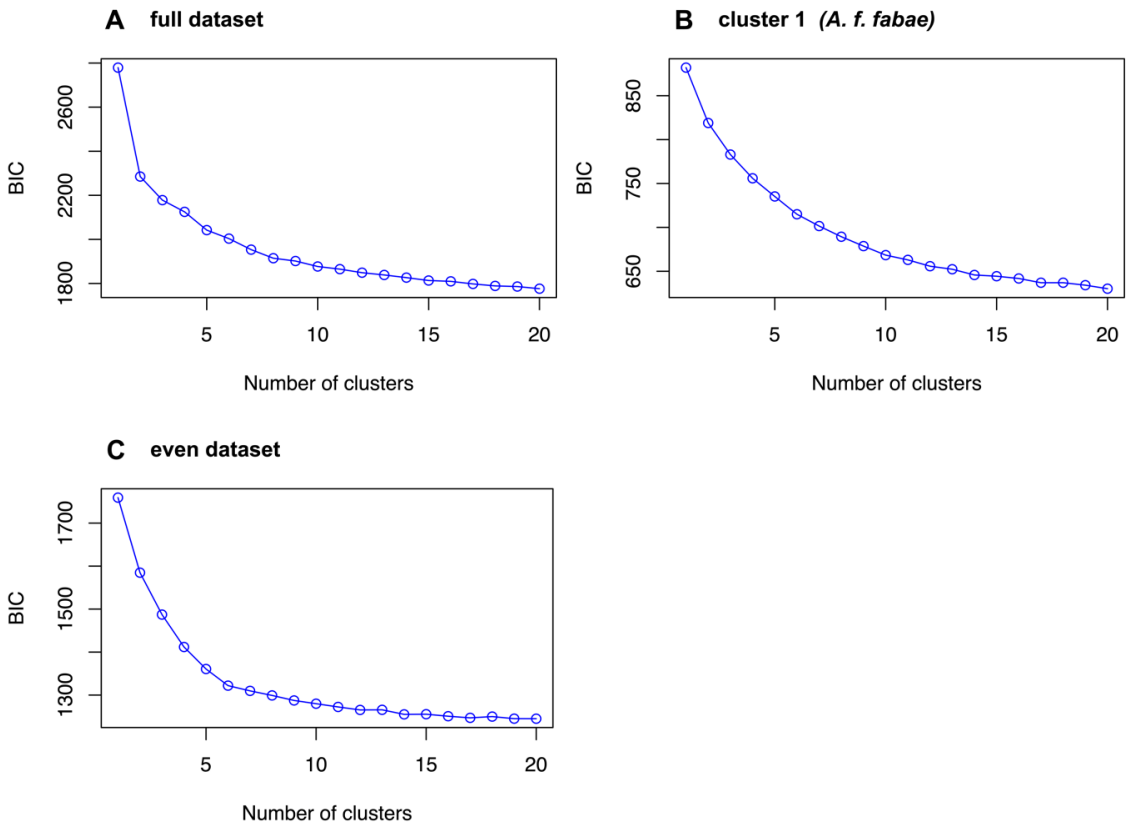


Figure S7: BIC values for k-means clustering solutions on (a) the full dataset, K=2 is suggested here, (b) on samples belonging to the presumed *A. f. fabae* cluster only, no substructure is suggested here, (c) on the even data subset, K=6 is suggested here (the "optimal" number of clusters might be indicated by minimal values or an "elbow" in the curve).

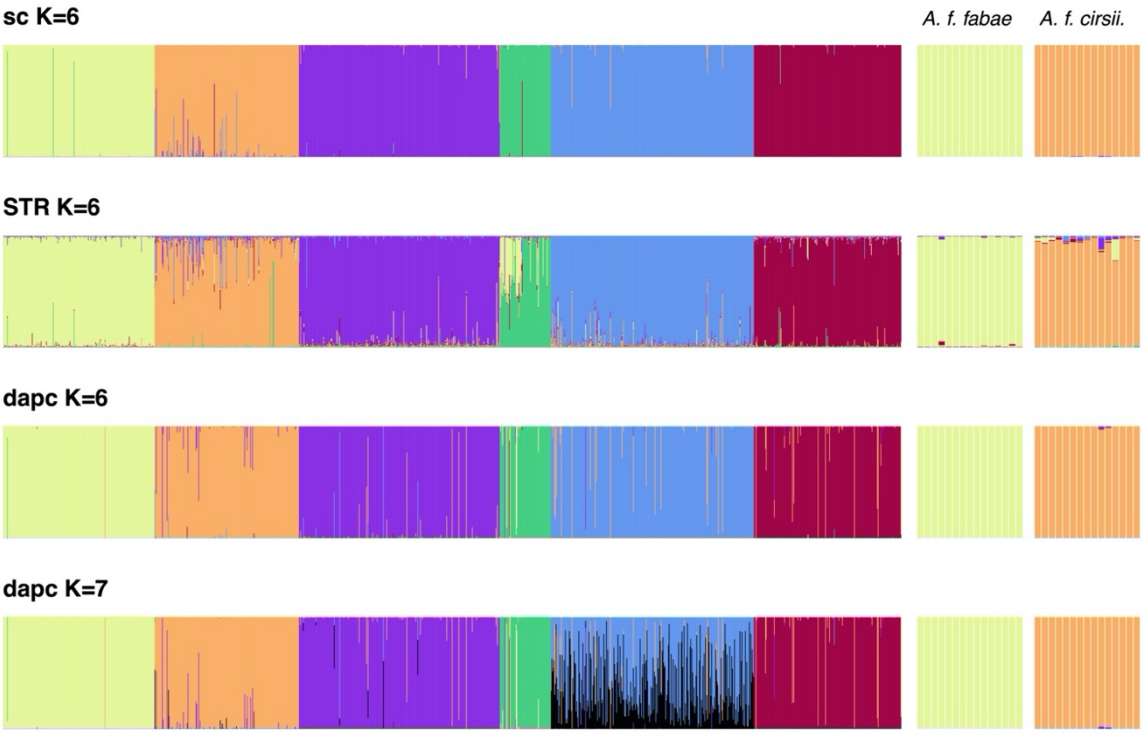


Figure S8: Clustering results from *snapclust* (sc), STRUCTURE (STR) and DAPC applied to the more balanced data subset. Each aphid individual is represented by a vertical bar, the proportion of this bar colored in a specific color is the likelihood that the sample belongs to a specific cluster (membership probability). For each K, the wide boxes to the left show all 1333 samples used in the analysis next to each other. For all solutions the samples are ordered by their most likely cluster in the *snapclust* K=8 solution in the full data analysis. The two narrow boxes to the left zoom in on the reference samples from *A. f. fabae* and *A. f. cirsiiacanthoides*, respectively.

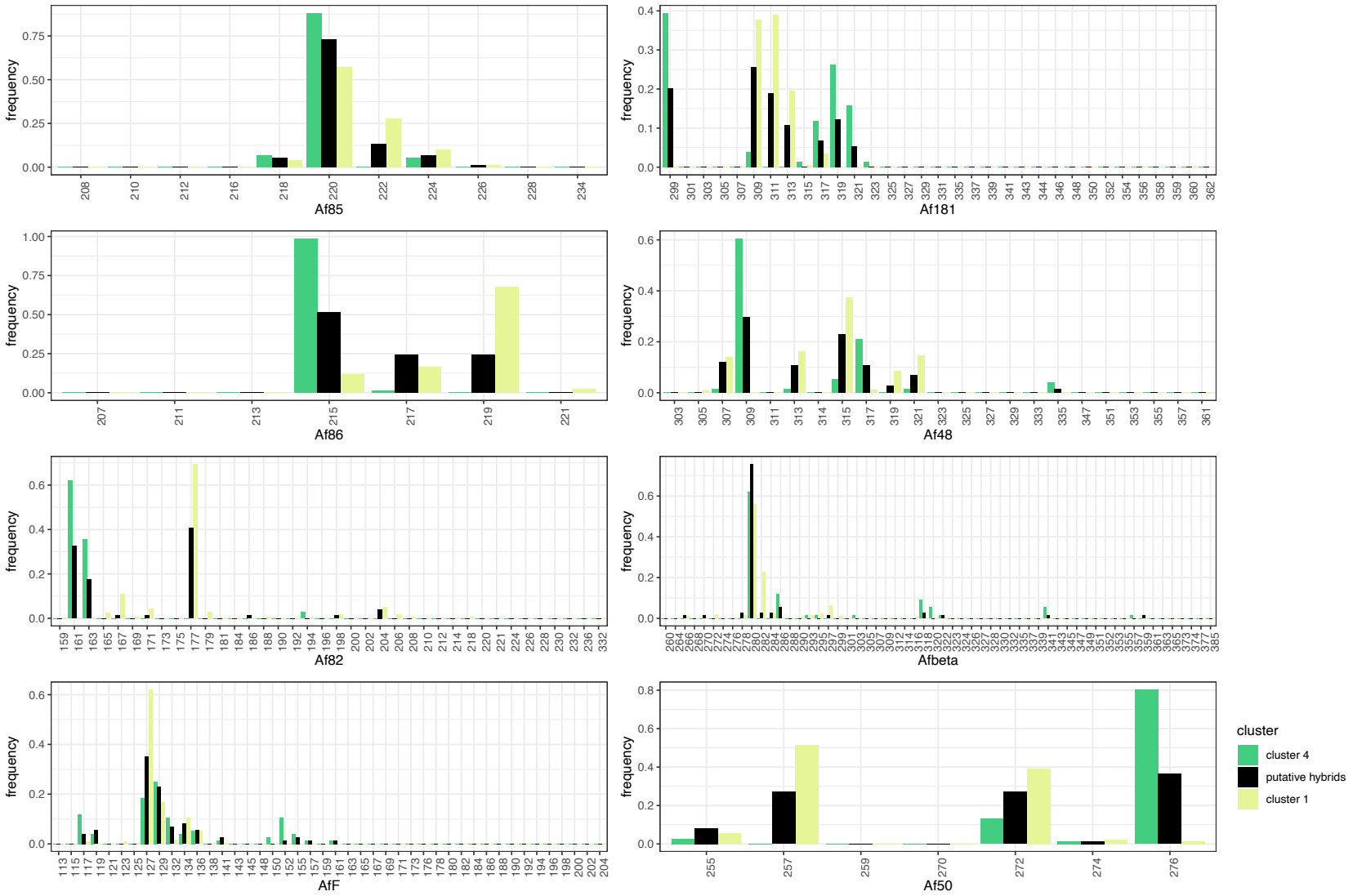


Figure S9: Allele frequencies in cluster 1 (yellow, *A. f. fabae*) and cluster 4 (green, supposed *A. viburni*), and their putative hybrids (black).

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General Discussion

Defensive symbiosis is a fascinating phenomenon and an excellent example for the context-dependency of costs and benefits in species interactions (Bronstein, 1994). It adds an additional and often neglected level of variation to studies of host-parasite interactions (Vorburger & Perlman, 2018). Following the key discovery of the protective effects of *Hamiltonella defensa* in pea aphids twenty years ago (Oliver *et al*., 2003), interactions with defensive microbial symbionts have been discovered in various other animals (Florez *et al*., 2015). At the same time, research on defensive symbiosis specifically in aphids has advanced in big steps. A multitude of studies have contributed to revealing patterns and mechanisms underlying *H. defensa*-conferred resistance, and have improved our understanding of the ecological and evolutionary dynamics between aphids and parasitoids. There is, nevertheless, still a lot to learn. In particular, many ecological and evolutionary predictions based on laboratory experiments remain to be assessed for their pertinence under natural conditions. With my PhD work, I aimed to contribute to this by collecting and analyzing field data from natural insect communities. The core of my thesis is a field survey on the seasonal dynamics of defensive symbiosis and parasitism risk (Chapter II): in a large team-effort, we collected data on endosymbiont prevalence and parasitoid abundance in three local populations of the black bean aphid *A. f. fabae*. Confined to one geographic area – Zurich, Switzerland – and a single aphid species, this survey puts just a small part of the entire natural community under the magnifying glass. Nevertheless, for this part it is comprehensive and well-resolved in time, and therefore allows to reflect on the available knowledge on *H. defensa* in a real-life context. In the following, I briefly sum up the main conclusions that result from my work, and their implications for future studies on defensive symbiosis in aphids.

Seasonal dynamics of *H. defensa* frequency

Balancing selection is thought to maintain defensive symbionts at intermediate prevalence in many aphid populations (Oliver *et al*., 2014). Costs and benefits of *H. defensa* likely vary with seasons, which bring along differences in weather, availability and quality of host plants, and enemy pressure. In caged aphid populations, infection with *H. defensa* is

a liability in the absence of parasitoids, but a selective advantage in their presence. This becomes evident from a decrease or increase in the symbiont prevalence, respectively, when starting from aphid populations with intermediate *H. defensa* frequencies (Dykstra *et al*., 2014; Hafer-Hahmann & Vorburger, 2020; Oliver *et al*., 2008). These changes can be noted within very few generations, suggesting that *H. defensa* frequencies in a natural aphid population could vary in response to variable parasitism pressure on the time scale of a single growing season.

Our conclusions from monitoring the seasonal dynamics of *H. defensa* in *A. f. fabae* are in line with the very limited number of comparable field studies – all of them on pea aphids–, where suggestive evidence for parasitoid-mediated selection tended to be detectable, but never in clear-cut, straightforward patterns (Ives *et al*., 2020; Leclair *et al*., 2021; Smith *et al*., 2015; Smith *et al*., 2021). One could argue that this is simply what has to be expected when moving from small and simplified lab communities to complex natural communities in the field. However, it is worth reflecting on the possible causes of the discrepancy if we are to better understand the ecological role of defensive symbionts in natural populations. In our survey, parasitoid abundance showed a spatially consistent early-summer peak in both years, and considering the two variables in isolation, we found a positive correlation between parasitism risk and *H. defensa* frequency at a time lag of two months. However, *H. defensa* frequencies were more erratic than one would expect if they were mainly determined by parasitoid abundance. In fact, they correlated more closely with the number of heat days experienced by previous aphid generations than with parasitism risk (Chapter II). We therefore conclude that while likely important, parasitism might not be the only driver of *H. defensa* frequency dynamics in black bean aphids.

One possible reason for the lack of a clear association between parasitism risk and *H. defensa* prevalence may be the fact that the symbiont does not provide a general protection against parasitoids: it was only protective against one parasitoid species, albeit the one that clearly posed the highest risk (*L. fabarum*, Chapter III). Since the resistance conferred by *H. defensa* against *L. fabarum* acted in a genotype-specific manner, the strength of selection for *H. defensa*-infected aphids will further depend on the genotypic composition of the *L. fabarum* population, which we did not assess comprehensively. It is also possible that temperature is indeed causally linked to the dynamics of *H. defensa* prevalence, either indirectly or directly. An indirect effect could result from high temperatures weakening the

strength of *H. defensa*-conferred protection against parasitoids (e.g. Bensadia *et al*., 2006; Cayetano & Vorburger, 2013a; Higashi *et al*., 2020). This would reduce the strength of parasitoid-mediated selection when periods of high parasitism coincide with periods of high temperature. A direct effect could result from *H. defensa* actually increasing heat tolerance, as has been suggested before for this (Russell & Moran, 2006) and other aphid symbionts (Chen *et al*., 2000; Guay *et al*., 2009; Heyworth *et al*., 2020; Montllor *et al*., 2002), or if temperature affected the reliability of the symbiont's maternal transmission, as has been observed for *Wolbachia* in *Drosophila melanogaster* (Hague *et al*., 2022). Finally, we should not discount the possibility that *H. defensa* has positive or negative ecological effects on *A. f. fabae* that were not considered here. For example, *H. defensa* can be a liability when facing predators (Dion *et al*., 2011; Polin *et al*., 2014), or affect the nutritional value of aphid honeydew with consequences for the mutualism between aphids and tending ants (Schillewaert *et al*., 2017). Generally, facultative endosymbionts can provide multiple functions (Guo *et al*., 2017), and we cannot rule out that certain *H. defensa* strains provide benefits that have yet to be discovered. The dynamics of *H. defensa* frequencies may thus be shaped by net selection forces determined by a mixture of parasitism risk and other factors. I therefore propose to widen the focus of future research on the symbiosis between *H. defensa* and *A. f. fabae* to include non-defense traits.

Specificity of *H. defensa*-conferred resistance

H. defensa-conferred resistance to *L. fabarum* is genotype-specific: the outcome of an attack by *L. fabarum* on an aphid carrying *H. defensa* depends on the specific combination of parasitoid and symbiont genotypes (G × G interactions). This has been reported before (Cayetano & Vorburger, 2013b; Cayetano & Vorburger, 2015; Schmid *et al*., 2012; Vorburger & Rouchet, 2016). However, to what extent this specificity can drive host-parasitoid coevolution (Hafer & Vorburger, 2019) hinges on how sensitive G × G interactions are to environmental variation; and on whether they indeed play out in natural communities in the field. I addressed these issues with the two experiments reported in Chapters I and III. These experiments confirmed the consistency of G × G interactions between *H. defensa* and *L. fabarum*, which is, first of all, valuable and reassuring in regard of the reproducibility of former results. In Chapter I, I additionally show that different host plants – while affecting the fitness of aphids and parasitoids – do not significantly alter

direction and strength of the recorded G × G interactions. In combination with the results from Cayetano and Vorburger (2013b), who showed that G × G interactions in the same system are stable at variable temperatures, this demonstrates the robustness of the observed genotypic interactions over different biotic or abiotic environments. However, the studies cited above, including my experiment in Chapter I, confront somewhat arbitrary combinations of *H. defensa* strains and parasitoids with no history of co-adaptation. This leaves open whether this extent of variation is actually found at the level of local field communities, which is another necessary pre-condition for dynamic symbiont-driven coevolution to occur (Vorburger & Perlman, 2018). In Chapter III, I addressed this question by exposing combinations of *H. defensa* strains and parasitoids to each other that actually co-occur in in the field, as determined in Chapter II. I demonstrate that the genetic variability in *H. defensa* strains and *L. fabarum* genotypes present in the local insect community indeed results in genotype-specific outcomes in parasitism assays. Chapter III therefore shows that strong genotype-specificity of symbiont-conferred resistance can be a hallmark of wild *A. f. fabae* populations. This, in turn, lends substance to the notion that *H. defensa* is a major player of dynamic aphid-parasitoid coevolution in nature (Kwiatkowski *et al*., 2012; Vorburger & Perlman, 2018).

Local adaptation of aphids to parasitoids and the role of *H. defensa* in the natural insect community

Many scientists attribute defensive symbionts a high potential to drive host adaptation, but convincing evidence is still limited. The maybe best-known example for 'adaptation by symbiosis' in the field regards the rapid spread of defensive *Spiroplasma* in *Drosophila neotestacea* across North America in response to the introduction of a parasitic nematode (Jaenike *et al*., 2010). In Chapter III, I show that *H. defensa* protects its aphid host against *L. fabarum*, the most frequent parasitoid species we recorded in the field (70% of collected parasitoids). This observation suggests the symbiont-driven adaptation of aphids to parasitoids, a pattern found also by Wu *et al*. (2022). Generally, this seems to be less frequently reported in animals interactions than the opposite pattern, i.e. parasite local adaptation to hosts (Greischar & Koskella, 2007; Hoeksema & Forde, 2008; Lively *et al*., 2004). Likely explanations for my finding are the higher migration ability and slightly shorter generation time of aphids compared to parasitoids (Gandon & Michalakis, 2002).

Could (symbiont-driven) host adaptation therefore be a general pattern in aphid-parasitoid interactions? Investigating on this is not trivial, since an experiment testing for patterns of aphid resistance like ours in Chapter III needs to be preceded by a survey on symbiont and parasitoid diversity, which implies a lot of work. Nevertheless, such experiments could accompany field surveys that hopefully will follow on more aphid species and different places of the world in the future.

On a community level, resistance that is specific to the dominant parasitoid may allow a larger number of additional parasitoid species or predators to profit from a given aphid resource. For example, both Hrcek *et al*. (2016) and Rothacher *et al*. (2016) showed that the presence of *H. defensa* resulted in a higher diversity of parasitoid species exploiting the aphids, because the specific protection provided by the symbiont broke the dominance of the most abundant parasitoid. This suggests a pivotal role of *H. defensa* in promoting food web structure and complexity (McLean *et al*., 2016).

Population genetics shed light on the composition of the *Aphis fabae* species complex

The diversity present in natural communities may often go beyond what our eyes can see. More than 100 years ago, aphidologists have detected that not every aphid that looks like a black bean aphid behaves the same (Börner & Janisch, 1922): different *A. fabae* individuals can be categorized as belonging to different, morphologically cryptic subspecies depending on their preference for certain summer host plants (e.g. Müller, 1982). In contrast, these subspecies seem to share their taste for the same winter host plant, the spindle tree *Euonymus europaeus* (Blackman & Eastop, 2017). For Chapter II, we therefore genotyped all samples collected from this host, which allowed us to separate our focal subspecies *A. f. fabae* from other taxa. The resulting dataset, however, comprised more information than that. For Chapter IV, I complemented it with samples from various other host plants. This allowed me to show that there are indeed four genetically distinct groups of *A. fabae* that regularly feed on the winter host *E. europaeus*. In addition, I determined two genetic groups that feed exclusively on another winter host, *Viburnum opulus*. The six genetic groups I found in total clearly differ in their preferences for different summer hosts, and some of them show strikingly different prevalences of the facultative symbionts *H. defensa* and *R. insecticola*. Both findings are suggestive of divergent ecological selection acting on the different *A. fabae* subspecies. Indeed, the fact

that multiple closely related taxa remain genetically distinct despite mating on a common winter host is remarkable, and suggests strong selection against hybrids. There is evidence for prezygotic mechanisms preventing reproduction between subspecies (Raymond *et al*., 2001; Thieme & Dixon, 1996), but in Chapter IV, I show that hybrids nevertheless occur in the field. However, hybrids are quite rare, and they occur mostly in spring, i.e. shortly after sexual reproduction, and most of them fail to return to the winter host plant in autumn. This suggests that postzygotic selection against hybrids is reinforcing genetic divergence between *A. fabae* subspecies.

There are multiple directions in which one could expand and improve on Chapter IV. If I had the possibility to do so, I would start by searching for black bean aphids feeding on the diagnostic summer hosts of *A. f. solanella* and *A. f. euonymi*, which I suppose represent the two genetic groups that we recovered on *E. europaeus* in addition to *A. f. fabae* and A. *f. cirsiiacanthoides*. Finding them on black nightshade, *Solanum nigrum* (for *A. f. solanella*) and on *E. europaeus* during summer (for *A. f. euonymi* that is monoecious and feeds on this shrub all year round) would confirm my hypothesis. This can still be done: as I found that both temporal and spatial differentiation within subspecies is very low compared to the differentiation between subspecies (Chapter IV), such samples – or any samples taken in the same geographic area – could probably be added on top of my dataset and analyzed jointly without any problems. For future collections it would be important to extract the DNA from at least some of the individuals non-destructively and preserve their bodies for morphological analysis, e.g. as microscope slides (Favret, 2005). This would have helped to identify the *V. opulus*-specific genetic cluster that I suspect to represent *A. viburni*, a species very similar to *A. fabae* for the naked eye in the field, but distinguishable by microscopic examination if I had preserved some specimens. More generally, I showed that microsatellite genotyping is suitable to distinguish black bean aphid taxa for which the resolution capacity of CO1 barcoding and/or morphology ends (Coeur d'acier *et al*., 2007; Coeur d'acier *et al*., 2014). Hence it would be worthwhile to design a study involving parallel genetic (using microsatellites) and morphological assessment of black bean aphids s. s. and s. l. collected from a wider range of their >100 host plants. This could be interesting from an ecological point of view to understand which plants are more 'specialist hosts' and which are suitable for a diversity of subspecies, and it would permit to further elucidate the still somewhat obscure taxonomy of the *A. fabae* complex.

Finally, it could be interesting to investigate the reasons for the variable endosymbiont frequencies we detected in different *A. fabae* subspecies. As some endosymbionts can provide aphids with the ability to feed on host plants that would otherwise be unsuitable (Hosokawa *et al.*, 2007; Tsuchida *et al.*, 2004; Wagner *et al.*, 2015), I wonder whether there could be a direct causal relationship between endosymbiont prevalence and the subspecies' variable performances on different host plants. To study this, I would start by considering those taxa with a very high prevalence of a particular symbiont, such as the presumed *A. viburni* (all collected samples carried *H. defensa*), or *A. f. euonymi* (92% carried *R. insecticola*). I would also be curious whether endosymbiont haplotypes, including those of the obligate symbiont *B. aphidicola*, are different among *A. fabae* taxa. Such information could provide more hints on their time of divergence, or the frequency of hybridization among taxa.

Conclusion

With my PhD thesis, I confirm the persistence of genotype-by-genotype interactions between *H. defensa* and the parasitoid *L. fabarum* in a variable environment (Chapter I), and I show that there is variation in both the defense provided by *H. defensa* strains and the susceptibility of parasitoids to this defense at the scale of a local population (Chapter II and III). I present an unprecedentedly fine-scaled overview on the parasitoid and endosymbiont communities in a natural population of *A. f. fabae* (Chapter II), which suggests that dynamics in the frequency of *H. defensa* might not be uniquely related to the risk imposed by parasitoids (Chapter II). Finally, I provide clear evidence for the genetic divergence of *A. fabae* subspecies feeding on different host plants (Chapter IV). These subspecies are also characterized by diverging facultative endosymbiont frequencies, which closes the loop by hinting at the dynamism of costs and benefits experienced by symbiont-infected aphids living in a diverse natural environment (Chapter IV).

Personally, studying symbiont-conferred resistance in the field taught me also many things that may not be put in a scientific publication, but that helped me develop a comprehensive understanding of my model system more than any laboratory experiment could. Following 'my' aphid populations throughout the seasons and realizing how they develop in synchrony with the vegetation, the agricultural calendar, and their natural antagonists, was an invaluable experience that aroused my curiosity and raised my awareness for the

involved ecological processes. Our field survey may have produced as many questions as it answered, but this can be seen as one of its major values: it challenges potentially simplistic ideas and motivates new research by reminding us that there is still a lot to discover on defensive symbiosis in the wild.

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Scientific publications

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- Gimmi, E., & Vorburger, C. (2021). Strong genotype-by-genotype interactions between aphid-defensive symbionts and parasitoids persist across different biotic environments. *Journal of Evolutionary Biology*, 34(12), 1944-1953. https://doi.org/10.1111/jeb.13953
- Stritt, C., Gimmi, E. L., Wyler, M., Bakali, A. H., Skalska, A., Hasterok, R., ... & Roulin, A. C. (2022). Migration without interbreeding: evolutionary history of a highly selfing Mediterranean grass inferred from whole genomes. *Molecular Ecology*, 31(1), 70-85. https://doi.org/10.1111/mec.16207
- Stritt, C., Wyler, M., Gimmi, E. L., Pippel, M., & Roulin, A. C. (2020). Diversity, dynamics and effects of long terminal repeat retrotransposons in the model grass *Brachypodium distachyon*. *New Phytologist*, 227(6), 1736-1748. https://doi.org/10.1111/nph.16308

Conference attendance and presentations (selection)

- ESEB, August 2022, Prague: poster "*Defensive symbiosis in the wild*"
- Bapoa, Mai 2022, Nice: poster "*Genotypes and symbiotypes in the* Aphis fabae *complex*"
- Biology, February 2022, Basel: poster "*Defensive Symbiosis in the wild*"
- Bapoa Webinar, September 2021, virtual: presentation "*Defensive symbiosis in the wild: a field study on seasonal dynamics of aphid friends and foes*"
- Evolution, June 2021, virtual: presentation "*Robust genotype-by-genotype effects between aphid endosymbionts and parasitoids on variable host plants*"
- Entomo.ch, March 2021, virtual: presentation "*Defensive symbionts protect aphids from parasitoid wasps depending on stable genotype-by-genotype interactions*"

Teaching and Supervision

- Co-Supervisor: research project of Severin Frei (Msc Student ETH, Biology), fall semester 2022/2023
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