

Symbiont-conferred resistance to parasitoids in aphids – challenges for biological control

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Remark:

Several Latin genus names occurring repeatedly in this manuscript start with the letter A (*Acyrtosiphon*, *Aphis*, *Aphidius*, *Aphelinus*, *Arsenophonus*). To avoid confusion, they were always written out rather than abbreviated as *A.* after first mention.

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Abstract

Parasitoid wasps are frequently employed to control pest aphids in greenhouse crops. However, aphids can be infected with heritable bacterial endosymbionts that strongly increase their resistance to parasitoid wasps. These defensive symbionts have the potential to compromise the effectiveness of biological control. Unfortunately, we still lack the necessary studies to assess their effects at the scale of greenhouses, but laboratory experiments indicate clearly that defensive symbionts represent a problem. Selection by parasitoids results in the rapid evolution of symbiont-conferred resistance, to the point that symbiont-protected aphid populations can escape control by parasitoids. The limited evidence available from field experiments is slightly less disquieting, suggesting that even though it is operational under natural conditions, protection by defensive symbionts does not always provide a net fitness benefit to the aphid hosts. Factors that can limit the success of symbiont-protected aphids are physiological and ecological costs of harboring defensive symbionts, as well as counteradaptations by parasitoids. Based on a review of the pertinent literature, I derive recommendations for mitigating the challenges resulting from the presence of defensive symbionts for the biological control of pest aphids. These include selective breeding of parasitoids as well as clever release strategies, potentially in combination with other biocontrol agents.

Keywords. Aphids, bacterial endosymbionts, biological control, defensive symbiosis, greenhouses, *Hamiltonella defensa*, parasitoids

1. Background: Aphid control by parasitoids and the discovery of defensive symbionts

Aphids are important agricultural pests of cereals, vegetable and fruit crops worldwide. They harm plants directly by feeding on phloem sap, and they transmit numerous plant viruses (Katis et al., 2007), including some of the economically most important ones (Tomlinson, 1987). Yield losses caused by aphids in Europe alone have been estimated to be in the range of hundreds of thousands or even millions of tons for certain crops like wheat, potatoes or sugar beets (Wellings et al., 1988), resulting in large economic damage (Dedryver et al., 2010).

Natural enemies that can reduce the negative impact of aphids include entomopathogenic fungi, predators such as adults and larvae of ladybird beetles, larvae of lacewings, hoverflies and predatory midges, as well as parasitoid wasps from the subfamily Aphidiinae (Hymenoptera: Ichneumonoidea: Braconidae) and the genus *Aphelinus* (Hymenoptera: Chalcidoidea: Aphelinidae). Due to their short generation time and high fecundity, parasitoids can be very effective in controlling aphids, and there is evidence that at least in certain crops such as cereals, parasitoids may be the most important group of natural enemies of aphids (Schmidt et al., 2003). Aphid parasitoids have indeed been used in classical importation biological control. Examples include the introduction of the Asian parasitoid *Binodoxys communis* in North America with the aim to control the invasive soybean aphid, *Aphis glycines* (Ragsdale et al., 2011), or the introduction of *Lysiphlebus testaceipes* from Cuba in France to control the exotic *Citrus* pests *Toxoptera aurantii* and *Aphis citricola* (Sary et al., 1988). However, active release of mass-reared parasitoids as an immediate treatment against aphid infestations is rarely attempted under field conditions, because the large numbers of individuals required and their rapid loss from treated sites by dispersal make this approach economically unviable compared to the application of insecticides (Boivin et al., 2012). Under

field conditions, aphid parasitoids are mainly promoted by cultivation measures enhancing the availability of alternative, non-pest hosts in the vicinity of crops, e.g. non-crop strips (Langer and Hance, 2004; Frère et al., 2007). The situation is different in protected crops, where the confined space allows for successful aphid control with parasitoids at affordable costs. Early inoculative or inundative releases of parasitoids and the application of banker plant systems (see Frank, 2010) have therefore become the methods of choice for aphid control in the greenhouse production of fruits and vegetables, supporting a growing industry of commercial suppliers of biocontrol agents (van Lenteren, 2012).

In this context, it came as a bit of a shock when the first demonstration of a defensive symbiosis in aphids was published (Oliver et al., 2003), showing that pea aphids (*Acyrtosiphon pisum*) can harbor heritable infections with bacterial endosymbionts that increase their resistance to the parasitoid *Aphidius ervi*, a commonly employed biocontrol agent. Two bacteria later named *Hamiltonella defensa* and *Serratia symbiotica* (Moran et al., 2005b) provided strong and moderate protection, respectively. Although a fascinating evolutionary phenomenon in its own right (Lively et al., 2005; Jones et al., 2011), it became clear immediately that defensive symbiosis is of applied importance as well, because it has the potential to compromise the effectiveness of biological control with parasitoids (Oliver et al., 2005). In this article I provide a coarse overview of the defensive symbionts known from pest aphids, I discuss the problems they may cause for biological control based on the evidence already available from laboratory and field experiments, and I derive recommendations to mitigate these problems.

2. The Menagerie

2.1. Facultative symbionts of pea aphids

The pea aphid is a long-standing model system used in numerous laboratories. It played an important role in the description of the primary symbiosis of aphids with the obligate bacterial endosymbiont *Buchnera aphidicola*, and it was among the first aphids in which facultative endosymbionts were discovered by microscopy (Buchner, 1965; Griffiths and Beck, 1973; McLean and Houk, 1973) and later identified by sequencing of 16S ribosomal RNA genes (Unterman et al., 1989). It was also the first aphid for which symbiont-conferred resistance to parasitoids was described (Oliver et al., 2003). Consequently, the community of facultative bacterial endosymbionts occurring in pea aphids is much better characterized than in any other aphid species. Table 1 lists the facultative bacterial endosymbionts presently known from pea aphids and the phenotypic effects they may exert on their hosts. This list is incomplete because future research may well discover more endosymbionts, and additional phenotypic effects of those already known will undoubtedly be documented. Nevertheless, it is already clear from this incomplete list that protection against natural enemies such as parasitoids and entomopathogenic fungi is not restricted to single symbiont taxa, and that the same symbiont can have multiple ecologically relevant effects (e.g. Cayetano et al., 2015; Heyworth and Ferrari, 2015). Some of these effects are beneficial to the host (protection against natural enemies, increased stress tolerance), others are detrimental (reduced lifespan and fecundity in the absence of natural enemies). This implies that it will depend on the ecological context (e.g. host plant quality, risk of parasitism, etc.), whether the possession of any give symbiont represents a net benefit or a net liability to the aphid host.

2.2 What about other aphid pests?

The pea aphid is an important biological model and it has been included by Blackman and Eastop (2007) in a list of the 14 aphid species of most agricultural importance, because it can cause significant damage in pea and alfalfa crops, for example. Yet compared to some other global aphid pests, it is probably of limited importance. What about the real nasties like the polyphagous *Myzus persicae* and *Aphis gossypii*, or the main cereal pests like *Rhopalosiphum padi*, *Diuraphis noxia* or *Schizaphis graminum* – are they also protected by defensive endosymbionts? It is still too early to provide a comprehensive answer to this question, but fortunately, more and more pest aphids are getting screened for endosymbionts, and a broad-scale survey using symbiont detection with diagnostic PCR (Henry et al., 2015) as well as a recent review by Zytynska and Weisser (2016) are beginning to shed light on this issue. I refer to these works for information about individual aphid species. The general picture starting to emerge is that the 'common suspects' like *H. defensa*, *R. insecticola* and *Serratia symbiotica* occur in numerous pest aphids, that some species of aphids can harbor additional symbionts not documented from pea aphids, and that phenotypic effects exerted by particular symbionts in pea aphids cannot always be generalized to other aphid species. To give some examples, *H. defensa* protects pea aphids against parasitoid wasps and has been detected in >50 additional aphid species (Henry et al., 2015; Zytynska and Weisser, 2016). While it is also protective against parasitoids in *Aphis fabae* and *Aphis craccivora* (Schmid et al., 2012; Asplen et al., 2014), other studies failed to demonstrate protection against parasitoids, e.g in the cereal aphid *Sitobion avenae* (Łukasik et al., 2013a) or in the potato aphid *Macrosiphum euphorbiae* (Clarke et al., 2016). Whether this is generally true for *H. defensa* in these species or whether this only applies to the particular strains included in these studies needs to be verified, because also in pea aphids it is possible to find strains that provide little or no protection (McLean and

Godfray, 2015; Leclair et al., 2016). Endosymbionts of the genus *Arsenophonus* have not been described from pea aphids, but seem to be common in aphids of the genus *Aphis* (Jousselin et al., 2013). They have been shown to modify dietary breadth in the polyphagous pest aphid *Aphis craccivora* (Wagner et al., 2015). Another facultative endosymbiont unknown from pea aphids is SMLS, which was discovered in Chinese *Sitobion miscanthi* (Li et al., 2011; 2016). Whether it has any ecologically relevant phenotypic effects on the host remains to be clarified. The important pest aphids *M. persicae* and *Aulacorthum solani* are interesting because they are rarely infected with facultative endosymbionts compared to other aphid species (von Burg et al., 2008; Henry et al., 2015). On the other hand, a strain of *R. insecticola* discovered in Australian *M. persicae* is strongly protective against parasitoid wasps (Vorburger et al., 2010), a trait generally not observed in *R. insecticola* strains from other aphid species (Oliver et al., 2003; Vorburger et al., 2009; Hansen et al., 2012).

3. Potential problems for biological control: laboratory and field evidence

Based on the limited evidence available, it appears that the occurrence and phenotypic effects of facultative bacterial endosymbionts differ among aphid species, but considering the ubiquity of these symbionts and the rapidly accumulating evidence that many of them can be protective, it is probably safest to assume for the moment that defensive symbiosis may occur in all major pest aphids. Hence it is important to ask whether this phenomenon can cause significant problems for biological control. Unfortunately, there is still a large discrepancy between the relatively comprehensive information available from laboratory experiments and the very limited information from field studies, as well as the virtual absence of studies at the greenhouse scale, where the problem might be most relevant and the information most urgently needed.

3.1. Evidence from laboratory experiments

The simplest experiments to demonstrate protection by bacterial endosymbionts involve comparing the susceptibility to parasites between lines with the same genetic background in the presence and absence of the symbionts. In aphids, such lines can be generated either by curing an infected clone of the symbiont with antibiotics or by introducing new heritable infections in previously uninfected clones by microinjection (Oliver et al., 2010). These approaches have been used successfully to demonstrate protection against parasitoids by *H. defensa*, *S. symbiotica* and X-type symbionts in pea aphids (Oliver et al., 2003; Heyworth and Ferrari, 2015), by *H. defensa* in *Aphis fabae* and *Aphis craccivora*, (Schmid et al., 2012; Asplen et al., 2014), and by a strain of *R. insecticola* in *M. persicae* (Vorburger et al., 2010). If we take such laboratory assays of resistance to parasitism as a guideline, there is no escaping the conclusion that defensive endosymbionts do have the potential to compromise biological control with parasitoids. There is variation in the strength of protection provided by different symbiont strains (e.g. Oliver et al., 2005; Cayetano et al., 2015), and protection may also depend on the species or even the genotype of the attacking parasitoid (e.g. Asplen et al., 2014; Cayetano and Vorburger, 2015; McLean and Godfray, 2015), but often the effects are very strong. For example, *H. defensa* can reduce parasitism by *Aphidius ervi* in pea aphids by up to 80% (Oliver et al., 2005), and in some cases even more (Oliver et al., 2009; McLean and Godfray, 2015). In black bean aphids (*Aphis fabae*), protection against their main parasitoid *Lysiphlebus fabarum* can be of similar magnitude (Schmid et al., 2012; Cayetano et al., 2015), and even complete resistance is commonly seen (Vorburger et al., 2009). Similarly, a protective strain of *R. insecticola* found in *M. persicae* provides essentially complete

resistance to this important pest against *Aphidius colemani* and *Diaeretiella rapae* (von Burg et al., 2008; Vorburger et al., 2010).

A straightforward prediction from these results is that symbiont-protected aphids should be under strong positive selection in the presence of parasitoids. This has been confirmed in a number of laboratory cage experiments with mixed populations of protected and unprotected aphids. Oliver et al. (2008) showed that pea aphid infection with *H. defensa* increased from 33% to near-fixation over the course of 12 weeks in cages containing *Aphidius ervi* (but decreased in cages without parasitoids). Similarly, a *R. insecticola*-protected clone increased from 10% frequency to nearly 100% in cage populations of *M. persicae* in the presence of *Aphidius colemani* over the course of only 8 weeks (Herzog et al., 2007). A very recent laboratory cage experiment by Sanders et al. (2016) addressed the influence of defensive symbionts in a complex but stable community of three aphid species growing on *Vicia faba* (*Acyrtosiphon pisum*, *Aphis fabae* and *Megoura viciae*), each with a specialized parasitoid (*Aphidius ervi*, *Lysiphlebus fabarum* and *Aphidius megourae*, respectively). Replacing the pea aphid clone with the same clone carrying an infection with a protective *H. defensa* strain completely disrupted the stability of the community. Protected pea aphids were so resistant that they escaped control by *Aphidius ervi* and subsequently outcompeted the other two aphid species to the point of extinction, thus removing their parasitoids from the cages as well (Sanders et al., 2016). The final result were cages infested with high densities of pea aphids only. Finally, in an unpublished experiment by Käch (2014), the frequency of *H. defensa*-protected clones in caged populations of black bean aphids increased rapidly from 33% to near-fixation in the presence of their parasitoid *L. fabarum*, resulting in an escape from control and even frequent extinctions of the parasitoids. All of these results are quite discouraging from a biocontrol perspective and imply that selection for resistance conferred by heritable endosymbionts may indeed disrupt aphid control by parasitoids. The important

question is whether such findings can indeed be scaled up from simple laboratory cage environments to the more complex situation of greenhouses, where biocontrol interventions take place. Unfortunately, this question is still largely unanswered.

3.2. Evidence from the field

There is some comparative evidence that defensive endosymbionts provide effective protection and reduce parasitism under field conditions. Already before defensive symbionts of aphids were described, laboratory assays have shown that pea aphids specialized on alfalfa are more resistant to their parasitoid *Aphidius ervi* than pea aphids specialized on clover (Hufbauer and Via, 1999). This is paralleled by lower mortality from *Aphidius ervi* in alfalfa than in clover fields (Smith et al., 2015), and presumably related to the fact that the alfalfa host race is more frequently infected with *H. defensa* than the clover host race (Frantz et al., 2009; Ferrari et al., 2012; Bilodeau et al., 2013; Smith et al., 2015). Temporal correlations between the frequency of infection with *H. defensa* and parasitism by *Aphidius ervi* are also indicative of protection in the field (Smith et al., 2015). A similar situation occurs in the cowpea aphid, *Aphis craccivora*, in which different host-associated populations also differ in the endosymbionts they possess. Cowpea aphids collected from alfalfa are predominantly infected with *H. defensa* and show low rates of parasitism by parasitoid wasps in the field, whereas cowpea aphids from locust (*Robinia pseudoacacia*) predominantly harbor a different facultative symbiont, *Arsenophonus*, and are more frequently parasitized by wasps (Brady and White, 2013). Cowpea aphids harboring *H. defensa* have indeed been shown to be more resistant to some (not all) parasitoids in the laboratory (Asplen et al., 2014). The lower parasitism of cowpea aphids from alfalfa is therefore suggestive of symbiont-conferred protection in the field, but it provides no proof because the presence or absence of *H. defensa*

is confounded with other factors that could influence parasitism rates, such as host genetic variation, host plant effects or a difference in parasitism risk between habitats.

Direct experimental evidence for the role of defensive symbionts in the field is surprisingly scarce and mainly comes from two recently published field experiments. Rothacher et al. (2016) set up replicated field plots containing broad beans (*Vicia faba*) with colonies of genetically identical *Aphis fabae* (a single clone) that either did or did not harbor *H. defensa*, and they followed their colonization by parasitoids over an entire growth season. Plots with *H. defensa*-free aphids yielded approx. fifteen times more mummies, i.e. successfully parasitized aphids, than plots with *H. defensa*-positive aphids (Fig. 1). Interestingly, the presence of *H. defensa* also had a strong effect on the species composition of emerging parasitoids. The dominant parasitoid, *L. fabarum*, comprised 84.5% of all identified parasitoids, but it was only obtained from plots with *H. defensa*-free aphids (Rothacher et al., 2016). These results demonstrate that protection by *H. defensa* is operational under natural conditions, and protection appears to be particularly effective against the parasitoids posing the highest risk. In another field experiment, Hrček et al. (2016) placed potted plants (*Lotus pedunculatus* and *Trifolium pratense*) carrying pea aphids with or without defensive symbionts into natural meadows for 10 days and returned them to the laboratory to score parasitism. One comparison was between a clone infected with *H. defensa* and the same clone cured from this symbiont, and it showed as well that *H. defensa* reduced parasitism, particularly by wasps of the genus *Aphelinus*. The other comparison was between a clone infected with *R. insecticola*, which is protective against fungal pathogens in the lab, and its cured counterpart. This comparison showed significant protection by *R. insecticola* against fungal pathogens in the field.

Both of these field experiments thus demonstrated that symbiont-conferred protection is effective in the field, but remarkably, both studies also found that this protection did not result in an overall benefit to the hosts. In the study by Rothacher et al. (2016), black bean aphids

with *H. defensa* did not reach higher population sizes than those without (Fig. 1), and in the study by Hrček et al. (2016), overall survival of pea aphids was not affected by *H. defensa* and even reduced significantly by *R. insecticola*.

It is possible that selection by parasitoids was simply not strong enough at these field sites to make a difference (Rothacher et al., 2016), but there were also indications that the protection might have been counteracted by costs of harboring the defensive symbionts, resulting in no net benefit of their possession (Hrček et al., 2016). From a biocontrol perspective this is important, because it implies that there are factors that can mitigate the problem of controlling symbiont-protected aphids. These are discussed in the next section.

4. Factors mitigating the problem

4.1. Costs of symbiont-conferred resistance

Resistance to parasitoids does not come for free to the insect host. We distinguish between constitutive costs of possessing the ability to resist and induced costs of using this ability when attacked (Schmid-Hempel, 2003). For example, flies (*Drosophila melanogaster*) selected for an increased ability to encapsulate parasitoid eggs suffer from reduced competitive ability (Kraaijeveld and Godfray, 1997), which is indicative of a constitutive cost, and flies mounting a successful immune response against parasitoids suffer from reduced starvation resistance (Hoang, 2001), which is consistent with an induced cost. The same might apply if resistance is not encoded by the host but conferred by bacterial endosymbionts.

In the case of the defensive symbiont *H. defensa*, evidence has accumulated that its possession is indeed associated with constitutive costs to the aphid host. When competing with uninfected members of the same clone in cages, pea aphids infected with *H. defensa*

declined over time in the absence of parasitoids, even though *H. defensa* did not have any significant effects on life-history traits measured in a separate experiment (Oliver et al., 2008). Such effects were, however, observed in black bean aphids. Experimental infections with various isolates of *H. defensa* significantly reduced aphid lifespan, which translated into reduced lifetime fecundity compared to uninfected aphids (Vorburger and Gouskov, 2011). Other studies hinting at costs of resistance conferred by *H. defensa* include Simon et al. (2011) in pea aphids and Dykstra et al. (2014) in *Aphis craccivora*.

Constitutive costs of symbiont-mediated resistance can also be ecological rather than physiological, because the symbionts may affect more than one ecologically relevant trait. For example, pea aphids infected with *H. defensa* exhibit a reduction of defensive behaviors against natural enemies (Dion et al., 2011a), which can result in increased susceptibility to predators like ladybird beetles (Polin et al., 2014). This could be an important effect when biological control of aphids employs a combination of parasitoids and predators (see below).

Costs of heritable defensive symbionts may also be induced upon attack by parasitoids. This could occur if parasitoid attacks triggered the deployment of defenses that do not discriminate sufficiently between host and parasitoid tissue. Unfortunately, knowledge about the mechanisms and dynamics of symbiont-mediated resistance is still very limited. Only in *H. defensa* has protection against parasitoids been linked to a specific mechanism, the presence of a toxin-encoding bacteriophage named APSE in the symbiont's genome (Oliver et al., 2009). It appears that these eukaryote-targeted toxins are expressed constitutively (Moran et al., 2005a), but APSE copy numbers do show signs of induction in that they tend to increase relative to *H. defensa* cell numbers in parasitized aphids (Martinez et al., 2014). Possibly this reflects increased *H. defensa* cell lysis by APSE and hence increased toxin release in response to parasitism.

A suggestive example of induced costs was recently published by Leclair et al. (2016). Pea aphids of a specialized biotype associated with the host plant *Genista tinctoria* are invariably infected by *H. defensa* and fully protected against *Aphidius ervi*, but this protection comes at a very high price. Following a parasitoid attack, the aphids survive because the development of the parasitoid larva is arrested at some point, but the aphids are also harmed in the process and lose virtually all their embryos (Leclair et al., 2016). Similarly, individuals of a strongly resistant clone of *M. persicae* protected by a defensive strain of *R. insecticola* were nearly sterilized after surviving attacks by the parasitoid *Aphidius colemani* (Vorbürger et al., 2008b). High induced costs can also counterbalance the benefits of symbiont-mediated protection and thus prevent or at least delay the fixation of defensive symbionts under selection by parasitoids (Kwiatkowski and Vorbürger, 2012).

4.2. Parasitoids can also harm resistant aphids

The strength of resistance conferred by some strains of defensive endosymbionts is astounding, but possessing a resistance-conferring symbiont does not mean aphids are completely unaffected by parasitoids. The example of Leclair et al. (2016) mentioned above showed that in certain cases the resistance comes at very high induced costs, such that aphids resisting a parasitoid still lose virtually all their fitness. While this may be the exception rather than the rule, it is reasonable to assume that being stabbed by parasitoids is harmful to aphids even if the parasitoid eventually fails to develop. Negative effects of failed parasitism attempts have been observed in aphids (Vorbürger et al., 2008a; 2008b), although one study on *Aphis fabae* reported the counterintuitive result that *H. defensa*-protected aphids surviving a single parasitoid attack enjoyed a slight fitness increase compared to controls (Vorbürger et al., 2013). The latter can probably not be generalized because it was found in a single host

clone-symbiont strain combination, and even in that case multiple oviposition attempts would presumably still be harmful. The general disturbance from foraging parasitoids is also likely to reduce aphid fitness. Aphids respond to the perceived presence of parasitoids by flight, either by dropping off the host plant or by crawling away from the feeding site (Villagra et al., 2002; Dion et al., 2011a). Frequent disturbance can thus prevent aphids from feeding and may eventually result in starvation. This was suggested in a still unpublished experiment by Hertäg (2016), in which caged populations of two aphid species (*Aphis fabae* and *M. persicae*) competed on the same host plants in the presence of a joint parasitoid, *Aphidius colemani*. When both species were unprotected by symbionts, they were successfully controlled and driven to extinction by *Aphidius colemani*. When both species were protected by a defensive strain of *R. insecticola*, the aphids escaped control and both aphid species persisted. When only one of the species harbored the symbiont, however, control was successful again and the unprotected as well as the symbiont-protected species were suppressed. Apparently, the susceptible aphid species supported such high densities of parasitoids that they killed the resistant aphids as well, presumably by their stabbing and/or by disturbing them to the point of starvation.

This has implications for biological control in greenhouses. If very high densities can be achieved in a greenhouse, parasitoids may be able to control symbiont-protected aphids as well. However, obtaining such high densities by inundative releases is likely to be prohibitively expensive. As the example by Hertäg (2016) showed, high parasitoid densities can be obtained if a susceptible aphid population of sufficient size is present in the same environment. This could be achieved with banker plant systems, although I suspect that conventional systems would have to be greatly enlarged to cover a substantial proportion of the available greenhouse space for producing parasitoid densities high enough to control

resistant aphids on the crop simply by 'stressing them to death'. Also this would come at a significant economic cost.

4.3. Parasitoid adaptation to symbiont-conferred resistance

The evolution of resistance to parasitoids via selection for symbiont-protected clones is in a sense similar to the evolution of insecticide resistance that has plagued the control of aphids and other arthropod pests for decades (Devonshire et al., 1998; Hoy, 1998; ffrench-Constant et al., 2004). An important difference, however, is that parasitoids have the capacity for counteradaptation to their hosts' defenses, including defenses conferred by endosymbionts. This has been demonstrated using experimental evolution in the laboratory. When forced to develop on pea aphids protected by *H. defensa*, experimental populations of *Aphidius ervi* showed a rapid increase in infectivity, reaching similar rates of parasitism as on unprotected aphids within only four generations (Dion et al., 2011b). Using a similar approach with black bean aphids and *L. fabarum*, Rouchet and Vorburger (2014) also documented rapid counteradaptation of parasitoids to the presence of *H. defensa* in their hosts, although even after 11 generations of experimental evolution, parasitism success remained somewhat lower than on unprotected aphids. More importantly, parasitoid counteradaptation was highly specific to symbiont strains, which was discovered because the three isolates of *H. defensa* employed in the experiment comprised two different genotypes. Parasitoids experimentally adapted to aphids infected with the first genotype remained uninfective on aphids harbouring the second, and *vice versa* (Rouchet and Vorburger, 2014).

The rapid response to selection seen in these experimental evolution studies suggests that natural populations of aphid parasitoids harbor considerable genetic variation for the ability to overcome symbiont-mediated resistance. This is also supported by assays using numerous

field-collected lines of *L. fabarum* (e.g. Cayetano et al., 2015; Vorburger and Rouchet, 2016), and it presumably reflects the diversity of symbiont-protected and unprotected hosts these parasitoids encounter in their natural environment. A high evolutionary potential to adapt to defensive symbionts is encouraging for biological control with parasitoids. It implies that parasitoids may be able to track the evolution of symbiont-conferred resistance in aphids at least partially, and that parasitoid performance against symbiont-protected aphids could be improved by selective breeding. That said, the high specificity observed in the *Aphis fabae*-*L. fabarum* interaction (Rouchet and Vorburger, 2012; 2014) also implies that different strains of defensive symbionts may require specific counteradaptations by the parasitoids, such that there will be no 'silver bullet' to fight any symbiont-protected aphids. Furthermore, it is questionable whether the current approaches for the production of parasitoids for biological control of aphids do maintain the genetic variation required for parasitoids to adapt to the presence of defensive symbionts in their aphid hosts (see 'Practical Recommendations' below).

5. Research directions

Research on defensive symbioses is a young field but growing rapidly, not least because of the public health benefits that are hoped for from employing endosymbionts to reduce disease transmission by arthropod vectors (Bull and Turelli, 2013). Swift progress can be expected especially for the mechanistic understanding of defensive symbioses. Sequencing technology has developed to the point that entire bacterial genomes can be sequenced quickly and at affordable costs, e.g. to look for genomic differences between protective and non-protective strains of endosymbionts. Such approaches are being applied to defensive endosymbionts of aphids and are starting to yield useful insights (Oliver et al., 2009; Hansen et al., 2012). It is important to realize, however, that comparative genomics can only help to generate

hypotheses about molecular mechanisms underlying the phenotypes exerted by endosymbionts. The functional testing of these hypotheses remains a tedious process because endosymbiotic bacteria are generally not culturable and therefore not amenable to genetic manipulation.

A functional understanding of the defensive symbioses occurring in aphids will undoubtedly have its uses for biological control as well, but detailed knowledge about the molecular mechanisms employed by the different bacteria may not be the most crucial information. With respect to challenges arising from defensive symbiosis for biological control, I see mainly two knowledge gaps that should be closed soon to make progress. One is the frequency of infection and the strain diversity of defensive symbionts found in the major pest aphids at a global scale, and the other is the evolution of symbiont-conferred resistance that may occur under current biological control with parasitoids.

5.1. Global aphid pests and their defensive symbionts

Of the approximately 4000 aphid species estimated to occur worldwide, only about 100 are considered pests (Dedryver et al., 2010), and even fewer cause major economic damage at a global scale. It is for these species that we most urgently need comprehensive knowledge about protection by defensive symbionts to inform biological control strategies. Blackman and Eastop's (2007) list of '14 aphid species of most agricultural importance' would be an obvious starting point, although there might be good reasons to include additional species as well.

It is relatively straightforward to screen aphids for the presence of known bacterial endosymbionts using diagnostic PCR with symbiont-specific primers. Such primers are available for numerous endosymbiont species and typically amplify part of the 16S ribosomal RNA gene, (e.g. Ferrari et al., 2012). although other genomic targets could also be used. By

multiplexing primer pairs for multiple symbionts in a single PCR reaction, cost-effective surveys of relatively large samples are readily possible (Peccoud et al., 2014; Vorburger et al., 2017). However, by using diagnostic PCR, only previously characterized endosymbionts can be detected, and mutations in the primer binding sites of more divergent strains can lead to false negatives even for known symbionts. Unless PCR amplicons are sequenced, diagnostic PCR will only provide presence-absence data, but no information about strain variation. This is a problem because the same symbiont taxa can comprise protective and unprotective strains against parasitoids and other natural enemies (e.g. Hansen et al., 2012; Łukasik et al., 2013c; McLean and Godfray, 2015; Leclair et al., 2016). For a global survey of symbiont infections in pest aphids, a standardized methodology should be developed that overcomes these shortcomings while remaining easy and affordable to implement.

A promising approach for this purpose is deep sequencing of PCR amplicons generated with generic primers, which has already been applied to variable regions of the 16S ribosomal RNA gene in pea aphids (Russell et al., 2013; Gauthier et al., 2015) and in multiple species of the genus *Cinara* (Jousselin et al., 2016). Endosymbionts typically occur at such high densities that they can be distinguished from environmental bacteria based on sequence counts (Jousselin et al., 2016), the use of generic primers allows for the discovery of previously unknown symbionts, and the large amounts of sequence data can be used to quantify strain diversity and reconstruct evolutionary relatedness among symbiont strains. Ideally, multiple bacterial loci would be targeted by amplicon sequencing, and for symbionts where this is possible, it could even be considered to slot in loci that are known or suspected to affect the defensive phenotype (e.g. APSE phage variants in *H. defensa*, Weldon and Oliver, 2016). A concerted effort to obtain a comprehensive picture of the occurrence and strain diversity of facultative endosymbionts in the major pest aphids worldwide would do a great service to the

research community interested in defensive symbiosis, and it would provide an invaluable resource for efforts to improve biological control of these global pests.

5.2. The evolution of symbiont-conferred resistance under current biocontrol practice

Laboratory cage experiments have shown that selection by parasitoids can lead to the rapid evolution of symbiont-mediated resistance, sometimes to the point that parasitoids are no longer able to control the resistant, symbiont-protected aphids (see above). It is important to establish if and to what extent this process is ongoing and potentially compromising pest control under biocontrol practices currently in use in protected crops. A simple approach to obtain at least a preliminary insight would be to compare the prevalence of defensive symbionts in greenhouse populations of pest aphids with those of field populations in the same geographic region. More informative would be to follow the temporal dynamics of infections with symbionts in greenhouse infestations, starting prior to the first release of parasitoids and continuing through the biocontrol intervention. This represents a significant effort because such a monitoring would need to be replicated (at the level of greenhouses) and compared with appropriate controls, ideally with untreated greenhouses containing the same crop, or alternatively, with greenhouses receiving conventional insecticide treatment, if no control is not an option for economic reasons. This monitoring could be complemented by collecting a random sample of clones at the beginning and at the end of biocontrol interventions and testing their susceptibility to the parasitoids that were released, in order to quantify eventual resistance evolution also at the phenotypic level. I realize that this may seem challenging, but I see no alternative to greenhouse-level experimentation if we are to obtain a realistic appreciation if and to what extent the evolution of symbiont-conferred resistance in pest aphids is hampering biological control with parasitoids under current practice.

6. Practical recommendations

Despite the need for further research described above, the knowledge currently available allows for some practical recommendations for biological control of pest aphids in the face of their protection by defensive symbionts. These recommendations concern the production of parasitoids for biological control as well as their application in the crop.

6.1. Recommendations for parasitoid production

It has been demonstrated that natural populations of aphid parasitoids harbor substantial genetic variation for the ability to overcome symbiont-conferred resistance and are thus able to adapt to the presence of defensive symbionts in their hosts. In an experimental setting, this counteradaptation by parasitoids happened surprisingly quickly, resulting in substantial improvement of parasitoid infectivity on symbiont-protected hosts over just 4-5 generations (Dion et al., 2011b; Rouchet and Vorburger, 2014). That is a timescale relevant for real biocontrol interventions, suggesting that released parasitoids can adapt to symbiont-protected aphids, provided they possess the required evolvability. A first recommendation for parasitoid production is therefore very simple: Maintain high genetic diversity. This can be achieved by starting the breeding stocks from a broad genetic basis of numerous and diverse field accessions, and by maintaining breeding stocks at a high effective population size to prevent the erosion of this genetic diversity over time.

More specifically, it would be important that the genetic variation of commercially produced parasitoids comprises alleles relevant for overcoming symbiont-conferred resistance. This may be more difficult to achieve because mass-rearing of parasitoids takes

place under very standardized conditions on highly susceptible (hence unprotected) aphids to maximize yield. Such conditions could potentially select against alleles that are beneficial on symbiont-protected hosts, although this remains to be tested. One recommendation to counteract this risk is employing repeated admixture from the wild. Present evidence suggests that natural populations of aphid parasitoids do possess the necessary genetic variation for adaptation to hosts with defensive symbionts (e.g. Dion et al., 2011b; Vorburger and Rouchet, 2016). Regular supplementation with parasitoids from the field could thus ensure that this variation also remains present in commercial breeding stocks. It is worth considering, though, that this approach may not be without risks. Parasitoids can themselves have endosymbionts. *Wolbachia*, for example, is common in hymenopteran parasitoids, and some strains of *Wolbachia* manipulate host reproduction by inducing cytoplasmic incompatibility (Cook and Butcher, 1999). This could cause problems in parasitoid rearing. Keeping new field accessions separate initially to do the necessary tests to exclude this risk would be a sensible precaution.

An alternative and possibly more effective strategy would be to breed part of the parasitoid population on symbiont-protected aphids. The yield from such cultures would be low initially, but laboratory selection for a sufficient number of generations should accomplish rates of mummification comparable to normal breeding (Dion et al., 2011b; Rouchet and Vorburger, 2014). Mixing these with conventionally produced mummies could ensure that each release contains individuals preadapted to symbiont-protected aphids. The main problem for this approach would be the high genetic specificity of symbiont-conferred resistance (e.g. Schmid et al., 2012). It is demonstrably feasible to adapt parasitoids to particular strains of defensive symbionts by selection, but this would not necessarily improve their effectiveness if the targeted aphid infestation is protected by different strains (Rouchet and Vorburger, 2014).

Let me therefore develop the thought of selective breeding even further. If symbiont-conferred resistance to parasitoids in the most problematic pest aphids of greenhouse crops is

due to an overseeable number of symbiont species or strains – future research will have to show if this is the case – specialized parasitoids could be produced against the main defensive symbionts. The business of controlling aphids with parasitoids might then develop from one of mass-production and release to one of diagnosis and delivery of customized biocontrol agents. Analyzing a sample of the early colonizers would tell which defensive symbionts protect the aphids and inform the choice of parasitoids specifically suited to control them. These are clearly still dreams of the future, but the increasing ease, speed and affordability of molecular diagnostics could eventually make such a targeted approach feasible.

6.2. Recommendations for parasitoid application

Laboratory cage experiments have shown that parasitoid releases can select very rapidly for symbiont-protected aphids (see above). Assuming that this observation is transferable to greenhouse environments, the challenge for biological control is to keep an increasingly resistant aphid population in check. A possible way to avoid the application of insecticides when aphids are starting to escape control by parasitoids would be the combined release of parasitoids and predators. Ideally, early release of parasitoids would keep aphid densities low and predators would take out the resistant part of the aphid population.

There is some evidence that facultative endosymbionts of aphids can also have negative effects on predators. Larvae of the ladybird beetle *Hippodamia convergens* show somewhat higher mortality when they are fed pea aphids containing *H. defensa* or *S. symbiotica* than when they are fed pea aphids without these symbionts (Costopoulos et al., 2014). However, this does not deter beetle feeding, hence they remain effective as biocontrol agents against symbiont-protected aphids. Because pea aphids infected with *H. defensa* show reduced

defensive behavior (Dion et al., 2011a), predators might even be more effective against aphids possessing this symbiont (Polin et al., 2014).

What might hamper the success of combined parasitoid and predator releases, on the other hand, is intraguild predation (Polis et al., 1989). It is known that by consuming developing parasitoids inside aphids and mummies or via behavioral effects, predators can disrupt aphid control by parasitoids (e.g. Raymond et al., 2000; Snyder and Ives, 2001). However, examples where the combined effects of parasitoids and predators surpass that of parasitoids alone can also be found in the literature (e.g. Snyder et al., 2004; Gontijo et al., 2015). I would argue that the negative effects of intraguild predation are diminished if a high proportion of the aphid population carries defensive symbionts, because the majority of parasitoids will anyway fail to develop in protected hosts, and that the problem could possibly be mitigated further by clever timing of parasitoid and predator releases. Using parasitoids against the buildup of aphid populations initially, and employing predators against the resistant, symbiont-protected aphids spared by parasitoids at a later stage, might be a successful strategy. Empirical tests are needed to either support or refute this conjecture.

The final recommendation is simply to aim for high parasitoid densities in biological control of pest aphids. If numerous enough, parasitoids can also control resistant aphids by 'stressing them to death' (Hertäg, 2016). I am aware that this recommendation is difficult to implement because parasitoids are expensive to purchase. Nevertheless, relatively high parasitoid-to-aphid ratios are beneficial even if aphids are unprotected by symbionts, and any measures to augment these ratios would also help to control symbiont-protected aphids. These could include releasing the parasitoids early enough to hit beginning infestations while densities are still low (Neuville et al., 2016), which is already practiced (Van Driesche and Heinz, 2004), or the supply of food resources to augment parasitoid lifespan and fecundity (Tylianakis et al., 2004). Particularly promising seems the application of banker plant systems

(Frank, 2010). They allow the early establishment of parasitoid populations to meet the first colonizers of the targeted pest aphids, and they can ensure the continued availability of a susceptible host population in the greenhouse. This could be important to keep the pressure up throughout the crops' growth period. Selection by parasitoids can result in the rapid increase of symbiont-conferred resistance in the pest aphid (Herzog et al., 2007; Oliver et al., 2008; Sanders et al., 2016), which in turn can entail a decline of parasitoid densities in the absence of alternative, susceptible hosts (Käch, 2014). Banker plant systems may have the potential to prevent this decline, but there are still many uncertainties. For one, it is currently unclear how large banker plant systems would need to be to fulfil this role. They may require too much space to be economically viable. Another concern is that the evolution of symbiont-conferred resistance is a real risk also in alternative hosts on banker plants, although this could probably be prevented easily by supplying alternative hosts from carefully controlled stocks.

7. Conclusions

After the discovery of the first defensive symbionts in pea aphids, research on defensive symbiosis in aphids has burgeoned and continues to grow. Although still far from comprehensive, current knowledge suggests that facultative bacterial endosymbionts occur in most, if not all, pest aphids, and that several of them can provide their hosts with protection against parasitoids and other natural enemies. Laboratory experiments indicate that protection against parasitoids can be strong enough to represent a challenge for biological control. Parasitoids rapidly select for protection by symbionts such that the remaining resistant aphids can escape control by parasitoids. The limited evidence available from field experiments paints a somewhat different picture. While clearly effective under natural conditions, protection by defensive symbionts may not always result in selection for infected aphids.

Likely mitigating factors include physiological or ecological costs associated with the possession of defensive symbionts. However, most parasitoid releases for controlling pest aphids do not take place in the field, but in greenhouses. A key question to answer is therefore whether with respect to the evolution of symbiont-conferred resistance, the situation in greenhouse crops comes closer to a field or a laboratory situation. Unfortunately, this is still an open question. My gut feeling is that the confined space, the simplified habitat with typically a single crop at a high density, and the relatively constant and benign physical conditions make the greenhouse environment sufficiently similar to laboratory environments to justify concerns about the evolution of symbiont-conferred resistance. However, that the term gut feeling is used in a scientific review is in itself disconcerting, and such an important questions should certainly not rely on the gut feeling of a single researcher with virtually no practical experience in applied biocontrol. This review is therefore also a plea for increased research on the effects of defensive symbiosis in aphids at a greenhouse scale.

To end on a positive note: Even if defensive symbionts turn out to be a major challenge for biological control of aphids in greenhouses, recent research results imply that clever breeding and application of biocontrol agents have the potential to mitigate the problem. Defensive symbiosis is such an active field of research at the moment that new ways to address the challenge that were not discussed in this review will become evident in due course.

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Table 1. List of facultative bacterial endosymbionts presently known to occur in pea aphids with demonstrated phenotypic effects on their hosts (where applicable) and key references.

Facultative endosymbiont	Phenotypic effects	References
<i>Hamiltonella defensa</i>	Protection against parasitoids	(Oliver et al., 2003, 2005; Ferrari et al., 2004; McLean and Godfray, 2015)
	Protection against heat stress	(Russell and Moran, 2006)
	Reduced lifespan	(Simon et al., 2011)
	Reduced fecundity	(Simon et al., 2011)
	Reduced competitive ability	(Oliver et al., 2008)
	Reduced defensive behavior	(Dion et al., 2011a; Polin et al., 2014)
<i>Regiella insecticola</i>	Protection against fungal pathogens	(Ferrari et al., 2004; Scarborough et al., 2005; Parker et al., 2013)
	Protection against parasitoids	(Hansen et al., 2012)
	Host plant specialization	(Tsuchida et al., 2004; Ferrari et al., 2007)
	Reduced heat tolerance	(Russell and Moran, 2006)
	Reduced production of winged morphs	(Leonardo and Mondor, 2006)
	Reduced production of sexual morphs	(Leonardo and Mondor, 2006)

<i>Serratia symbiotica</i>	Protection against parasitoids	(Oliver et al., 2003)
	Protection against heat stress	(Montllor et al., 2002; Russell and Moran, 2006)
	Delayed development	(Koga et al., 2004)
	Reduced body weight	(Koga et al., 2004)
	Reduced competitive ability	(Oliver et al., 2008)
<i>Rickettsia</i>	Protection against fungal pathogens	(Łukasik et al., 2013b; Łukasik et al., 2013c)
	Reduced fecundity	(Sakurai et al., 2005; Simon et al., 2007; Łukasik et al., 2013b)
	Reduced lifespan	(Simon et al., 2007)
	Reduced body weight	(Sakurai et al., 2005)
<i>Rickettsiella</i>	Protection against fungal pathogens	(Łukasik et al., 2013b)
	Alteration of body color	(Tsuchida et al., 2010; Tsuchida et al., 2014)
	Increased body weight	(Tsuchida et al., 2014)
	Reduced time to reproduction	(Tsuchida et al., 2014)
<i>Spiroplasma</i>	Protection against fungal pathogens	(Łukasik et al., 2013b; Łukasik et al., 2013c)

	Reduced lifespan	(Fukatsu et al., 2001; Simon et al., 2011)
	Reduced fecundity	(Fukatsu et al., 2001; Simon et al., 2011)
	Male killing	(Simon et al., 2011)
X-type/PAXS	Protection against fungal pathogens	(Heyworth and Ferrari, 2015)
	Protection against parasitoids	(Guay et al., 2009; Heyworth and Ferrari, 2015; 2016)
	Protection against heat stress	(Guay et al., 2009; Heyworth and Ferrari, 2015)
	Reduced fecundity	(Heyworth and Ferrari, 2015)
<i>Wolbachia</i>	Unknown	(Russell et al., 2013; Wang et al., 2014; Gauthier et al., 2015)

Figure legends

Fig. 1. Defensive symbionts protect aphids against parasitoids in the field. Counts of black bean aphids (*Aphis fabae*) and successfully parasitized aphids (Mummies) from a field experiment in which plants (*Vicia faba*) colonized by aphids that were (A) uninfected, or (B) experimentally infected with the facultative endosymbiont *Hamiltonella defensa* were exposed to natural colonization by parasitoids over one field season (May – September 2013). Each treatment was applied to five replicate plots. Values represent natural log-transformed counts \pm 1 SE. Reproduced with permission from Rothacher et al. (2016).

Figure 1

