

Received Date : 31-May-2016

Revised Date : 18-Aug-2016

Accepted Date : 29-Sep-2016

Article type : Standard Papers

Editor: Hefin Jones

Section: Evolutionary Ecology

**The outcome of competition between two parasitoid species is influenced by a facultative symbiont of their aphid host**

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Running headline: Symbiont affects multiparasitism

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1111/1365-2435.12781

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## Summary

1. Symbiotic bacteria can act to protect their host against natural enemies. Where this protection is asymmetric against different natural enemies protection conferred by symbionts has the potential to mediate interactions between natural enemies, as well as between enemies and the host.

2. In pea aphids (*Acyrtosiphon pisum*), resistance against parasitoid wasps can be conferred by facultative symbiotic bacteria. We investigated whether the outcome of competition between two parasitoid species can be influenced by the presence of a defensive symbiont in the host.

3. We exposed pea aphids from a single clonal line, with and without a strain of the protective endosymbiont *Hamiltonella defensa*, to multiparasitism by the parasitoid wasps *Aphelinus abdominalis* (Aphelinidae) and *Aphidius ervi* (Braconidae), and recorded the outcome. The symbiont strain is known to impact *A. abdominalis* more strongly than *A. ervi*.

4. We found that the presence of a strain of the protective endosymbiont *Hamiltonella defensa* can reverse the outcome of competition between the wasps. In the absence of the symbiont, *A. ervi* gains very little success when attacking an aphid previously parasitized by *A. abdominalis*. However, where the aphids possessed the symbiont, *A. abdominalis* did not develop successfully, and the success rate of *A. ervi* was significantly increased.

5. Our results show that defensive facultative symbionts are able to influence community interactions at the trophic level above their host.

**Keywords:** aphid, facultative symbiosis, intrinsic competition, parasitoid, resistance, symbiont

## Introduction

A large proportion of insects carry symbiotic bacteria (Duron & Hurst 2013). In some cases, the relationship is obligate and mutualistic (for example, bacteria may provide essential nutrients missing in their hosts' diets (Douglas 2009)) while in others it is facultative, with not all individuals infected. Facultative symbionts can have important effects on their hosts' biology, including imparting resistance to natural enemies (Haine 2008; Brownlie & Johnson 2009). Symbiont-mediated defence has evolved across a broad taxonomic range of bacteria (Duron & Hurst 2013) and against a wide variety of natural enemies, including viruses (Hedges *et al.* 2008), fungal pathogens (Scarborough, Ferrari & Godfray 2005; Łukasik *et al.* 2013b), predators (Kellner 1999; Piel, Höfer & Hui 2004) and parasitoids (Oliver *et al.* 2003; Oliver, Moran & Hunter 2005).

The majority of experimental work on understanding symbiont-mediated protection has focussed on interactions between one host and one natural enemy (Brownlie & Johnson 2009). Protective facultative symbionts can have an important effect on the outcome of these interactions, with carriage of some symbionts providing almost complete immunity (Oliver, Moran & Hunter 2005). However, symbionts cannot provide protection against all natural enemies and thus their presence can alter the relative value of their host to its different adversaries. This will be particularly true if they alter the outcome of competition between natural enemies when two species attack or infect the same host. As yet we have little data to assess the possible effects of symbionts on insect community structure and food webs, but their impact on interactions in the laboratory suggests that their influence might be significant (Ferrari & Vavre 2011; Oliver, Smith & Russell 2014).

The pea aphid (*Acyrtosiphon pisum*) has become a model system for research on facultative symbiosis. Seven different facultative symbionts have been recorded commonly from pea aphids, and of these, six have been shown to affect host resistance to natural enemies. At least

some strains of four species confer protection against fungal pathogens (Scarborough, Ferrari & Godfray 2005; Łukasik *et al.* 2013b), while two can defend their host against parasitoid wasps (Oliver *et al.* 2003; Oliver, Moran & Hunter 2005). Of the latter, *Hamiltonella defensa* most strongly increases aphid resistance to parasitoids (Oliver, Moran & Hunter 2005), probably via the production of phage-encoded toxins (Degnan & Moran 2008b; Degnan & Moran 2008a; Oliver *et al.* 2009). The different types of phage carried by this symbiont appear to vary in the protection they confer against different parasitoid species (Oliver, Moran & Hunter 2005; McLean & Godfray 2015), although the mechanistic basis for this variation is not yet understood.

Pea aphids are attacked by a range of hymenopteran parasitoid species, which can compete for access to hosts (Müller *et al.* 1999; van Veen *et al.* 2008). Competition between parasitoids attacking the same host can be extrinsic or intrinsic (Harvey, Poelman & Tanaka 2013; Cusumano, Peri & Colazza 2016). Extrinsic competitive interactions are chiefly behavioural and involve adult parasitoids either directly interacting with each other or responding to semiochemicals deposited by other individuals. Intrinsic competition involves the immature parasitoids as they develop on or inside a common host. If two parasitoids of the same species lay eggs on the same host then ‘superparasitism’ is said to occur while if the parasitoids are of different species it is referred to as ‘multiparasitism’ (Godfray 1994).

When multiple solitary parasitoids are laid in or on a single host, only one will be able to emerge successfully. In some instances, different parasitoid species can be ranked in a fixed competitive hierarchy (Chow & Mackauer 1984; Bai & Mackauer 1991), although the relative timing of the attacks may also be important (Chow & Mackauer 1985; Bai & Mackauer 1991; Moretti & Calvitti 2008). In most circumstances even the superior competitor suffers from the presence of a rival, for example because of increased development time (McBrien & Mackauer 1990; Harvey, Gols & Strand 2009), and this

appears to have driven the evolution of discrimination and avoidance of previously parasitized hosts in a number of species (Bai & Mackauer 1991). In addition, both the host insect species, and the plant species on which the host is feeding, can influence the outcome of intrinsic competitive interactions between parasitoids (Poelman *et al.* 2014). The observation that particular defensive symbiont strains protect against different parasitoid species (Asplen *et al.* 2014; Cayetano & Vorburger 2015; McLean & Godfray 2015) suggests that their presence may also influence the outcome of multiparasitism, though whether this occurs is presently unknown.

In this study, we test whether the result of intrinsic competition between two taxonomically distant parasitoid species (*Aphidius ervi* [Braconidae] and *Aphelinus abdominalis* [Aphelinidae]) can be affected by the presence of protective endosymbiotic bacteria in their common host, and thus whether endosymbionts may impact on community interactions at higher trophic levels. We are unaware of previous studies of multiparasitism by these species and so first investigated which species tended to win when parasitising first or second before going on to explore how this was affected by the presence of a defensive symbiont.

## Materials and Methods

### *Experimental organisms*

The pea aphid clone used in our experiments was collected from *Lotus pedunculatus* in Berkshire, UK during summer 2008 and had subsequently been maintained in the laboratory on leaves of *Vicia faba* at 14°C with 70-80% humidity and a 16:8h light:dark cycle.

Immediately after collection, the aphid clone was tested for all known pea aphid secondary symbionts using diagnostic primers (see Russell and Moran (2005) for primer details) and was found to carry only *H. defensa*. The clone's genetic affiliation to the pea aphid biotype feeding on *L. pedunculatus* was confirmed using microsatellite typing (Peccoud *et al.* 2009).

Three years prior to the work reported here, a sub-line of “cured” aphids without the secondary symbiont was created using oral administration of antibiotics as described in McLean *et al.* (2011). The infection status of both the naturally-infected and the cured lines were reconfirmed immediately before the start of the experimental work (using primers and conditions in Henry *et al.* (2013)). A previous study had shown that the strain of *H. defensa* carried by our experimental clone gives almost complete protection against the parasitoid *A. abdominalis* and weaker protection against *A. ervi* (McLean & Godfray 2015).

The *A. ervi* and *A. abdominalis* wasps used in the experiment were taken from inbred cultures maintained in our laboratory (*A. abdominalis* for four months; *A. ervi* for more than five years) on a pea aphid clone (referred to below as the ‘stock clone’) that was highly susceptible to both wasp species and lacked any known secondary symbionts. The stock clone was collected from *Lathyrus pratensis*, and is not closely related to the experimental clone. Cultures of both wasp species were kept in a controlled temperature room at 20°C with 70-80% humidity and a 16:8h light:dark cycle; these were also the conditions under which the experiments were carried out. Wasps for experiments were obtained by placing mated females for 24 hours in a 30cm<sup>3</sup> Perspex cage (*A. ervi*) or 9cm Petri dishes (*A. abdominalis*) with stock clone aphids feeding on *V. faba* plants or leaves, respectively. The difference in treatment here is due to the differences in size and behaviour between the two species: *A. ervi* are unsystematic in their manner of attack, and benefit from a larger cage to permit flying, while *A. abdominalis* are smaller and more methodical in approach, and rarely fly. Using Petri dishes rather than cages also reduces the chance that *A. abdominalis* individuals can escape to invade where they are not wanted, an activity at which this species is notoriously successful. Once mummies were visible (after one week for *A. abdominalis* and 10 days for *A. ervi*) they were placed in individual “size 0” gelatine capsules so that virgin female wasps could be obtained on eclosion. The haplodiploid sex determination of Hymenoptera means

that all offspring of virgin females are male, and using virgin females for experiments prevents any differences in the competitive ability of the sexes from influencing the results. After emergence, female wasps were provided with 30% honey solution and were allowed experience ovipositing on stock clone aphids for 24 hours. Wasps were isolated from aphids 30 minutes before the start of the experiments.

### *Experimental design*

We compared the outcome of multiparasitism in aphids carrying *H. defensa* and genetically identical aphids that carried no symbionts. Wasps were allowed to attack aphids at two time points: the first at four days old, an age when preliminary experiments had shown that symbionts can confer protection, and the second three days later. In cases of multiparasitism, oviposition order may be important and so we carried out two separate experiments where each of the two wasp species was allowed to oviposit first. The three-day gap between ovipositions was chosen so that the larva of the first wasp escapes the surrounding serosal membrane (*Aphidius*) (Bai & Mackauer 1991) or hatches (*Aphelinus*) (Lundie 1924; Bai & Mackauer 1991; Christiansen-Weniger 1994) just as the second egg is laid. We reasoned this was most likely to equalise the first-oviposition advantage.

The experimental design is summarised in Table 1. In Experiment 1, aphids were attacked by *A. abdominalis* first and by *A. ervi* second. Control aphids were exposed to one wasp only at the same time points (*A. abdominalis* at day four, *A. ervi* at day seven) to distinguish the effects of oviposition time and multiparasitism (Table 1). In Experiment 2 we assessed the outcome of multiparasitism when *A. ervi* oviposited first, and likewise included controls of both wasps at the appropriate time point (Table 1).

Before the experiment, adult female aphids of both the naturally-infected and cured lines were placed in 9cm Petri dishes containing a single leaf of *Vicia faba* with the petiole inserted

into 2% agar to keep it fresh. After 24 hours the adult aphids were removed and their offspring retained for the experiments.

In order to maximise the probability that each aphid received its intended parasitism treatment, all ovipositions were observed. For parasitism by *A. abdominalis*, aphids were placed in a 9cm Petri dish with a leaf of *Vicia faba* and watched for 150 minutes; this wasp species usually requires aphids to be stationary (feeding) for successful oviposition.

Oviposition events were timed and only those where the female had her ovipositor inserted in the aphid for sufficient time to lay an egg (a minimum of 80 seconds) were assumed to be successful. Oviposition can be interrupted by host behaviour that dislodges the wasp or by interaction with another aphid, and this typically results in no egg being laid (Gerling, Roitberg & Mackauer 1990). In approximately half of all dishes the parasitoid used a host for food rather than for oviposition (host-feeding) and these aphids, which invariably die shortly afterwards (Bai & Mackauer 1990), were not retained. For parasitism by *A. ervi*, aphids were placed in a 5cm Petri dish without any leaf material; this wasp species is very successful at attacking moving aphids. The dishes were watched for 120 minutes and aphids were removed immediately following oviposition, which takes less than a second in this species. *A. ervi* does not practice host-feeding.

Experiment 1 was carried out in two temporal blocks, with five replicates of each treatment in each block (one block had only four replicates for the *A. ervi* control with symbiont-infected aphids). All replicate dishes were initiated with 12 individual aphids and a single wasp. After parasitism the aphids were kept in 9cm Petri dishes with leaves of *V. faba*, as before, and observed daily for signs of parasitism. The two parasitoid species form different and distinctive mummies: *A. ervi* mummies are swollen and golden, and appear approximately 10-12 days after oviposition, while *A. abdominalis* mummies are black and appear around eight days after oviposition. The numbers of mummies and live aphids were recorded for all



treatments and successful parasitism was defined as the formation of the distinctive mummy.

Experiment 2 was smaller, with a single block containing four replicates in both of the two

*A. abdominalis* control treatments, five replicates for the multiparasitism treatment with symbiont-infected aphids, seven replicates for the *A. ervi* control with symbiont-infected aphids, and six replicates in both the multiparasitism treatment and *A. ervi* control with symbiont-free aphids. Ten aphids were used per dish but in other respects the protocol was identical to Experiment 1.

### *Statistical analysis*

We used generalized linear modelling implemented in R v 3.0.2. (R Development Core Team 2013) to compare the effects of symbiont status (cured or infected) and parasitism treatment (multiparasitism or single parasitism) on rates of successful parasitism by each wasp species.

The response variable is a proportion (number of mummies of the focal species divided by the sum of all parasitized and surviving aphids) and a quasibinomial error distribution was used, which takes into account any overdispersion in the data. The R package ‘multcomp’ (Hothorn, Bretz & Westfall 2008) was used to carry out multiple comparisons using Tukey contrasts.

## **Results**

### *Experiment 1: Oviposition by *Aphelinus abdominalis* precedes *Aphidius ervi**

First we consider the outcome of single parasitism (the control aphids) in the presence and absence of symbiont infection. In our experimental design both *A. abdominalis* and *A. ervi* were efficient parasitoids with oviposition in symbiont-free aphids leading to successful mummy formation in 84% (Fig. 1a) and 67% (Fig. 1b) of cases respectively. Comparison of the success rates of each parasitoid species when attacking symbiont-free and infected aphids

supports our previous assessment of the defensive phenotype of this *H. defensa* isolate. This confirmation is necessary because our previous assays used timed exposures in which self-superparasitism and behavioural defence was not prevented, rather than direct observations of single parasitism events. *A. abdominalis* seems incapable of developing in the presence of this symbiont, a highly significant effect ( $F_{1,37}=272.77$ ,  $P<0.001$ ; Fig. 1a), while the performance of *A. ervi* was significantly reduced ( $F_{1,35}=6.70$ ,  $P=0.014$ ; Fig. 1b), though not as severely as for the aphelinid.

We next consider the outcome of multiparasitism. When a symbiont-free aphid was attacked first by *A. abdominalis* and then by *A. ervi*, mummies of the first wasp species were produced 96% of the time, while of *A. ervi* only 2% (Fig. 1c). Compared to when it parasitises alone, *A.abdominalis* actually did slightly better when subsequently attacked by *A. ervi* (96% success versus 84%;  $F_{1,36}=4.90$ ,  $P=0.034$ ). In contrast, *A. ervi* suffered greatly (2% success versus 67%;  $F_{1,35}=6.70$ ,  $P=0.014$ ) from the prior presence of *A. abdominalis* (Figs 1b & 1c).

Our data support the hypothesis that symbiont carriage affects the outcome of multiparasitism (Fig. 1c). No *A. abdominalis* in the multiparasitism treatment developed successfully in the presence of the symbiont (as in the single parasitism case) and 44% of the aphids went on to produce *A. ervi* mummies. There was therefore a substantial advantage to *A. ervi* when multiparasitising in the presence of the symbiont rather than in cured aphids (44% success versus 2% mummies formed;  $z$  value = -2.58,  $P=0.043$ ). In fact, when parasitizing infected aphids, *A. ervi* produced more mummies in multiparasitized symbiont-infected aphids compared to when it was the sole parasitoid (44% vs 23%; Figs 1b, 1c), though this effect was not significant when adjusted for multiple comparisons ( $z$  value=-1.59,  $P=0.361$ ).

## Experiment 2: Oviposition by *Aphidius ervi* precedes *Aphelinus abdominalis*

Here we consider the outcome of parasitism with the order of oviposition reversed relative to Experiment 1. Again, our controls showed that *A. abdominalis* never developed successfully in the presence of the symbiont, and this was true whether or not the aphid was previously parasitized by *A. ervi* ( $F_{1, 17}=72.08$ ,  $P<0.001$ ; Figs 1d, 1f). However, we no longer saw an effect of the symbiont on successful parasitism by *A. ervi* in the absence of multiparasitism ( $F_{1, 21}=62.50$ ,  $P=0.600$ ), probably due to the difference in aphid age (four days younger than Experiment 1) (McLean & Godfray 2014).

When an aphid without the symbiont was attacked first by *A. ervi* and then by *A. abdominalis*, mummies of *A. ervi* were produced 39% of the time, while the figure for *A. abdominalis* was 36%. This represents a substantial cost of multiparasitism to *A. abdominalis* compared to when it developed alone in a host (95% v 36%;  $F_{1, 17}=14.42$ ,  $P=0.002$ ; Fig. 1c, 1d) or when it was the first wasp to oviposit under multiparasitism (96% success; Fig. 1c).

When attacking aphids without the symbiont, *A. ervi* was less successful in previously parasitized hosts than when parasitising alone, although the difference is not statistically significant (39% success multiparasitized versus 77% single;  $z$  value=1.53,  $P=0.419$ ; Fig. 1e, 1f). The results were similar in infected aphids (60% success multiparasitized versus 45% single;  $z$  value=-0.75,  $P=0.877$ ) and the interaction is not significant ( $F_{1, 20}=2.71$ ,  $P=0.115$ ; Figs 1e, 1f). Comparison of Experiment 1 and Experiment 2 shows that in the absence of the symbiont, the cost of multiparasitism for *A. ervi* was dependent on oviposition order (*A. ervi* first 39% success versus *A. ervi* second 2%; Fig. 1c, 1f).

## Discussion

Our results show that the protective endosymbiont *H. defensa* can reverse the outcome of competition following multiparasitism by *A. ervi* and *A. abdominalis* (Fig. 1c, 1f). We found that *A. ervi* is generally the weaker competitor and its rate of successful parasitism was only 2% when ovipositing into a symbiont-free aphid previously parasitized by *A. abdominalis*.

The presence of the symbiont increased its success rate to 67%, and hence *H. defensa* has a strong effect on the outcome of competition between these two wasps. When *A. abdominalis* attacked symbiont-free aphids previously parasitized by *A. ervi*, it succeeded in developing in 36% of aphids. Although this is costly relative to symbiont-free aphids which have not been previously parasitized (which give around 85-95% success), it is much better for *A. abdominalis* than ovipositing in an aphid with the protective symbiont, which never led to successful development in our study.

Both parasitoid species are common and co-occur in the UK (van Veen *et al.* 2008) and elsewhere, and almost certainly compete for hosts in the field. We have observed parasitoids of both these genera attack the same aphid colony at the same time in recent field experiments (Hrček, McLean & Godfray *in submission*). Multiparasitism involving *A. ervi* and a different aphelinid, *Aphelinus asychis*, was studied by Bai and Mackauer (1991), though here *A. ervi* was nearly always the successful competitor irrespective of the order of attack, indicating that subtle differences in biology can determine the outcome of competition. It should be noted that we used single inbred lines of wasps for our experiments, and competitive outcomes could also vary between different genetic lines of the parasitoids.

We found that a defensive symbiont that has a stronger effect on the superior competitor can affect the outcome of multiparasitism. *A. ervi* is such a poor competitor that one would expect it to avoid parasitizing an aphid already containing an *A. abdominalis* larva unless the aphid

also carried *H. defensa*. This species is able to distinguish hosts previously parasitized by another aphelinid, *A. asychis*, using external cues (probably semiochemicals left by the first wasp to help it avoid self-superparasitism) (Bai & Mackauer 1991), though it has yet to be shown whether this ability extends to the congeneric *A. abdominalis*. There is also evidence that *A. ervi* is capable of detecting the presence of *H. defensa* (Łukasik *et al.* 2013a). However, the variation in the protection conferred by different symbiont strains (Oliver, Moran & Hunter 2005; Martinez, Weldon & Oliver 2013; McLean & Godfray 2015) means that their effects on the outcome of multiparasitism are likely to be strain-specific. This could reduce the advantage of symbiont detection by *A. ervi*, or select for the wasp to distinguish amongst strains were this to be possible.

Superparasitism or multiparasitism with low probabilities of success may still be adaptive if the opportunity costs in terms of wasted time or eggs are low (reviewed in Godfray 1994; Harvey, Poelman & Tanaka 2013). Before this was appreciated biologists were puzzled by these behaviours in solitary parasitoids and generally considered them maladaptive. One idea, the multiple target hypothesis, held that laying several eggs benefitted the wasp as it exhausted the host immune system (Salt 1968) though the evidence in its support (Puttler & van den Bosch 1959; Puttler 1974; Berberet, Willson & Odejar 1987) could more easily be explained by the increased probability of at least one egg surviving when several are laid (Vinson 1990). The first convincing evidence for the multiple target hypothesis was recently provided by Oliver *et al.* (2012) who showed that *A. ervi* benefitted from laying multiple eggs (self-superparasitism) in aphids protected by a strain of *H. defensa* that potentially provided strong protection against this wasp. In our experiments, we found that *A. abdominalis* had slightly but significantly greater success in cured hosts previously attacked by *A. ervi* which might reflect some benefit of multiple oviposition in overcoming the aphid's intrinsic defences. However, multiparasitism did not have an effect on the performance of either wasp

in symbiont-infected aphids: *A. abdominalis* never succeeded, and the small advantage to *A. ervi* was not significant.

Aphids and their natural enemies have provided insights into the ways in which natural insect communities are structured by trophic and non-trophic (indirect) interactions (Müller *et al.* 1999; Müller & Godfray 1999; van Veen, Morris & Godfray 2006; van Veen *et al.* 2008; Petermann *et al.* 2010). In a typical ecological setting the pea aphid competes with several other aphid species (and numerous other herbivores) and is attacked by four or five parasitoid wasps (themselves attacked by up to ten secondary parasitoids), two or three fungal pathogens, and a relatively large guild of predators (van Veen *et al.* 2008). Our results demonstrate one means by which bacterial symbionts can affect interactions within the wider community, not only through the direct effects they have on the success of parasitism (Oliver *et al.* 2003; Oliver, Moran & Hunter 2005), but also by altering the outcome of competitive intraguild interactions at a trophic level above their host. Given the diversity of symbiont species and the multiple affects they have on host biology (Oliver *et al.* 2010; Henry *et al.* 2013; Henry *et al.* 2015), we expect many more cases of indirect symbiont effects to be discovered in the coming years. Determining how facultative symbionts influence the complex web of insect community interactions is both a fascinating and daunting challenge.

### **Data accessibility**

Data deposited in the Dryad Digital Repository: doi:10.5061/dryad.15998 (McLean & Godfray, 2016).

## Acknowledgements

The authors would like to thank Jan Hrčák, Ben Parker and Lee Henry for useful discussion.

This work was funded by the Natural Environment Research Council (NE/K004972/1).

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**Table 1.** Overview of experimental design. Aphids, with and without the secondary symbiont *Hamiltonella defensa*, were exposed to parasitoids either once or twice. The table shows which wasp species were allowed to attack the aphid in the two exposure periods.

Treatment	First exposure period Aphids 3 <sup>rd</sup> instar (day 4)	Second exposure period Aphids adult (day 7)
<b>Experiment 1</b>		
Multiparasitism	<i>Aphelinus abdominalis</i>	<i>Aphidius ervi</i>
<i>Aphelinus</i> control	<i>Aphelinus abdominalis</i>	None
<i>Aphidius</i> control	None	<i>Aphidius ervi</i>
<b>Experiment 2</b>		
Multiparasitism	<i>Aphidius ervi</i>	<i>Aphelinus abdominalis</i>
<i>Aphidius</i> control	<i>Aphidius ervi</i>	None
<i>Aphelinus</i> control	None	<i>Aphelinus abdominalis</i>

## Figure legend

**Figure 1.** Outcome of parasitism in aphids with and without the symbiont *Hamiltonella defensa*.

Parasitism is defined as the proportion of aphids which formed parasitoid mummies (=successful pupation). Light bars represent *Aphidius ervi*; dark bars represent *Aphelinus abdominalis*. In Experiment 1 (A–C), *A. abdominalis* attacked when aphids were 4 days old (third instar) and *A. ervi* when aphids were 7 days old (fourth instars or adults); in Experiment 2 (D–F), *A. ervi* attacks when aphids were 4 days old and *A. abdominalis* when aphids were 7 days old. Results are shown for controls with *A. abdominalis* only (A, D), controls with *A. ervi* only (B, E) and for multiparasitism (C, F). Error bars denote standard error of the mean.

