

**Faithful vertical transmission but ineffective horizontal
transmission of bacterial endosymbionts during sexual
reproduction of the black bean aphid, *Aphis fabae***

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Abstract. 1. Insects are commonly infected with bacterial endosymbionts. In addition to the costs and benefits associated with harboring these symbionts, their rates of vertical and horizontal transmission are important determinants of symbiont prevalence.

2. Aphids are cyclical parthenogens and show virtually perfect maternal transmission of endosymbionts during asexual reproduction. Less clear is the role of the annual sexual generation, during which overwintering eggs are produced. Data from pea aphids (*Acyrtosiphon pisum* Harris) suggest that maternal transmission failures and horizontal transmission via males may occur under sexual reproduction at least occasionally. No such data exist for other aphid species.

3. Here we estimated rates of maternal and paternal transmission of facultative endosymbionts during sexual reproduction in the black bean aphid, *Aphis fabae* (Scopoli). Crosses were performed between clones infected with *Hamiltonella defensa*, clones infected with *Regiella insecticola* and clones without facultative endosymbionts. Eggs were overwintered under three different conditions.

4. Only one of 205 offspring from crosses testing for maternal transmission failed to inherit the symbiont present in the maternal clone, and in crosses testing for horizontal transmission, only one of 412 offspring acquired a facultative symbiont from the father.

5. These results show that in *A. fabae*, maternal transmission of *H. defensa* and *R. insecticola* is extremely reliable also during sexual reproduction, indicating that maternal transmission failures are unlikely to exert a significant influence on frequencies of infection. Paternal transmission of endosymbionts was exceedingly rare, suggesting that this route of horizontal transmission may be less important than hitherto assumed.

Keywords. *Aphis fabae*, diapause, endosymbionts, *Hamiltonella defensa*, maternal transmission, *Regiella insecticola*, symbiosis

Introduction

Aphids are host to heritable bacterial endosymbionts. The obligate endosymbiont *Buchnera aphidicola* is indispensable for aphids because it provides essential amino acids that are lacking from the phloem sap diet (Douglas, 1998). Besides *B. aphidicola*, aphids may possess one to several other, facultative endosymbionts, such as *Hamiltonella defensa*, *Regiella insecticola* or *Serratia symbiotica* (Moran et al., 2005). These are not strictly required for aphid survival, but they can confer important ecological benefits to their hosts, such as resistance to parasitoids or pathogenic fungi (Oliver et al., 2003; Scarborough et al., 2005; Vorburger et al., 2010; Łukasik et al., 2013), increased thermal tolerance (Montllor et al., 2002; Russell & Moran, 2006) or improved performance on particular host plants (Tsuchida et al., 2004; Wagner et al., 2015). Unlike *B. aphidicola*, whose phylogeny is congruent with that of its aphid hosts, indicating exclusively vertical transmission (Moran et al., 1993; Clark et al., 2000), facultative endosymbionts appear to undergo horizontal transmission at least occasionally (Sandström et al., 2001; Russell et al., 2003). Different routes of horizontal transmission are available to endosymbionts. They can be transmitted via contaminated ovipositors of parasitoids wasps (Gehrer & Vorburger, 2012), and a study on whiteflies suggests that oral transmission via the host plant may also be possible (Caspi-Fluger et al., 2012), although this is yet to be demonstrated in aphids. Particularly intriguing is a result by Moran and Dunbar (2006), showing that when pea aphids (*Acyrtosiphon pisum* Harris) undergo sexual reproduction in autumn, male-to-female horizontal transmission of *H. defensa* and *R. insecticola* is possible during mating via the male's ejaculate. This occurred in two out of nine experimental crosses analyzed (Moran & Dunbar, 2006). However, a later study suggested that this route may not always be so effective in pea aphids (Peccoud et al., 2014),

and it is currently unknown whether sexual transmission can occur in other aphid species as well.

Despite their capacity for horizontal transmission and the ecological benefits they provide, facultative endosymbionts tend not to get fixed in aphid populations and typically occur at intermediate frequencies (Tsuchida et al., 2002; Vorburger et al., 2009; Russell et al., 2013). This may in part be explained by costs associated with the possession of these symbionts under certain environmental conditions. For example, *H. defensa* provides strong protection against parasitoid wasps (Oliver et al., 2003; Schmid et al., 2012), but appears to have a negative effect on aphid fitness in the absence of parasitoids (Oliver et al., 2008; Vorburger & Gouskov, 2011). The prevalence of facultative endosymbionts may also be reduced if vertical transmission is unreliable. However, this does not seem to be the case during the parthenogenetic generations over the growth season, when live-bearing females give birth to clonal offspring. At least under laboratory conditions, vertical transmission is virtually perfect (Darby & Douglas, 2003), and parthenogenetic lines can be maintained for hundreds of generations without losing their symbionts (C. Vorburger, personal observation). This may be different during the single sexual generation of the aphid lifecycle (cyclical parthenogenesis). After mating in autumn, sexual females lay overwintering eggs, and the endosymbionts have to survive the winter to be present in the asexual fundatrices hatching from the eggs the next spring. Crosses done in pea aphids suggest that vertical transmission of facultative endosymbionts during sexual reproduction can be less than perfect, and that one endosymbiont in particular, *S. symbiotica*, can be lost frequently (Moran & Dunbar, 2006; Peccoud et al., 2014). Similar data are not available for any other aphid species.

Here we address the issue of symbiont transmission during sexual reproduction in the black bean aphid, *Aphis fabae* (Scopoli). The black bean aphid can carry many of the same bacterial endosymbionts as the pea aphid and has become an important additional model to study their

role in aphid ecology and evolution (Zytynska & Weisser, 2016). In particular, it has been used to demonstrate how the defensive endosymbiont *H. defensa* influences aphid coevolution with parasitoids (*Lysiphlebus fabarum*) by increasing the heritable variation as well as the specificity of resistance to parasitoids (Vorburger et al., 2009; Rouchet & Vorburger, 2012; Schmid et al., 2012) and by mediating trade-offs between parasitoid resistance and other components of fitness (Vorburger & Gouskov, 2011; Cayetano et al., 2015). We carried out a crossing experiment with black bean aphids designed to quantify vertical transmission rates of *H. defensa* and *R. insecticola* within matrilineal lines via the egg stage, as well as horizontal transmission rates from male to female. The results show that also during sexual reproduction, vertical transmission of these facultative endosymbionts is virtually perfect in black bean aphids, and that horizontal transmission via sex is exceedingly rare.

Material and methods

Study organisms

The nominal subspecies of the black bean aphid, *Aphis fabae fabae*, is a common pest of broad bean (*Vicia faba* L.), sugar beet (*Beta vulgaris* L.) and other crops, particularly in temperate regions of the northern hemisphere (Blackman & Eastop, 2000). In central Europe, *A. f. fabae* reproduces by cyclical parthenogenesis with host alternation. In autumn, winged individuals migrate from the herbaceous secondary hosts occupied by the live-bearing parthenogenetic summer generations back to the primary host, the European spindle tree (*Euonymus europaeus* L.). Mating between sexual morphs takes place on spindle, where the egg-laying sexual females (oviparae) also deposit the overwintering eggs. A first generation of

parthenogenetic females (fundatrices) hatches from these eggs in spring and their descendants eventually migrate again to secondary host plants.

For the crossing experiment described here, six clones of *A. f. fabae* were used. All were collected in June and July 2006 in Switzerland and Germany and since then maintained in the laboratory on broad beans under summer-like conditions that ensure continuous parthenogenetic reproduction (18 – 20°C, 16 h photoperiod). Two clones (nrs. 256 and 401) were uninfected with any known facultative endosymbionts of aphids, two clones (nrs. 329 and 343) were naturally infected with *H. defensa*, and two clones (nrs. 326 and 333) were naturally infected with *R. insecticola*.

Experimental procedures

To initiate sexual reproduction, we set up two broad bean seedlings with two young adult females for each clone (generation G0) and exposed them to conditions of reduced day length and temperature (10 h light:14 h dark at 15°C). These conditions simulate the approaching winter and reliably induce the production of sexual morphs in *A. fabae* (Hardie & Vaz Nunes, 2001). The G0 females were allowed to reproduce parthenogenetically for several days and then discarded. When their offspring (generation G1) reached adulthood, they were transferred in groups of four to new plants (six plants per clone) and allowed to reproduce for a total of 20 days. Every five days the G1 adults were transferred to new plants and their offspring (generation G2) on the old plants retained and reared to adulthood. Most of the G2 offspring produced over the first 10 days developed into winged females (gynoparae, which later give birth to oviparae), whereas those produced later developed into winged males.

When the first gynoparae were adult, we collected twigs of the primary host plant *E. europaeus* in the field, carefully checked them to ensure they were free of aphids, and inserted

them into Falcon tubes filled with wet vermiculite. We prepared several twigs per clone (depending on the number of gynoparae available), and each twig was colonized with five adult gynoparae and sealed in a cellophane bag. The gynoparae then gave birth to the next generation (G3) of sexual, egg-laying females (oviparae). Once the oviparae were adult, crosses were performed by placing three adult oviparae from one clone with two adult males from another clone on new twigs of *E. europaeus*, where the aphids mated and deposited their eggs on the stems at the base of buds (Fig. 1). In a first set of crosses, females from all four symbiont-infected clones were mated with males from both uninfected clones to quantify the fidelity of maternal symbiont transmission during sexual reproduction. In a second set of crosses, females from both uninfected clones were mated with males from all clones with facultative symbionts to test for horizontal male-to-female transmission at mating. All crossing combinations and the number of crosses per combination are detailed in Table 1.

When the oviparae and males had died and the twigs shed their leaves, we counted the number of black eggs on each twig. Black color indicates healthy eggs that have started development (Fig. 1), whereas unfertilized eggs or eggs failing to develop for any other reason remain pale or decay. To spread the risk of losing crosses and to evaluate methods for future crossing experiments, the egg-bearing twigs from each crossing combination were assigned to three different overwintering treatments. For the first treatment, twigs with eggs were surface sterilized by immersing them for 2 min in diluted bleach (0.5% sodium hypochlorite concentration) to avoid fungal growth (Via, 1992), rinsed with distilled water and then placed in a sterile petri dish containing a moist filter paper that had also been bleached and rinsed. Dishes were sealed with parafilm and placed in a climate-controlled cabinet with a 10 h photoperiod first at a constant 10°C for 5 days, after which the temperature was set at 4°C during the photoperiod and 1.5°C during the scotoperiod for 70 days. After this simulated winter, hatching of the eggs was induced by changing the rearing

conditions to 16 h light and 8 h dark at 14°C. When the eggs started to hatch, each egg-bearing twig was attached to a fresh spindle twig with young leaves inserted in a vermiculite-filled falcon tube and sealed in a cellophane bag, allowing hatchlings to crawl onto fresh leaves and start feeding. When they had grown to sufficient size (2nd to 4th instar), aphids were isolated individually on small spindle twigs and reared until they were adult and had produced some offspring. Fundatrices were then collected into 1.5 ml Eppendorf tubes and frozen at -20°C for later analysis. Not all hatchlings survived to adult fundatrices, we therefore checked developing aphids daily such that dead individuals could be collected quickly and were still suitable for symbiont detection.

The second overwintering treatment was identical to the first except that we omitted the bleaching and simply placed the untreated twigs into Petri dishes with a filter paper moistened with distilled water. For the third treatment, aphid eggs were simply exposed to the natural winter conditions outdoors. We inserted the spindle twigs with eggs into narrow (1 cm diameter) vermiculite-filled tubes, arranged the tubes in a tube rack and placed the rack inside an insect rearing cage (BugDorm 42222-F, MegaView Science, Taiwan) to prevent colonization by other arthropods. The cage was then mounted on 3 November 2011 at 2 m height on the stem of a small tree in a hedgerow on the institute's campus. It remained outdoors until it was returned to the laboratory when hatching started on 13 March 2012. After that, hatchlings were treated identically to those of the other overwintering treatments. The winter these eggs had spent in the field was relatively mild except for a severe cold spell from late January to mid-February, when temperatures did not rise above 0°C for 16 days and the lowest temperature measured at the nearest weather station reached -16.9°C (Supplementary online material, Fig. S1).

Endosymbiont detection

Presence or absence of secondary symbionts in fundatrices was determined by diagnostic PCR reactions, amplifying part of the 16S ribosomal RNA gene with symbiont-specific primers. DNA was prepared by grinding aphids in between 30 μ l (for the smallest nymphs) and 100 μ l (for adults) of 5% Chelex solution with 5 μ l of Proteinase K (10 mg/ml). After incubation at 37°C for at least 6 h, samples were incubated at 65°C for 15 min and finally boiled for 6 min. DNA preparations were stored at -20°C until use. PCR reactions were carried out in 10 μ l volumes with cycling conditions as described in Ferrari et al. (2012), using the universal forward primer 10F (Sandström et al., 2001) either with the *H. defensa*-specific reverse primer T419R or the *R. insecticola*-specific reverse primer U433R (Ferrari et al., 2012) to detect the respective symbionts. All PCRs included a negative and a positive control. PCR products were visualized on 2% agarose gels stained with ethidium bromide. Negative results were followed up by an additional PCR amplifying a fragment of the 16S rRNA gene of the obligate endosymbiont *B. aphidicola* (primers 16SA1 and Buch16S1R; Fukatsu et al., 2001). Samples in which the *B. aphidicola* 16S rRNA gene could not be detected (only 4 cases overall) were considered failed extractions that did not contain any amplifiable symbiont DNA and were thus excluded from all analyses.

Statistical analyses

For the crosses to quantify vertical transmission as well as for the crosses to detect horizontal transmission of facultative endosymbionts we analyzed hatching success (the proportion of fertilized eggs from which live fundatrices hatched) with generalized linear models in R version 3.1.3 (R Core Team, 2015), using quasibinomial fits to account for

overdispersion. We tested for the fixed effects of maternal clone, paternal clone and overwintering treatment as well as their two- and three-way interactions.

Horizontal transmission and vertical loss of facultative endosymbionts were too rare for meaningful statistical analysis. However, we calculated a 95% binomial confidence interval (Wilson's score interval) around the observed rates using the PropCIs package in R.

Results

Hatching success of overwintering eggs

From the crosses between females infected with facultative endosymbionts and uninfected males we overwintered 715 presumably fertilized eggs, from which 205 fundatrices were obtained (28.7% hatching). The hatching success varied significantly among maternal clones ($F_{3, 15} = 5.326$, $P = 0.011$) and was lowest for eggs from clone 333 (Fig. 2a), but it was not influenced significantly by the paternal clone ($F_{1, 15} = 0.761$, $P = 0.397$). The overwintering treatment also had a significant influence on the hatching success ($F_{2, 15} = 18.126$, $P < 0.001$; Fig. 2b). The highest proportion of eggs hatched after overwintering in petri dishes without prior bleaching (41.3%), whereas only a very low proportion hatched when twigs with eggs were surface-sterilized with bleach prior to sealing them in dishes (10.1%). The hatching success of the outdoor overwintering treatment was intermediate (26.6%). We observed no significant interactions between these effects on hatching success (complete analysis available as Supplementary online material, Table S1).

The outcome was very similar for crosses between uninfected females and males possessing facultative endosymbionts, from which we overwintered 1172 fertilized eggs producing 412 fundatrices (35.2%). Hatching success differed significantly between the two

maternal clones ($F_{1, 52} = 4.223$, $P = 0.045$), but not among paternal clones ($F_{3, 52} = 1.143$, $P = 0.340$)(Fig. 2c), and it was influenced by overwintering treatment ($F_{2, 52} = 10.791$, $P < 0.001$), following the same pattern as for the other crosses (Fig. 2d). Again, no significant interactions were observed (Table S2).

Endosymbiont transmission

Of the 205 fundatrices we obtained from mothers infected with either *H. defensa* or *R. insecticola*, all but one still possessed the maternal symbiont (Table 2a). A single individual from a cross between clones 343 and 401 tested negative for *H. defensa*. The egg from which it hatched had been overwintered under natural conditions outdoors. Over all offspring combined, this translates into a failure rate of vertical transmission of only 0.005 (0.001 – 0.027 95% CI), or of 0.008 (0.001 – 0.045) if only *H. defensa* is considered. Sixteen clonal lines derived from *H. defensa*-positive fundatrices and eight clonal lines from *R. insecticola*-positive fundatrices were kept until spring 2013 for further experiments on the symbionts' phenotypic effects in recombinant genetic backgrounds (R. Sieber & C. Vorburger, unpublished data). Diagnostic PCRs prior to these experiment (4 February 2013) confirmed symbiont presence, demonstrating that the fundatrix-derived clonal lines maintained stable heritable infections with their maternally acquired symbionts for a year (approx. 25 clonal generations).

From the crosses testing for sexual horizontal transmission of symbionts, 412 fundatrices were obtained (Table 2b). Like their mothers they did not possess any facultative endosymbionts, again with a single exception. One individual from a cross between clones 401 and 333 tested positive for *R. insecticola*. The egg from which it hatched had been overwintered indoors in a petri dish without prior bleaching. A single acquisition of a

facultative endosymbiont from the father would translate into a horizontal transmission rate of only 0.002 (0.001 – 0.014) overall, or of 0.005 (0.001 – 0.029) if only *R. insecticola* is considered.

Discussion

This crossing experiment showed that in black bean aphids, maternal transmission of the facultative bacterial endosymbionts *H. defensa* and *R. insecticola* is extremely reliable also under sexual reproduction. The endosymbionts are passed on to the eggs, persist in the diapausing egg stage, and re-establish a heritable infection in the hatching fundatrix and her descendants. The near-perfect maternal transmission of *H. defensa* and *R. insecticola* during sexual reproduction is consistent with a similar experiment on pea aphids (Peccoud et al., 2014). However, this may not be generalizable to all facultative endosymbionts of aphids. For *S. symbiotica*, another facultative endosymbiont not included in our study, Peccoud et al. (2014) found surprisingly high rates of loss (> 50%) during sexual reproduction of pea aphids. This symbiont may be special, however, because in addition to the non-cultivable, fully internalized endosymbiotic strains, cultivable strains have also been described that may occur as gut symbionts of aphids rather than true endosymbionts (Foray et al., 2014; Renoz et al., 2015). Such strains could feasibly be lost more frequently.

Another caveat about our maternal transmission results is that hatchling fundatrices from the same cross were feeding on the same spindle tree twig for a short period of time before being transferred to individual rearing. Considering that Caspi-Fluger et al. (2012) demonstrated horizontal transmission of a *Rickettsia* endosymbiont among whiteflies via the host plant, this could potentially have enabled horizontal transmission among hatchlings, although we are not aware of any evidence for plant-mediated transfer of endosymbionts in

aphids. Nevertheless, we cannot strictly exclude that some maternal transmission failures might have remained undetected because uninfected hatchlings reacquired the symbionts horizontally from their sisters. While this would be interesting to know from a mechanistic point of view, it does not invalidate our estimates of transmission rates, because hatchlings from multiple eggs developing in close proximity on the same twig corresponds to the natural situation experienced by black bean aphids in the field.

Our results from black bean aphids are also consistent with those of Peccoud et al. (2014) from pea aphids in that paternal transmission of facultative endosymbionts to the progeny was exceedingly rare. This differs from the results of Moran and Dunbar (2006), where in two out of nine crosses with pea aphids the paternal endosymbiont was acquired by a substantial fraction of the progeny. We cannot offer an explanation for these differences other than the rather trivial point that different symbiont strains and aphid clones were used in each experiment, and that they may vary in their propensities for horizontal transmission or for acquiring new symbionts, respectively. We are also confident that we did not miss any paternally acquired infections due to low titers. At least for *H. defensa* it was shown that endosymbiont populations show rapid exponential growth during aphid development (Schmid et al., 2012), such that they should have been readily detectable even if the initial inoculum in the egg was small. Thus, together with the results by Peccoud et al. (2014), our results suggest that sexual transmission may be less important as a route of lateral transfer for aphid endosymbionts than originally assumed. That said, even very low rates of horizontal transmission via this route can be important evolutionarily.

With just a single exception to the general pattern of maternal as well as paternal transmission it is of course difficult to exclude that these exceptions in our study resulted from experimental error (e.g. mislabeling of samples), although we did our best to avoid such errors. The interpretation of the experiment would hardly change, however, if we assumed the

two exceptions resulted from experimental error and the rates of maternal loss or paternal transmission were in fact zero. We would still conclude that these events may occur, albeit only rarely (95% CIs would be 0 – 0.018 and 0 – 0.009 for maternal loss and paternal transmission, respectively). Because part of the eggs were overwintered under natural conditions outdoors, we are confident that this conclusion is not only valid under laboratory conditions. Frequent loss during sexual reproduction is thus unlikely to be a significant factor in explaining why *H. defensa* and *R. insecticola* only occur at low to intermediate frequencies in black bean aphid populations (Vorburger et al., 2009). It is more likely that under certain environmental conditions (e.g. low risk of parasitism), the benefits are outweighed by the costs of harboring these symbionts (Oliver et al., 2008; Vorburger & Gouskov, 2011; Cayetano et al., 2015), such that their possession becomes a net liability and is selected against at least periodically.

The experiment also yielded some useful practical information for optimizing future crossing experiments with black bean aphids. Surface sterilization of pea aphid eggs with bleach was used successfully by Via (1992) to minimize fungal growth during the long cold treatment, resulting in a high hatching rate of 60% on average. In our case, overall hatching rates were lower, and bleaching clearly did more harm than good. Although the bleaching delayed the onset of fungal growth on the egg-bearing spindle tree twigs (C. Vorburger, personal observation), we observed some fungal growth also in petri dishes with bleached twigs towards the end of the overwintering period, and the hatching success of eggs was much lower compared to unbleached eggs that were otherwise treated identically. The concentration of the active compound sodium hypochlorite in our sterilization treatment was similar to that of Via (1992), but we did not detach the eggs from the plants they were deposited on for overwintering. It is possible that some of the bleach was absorbed by the spindle tree twigs, thus did not get removed by rinsing, and then exerted a negative effect on the eggs during the

overwintering treatment. Although the hatching success was somewhat lower than from unbleached petri dishes overwintered indoors, we were pleased that the outdoor overwintering treatment yielded numerous live fundatrices from all but two crossing combinations. This overwintering treatment was by far the least labor-intensive and did not require any indoor controlled temperature space. If economy of time and space are important, as for example in large crossing experiments with many different parental genotypes, the outdoor overwintering of eggs under natural conditions could thus be a valuable alternative to more involved procedures in the laboratory. A disadvantage of this method, however, is that the timing of crossing experiments is constrained by the natural seasons.

To conclude, knowledge about the effectiveness of maternal and paternal transmission of bacterial endosymbiont during sexual reproduction has so far been restricted to the pea aphid, *Acyrtosiphon pisum* (Moran & Dunbar, 2006; Peccoud et al., 2014). Here we extend this knowledge to another important models system, the black bean aphid, *A. fabae*, and two of its facultative endosymbionts, *H. defensa* and *R. insecticola*. Largely in accordance with Peccoud et al. (2014) we show that failures of vertical transmission via females as well as horizontal transmission via males can indeed occur in black bean aphids, but are exceedingly rare. Maternal transmission failures are thus unlikely to exert a significant influence on observed frequencies of infection in the field, and sexual acquisition of bacterial endosymbionts may occur less frequently than was hitherto assumed.

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448

Table 1. Summary of crossing combinations and the number of crosses per combination carried out to test for maternal/vertical and paternal/horizontal transmission of the facultative endosymbionts *Hamiltonella defensa* and *Regiella insecticola* during sexual reproduction of the black bean aphid, *Aphis fabae*.

Maternal clone	Paternal clone					
	256 (uninf.)	401 (uninf.)	329 (<i>H. def.</i>)	343 (<i>H. def.</i>)	326 (<i>R. ins.</i>)	333 (<i>R. ins.</i>)
256 (uninfected)	-	-	$n = 9$	$n = 15$	$n = 7$	$n = 11$
401 (uninfected)	-	-	$n = 8$	$n = 11$	$n = 6$	$n = 11$
329 (<i>H. defensa</i>)	$n = 5$	$n = 4$	-	-	-	-
343 (<i>H. defensa</i>)	$n = 5$	$n = 5$	-	-	-	-
326 (<i>R. insecticola</i>)	$n = 5$	$n = 5$	-	-	-	-
333 (<i>R. insecticola</i>)	$n = 5$	$n = 5$	-	-	-	-

Table 2. Summary of results from experimental crosses testing for maternal/vertical and paternal/horizontal transmission of the facultative endosymbionts *Hamiltonella defensa* and *Regiella insecticola* during sexual reproduction of the black bean aphid, *Aphis fabae*.

(a) Vertical transmission	Endosymbiont								
	<i>Hamiltonella defensa</i>				<i>Regiella insecticola</i>				Total
	Cross (maternal clone × paternal clone):	329×256	329×401	343×256	343×401	326×256	326×401	333×256	
Total number of hatchlings	28	11	38	46	47	28	2	5	205
Number of infected hatchlings	28	11	38	45	47	28	2	5	204
Number of uninfected hatchlings	0	0	0	1	0	0	0	0	1
(b) Horizontal transmission	Endosymbiont								
	<i>Hamiltonella defensa</i>				<i>Regiella insecticola</i>				Total
	Cross (maternal clone × paternal clone):	256×329	256×343	401×329	401×343	256×326	256×333	401×326	
Total number of hatchlings	34	94	14	75	23	76	58	38	412
Number of infected hatchlings	0	0	0	0	0	0	0	1	1
Number of uninfected hatchlings	34	94	14	75	23	76	58	37	411

Figure legends

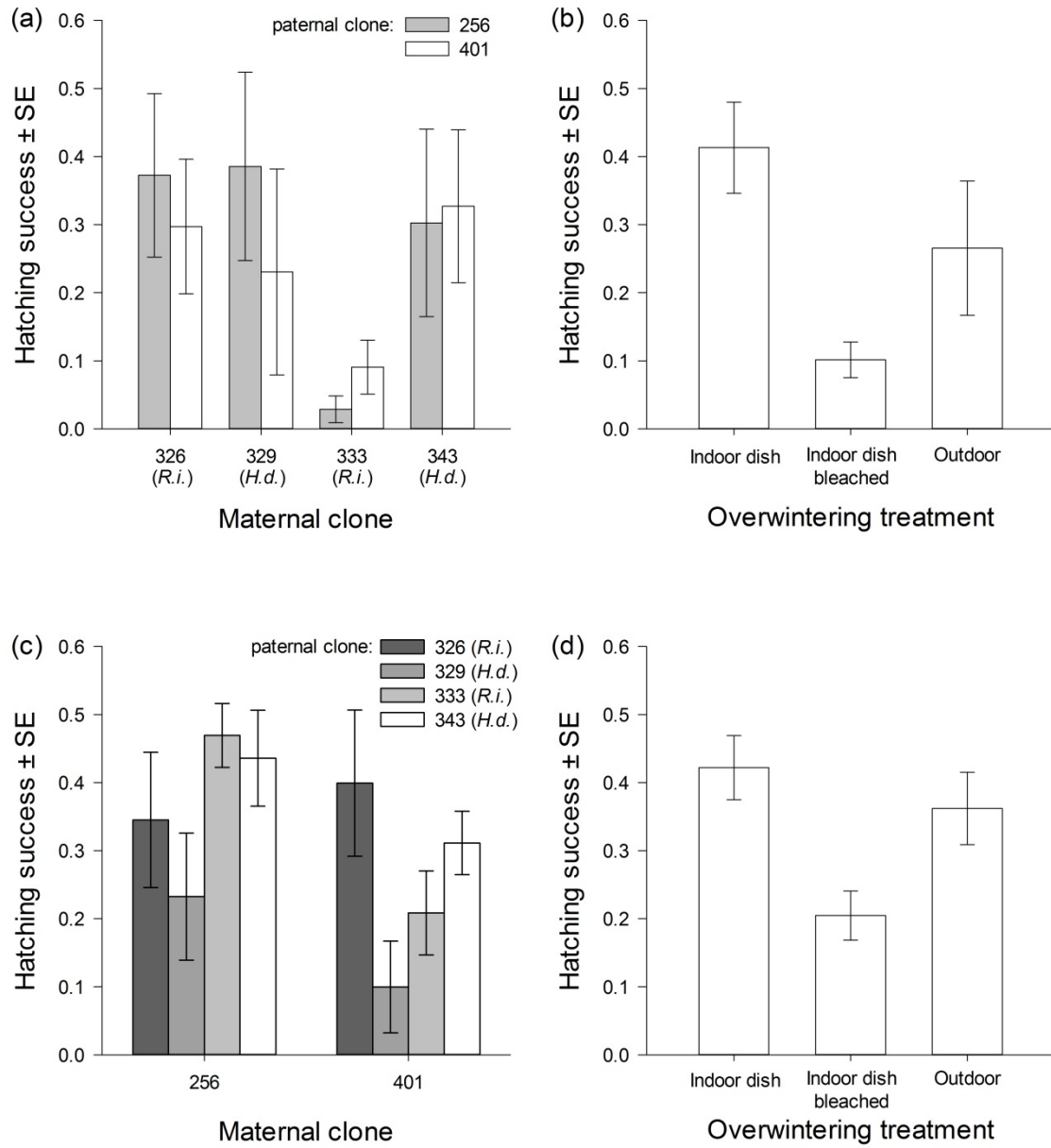
Fig. 1. Fertilized eggs of black bean aphids (*Aphis fabae*) deposited at the base of leaf buds on a twig of the European spindle tree (*Euyonymus europaeus*). Photograph by Christoph Vorburger.

Fig. 2. Bar plots depicting mean hatching success of eggs produced by crosses between females infected with facultative endosymbionts and uninfected males (top row) as a function of maternal and paternal clone (a) and overwintering treatment (b), and hatching success of eggs from crosses between uninfected females and symbiont-infected males (bottom row), also as a function of maternal and paternal clone (c) and overwintering treatment (d). *H.d.* = *Hamiltonella defensa*, *R.i.* = *Regiella insecticola*.

Figure 1



Figure 2



SUPPLEMENTARY ONLINE MATERIAL

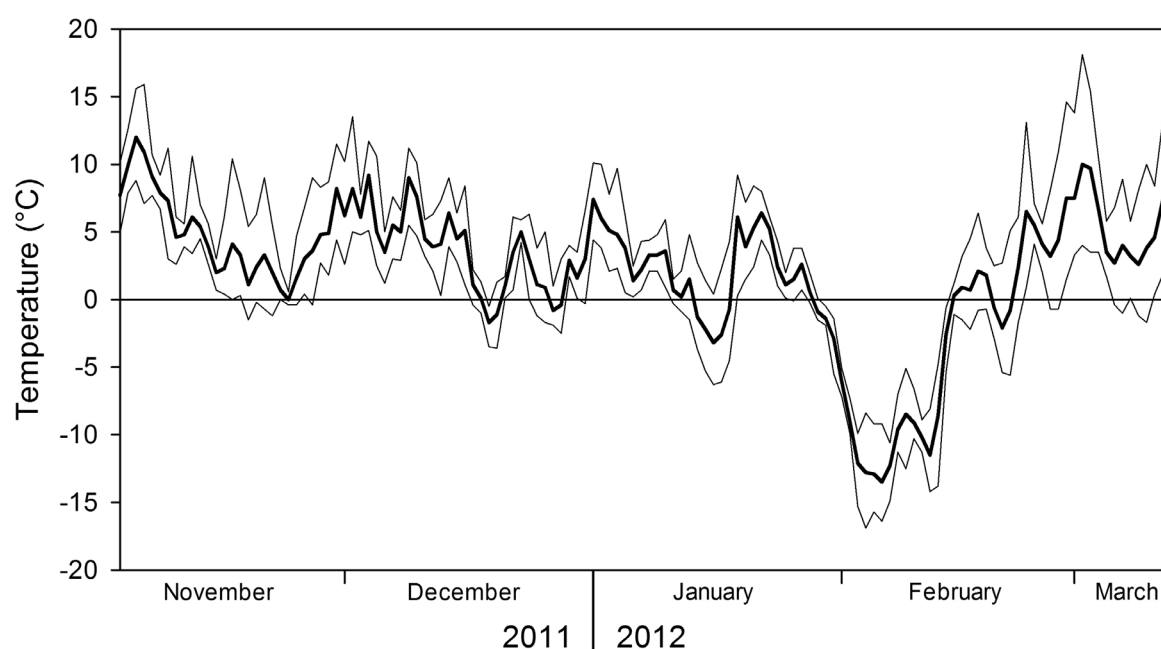


Fig. S1 Temperature during the period of overwintering black bean aphid eggs outdoors (3 November 2011 – 13 March 2012), measured at the nearest weather station of the Swiss Federal Office of Meteorology and Climatology, Meteoswiss (Station Zürich Fluntern, 47°22'40"N 8°33'56"E), at a distance of 4.5 km from the overwintering site. The bold line represents daily average temperature, the lower and upper regular lines represent daily minimal and maximal temperatures.

Table S1 Analysis of deviance table for the hatching success (proportion of eggs hatching) of crosses between uninfected females and males infected with facultative endosymbionts. The generalised linear model was a quasi-likelihood fit with logit link and binomial errors, using a dispersion parameter of 2.451395.

Effect	d.f.	Deviance	<i>F</i>	<i>P</i>
Maternal clone	1	10.352	4.223	0.045
Paternal clone	3	8.409	1.143	0.340
Overwintering treatment	2	52.908	10.791	< 0.001
Maternal × Paternal clone	3	15.075	2.050	0.118
Maternal clone × overwintering	2	12.482	2.546	0.088
Paternal clone × Overwintering	6	12.059	0.820	0.560
Maternal × Paternal clone × overwintering	6	17.852	1.214	0.314
Residual	52	150.440		

Table S2 Analysis of deviance table for the hatching success (proportion of eggs hatching) of crosses between females infected with facultative endosymbionts and uninfected males. The generalised linear model was a quasi-likelihood fit with logit link and binomial errors, using a dispersion parameter of 2.901315.

Effect	d.f.	Deviance	<i>F</i>	<i>P</i>
Maternal clone	3	46.357	5.326	0.011
Paternal clone	1	2.208	0.761	0.397
Overwintering treatment	2	105.180	18.126	< 0.001
Maternal × Paternal clone	3	4.194	0.482	0.700
Maternal clone × overwintering	6	14.113	0.811	0.578
Paternal clone × Overwintering	2	4.372	0.754	0.488
Maternal × Paternal clone × overwintering	6	24.708	1.419	0.271
Residual	15	50.689		