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# The direct effects of male killer infection on fitness of ladybird hosts (Coleoptera: Coccinellidae)

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## Abstract

Male killing bacteria are common in insects and are thought to persist in host populations primarily by indirect fitness benefits to infected females, whereas direct fitness effects are generally assumed to be neutral or deleterious. Here, we estimated the effect of male killer infection on direct fitness (number of eggs laid, as a measure of fecundity, together with survival) and other life-history traits (development time and body size) in seven ladybird host/male killer combinations. Effects of male killers on fecundity ranged, as expected, from costly to neutral; however, we found evidence of reduced development time and increased survival and body size in infected strains. Greater body size in *Spiroplasma*-infected *Harmonia axyridis* corresponded to greater ovariole number and therefore higher *potential* fecundity. To our knowledge, this is the first report of direct benefits of male killer infection after explicitly controlling for indirect fitness effects. Neutral or deleterious fitness effects of male killer infection should not therefore be automatically assumed.

## Introduction

At least one-third of insect species are infected with maternally inherited, facultative endosymbiont bacteria, such as *Wolbachia*, *Rickettsia*, *Spiroplasma* and Flavobacteria, which manipulate host reproduction by cytoplasmic incompatibility, induced parthenogenesis or male killing (Werren *et al.*, 1995, 2008; Weinert *et al.*, 2007; Duron *et al.*, 2008; Hilgenboecker *et al.*, 2008; Engelstadter & Hurst, 2009). Reproductive manipulators will only persist in host populations if they provide female hosts, which are responsible for their transmission, with a fitness advantage over uninfected females, for example in the form of resource reallocation or inbreeding avoidance (Hurst *et al.*, 1994, 1997; Majerus, 2006; Engelstadter & Hurst, 2009; Elnagdy *et al.*, 2011).

Aphidophagous ladybirds (Coleoptera: Coccinellidae) are particularly prone to infection with facultative bacteria that kill males early during their development ('early male killers', Hurst, 1991; Majerus & Hurst, 1997; Majerus, 2006; Majerus & Majerus, 2012). Due to the ephemeral nature and the large relative size of their aphid prey, newly hatched ladybird larvae obtain a large indirect fitness benefit by consuming the eggs of undeveloped siblings from within their own clutch (i.e. 'sibling egg cannibalism' Kawai, 1978). In male killer-infected clutches, undeveloped male eggs provide a substantial resource for their sisters. The advantage to females from this resource helps to maintain the male killer in the host population. Imperfect vertical transmission and/or a cost of infection can be indirectly compensated by this resource reallocation, reduced local resource competition and/or by reduced inbreeding and inbreeding depression (Hurst, 1991; Hurst *et al.*, 1997; Majerus & Hurst, 1997; Elnagdy *et al.*, 2011).

By definition, egg hatch rates should be reduced in male killer-infected clutches due to death of male progeny, and infected females may also lay fewer eggs (e.g. Hurst *et al.*, 1994). Theoretical work therefore assumes that male killers should have neutral or mildly deleterious effects on the direct fitness of the maternal host

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and that indirect fitness benefits are required to compensate for any direct costs to allow male killer invasion (Smith & Dunn, 1991; Hurst *et al.*, 1994, 1997; Randerson *et al.*, 2000; Engelstadter & Hurst, 2007). Formally, male killer invasion is theoretically possible if $b > 1/(a(1 + s)) > 1$, where $b$ is the indirect fitness benefit to females as a consequence of male sibling mortality, $a$ is the vertical transmission efficiency and $s$ the direct effect on female survival and fecundity (Hurst *et al.*, 1997). Theoretically, a purely maternally inherited male killer cannot spread if transmission efficiency is < 67% (Hurst, 1991). Empirical data indicate that $a$ typically varies between 80% and 95% in ladybirds, suggesting transmission efficiency is generally high, although some cases have been reported of transmission < 50% (Majerus & Majerus, 2010, 2012). Estimates of the fitness parameters in Hurst *et al.*'s (1997) model are needed to fully understand the dynamics of male killer persistence in host populations.

Our goal in this study was to investigate the effect of male killer infection on direct fitness and other life-history traits in several species of aphidophagous coccinellids, after controlling for confounding indirect fitness effects due to resource reallocation through sibling egg cannibalism. $s$ is often assumed to be negative in this group (Hurst *et al.*, 1994; Majerus, 2006), and therefore, $a$ and $b$ are expected to be much greater than zero to allow male killer spread. Consistent with this assumption, direct costs, rather than benefits, have been reported in the small number of empirical studies that have examined the effects of male killing bacteria in coccinellids so far. For example, reduced survival and fecundity of adult females was found in *Adalia bipunctata* and *Propylea japonica* infected with *Rickettsia* (Hurst *et al.*, 1994; Majerus, 2001, 2003), *Harmonia axyridis* infected with *Spiroplasma* (Majerus, 2001, 2003) and *Hippodamia variegata* infected with Flavobacteria (Hurst *et al.*, 1999). Reduced fecundity was also observed in *Cheilomenes sexmaculatus* infected with $\gamma$-Proteobacteria and *Coccinula sinensis* infected with Flavobacteria (Majerus & Majerus, 2000; Majerus, 2001, 2003), and reduced female embryo survival has also been recorded in *H. axyridis* infected with *Spiroplasma* (Majerus, 2001, 2003). Finally, we recently reported reduced survival of neonate *A. bipunctata* larvae infected with *Spiroplasma*, but no effect of *Wolbachia* or *Rickettsia* (Elnagdy *et al.*, 2011).

Although the majority of previous studies on the effects of male killing bacteria in coccinellids have focused on fecundity and/or survival, male killers can potentially affect other life-history traits, such as development time (Hurst *et al.*, 1994; Martins *et al.*, 2010), dispersal (Bonte *et al.*, 2009; Goodacre *et al.*, 2009) and resistance to pathogens (Unckless & Jaenike, 2012). We therefore performed a series of rearing experiments to examine fecundity (in terms of the number of eggs laid) and survival of female parents, development time of immature offspring and body size of adult female offspring, for uninfected and infected individuals from seven coccinellid host/male killer combinations. A positive correlation between female body size and fecundity is well known in insects (Evans, 1982; Gilbert, 1984; Reiss, 1989; Honěk, 1993), and body size is also directly proportional to the number of ovarioles (the functional units of insect ovaries that contain the developing egg chambers, Honěk, 1993), suggesting body size and ovariole number are good indirect indicators of fecundity. We therefore also examined whether *Spiroplasma* had an effect on ovariole number for *H. axyridis*, which showed the greatest difference in body size between infected and uninfected strains. In line with theoretical and empirical work discussed above, we hypothesized that the effect of male killer infection on female fecundity and other life-history traits would be neutral to mildly deleterious. If confirmed, this would indicate that male killers are primarily maintained through indirect fitness benefits, in combination with high transmission efficiency.

## Materials and methods

### Ladybird cultures

Matrilines of five ladybird species were established (Table 1) by isolating mating pairs from mixed sex, single population stocks. Where possible, we used strains collected from the same geographical location at the same time to avoid differences in genetic background. However, for *A. bipunctata*, a second location (St. Petersburg) was included as *Spiroplasma*-infected individuals were not detected in the Moscow population (Table 1). Between three and 16 matrilines were established for the different host/male killer infection combinations to provide 20 female parents for the experiment (Table 1).

For all experiments, larvae were removed from their clutch before they had a chance to engage in sibling egg cannibalism to prevent larvae gaining an indirect fitness advantage from resource reallocation. Ladybirds were maintained in Petri dishes at 21 °C, with 14L : 10D, artificial lighting and 35% humidity, in a controlled environment room, and were fed daily on pea aphids, *Acyrthosiphon pisum* (Harris) (Hemiptera: Aphididae), and supplemented with artificial diet (Majerus *et al.*, 1989). For each experiment, the number of aphids varied between species (as the larger, more voracious species require more aphids to survive) but the same number was given to the different infection combinations within species. All females used in the matrilines were virgins and were 2–4 weeks post-eclosion. Comparisons between infected and uninfected strains of the same species were carried out concurrently to avoid any confounding environmental variables. Three data sets were obtained relating to

**Table 1** Origins and infection status of coccinellid cultures used in experiments.

| Host | Male killer | Origin | Collection date | Matrilines |
|---|---|---|---|---|
| *Adalia bipunctata* (Abip) | Uninfected | Moscow, Russia | September 1998 | 3 |
| | *Rickettsia* | Moscow, Russia | September 1998 | 3 |
| | *Wolbachia* | Moscow, Russia | September 1998 | 3 |
| | *Spiroplasma* | St. Petersburg, Russia | September 2005 | 3 |
| *Coccinula crotchi* (Ccro) | Uninfected | Fugi, Honshu, Japan | September 2005 | 7 |
| | Flavobacterium | Fugi, Honshu, Japan | September 2005 | 5 |
| *Coccinula sinensis* (Csin) | Uninfected | Kofu City, Honshu, Japan | September 2005 | 10 |
| | Flavobacterium | Kofu City, Honshu, Japan | September 2005 | 5 |
| *Coccinella undecimpunctata* (Cuni) | Uninfected | Jordan | October 2004 | 4 |
| | *Wolbachia* | Giza, Egypt | October 2004/August 2005 | 6 |
| *Harmonia axyridis* (Haxy) | Uninfected | Fuchu, Honshu, Japan | September 2005/June 2006 | 16 |
| | *Spiroplasma* | Fuchu, Honshu, Japan | September 2005/June 2006 | 10 |

Four-letter host species codes are given in parentheses after species name. 'Matrilines' number of matrilines used to produce 20 female parents.

(i) female parents, (ii) immature offspring and (iii) adult female offspring, as outlined below.

### Female parents

Twenty females and 20 males were mated for each host/male killer combination, with males consecutively rotated between females of the same host species on a daily basis, for 28 days in the case of *A. bipunctata*, *Coccinula unidecempunctata* and *H. axyridis* and 42 days in the case of *Coccinula crotchi* and *C. sinensis*, which had lower oviposition rates. The number of eggs laid per female was recorded daily during the mating experiment. Males were removed after the 28- or 42-day period. As female ladybirds store sperm and continue to lay eggs after mating ceases, we continued to record the number of eggs laid throughout the remaining lifespan. Date of death was recorded and the total lifespan from hatching (in days) used for survival analyses.

Egg number data were tested for normality using Shapiro–Wilk tests. As the data were not normally distributed ($W = 0.937$, $P < 0.0001$), nonparametric Wilcoxon rank-sum tests were used to test for the effect of infection on number of eggs laid for each host species. We tested whether there was a difference in survival between infection combinations for each host species using chi-squared tests to compare Kaplan–Meier survival curves. All statistical analyses were carried out using R v2.15.1 (R Development Core Team, 2012).

### Immature offspring

In the week following females being mated for the first time, 15 first instar larvae from each infected female and 35 first instar larvae from each uninfected female were removed from their clutch, before they had a chance to engage in sibling egg cannibalism. Larvae were fed aphids daily and reared individually to adulthood. We recorded (in days) (i) time from laying to hatching of egg ('hatch time'), (ii) time from hatching to pupation ('larval time'), (iii) time from pupation to emergence ('pupal time') and (iv) total development time for immature offspring ('total development time'). Linear mixed-effects (LMER) models were used to compare infection combinations (fixed effect) within species. Matriline was included as a random effect in the models to avoid issues with repeated measures. Data were log-transformed after checking for normality and homoscedasticity. *P*-values for analysis of variance (ANOVA) were computed for LMER models using the pamer.fnc function in the 'LMERConvenienceFunctions' Package for R (Tremblay, 2011). The amount of deviance explained by infection was calculated as the sum of squares of infection divided by the sum of squares total (Tremblay, 2011).

### Adult female offspring

Newly emerged adults were sexed following Randall *et al.* (1992). Females were then weighed to the nearest milligram within 24 h of eclosion and before being fed. In addition, the number of ovarioles was counted for *H. axyridis*, which showed the largest difference in body size between infected and uninfected individuals (see Results), to investigate the relationship between infection and ovariole number. Female *H. axyridis* were killed by placing them in absolute ethanol in 0.5 mL Eppendorf tubes and then rehydrated using phosphate-buffered saline (PBS), pH 7.4 (Gibco BRL, Paisley, UK), prior to abdominal dissection. A few drops of toluidine blue solution (Fisher Scientific, Loughborough, UK) were added to the abdominal tissue and left for 2 min. Excess stain was then absorbed with filter paper and the tissue rinsed in a few drops of PBS. Both ovaries were separated from the rest of the tissue, and total number of ovarioles counted. A total of 20 *Spiroplasma*-

infected *H. axyridis* females from 6 female parents and 27 uninfected females from 15 female parents were dissected. Weight and ovariole data were analysed using LMER models and ANOVAS on log-transformed data, as described for immature offspring.

## Results

### Female parents

On average, 15 of 20 female parents from each host/male killer combination laid eggs (Table S1). The outcome of Wilcoxon rank-sum tests on eggs laid over 28 or 42 days and over lifespan was identical (Fig. S1 and Table S1); therefore, only the results from eggs/lifespan are presented here. The number of eggs laid per lifespan by uninfected *A. bipunctata* females was significantly greater than for any of the infected females (Fig. 1a and Tables 2 and S1). Uninfected *C. crotchi* females also laid significantly more eggs than Flavobacteria-infected females, but note the small sample size for these combinations (Table S1). For *C. sinensis*, *C. unidecempunctata* and *H. axyridis*, infected females laid more eggs on average than uninfected females, but the differences are not significant (Fig. 1c,d,e and Tables 2 and S1).

There was a significant difference in survival between all pairwise comparisons for *A. bipunctata*, except for *Wolbachia* × *Spiroplasma* (Fig. 2 and Tables 2 and S1). Survival was significantly lower for uninfected *A. bipunctata* than for *Wolbachia*-, *Spiroplasma*- or *Rickettsia*-infected females. *Rickettsia*-infected *A. bipunctata* females also had lower survival than *Wolbachia*- or *Spiroplasma*-infected females (Fig. 2 and Tables 2 and S1). Differences in survival between infections within other host species (particularly *H. axyridis*) were small and nonsignificant (Fig. 2 and Tables 2 and S1).

### Immature offspring

There was a slight trend for faster total development time in infected relative to uninfected hosts; however, this difference was only significant for *H. axyridis* (Fig. 3 and Tables 2 and S1). When total development time was broken up into stages (hatch time, larval time, pupal time), the results were more complex (Figs S2–S4 and Tables S1 and S2). For example, in *Spiroplasma*-infected compared with uninfected *H. axyridis* hosts,

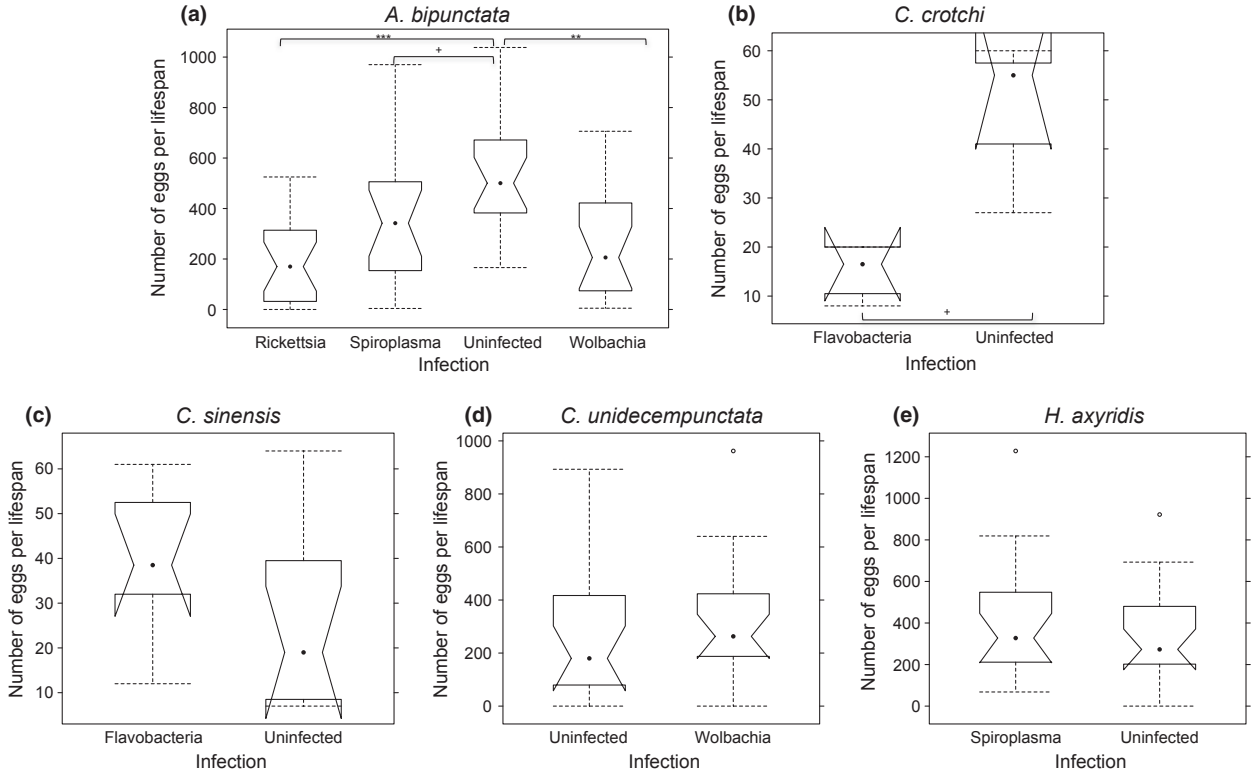


**Fig. 1** Number of eggs laid per lifespan for female parents. Notch box and whisker plots for number of eggs laid per lifespan of female parents. Closed circles correspond to medians, boxes correspond to the 25th and 75th percentile, and whiskers to maximum values or 1.5 times the interquartile range (when there are outliers present, represented by open circles). Symbols comparing combinations correspond to *P*-values for Wilcoxon rank-sum tests, where $^{+}P < 0.05$; $^{**}P < 0.001$; $^{***}P < 0.0001$. See Table 2 for full statistics.

**Table 2** Comparison of infection combinations for female parents, immature offspring and adult female offspring.

| | | Female parents | | Immature offspring | | Adult female offspring | |
|---|---|---|---|---|---|---|---|
| | | | | Development time | | Weight | |
| Host | M-Ks | Eggs/lifespan *W* (*P*) | Survival Chi-sq (*P*) | $F_{d.f.}$ (*P*) | Deviance | $F_{d.f.}$ (*P*) | Deviance |
| Abip | U×R | $^{-}55.5$ ($5.560 \times 10^{-5***}$) | $^{+}20.6$ ($5.560 \times 10^{-6***}$) | $3.3_{1,707}$ ($0.068^{NS}$) | 0.2 | $1.6_{1,403}$ ($0.21^{NS}$) | 0.4 |
| | U×W | $^{-}311.5$ ($0.0027^{**}$) | $^{+}27.8$ ($1.310 \times 10^{-7***}$) | $0.7_{1,795}$ ($0.420^{NS}$) | 0.03 | $^{+}17.9_{1,497}$ ($0.000^{***}$) | 3.1 |
| | U×S | $^{-}248.5$ ($0.047^{+}$) | $^{+}29.5$ ($5.690 \times 10^{-8***}$) | $3.6_{1,787}$ ($0.058^{NS}$) | 0.2 | $^{+}11.3_{1,493}$ ($0.000^{***}$) | 2.1 |
| | R×S | 129 ($0.094^{NS}$) | 16 ($6.23 \times 10^{-5***}$) | $0.1_{1,384}$ ($0.738^{NS}$) | 0.01 | $19.2_{1,378}$ ($0.000^{***}$) | 4.4 |
| | R×W | 173 ($0.341^{NS}$) | 14 ($0.0002^{**}$) | $0.1_{1,392}$ ($0.744^{NS}$) | 0.0 | $19.3_{1,382}$ ($0.000^{***}$) | 3.9 |
| | W×S | 149 ($0.377^{NS}$) | 0.1 ($0.710^{NS}$) | $0.2_{1,472}$ ($0.633^{NS}$) | 0.02 | $2.0_{1,472}$ ($0.158^{NS}$) | 0.4 |
| Ccro | U×F | $^{-}0$ ($0.049^{+}$) | 0.3 ($0.573^{NS}$) | $2.0_{1,72}$ ($0.167^{NS}$) | 2.2 | $^{+}4.5_{1,41}$ ($0.041^{+}$) | 8.5 |
| Csin | U×F | 62.5 ($0.137^{NS}$) | 0 ($0.990^{NS}$) | $0.1_{1,154}$ ($0.750^{NS}$) | 0.06 | $0.0_{1,113}$ ($0.845^{NS}$) | 0.0 |
| Cuni | U×W | 143.5 ($0.196^{NS}$) | 0.3 ($0.581^{NS}$) | $0.2_{1,339}$ ($0.646^{NS}$) | 0.04 | $^{+}21.2_{1,30}$ ($0.000^{***}$) | 7.2 |
| Haxy | U×S | 219 ($0.617^{NS}$) | 0.8 ($0.378^{NS}$) | $^{+}4.5_{1,597}$ ($0.033^{+}$) | 0.5 | $^{+}431.6_{1,402}$ ($0.000^{***}$) | 35.2 |

Host species codes are defined in Table 1. 'M-Ks' corresponds to infections compared, where 'U' is uninfected, 'W' *Wolbachia*, 'R' *Rickettsia*, 'S' *Spiroplasma* and 'F' Flavobacteria. A '$^{+}$' or '$^{-}$' symbol before the test statistic corresponds to the direction of the effect of male killer infection relative to uninfected hosts (i.e. a '$^{-}$' means the male killer has a negative effect). Female parents: comparison of number of eggs laid per lifespan and survival. '*W*' Wilcoxon rank-sum test statistic for differences between number of eggs laid over lifespan (corresponding to Fig. 1); 'Chi-sq', chi-squared test statistic for differences between survival curves (corresponding to Fig. 2). Immature offspring: comparison of total development time (corresponding to Fig. 3, see Table S2 for results broken down into different development stages). Adult offspring: comparison of weight (corresponding to Fig. 4). For both immature and adult female offspring, '*F*' ANOVA *F* comparing LMERs (see Materials and Methods); 'd.f.' lower denominator degrees of freedom; 'Deviance%' percentage deviance explained for each linear mixed-effects model term.

Exact *P*-values are given in brackets after the test statistic, $^{+}P < 0.05$; $*P < 0.01$; $**P < 0.001$; $***P < 0.0001$; and $^{NS}$not significant ($P > 0.05$). Note, lower-bound *P*-values corresponding to *F* are presented (which are more conservative than upper-bound values).

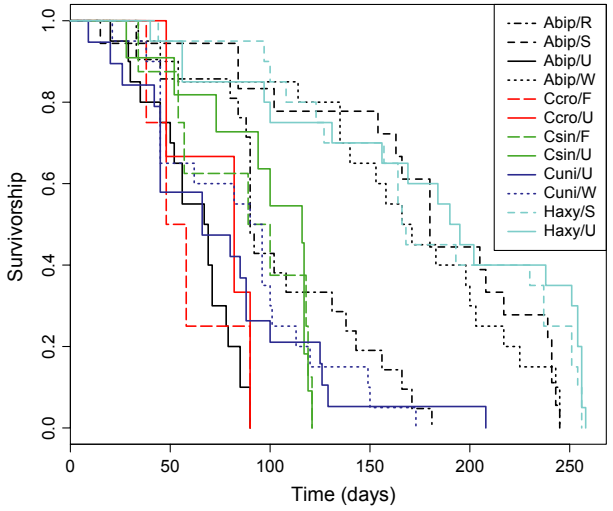


**Fig. 2** Survival of female parents. Kaplan–Meier survival curves for female parents. Host four-letter codes are defined in Table 1. Letters after '/' correspond to infection codes as follows: 'U' uninfected, 'S' *Spiroplasma*, 'W' *Wolbachia*', 'F' Flavobacteria and 'R' *Rickettsia*. See Table 2 for results of chi-squared test comparing survival curves within host species.

hatch time was significantly longer, larval time was significantly shorter, and pupal time was shorter but not significantly so, whereas for *A. bipunctata*, longer hatch and pupal times and shorter larval times for uninfected hosts cancelled each other out, so that overall there was no difference in total development time between infected and uninfected hosts (Figs S2 to S4 and Tables S1 and S2).

## Adult female offspring

We found a significant difference in the weight of adult female offspring between infection combinations for 7 of 10 comparisons (Table 2). In each case where the difference was significant, infected female offspring were larger than uninfected (Fig. 4 and Tables 2 and S1). *Wolbachia* and *Spiroplasma* were associated with increased body size in more than one host (*Wolbachia*: *A. bipunctata* and *C. unidecempunctata*; and *Spiroplasma*: *A. bipunctata* and *H. axyridis*, Fig. 4). For the significant comparisons, the percentage deviance explained ranged from 2.1% for *A. bipunctata: Wolbachia* vs. uninfected to 35.2% for *H. axyridis*: *Spiroplasma* vs. uninfected (Table 2). The effect of male killer infection on body size was therefore variable in magnitude, but substantial in the case of *Spiroplasma* infection on *H. axyridis*. *Spiroplasma*-infected *H. axyridis* females also had a significantly greater number of ovarioles than uninfected females [mean number of ovarioles, 48.10 (SD 5.119) and 40.185 (SD 4.507), respectively, $F_{1,24} = 21.552$, $P = 0.0001$, explained deviance = 24.021%, Fig. 5).

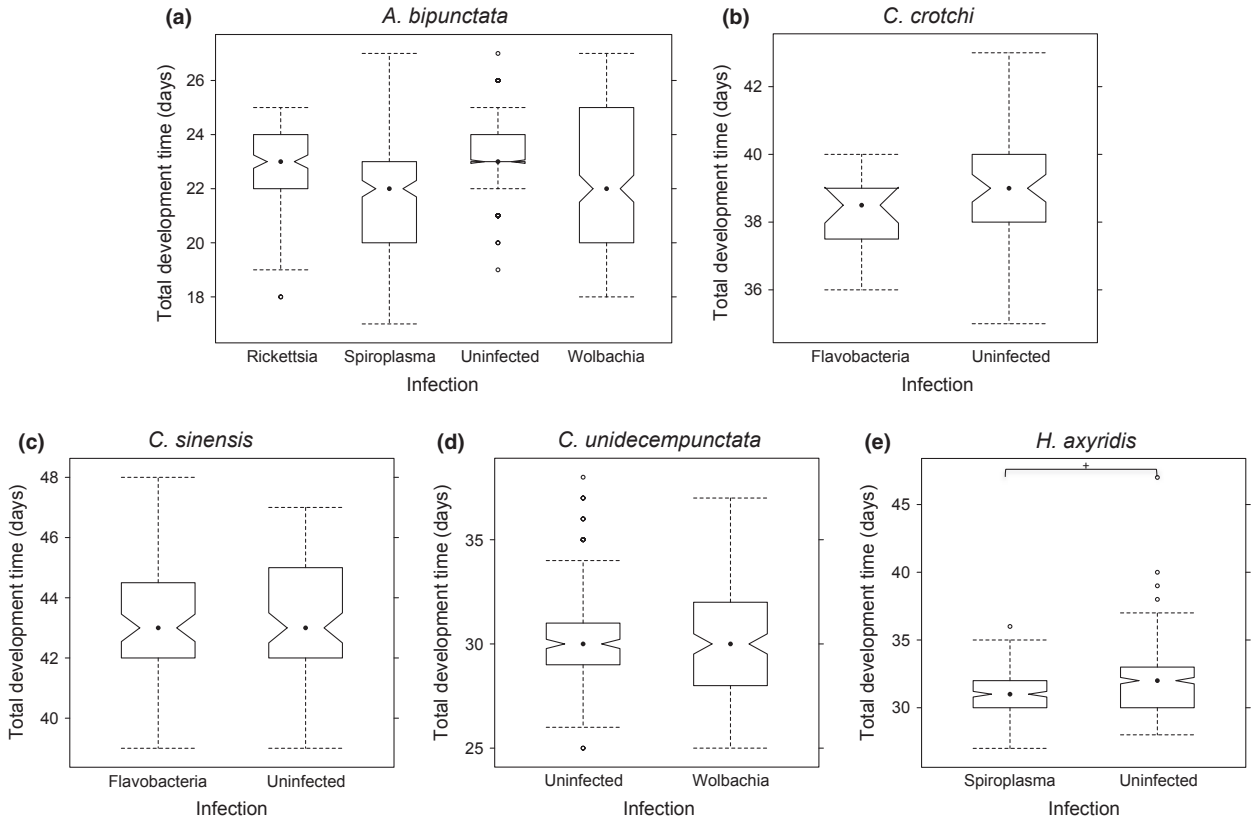


**Fig. 3** Total development time for immature offspring. Notch box and whisker plots for immature offspring total development time. Symbols comparing combinations correspond to ANOVA $F$ statistics comparing linear mixed-effects where $^{+}P < 0.05$. See Table 2 for full statistics.

## Discussion

Male killers are thought to be primarily maintained in host populations by strong indirect fitness benefits to infected females, which compensates for any direct costs associated with infection and imperfect vertical transmission (e.g. Hurst, 1991; Hurst *et al.*, 1997; Majerus & Hurst, 1997; Majerus, 2006). In coccinellids, the indirect fitness benefits of male killer infection are substantial and well understood, particularly from the point of view of resource reallocation (see e.g. Hurst *et al.*, 1997; Majerus, 2006 for reviews and Elnagdy *et al.*, 2011). Theoretical studies show that male killers that provide indirect fitness benefits can be maintained even if direct effects on fitness are neutral or mildly deleterious (Hurst, 1991; Smith & Dunn, 1991; Randerson *et al.*, 2000; Engelstadter & Hurst, 2007), and this is supported by previous empirical work (Hurst *et al.*, 1994; Majerus, 1999, 2001, 2003; Majerus & Majerus, 2000; Elnagdy *et al.*, 2011). Here, we confirmed that direct effects on female fecundity, in terms of the number of eggs laid, are neutral or deleterious in seven coccinellid host/male killer combinations. Effects of male killer infection on survival, development time and body size were neutral in the majority of cases; however, contrary to previous work, we found evidence for benefits to male killer infection for each of these three traits in certain host/male killer combinations and for ovariole number in *H. axyridis* infected with *Spiroplasma*, as discussed further below.

According to theory, if direct fitness effects are neutral, only a small indirect fitness benefit is required to maintain male killers in a population; however, if direct effects are deleterious, indirect fitness benefits must be very strong to compensate for direct fitness costs (Hurst *et al.*, 1997). Recently, we estimated the indirect fitness benefit obtained from resource reallocation via sibling egg cannibalism (in the form of a single-egg meal) in infected compared with uninfected strains of the same seven ladybird/male killer combinations studied here (Elnagdy *et al.*, 2011). In the present study, we estimated direct fitness in terms of the number of eggs per lifespan. We found that *Rickettsia*, *Spiroplasma* and *Wolbachia* all significantly reduced the number of eggs laid in their host, *A. bipunctata*, as did Flavobacteria in *C. crotchi*. If all else is equal, one would therefore expect $b$ to be much greater than zero in these four strains. We also found that the number of eggs laid by *C. sinensis*, *C. unidecempunctata* and *H. axyri-*

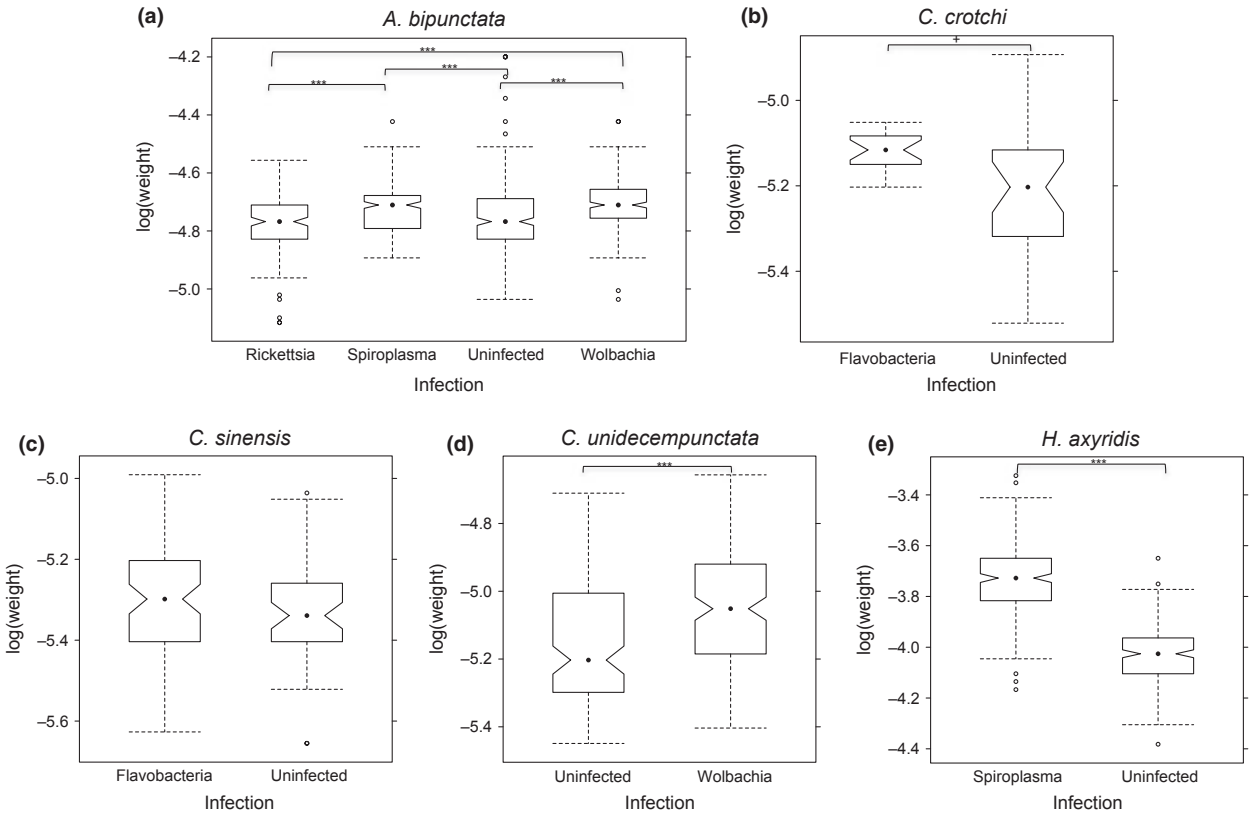


**Fig. 4** Adult female offspring weight. Notch box and whisker plots for adult female offspring weight. Symbols comparing combinations correspond to ANOVA *F* statistics comparing linear mixed-effects where $^{+}P < 0.05$; and $^{***}P < 0.0001$. See Table 2 for full statistics.

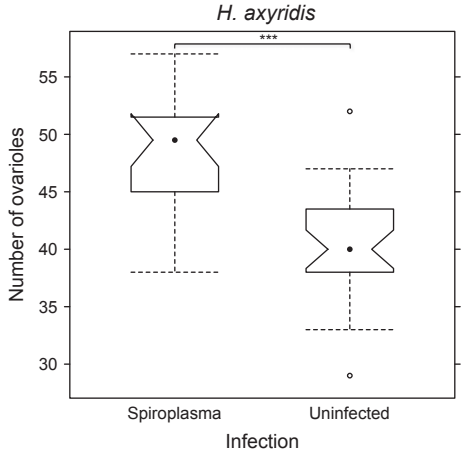


**Fig. 5** Number of ovarioles in adult offspring females for *Harmonia axyridis*. Notch box and whisker plots for ovariole number in adult offspring *H. axyridis* females. Symbols comparing combinations correspond to ANOVA *F* statistics comparing linear mixed-effects where $^{***}P < 0.0001$.

*dis* infected with Flavobacteria, *Wolbachia* and *Spiroplasma*, respectively, was slightly, but not significantly, greater than for uninfected individuals of the same species, and one would therefore expect *b* to be smaller in these combinations. In contrast to expectations, high direct fitness costs in *Rickettsia*- and *Spiroplasma*-infected *A. bipunctata* corresponded with the lowest values of *b* estimated by Elnagdy *et al.* (2011), whereas neutral direct fitness effects in *C. sinensis* infected with Flavobacteria corresponded with the greatest value of *b*. This suggests that the relationship between our estimates of *s* and *b* is far from simple, and that other indirect estimates of fitness, as well as transmission efficiency and prevalence estimates from the same strains, are needed to fully understand the relationship between the parameters in Hurst *et al.*'s (1997) model.

As mentioned, male killer infection had a direct negative effect on fecundity in *A. bipunctata* infected with *Rickettsia*, *Wolbachia* and *Spiroplasma* and in *C. crotchi* infected with Flavobacteria. This confirms that male killers from taxonomically diverse groups can have similar fitness effects, as reported previously (Hurst *et al.*, 1994, 1999; Majerus & Majerus, 2000; Majerus, 2001, 2003). A negative effect of *Rickettsia* infection on fecundity of *A. bipunctata* has also been previously reported (Hurst *et al.*, 1994). However, in contrast to the findings of Hurst *et al.* (1994), we found that survival was greater in *Rickettsia*-infected than in uninfected

*A. bipunctata* adult females. As the origins of the *A. bipunctata* used here and by Hurst *et al.* (1994) were geographically distant (Moscow, Russia and Cambridge, UK, respectively), a difference in the genetic background of the host, and/or the strain of *Rickettsia*, could explain the difference between survival results. Indeed, considerable divergence has been found between strains of *Rickettsia* associated with different mtDNA haplotypes in *A. bipunctata* (Jiggins & Tinsley, 2005). Future studies should investigate the role of, and attempt to control for, host genetic background, which is likely to play an important part in observed fitness effects. *Wolbachia* and *Spiroplasma* also significantly increased survival in *A. bipunctata*, suggesting the survival benefit to infection may compensate somewhat for reduced fecundity in this species. However, survival estimates obtained in the laboratory may not accurately reflect those in the field, and the finding of increased survival for male-killer infected strains of *A. bipunctata* should therefore be confirmed by studying wild populations.

The finding of greater survival in infected (relative to uninfected) *A. bipunctata* adult females for all three male killers was surprising, as in a previous study we found evidence of reduced survival of starved neonate *A. bipunctata* larvae infected with *Spiroplasma*, but no effect of *Wolbachia* or *Rickettsia* (Elnagdy *et al.*, 2011). The contradiction in survival effects between the two life stages is interesting, as one might expect selection pressure for increased survival to be greatest during developmental stages, which must be survived for female hosts to then mate (and transmit male killers) as adults. However, indirect fitness benefits from resource reallocation (in the form of sibling egg cannibalism) are greatest at the larval stage, and *Spiroplasma*-infected *A. bipunctata* larvae show a much greater response to a single-egg meal than do *Wolbachia*- or *Rickettsia*-infected larvae (Elnagdy *et al.*, 2011). The indirect fitness benefit from sibling egg cannibalism therefore compensates for any direct costs to larval survival (Elnagdy *et al.*, 2011).

Where costs and benefits of infection were seen in development time at the egg, pupal and larval stages, they generally cancelled each other out so that direct effects of male killer infection on total development time were generally neutral, as reported previously (e.g. Hurst *et al.*, 1994). However, *Spiroplasma* infection significantly reduced development time of *H. axyridis*, with the strongest effect seen at the larval stage. Male killing *Spiroplasma* has also been found to reduce development time in *Drosophila melanogaster*, but this benefit was probably mediated by indirect effects of reduced competition (Martins *et al.*, 2010). To our knowledge therefore this is the first report of a male killer directly reducing development time of its host.

Female body size and ovariole number are thought to be good indirect indicators of fecundity in insects (Evans, 1982; Gilbert, 1984; Reiss, 1989; Honěk, 1993), and male killers such as *Spiroplasma* and *Rickettsia* are transmitted transovarially and present at high concentration in the ovarioles of coccinellids (Sokolova *et al.*, 2002). We therefore hypothesized that female body size and the number of ovarioles should be lower in infected than in uninfected hosts. The greatest effect of male killer infection was on weight of adult female offspring, and contrary to expectations, infected hosts were significantly heavier than uninfected hosts in seven of the ten host/male killer combinations. Body size was not an accurate proxy for fecundity between generations; however, male killers that significantly decrease fecundity in female parents (e.g. *Spiroplasma* and *Wolbachia* in *A. bipunctata*) *increase* body size in their female offspring, suggesting the relationship between body size and fecundity is not straightforward. The strongest effect on weight of adult female offspring was seen for *Spiroplasma*-infected *H. axyridis*, which also exhibited significantly greater ovariole number compared with uninfected individuals. However, although *Spiroplasma*-infected individuals of *H. axyridis* laid 407 eggs compared with 336 laid by uninfected individuals, this difference was not significant. It would seem therefore that *Spiroplasma* has a positive effect on the *potential* fecundity of *H. axyridis*, but it is not clear why this was not reflected in a significantly greater number of eggs laid by female parents. The direct benefits to development time, body size and ovariole number of *Spiroplasma* in *H. axyridis* have potentially important implications as this host species is regarded as one of the world's most invasive insects. The role of *Spiroplasma* infection in invasion success certainly warrants investigation.

Together, these results indicate that direct effects of male killer infection in coccinellids vary throughout the different life stages and range from costly to neutral to beneficial. That effects vary between different life stages is not surprising, as many heritable microbes are present at low levels in larvae and then increase as the host becomes reproductively active (e.g. as seen in *Spiroplasma*-infected *Drosophila*, Anbutsu & Fukatsu, 2003). Positive direct fitness effects of cytoplasmic incompatibility–inducing *Wolbachia* have been documented in drosophilids (e.g. Fry *et al.*, 2004; Riegler & O'Neill, 2007; Weeks *et al.*, 2007) and *Aedes albopictus* (Dobson *et al.*, 2004), but the evidence for direct benefits of male killing bacteria is so far inconclusive. Male killing *Wolbachia* benefits female fecundity and pathogen resistance in *Drosophila innubila* (Unckless & Jaenike, 2012), whereas *Rickettsia* provides considerable fitness benefits to invasive *Bemisia tabaci* (Himler *et al.*, 2011), but in both cases, it is unclear how much of the observed fitness benefit is due to indirect rather than direct effects. Moreover, although *Rickettsia* distorts sex ratio in *B. tabaci*, whether it is a male killer has yet to be confirmed (Himler *et al.*, 2011). To our knowledge, our study is therefore the first to report direct benefits of male killer infection after controlling for any confounding effects on indirect fitness.

We are aware of two main limitations to our study, which warrant further investigation. Firstly, our estimates of fecundity and survival obtained in the laboratory are unlikely to correspond precisely to those in the wild, and field studies are therefore essential for estimating overall fitness (*s*) and further empirical investigation of the important model of Hurst *et al.* (1997). Secondly, female embryo survival can be reduced by male killer infection (Majerus, 2001, 2003; Elnagdy *et al.*, 2011); therefore, our estimates of fecundity, based on number of eggs alone, might not accurately reflect the true or 'realized' fecundity (i.e. the number of female offspring that survive to reproduce). Hatch rate could be regarded as closer to the realized fecundity, but is not particularly informative in this context as it is confounded by male killing (i.e. by definition, hatch rates in male killer-infected clutches are approximately half than those in noninfected clutches). More realistic estimates of fecundity, obtained by comparing the number of female offspring that survive to reproduce in infected and noninfected clutches, are required.

In summary, we confirmed that direct effects of male killer infection on fecundity of coccinellid hosts range from costly to neutral. However, we also report, for the first time, the evidence of direct benefits of male killer infection on survival, development time, body size and ovariole number. Neutral or deleterious direct fitness effects should not therefore be generally assumed, and direct benefits from male killer infection may be more important than previously realized. Further empirical work, ideally in wild populations, is needed to fully understand the respective roles of direct and indirect fitness effects in the dynamics of male killer persistence in host populations.

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## Supporting information

Additional Supporting Information may be found in the online version of this article:

**Figure S1** Notch box and whisker plots of number of eggs laid by female parents during the experiment (42 days for *Coccinula crotchi* and *Coccinula sinensis*, and 28 days for other species).

**Figure S2** Notch box and whisker plots of hatch time for immature offspring.

**Figure S3** Notch box and whisker plots of larval time for immature offspring.

**Figure S4** Notch box and whisker plots of pupation time for immature offspring.

**Table S1** Summary statistics for female parents, immature offspring and adult female offspring.

**Table S2** Results of linear mixed effects models for Immature offspring hatch time, larval time and pupal time.

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