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SHORT COMMUNICATION

Parasitoid bacterial symbionts as markers of within-host competitive outcomes: superparasitoid advantage and sex ratio bias

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Abstract. 1. Bacterial symbionts have the potential to alter insect fitness, which could influence insect competitive ability. To investigate this possibility, within-host competitions were staged between individuals of the parasitoid, *Encarsia inaron* Walker, that were differentially infected with the bacterial symbionts *Cardinium* and *Wolbachia*.

2. When parasitoids of different infection status parasitised the same whitefly host, there was no evidence that symbiont infection influenced the outcome of competition.

3. Using symbionts as markers, a significant advantage for the second (superparasitising) wasp was detected when eggs were deposited within 4 h of one another, probably as a result of ovicidal behaviour by the superparasitoid, but not when eggs were deposited 1 day apart. Additionally, the emerged sex ratio of superparasitoid offspring was male biased when eggs were deposited 4 h after the initial eggs, but had an even sex ratio when deposited 1 day later.

4. The present study demonstrates the potential utility of symbiont infection as a marking system for investigating within-host competition among parasitoids.

Key words. *Bemisia tabaci*, competition, facultative endosymbionts, ovicide, superparasitism.

Introduction

Facultative bacterial endosymbionts that are transmitted vertically, from mother to offspring, are common among arthropods (Duron *et al.*, 2008; Werren *et al.*, 2008; Oliver *et al.*, 2010). In contrast with infectious disease-causing microbes, vertically-transmitted endosymbionts share the fate of their arthropod host and are under selective pressure to minimise host fitness costs. They often provide benefits that improve the joint fitness of the host and the bacteria (e.g. Oliver *et al.*, 2010; Himler *et al.*, 2011), although counterexamples of negative or neutral consequences have also been documented (e.g. Fleury *et al.*, 2000; Charlat *et al.*, 2004).

In the whitefly parasitoid *Encarsia inaron* Walker (=*Encarsia partenopea*, Masi) (Hymenoptera, Aphelinidae), the fitness effects of symbiont infection are not straightforward (White *et al.*, 2011). This parasitoid is simultaneously infected with two common bacterial symbionts, *Wolbachia* and *Cardinium* (Perlman *et al.*, 2006); *Wolbachia* causes a well-characterised phenotype known as cytoplasmic incompatibility (White *et al.*, 2009), and *Cardinium* increases adult female longevity but decreases egg load (White *et al.*, 2011). Symbiont effects on larval fitness have not been investigated in this system, but symbionts have the potential to alter the outcome of competition within whitefly hosts. Deposition of two or more eggs within the same host (superparasitism) can be common in this solitary parasitoid (Gould *et al.*, 1992), making within-host competition a frequent occurrence. Symbiont contributions to differences in larval size and/or development rate could affect the outcome of competition in such systems (Huigens *et al.*, 2004). On the other hand, if symbionts do not affect larval fitness or competitive ability, then symbionts, which have near-perfect vertical transmission in this system (White *et al.*, 2011), can potentially be used as a non-biased marker of competitive outcomes between differentially infected wasps.

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The primary objective of this research was to examine whether bacterial endosymbionts affect the outcome of competition in hosts superparasitised by *E. inaron*. Additionally, symbionts were used as markers to more generally examine the outcome of competition as a function of (i) oviposition order, (ii) time between ovipositions, (iii) maternal mating status, and (iv) offspring sex.

Methods

Encarsia inaron is a minute parasitoid (<1 mm) of whiteflies. Laboratory cultures of *E. inaron* lacking one or both symbionts were generated using low doses of antibiotics (White *et al.*, 2009, 2011). All four cultures were maintained on sweet potato whitefly (*Bemisia tabaci* Gennadius) on cowpea (*Vigna unguiculata* L.), at an ambient lab temperature (~27 °C) and LD 16 : 8 h photoperiod, with infection status periodically verified using diagnostic polymerase chain reaction (PCR) (White *et al.*, 2009).

To test competitive outcomes between differentially infected parasitoids, superparasitised hosts were generated as follows. Three to six *E. inaron* of the same symbiont infection status were placed on an agar-embedded, excised cowpea leaf that was infested with sessile second to third instar *B. tabaci* nymphs (White *et al.*, 2009). As wasps assumed the upright 'drilling' posture on hosts, the leaf was marked ('Mark-It' markers, Bic Corp., Shelton, Connecticut) near the parasitised hosts. Wasps were removed from the leaf disk within 2 h. Unmarked whitefly hosts were then removed, and a second group of wasps with a contrasting infection status was observed on the marked hosts using an identical oviposition bout.

To validate the accuracy of this protocol, some singly and multiply marked hosts were dissected in Ringer's solution under 50× magnification and examined for the presence of parasitoid eggs. Of dissected singly marked hosts, 151/163 (93%) had at least one egg and 131/163 (80%) had exactly one egg. Of multiply marked hosts, 82/105 (78%) had at least two eggs. Therefore, the great majority of multiply-marked hosts received at least one egg from each mother. Furthermore, when dissections of superparasitised hosts occurred 1–2 days after egg deposition, physiological development of both eggs was usually evident, indicating that both remained viable in superparasitised hosts.

All remaining superparasitised hosts ($n = 489$) and a subset of singly parasitised hosts ($n = 218$) were reared out to estimate overall parasitism success. DNA was extracted from the winning wasp from superparasitised hosts, and tested with diagnostic PCR for the presence of *Wolbachia* and *Cardinium* (White *et al.*, 2009). All superparasitism contests were between wasps that differed in both *Wolbachia* and *Cardinium* infection status: either doubly-infected 'Both' versus uninfected 'None' or singly-infected *Cardinium* '*Card*' versus singly-infected *Wolbachia* '*Wol*'. The small percentage (12/301 = 4%) of offspring that gave inconsistent results (i.e. in a contest of Both versus None, the winning offspring had only *Cardinium*) were excluded from the dataset. These probably represented PCR amplification errors, but might be instances of imperfect maternal transmission or horizontal transfer of symbionts among hosts. This low 'error rate' indicates that bacterial symbionts are reliable markers in this system.

In addition to testing the effects of symbionts *per se* on competitive outcomes, the following factors were also tested. Oviposition order was tested using symbiont status to categorise each victorious offspring ($n = 289$) as originating from the first or second mother. Time between ovipositions was tested by initiating superparasitism bouts either directly after the initial oviposition bout ($n = 197$) or 1 day later ($n = 92$). Maternal mating status was tested by conducting competitions between offspring of mothers that either had had the opportunity to mate ($n = 169$), or had been isolated as pupae and remained virgins ($n = 62$). Finally, offspring of mated mothers were categorized by sex, and the secondary (emerged) sex ratio of victorious wasps was compared across categories. All comparisons were analysed statistically using χ^2 contingency table analysis. Because multiple superparasitised hosts were usually derived from each leaf disk (mean $= 2.25 \pm 0.13$ SE superparasitized hosts per disk), logistic regression analysis was also undertaken to account for this potential lack of independence. In all cases, the logistic and χ^2 analyses gave similar results, so only the χ^2 results are presented.

Finally, because two other members of the genus *Encarsia* have been observed to commit ovicide (Netting & Hunter, 2000; Collier *et al.*, 2007), observations were conducted to determine whether superparasitising *E. inaron* kill previously laid eggs. Individual second and third instar whiteflies were initially parasitised as described above, then slide mounted in observation arenas (Netting & Hunter, 2000), and exposed to a superparasitising *E. inaron* female within 4 h. These arenas allowed observation of within-host ovipositor activity at 200–400× magnification, and incidences of oviposition and/or ovicide were scored for each host.

Results and discussion

The overall rate of successful wasp emergence in superparasitised hosts was 51.3%, which did not differ significantly from the 52.9% success rate from singly parasitised hosts ($\chi^2_1 = 0.70$, $P = 0.70$). For superparasitised hosts, offspring of the superparasitoid were twice as likely to win the competition relative to offspring of the first wasp when the eggs were oviposited within 4 h of each other (Fig. 1a), but this advantage disappeared if one day elapsed between ovipositions (Fig. 1b). Symbiont infection status did not affect the outcome of competition either when superparasitism occurred quickly (Fig. 1a) or when a day elapsed between ovipositions (Fig. 1b). Therefore, symbionts either do not affect larval fitness in *E. inaron*, or the effects are too subtle to influence wasp competitive ability within superparasitised hosts. When combined with the low symbiont transmission error rate (<4%, as described above), bacterial symbionts appear to be stable markers that do not bias competitive outcome within *E. inaron*, and are suitable for investigating competition within superparasitised hosts.

Among mated wasps, first wasp progeny did not differ from an expected 50 : 50 sex ratio (50/98 = 51% male; $\chi^2_1 = 0.04$, $P = 0.84$), nor did superparasitoid progeny when 1 day separated the eggs (11/21 = 52% male; $\chi^2_1 = 0.05$, $P = 0.83$).

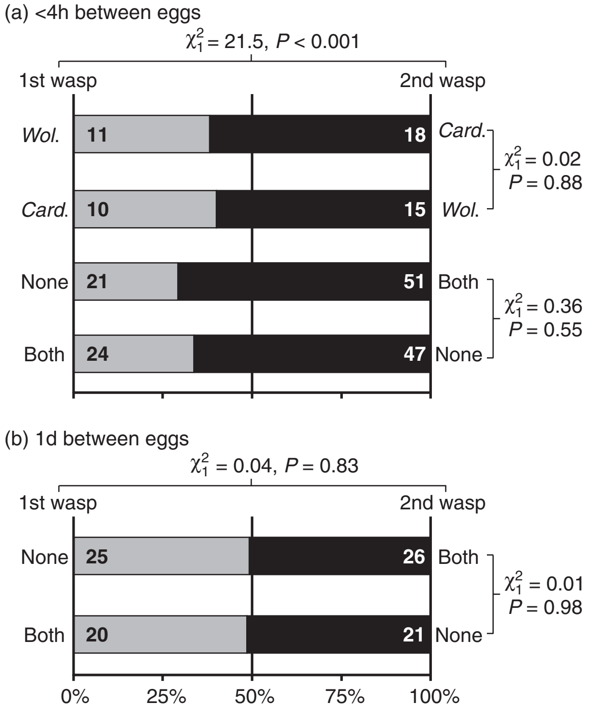

Fig. 1. Outcome of within-host competition between parasitoids with different symbiont infections when competing eggs were deposited (a) less than 4 h or (b) 1 day apart. For each horizontal bar, the first wasp's symbiont infection is shown on the left, the superparasitoid's on the right. The grey portion of each bar shows the percentage of first wasp victories, the black shows superparasitoid victories; sample sizes are indicated within the bars.

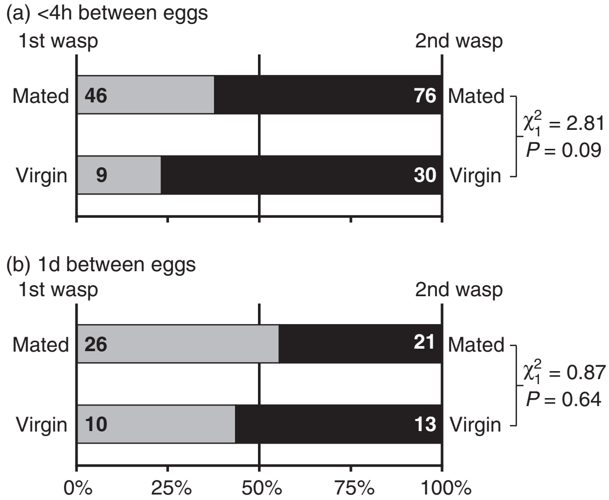

Fig. 2. Outcome of within-host competition when both mothers were mated or both were virgin, when competing eggs were deposited (a) less than 4 h or (b) 1 day apart. For each horizontal bar, the grey portion of the bar shows the percentage of first wasp victories, the black shows superparasitoid victories; sample sizes are indicated within the bars.

In contrast, superparasitoid progeny were significantly male biased when <4 h separated the eggs (53/77 = 68.8% male, $\chi_1^2 = 10.9$, $P = 0.001$). This percentage is similar to the percentage advantage to offspring of the superparasitising wasp (66.5%), suggesting that offspring sex might play a role in the superparasitoid advantage. Male-only competitions between offspring of virgin wasps allowed assessment of competitive interactions in the absence of potentially differential sex allocation by the mothers. Superparasitoid progeny won the competition approximately 75% of the time when male eggs were laid within 4 h of each other, and 50% of the time when the eggs were laid 1 day apart (Fig. 2). Neither of these success rates differed significantly from competitions between progeny of mated mothers (Fig. 2), suggesting that the observed superparasitoid advantage was not a function of differential survival or competitive ability of males versus females. Instead, the male bias in the superparasitoid progeny is probably a consequence of male bias in eggs deposited by the superparasitoid mother, perhaps in response to a perception that a previously parasitised host represents a lower quality resource (Van Baaren *et al.*, 1999). Speculatively, it is also possible that superparasitoids cannot detect previous parasitism that occurred 1 day earlier, and this is why the male bias in the superparasitoid progeny was no longer evident when a day separated oviposition events.

The superparasitoid advantage was probably attributable, at least in part, to ovicide. Observations of within-host ovipositor movements showed that, of 12 hosts in which the second wasp was observed to lay an egg, 3 were laid after piercing and killing the first egg, versus 9 that were laid in addition to the first egg without ovicide. Ovicide in *E. inaron* appears to occur at a lower frequency than in congeneric *E. formosa* (Netting & Hunter, 2000), but nevertheless would contribute to the superparasitoid advantage. *Encarsia inaron* is taxonomically quite distant from the other *Encarsia* species that have been demonstrated to commit ovicide (Manzari *et al.*, 2002), indicating that ovicide may be widespread in this genus, and more common for endoparasitoids than previously recognised. However, the disappearance of the superparasitoid advantage after 24 h suggests that there may be limitations to the utility of ovicide, probably based on physiological changes in the initial egg as it develops (S. Kelly, pers. obs.).

Finally, the present study demonstrates the potential utility of symbiont infection as a marking system for investigating within-host competition among parasitoids. In general, few markers are available for monitoring the outcome of within-host competition (but see Goubault *et al.*, 2003; Vorburger *et al.*, 2010). As a marking tool, bacterial symbionts such as *Wolbachia*, *Cardinium* or others show promise because (i) they are common among parasitoids (Floate *et al.*, 2006), (ii) they can be readily cured using antibiotic-laced honey (White *et al.*, 2009), and (iii) simple diagnostic protocols for symbiont detection are readily available (Simões *et al.*, 2011). For any system, care would need to be taken to ensure that the symbiont is a valid marker, with high vertical transmission, low horizontal transmission, and lacking fitness effects on parasitoids competing within a host. When these conditions hold, as they do for *E. inaron*, bacterial endosymbionts represent an alternative marking tool that is simpler to develop than microsatellite markers, and may be particularly useful in systems with low genetic diversity.

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