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Manipulation of oviposition choice of the parasitoid wasp, *Encarsia pergandiella*, by the endosymbiotic bacterium *Cardinium*

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Abstract

Reproductive manipulations of hosts by maternally inherited bacterial endosymbionts often result in an increase in the proportion of infected female hosts in the population. When this involves the conversion of incipient males to genetic or functional females, it presents unique difficulties for symbionts invading hosts with sex-specific reproductive behaviours, such as the autoparasitic *Encarsia pergandiella*. In sexual forms of this species, female eggs are laid in whitefly nymphs and male eggs are laid in conspecific or heterospecific parasitoids developing within the whitefly cuticle. Further, eggs laid in the 'wrong' host do not ordinarily complete development. This study explored the role of a bacterial symbiont, *Cardinium*, in manipulating oviposition behaviour in a thelytokous population of *E. pergandiella*. Oviposition choice was measured by the number and location of eggs deposited by both infected and uninfected adult wasps in arenas containing equal numbers of hosts suitable for the development of male and female wasps. Uninfected wasps included antibiotic-treated female wasps and (untreated) daughters of antibiotic-treated female wasps. The choices of wasps in the thelytokous population treatments were compared with those of a conspecific sexual population. We found that offspring of antibiotic-cured thelytokous wasps reverted to the behaviour of unmated sexual wasps, laying their few eggs almost exclusively in hosts appropriate for male eggs. Infected thelytokous wasps distributed their eggs approximately evenly between host types, much like mated sexual female wasps. The antibiotic-treated female wasps exhibited choices intermediate to wasps in the other two treatments. The change in the observed behaviour appears sufficient to allow invasion and persistence of *Cardinium* in sexual populations. Lastly, our results suggest a reduction in host discrimination as a possible mechanism by which *Cardinium* influences this change.

Introduction

In the past 25 years, we have gained substantial insight into the diversity and scope of inherited bacterial symbioses in arthropods (e.g. O'Neill *et al.*, 1997; Stouthamer *et al.*, 1999; Bandi *et al.*, 2001). Symbionts can have a profound impact on the ecology and evolution of their hosts. For instance, symbionts may mediate ecological interactions, supplement nutrient-poor diets (Russell & Moran, 2006) or dramatically manipulate host reproduction (O'Neill *et al.*, 1997; Stouthamer *et al.*, 1999).

Symbionts that are found within cells are often transmitted vertically – from mother to offspring. When inherited in this way they may invade a host population either by contributing directly to the fitness of their hosts (Bull, 1983) or by manipulating host reproduction in ways that increase the frequency or fitness of infected females, often at the expense of males (O'Neill *et al.*, 1997). A well-known example of the latter type of symbiont is *Wolbachia*, an alphaproteobacterium that has been found to be pervasive in arthropods (e.g. Werren

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et al., 1995; Jeyaprakash & Hoy, 2000). Largely vertically transmitted, *Wolbachia* has been shown to manipulate host reproduction in four main ways: by cytoplasmic incompatibility (CI, where uninfected females that mate with infected males have lowered fecundity relative to infected female wasps), through male killing (where male offspring die, often early in embryogenesis), through feminization (where genotypic male offspring develop as phenotypic female offspring) and by parthenogenesis induction (PI, where genotypic male offspring are converted to genotypic female offspring) (reviewed in O'Neill *et al.*, 1997; Werren, 1997; Stouthamer *et al.*, 1999).

A newly described bacterial symbiont, *Cardinium*, of the Bacteroidetes (= CFB group), has been found to play a role in three of these four reproductive manipulations in arthropods. *Cardinium* causes feminization in *Brevipalpus phoenicis* mites (Weeks *et al.*, 2001), is associated with parthenogenesis in *Aspidiotus nerii* (Provencher *et al.*, 2005) and in some species of *Encarsia* parasitoid wasps (Zchori-Fein *et al.*, 2001), causes PI in *E. hispida* (Zchori-Fein *et al.*, 2004) and causes CI in a sexual population of *Encarsia pergandiella* (Hunter *et al.*, 2003). *Cardinium* is also known to enhance the fecundity of the predatory mite *Metaseiulus occidentalis* (Weeks & Stouthamer, 2003). Recent surveys have found *Cardinium* to be common in arthropod hosts: 6–7.2% of sampled arthropods, distributed in the Hymenoptera, the Hemiptera and the Acari, harbor *Cardinium* (Weeks *et al.*, 2003; Zchori-Fein & Perlman, 2004).

The subject of this paper is a *Cardinium*-infected all-female population of the parasitoid wasp, *E. pergandiella*. Normally, in haplodiploid systems such as the Hymenoptera, male wasps are produced from unfertilized eggs and female wasps from fertilized eggs. This reproductive biology is called arrhenotoky, and will be referred to as 'sexual' here. PI in these systems requires that haploid incipient male eggs be converted into diploid female eggs. In PI *Wolbachia*, cytogenetic studies have shown this to be the result of interruptions in the early stages of mitosis (Stouthamer & Kazmer, 1994; Gottlieb *et al.*, 2002; Pannebakker *et al.*, 2004). This type of parthenogenesis is termed thelytokous parthenogenesis or thelytoky (the asexual production of females) to distinguish it from the normal asexual production of males found in the Hymenoptera.

Sexual populations of *E. pergandiella* are autoparasitoids (Gerling, 1966; Walter, 1983; Hunter & Woolley, 2001). The hosts for female eggs of sexual *E. pergandiella* are nymphal whiteflies (the *primary* hosts), minute sap-sucking Hemiptera with sessile scale-like immature stages. The hosts for male eggs *E. pergandiella* are pupal parasitoid wasps developing within the whitefly cuticle (the *secondary* hosts). These sex-specific host relationships appear to be obligate, as evidenced by the failure of eggs laid in inappropriate hosts to develop. For example, the haploid eggs laid (rarely) by virgin sexual female wasps in primary hosts (Gerling, 1966; Hunter, 1999) do not develop, nor do those eggs laid by thelytokous wasps (where all are expected to be female offspring) in secondary hosts (Zchori-Fein *et al.*, 2001). However, the requirements that are met by these hosts and mechanisms that maintain this specificity are unknown.

Autoparasitoid dependence on particular host types for the development of each sex places a unique obstacle in the path of a PI symbiont. The conversion of haploid, incipient male eggs to diploid female eggs creates a mismatch between offspring sex and host type. To successfully invade an autoparasitoid population, symbionts in infected females must either: (a) challenge the autoparasitoid developmental requirements, inducing the development of female offspring in secondary hosts (where unfertilized male eggs are usually laid); or (b) induce a change in the female wasp's host selection behaviour, causing the female wasp to lay unfertilized eggs into primary hosts, where thelytokous female wasps may then develop. Evidence of a change in oviposition behaviour accompanying *Cardinium* infection has been found in thelytokous *E. pergandiella* (Zchori-Fein *et al.*, 2001).

In no-choice tests by Zchori-Fein *et al.* (2001), infected *E. pergandiella* wasps laid similar numbers of eggs in arenas containing either primary hosts or only secondary hosts. Antibiotic-cured female wasps oviposited in a similar number of secondary hosts as infected female wasps, but laid very few eggs in primary host arenas. When arenas were incubated to assess development, infected wasps produced offspring (all female) on primary hosts. Very few offspring were produced by cured female wasps, and those that did were female wasps emerging from the rare eggs laid in primary hosts. Neither treatment produced offspring in secondary hosts. The failure to restore male production after antibiotic treatment is curious and unlike the pattern found in many PI *Wolbachia* systems, where male wasps are regularly produced by cured female wasps (Stouthamer *et al.*, 1990; Zchori-Fein *et al.*, 1992, 1995; Kajita, 1993; Pijls *et al.*, 1996; Hunter, 1999; Arakaki *et al.*, 2000; Giorgini, 2001; Pannebakker *et al.*, 2005). In PI *Wolbachia*, this production of male wasps by cured female wasps is considered as evidence of *Wolbachia*'s involvement in the induction of parthenogenesis via restoration of diploidy to haploid eggs. Production of male wasps following antibiotic treatment has been shown in *Cardinium*-infected *Encarsia hispida* (Hunter, 1999; Giorgini, 2001; Zchori-Fein *et al.*, 2004). The inability of *E. pergandiella* to produce male offspring after antibiotic treatment appears to be more representative of *Cardinium*-infected thelytokous *Encarsia* (Giorgini, 2001; Hunter & Zchori-Fein, 2006) and may be the result of relaxed selection on the development of male wasps (Zchori-Fein *et al.*, 2001). There are, however, other possible explanations. For instance, removing the bacteria may not be sufficient to restore normal cell division

in developing offspring, because of residual bacterial effects continuing to restore diploidy (Arakaki *et al.*, 2000). Alternatively, there may be a current lack of involvement of the bacteria in the process.

This study focuses on and further explores the apparent change in oviposition behaviour observed by Zchori-Fein *et al.* (2001). The previous study used no-choice arenas; we used choice arenas with equal numbers of evenly distributed primary and secondary hosts. All hosts were dissected to determine the relative proportions of each host used. The use of uniform arenas eliminates the possible changes in oviposition that may have been associated with the different presentations of the two host types used in the previous study. This includes the possibility of laying eggs in less acceptable hosts that might occur when female wasps are faced with no alternative host type. Both host types are likely encountered together in nature (Hunter & Godfray, 1995; Hunter & Kelly, 1998; Bográn & Heinz, 2002). Additionally, we examined the oviposition behaviour of a conspecific sexual population (*E. pergandiella* from Texas) so we could compare the behaviour of the thelytokous population with that of wasps representing its likely ancestral state. Finally, we evaluated F_1 (untreated) offspring of cured thelytokous females to obviate concerns about antibiotic effects on the observed egg-laying behaviour. Previous studies of thelytokous species in which the symbiont has become fixed in the population have not been able to address concerns about antibiotic effects caused by the production of only infertile F_1 male wasps after curing (Zchori-Fein *et al.*, 1992, 1995; DeBarro & Hart, 2001; Gottlieb & Zchori-Fein, 2001). Although extremely rare, these F_1 female wasps allowed us to separate antibiotic treatment and infection status as potential factors influencing oviposition behaviour.

Materials and methods

Origin and identity of cultures

Encarsia pergandiella was raised on whitefly (*Bemisia tabaci*) hosts on cowpea (*Vigna unguiculata*) (25 °C 16L/8D, ambient humidity). Sexual *E. pergandiella* originated from a Texas population maintained in the laboratory since 2003 and thelytokous *E. pergandiella* originated from a Brazilian population maintained in the laboratory since 1993. Both are infected with *Cardinium*. In the sexual population, *Cardinium* infection is associated with CI (Hunter *et al.*, 2003). We note that this symbiont phenotype is expected to be asymptomatic at fixation (as it is in our laboratory culture).

Antibiotic curing

Encarsia pergandiella adults were collected within 12 h of emergence and exposed to a honey + rifampicin (50 mg mL^{-1}) mixture for 48 h at 25 °C [14L/10D, 65% relative humidity (RH)]. After this time the adults were either placed in vials with honey until they were used in experiments or introduced to whitefly-infested plants for the production of offspring (see below).

Creation of treatment groups

Thelytokous E. pergandiella

Parental (F_0) treatment groups for each block originated from a common pool of infected female wasps. Treatments were infected (fed only honey) and cured (treated with antibiotic as described above). All of the F_0 wasps were used for experiments within 5 days of emergence. The F_1 wasps were untreated, adult progeny of antibiotic-cured female wasps placed on primary hosts at 22 °C (15L/9D, ambient humidity). Cured female wasps produced very few offspring, so the relatively few F_1 adults were offspring collected from hundreds of cured adults placed on 21 whitefly-infested plants. At 16 days, all leaves were examined for evidence of F_1 *E. pergandiella* pupae and each pupa was isolated into a 1.2-mL vial with a small drop of honey. Vials were checked for the emergence of adult wasps every 24 h and emerged F_1 wasps were left in their respective vials for 5–7 days until the time of the experiment.

Sexual E. pergandiella

F_0 treatment groups for the sexual *E. pergandiella* experiment also originated from a common pool of infected female wasps. Treatments were created in the same way as described for the thelytokous population. For this population, we also created an additional treatment of infected, mated female wasps. Each female wasp in this treatment was individually mated with a male wasp of the same age.

Experimental set-up

To assess host choice in each treatment, individual primary and secondary hosts were punched out of cowpea leaves using a 0.5-cm diameter sharpened brass tube. Primary hosts were third to fourth instar nymphal whiteflies (*B. tabaci*), and secondary hosts were early pupal *Eretmocerous eremicus* wasps (pre-red eye) raised on whitefly hosts at 25 °C 16L/8D, ambient humidity.

Oviposition arenas consisted of a grid of alternating primary and secondary hosts (32 total hosts, 16 of each type) on a damp piece of filter paper in a 35-mm Petri dish. Each leaf disc was attached to the filter paper by coating the opposite side of the disc with a dilute honey/water solution. Prior to the beginning of the experiment, the filter paper in each arena was allowed to dry enough so as not to impede the locomotion of the wasps. Each wasp was placed individually into an arena, and allowed to oviposit for 6 h (25 °C, 14L/10D, 65% RH). After 6 h, wasps were removed from the arenas and held for

polymerase chain reaction (PCR) confirmation of infection status.

Arenas were placed into an environmental chamber (25 °C, 14L/10D, 65% RH) for 24 h to allow the eggs to increase in size. *E. pergandiella* eggs are hydropic, increasing vastly in size over the first 24 h (Gerling, 1966), even in inappropriate hosts (S.G. Kenyon, unpublished). Counting these larger, more developed eggs reduced errors in egg detection. After 24 h, the arenas were moved to 4 °C to slow down the development and all hosts in each arena were dissected to count the wasp eggs. The number of hosts used (of each type) and eggs laid (in each host type) were counted for each individual wasp. *E. pergandiella* female wasps lay only one egg at a time, and each host supports the development of only one offspring (Gerling, 1966).

The experiment on the thelytokous (Brazil) population of *E. pergandiella* was conducted in three blocks. The first block was in March 2004, the second in April 2004 and the third in October 2004. The one block of the sexual (Texas) population of *E. pergandiella* was performed in March 2005.

PCR assay/DNA extractions

To determine whether individual wasps were infected, wasps were placed in 0.5-mL centrifuge tubes and frozen in liquid nitrogen. A lysis solution (50 ul of 20 mg ul^{-1} proteinase K, 5 ul of 1.0 M Tris, 1 ul of 0.5 M EDTA, 5 ul of Nonidet/NP40 detergent and 940 ul of ddH_2O) was added, and the wasps were ground using plastic pestles. The resulting homogenate was placed in a 65 °C water bath for 2 h to lyse, and then heated to 95 °C for 10 min to denature. These lysed samples were then placed at −20 °C until a PCR was run to determine the presence of *Cardinium*. Previous studies have shown that *Cardinium* is the only intracellular bacterium associated with both thelytokous and sexual populations of *E. pergandiella* (Zchori-Fein *et al.*, 2001; Hunter *et al.*, 2003).

Polymerase chain reactions were carried out in an Eppendorf Mastercycler (Eppendorf AG, Westbury, NY, USA). Ten-μL reactions (5.65 μL of purified water, 1 μL of 10× buffer, 0.8 μL of 10 mM dNTPs, 0.5 μL of 25 mM $MgCl_2$, 0.5 μL of 5 pmol μL^{-1} forward primer, 0.5 μL of 5 pmol μL^{-1} reverse primer, 0.05 μL of 5 U μL^{-1} Eppendorf MasterTaq polymerase and 1 μL of sample) were run, both to detect successful DNA extraction with mitochondrial primers (mtd10 5′-TTGATTTTTTGGTCATCCAGAAGT-3′, mtd12 5′-TCCAATGCACTAATCTGCCATATTA-3′) and to test for the presence of *Cardinium* DNA with *Cardinium*-specific primers (cloF 5′-GCGGTGTAAAATGAGCGTG-3′, cloR1 5′-ACCTMTTCTTAACTCAAGCCT-3′) (Weeks & Stouthamer, 2003). Positive and DNA-free negative controls were used in every run. Each PCR was run for one cycle of 94 °C for 4 min, 35 cycles of (94 °C for 1 min, 55 °C for 1 min, 72 °C for 1 min) and a final cycle of 72 °C for 6 min followed by a 10 °C hold. Reactions were run on a 1% agarose gel stained with ethidium bromide.

Analysis

Logistic regression analyses were used to compare proportions of eggs allotted to each host type and to compare the hosts of each type used, both with respect to block and treatment. Logistic regression allows proportional data to be analysed directly without transformation. The error distributions of proportions of primary and secondary hosts were assumed to be binomial. ANOVA was used to compare differences in total hosts parasitized, in eggs laid, as well as the mean number of eggs per host, all with respect to block and treatment. Tukey's HSD *post hoc* tests ($P < 0.05$) were performed to compare within treatments and blocks. Both types of analyses were performed using JMP IN 4.1 (SAS Institute Inc., Cary, NC, USA).

Results

Oviposition patterns

Some broad differences were seen in basic measures of oviposition behaviour (average number of total eggs laid and total hosts used). These differences directly influence what inferences can be made about host choice and discrimination. Infected and cured wasps of the thelytokous population laid similar numbers of eggs and used similar numbers of hosts, whereas wasps in the F_1-cured treatment were less fecund, laying significantly fewer eggs (ANOVA: $F_{2,99} = 37.19$, $P < 0.0001$) on significantly fewer hosts (ANOVA: $F_{2,101} = 35.06$, $P < 0.0001$) (Fig. 1a). In the sexual population, the mated female wasps infected with *Cardinium* laid significantly more eggs (ANOVA: $F_{2,30} = 5.16$, $P = 0.012$) and used almost double the number of hosts (ANOVA: $F_{2,30} = 6.64$, $P = 0.004$) as unmated wasps. Both F_0 thelytokous wasps and unmated sexual wasps appeared to lay similar numbers of eggs, but the unmated sexual wasps distributed these eggs across a greater number of hosts (Fig. 1).

Host choice

There are two ways to measure choice: either by the number of eggs laid in each host type or by the number of hosts parasitized of each host type. *E. pergandiella* lays one egg with each oviposition, so each egg represents a single choice. Only one wasp can develop in each host; therefore hosts parasitized of each type represent choices that reflect potential successful development. Patterns of host use were similar to those seen in the number of eggs laid (Fig. 1). The significance of oviposition choice is best understood in the context of the benefit to the female wasp laying the eggs, progeny development, which is represented by host use. We note that only certain

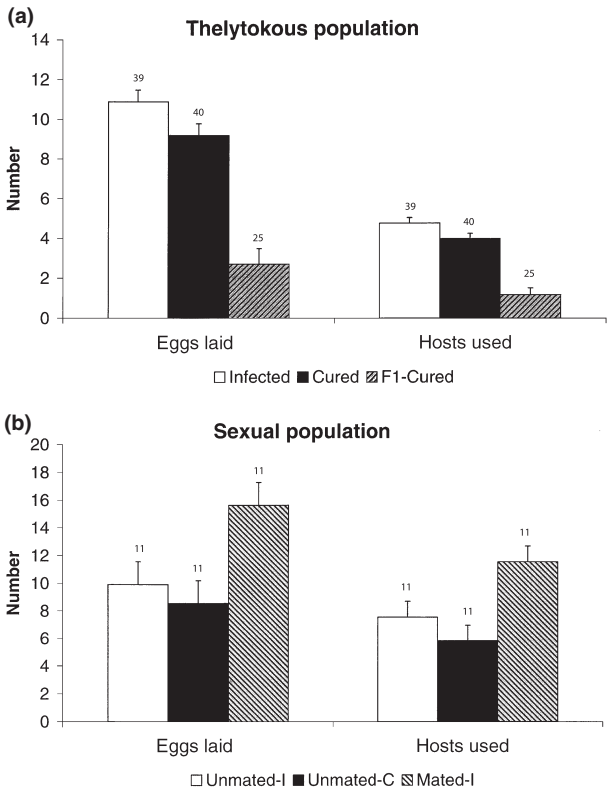

Fig. 1 Oviposition patterns. (a) Mean eggs laid and hosts parasitized for thelytokous treatments. White bars indicate F_0-Infected female wasps (mean ± SE), black bars indicate F_0-Cured female wasps (mean ± SE) and hatched bars indicate F_1-Cured female wasps (mean ± SE) for each parameter. (b) Mean eggs laid and hosts parasitized for sexual (arrhenotokous) treatments. White bars indicate Unmated-I(nfected) female wasps (mean ± SE), black bars indicate Unmated-C(ured) female wasps (mean ± SE) and hatched bars indicate Mated-I(nfected) female wasps (mean ± SE) for each parameter. Numbers above bars indicate replicates.

choices would be successful if eggs were allowed to develop. For thelytokous female wasps, only primary hosts support offspring development, while for sexual female wasps, the suitability of the host types is dependent on whether or not the egg is fertilized.

In the thelytokous population, the proportion of each host type parasitized was compared for each treatment using a binomial model. The model was significant (LR-$\chi^2_{\text{WholeModel}} = 45.68$, d.f. = 3, $P < 0.0001$), indicating a good fit of the data. In the model, both block (LR-$\chi^2_{\text{blk}} = 40.11$, d.f. = 2, $P < 0.0001$) and treatment (LR-$\chi^2_{\text{trt}} = 19.07$, d.f. = 2, $P < 0.0001$) were highly significant. Infected female wasps parasitized both host types, using more primary hosts than did female wasps of other treatments. Cured female wasps continued to use both host types but used a higher proportion (68–84%) of secondary hosts in each block. Cured offspring of antibiotic-treated female wasps increased secondary host use to an even greater extent, laying 95–98% more of their

Table 1 Logistic regression equation coefficients.

	Regression coefficient
(a) Thelytokous	$B_0 = 1.26$, $P = 0.0004$*
	$B_{F0I} = -1.49$, $P < 0.0001$*
	$B_{F0C} = -0.44$, $P = 0.23$
	$B_{F1C} = 1.93$, $P = 0.0051$*
	$B_{Block1} = -0.90$, $P < 0.0001$*
	$B_{Block2} = 0.23$, $P = 0.16$
	$B_{Block3} = 0.67$, $P < 0.0001$*
b) Sexual	$B_0 = -1.94$, $P < 0.0001$*
	$B_{Mated} = 1.64$, $P < 0.0001$*

Regression coefficients and their significance for the regression equation of (a) thelytokous and (b) sexual wasp populations. Within the equation, each coefficient either contributes one unit ($x = 1$) or not at all ($x = 0$) to the final logit depending on the treatment or block being considered.
*Indicates a significant *p*-value.

eggs in secondary hosts. Chi-squared values for block and treatment were calculated using likelihood ratio tests, and those values for individual regression coefficients are shown in Table 1. Worth noting is that the regression coefficient for F_0-cured female wasps was not significant, so all variation in this treatment was caused by the variation in block and that experienced by all wasps (represented by the intercept). Likewise, block 2 did not contribute significantly to the variation between groups. Raw treatment proportions of secondary hosts in each block of the thelytokous population are shown in Fig. 2a to help visualize the results of the logistic regression. ANOVA results in Fig. 3a support these findings, showing how each host type was used, by treatment, in each block.

Sexual *E. pergandiella* laid nearly their entire complement of eggs in secondary hosts when unmated (regardless of antibiotic curing) and partitioned their eggs evenly between host types when mated (Figs 2b and 3b). Logistic regression analyses were significant (LR-$\chi^2 = 72.93$, d.f. = 1, $P < 0.0001$) with mating status as a factor (see Table 1 for regression coefficients). Infection status is not included in the model as it was not found to have a significant explanatory value for how hosts were used. Being mated corresponded to an 89–99% increase in primary host use. Interestingly, sexual treatments did not differ significantly in the number of eggs laid in secondary hosts ($F_{2,30} = 0.66$, $P = 0.52$) and the proportional difference between mated and unmated female wasps can be wholly ascribed to mated female wasps laying additional eggs in primary hosts ($F_{2,30} = 31.70$, $P < 0.0001$) (Fig. 3b).

Superparasitism

Only one *E. pergandiella* wasp develops from a given host, so > 1 egg laid in a host is considered superparasitism. There were no significant differences in the number of

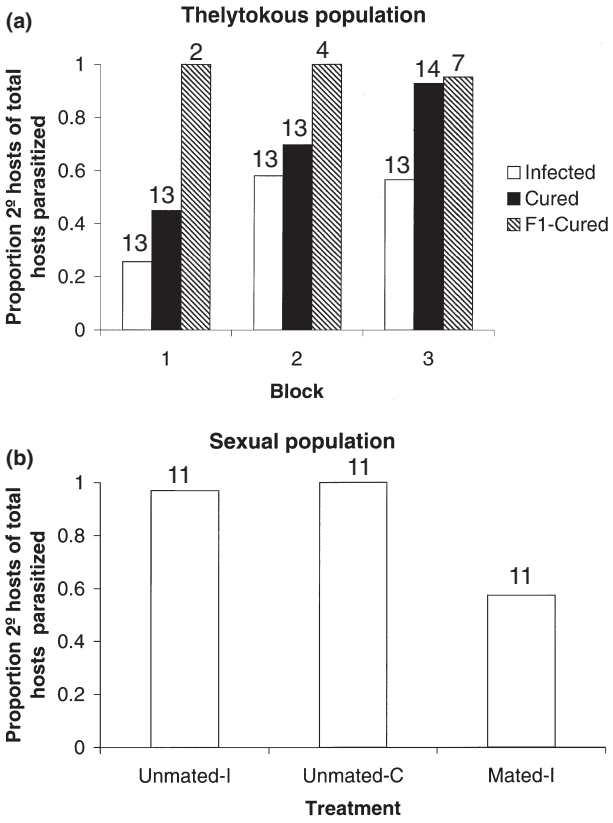

Fig. 2 Proportion secondary hosts of total hosts parasitized. In thelytokous treatments (a) white bars indicate F_0-Infected, black bars indicate F_0-Cured and hatched bars indicate F_1-Cured offspring in each of three blocks. The sexual (arrhenotokous) treatments (b) are Unmated-I(nfected), Unmated-C(ured) and Mated-I(nfected). Numbers above bars indicate replicates. In block 1: 2/2 laid eggs; in block 2: 4/13 laid eggs; and in block 3: 7/10 laid eggs. Only those wasps laying eggs were used to calculate proportions.

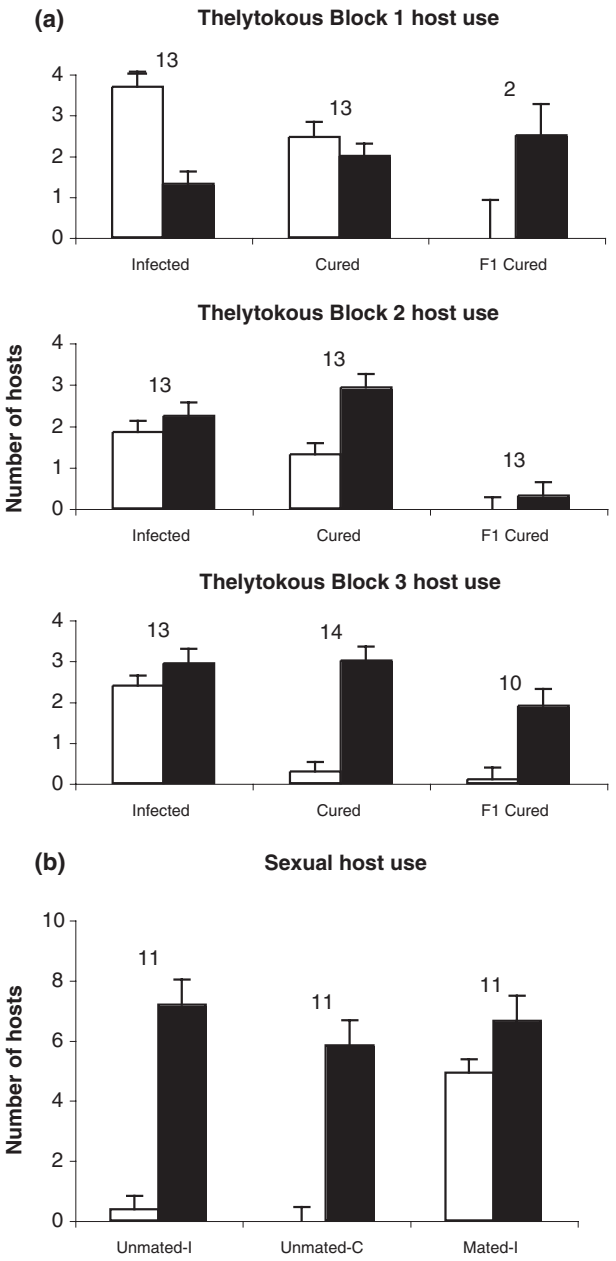

Fig. 3 Distribution of parasitism between host types. White bars indicate primary hosts parasitized (±SE) and black bars indicate secondary hosts parasitized (±SE) in each treatment. Thelytokous blocks (a) include F_0-Infected and Cured treatments as well as the F_1-Cured (untreated) offspring of cured female wasps. The sexual (arrhenotokous) treatments (b) are Unmated-I(nfected), Unmated-C(ured) and Mated-I(nfected). Numbers above bars indicate replicates.

eggs laid per host among treatments within either population (Fig. 4). However, thelytokous wasps laid a significantly higher mean number of eggs per host (2.13 ± 0.09) than did female wasps in the sexual population (1.34 ± 0.16, $F_{1,169} = 18.57$, $P < 0.0001$). This was in spite of an equal or greater number of total eggs laid in the sexual population, which would be expected to contribute to a greater level of random superparasitism in the sexual wasps.

Discussion

Change in oviposition behaviour in response to curing of *Cardinium* infection

The results of this study indicate that thelytokous *E. pergandiella* female wasps change their oviposition behaviour when free of *Cardinium*, shifting the distribution of eggs towards secondary hosts. Antibiotic-cured female wasps used a greater proportion of secondary hosts than did infected female wasps and untreated offspring of antibiotic-cured female wasps laid eggs almost exclusively in secondary hosts. This preferential use of secondary hosts was also observed, as expected, by

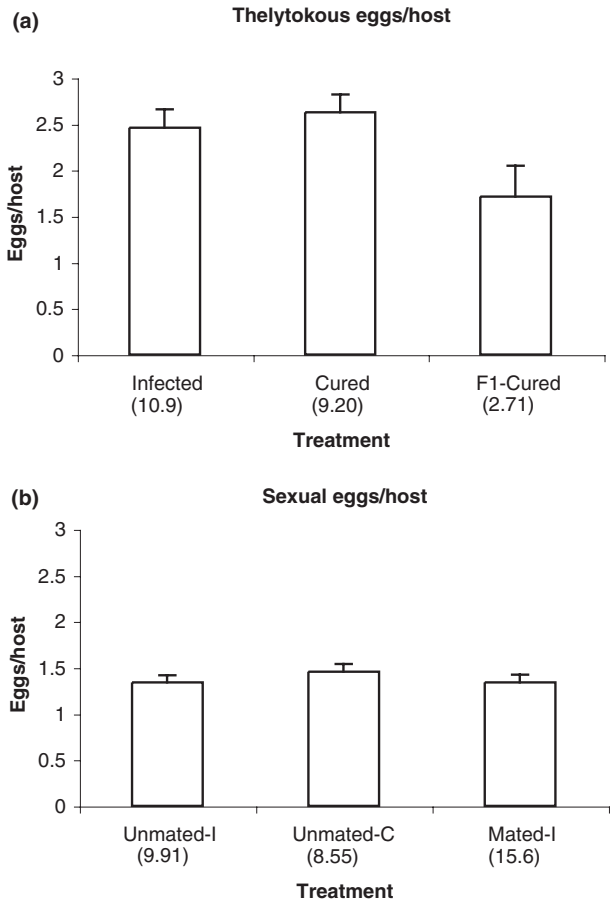

Fig. 4 Numbers of eggs/host for thelytokous and sexual populations. Bars indicate eggs/host (±SE). Thelytokous blocks (a) include F_0-Infected ($n = 39$) and Cured ($n = 40$) treatments as well as the F_1-Cured (untreated) offspring of cured female wasps ($n = 25$). The sexual (arrhenotokous) treatments (b) are Unmated-I(nfected), Unmated-C(ured) and Mated-I(nfected). All sexual treatments had 11 replicates. Parenthetical numbers below treatment labels indicate the mean number of eggs laid.

unmated female wasps of a conspecific sexual population. These findings implicate the symbiotic *Cardinium* bacteria in a critical change in oviposition behaviour that, because of obligate sex-specific host development, is likely responsible for the persistence of the thelytokous population in nature. Because we used untreated F_1 offspring of antibiotic-cured female wasps, the observed change was not confounded by antibiotic effects, a frequent problem for studies of PI systems in which sexual function cannot be restored (Zchori-Fein *et al.*, 1992, 1995; DeBarro & Hart, 2001; Gottlieb & Zchori-Fein, 2001; Stouthamer & Mak, 2002; Weeks & Breeuwer, 2003).

Comparing thelytokous F_1 female wasps to the infected female wasps gives us insight into the degree of change in oviposition behaviour (Fig. 2a). Wasps that are *Cardinium*-free throughout their lives act much like unmated sexuals (Fig. 2b), parasitizing only secondary hosts with the exception of rare 'mistakes'. This is a striking shift from the relatively equal use of both host types in F_0-infected female wasps, implicating *Cardinium* as the reason for the acceptance of primary hosts by infected female wasps. Curing of the thelytokous population with antibiotics resulted in variable oviposition behaviour. While PCR indicated that these wasps were no longer infected, these recently cured wasps may have retained residual bacterial products or been influenced by bacterial involvement prior to curing. We might, therefore, predict that the behaviour of the antibiotic-cured female wasps would be intermediate between that of the infected female wasps and the F_1 offspring of cured female wasps. Despite a significant block-to-block variation, this was indeed the case (Figs 2a and 3a). Although antibiotic-cured female wasps of all blocks laid eggs in both host types, they laid a higher proportion in secondary hosts than did infected female wasps within each block, and their continued use of primary hosts differentiates their behaviour from that of the F_1-cured female wasps. The variability in the response of these cured female wasps to their host environment is perhaps not surprising. These female wasps likely ingested variable doses of rifampicin (depending on the number of times they fed) and experienced an abrupt change in infection status. This is in contrast to that of the other two treatments where the infection status (infected or cured) was constant throughout the life of the female wasp.

Parthenogenesis-inducing symbionts are predicted to invade a host population by causing infected, unfertilized eggs to develop into female offspring (Stouthamer *et al.*, 1999). In an autoparasitic lineage, this change creates a mismatch between the egg-laying behaviour of an unmated female wasp and the appropriate developmental location for these unfertilized eggs that will develop as female wasps. Perhaps the easiest solution to imagine is that somehow these unfertilized eggs are able to develop in secondary hosts. There have been observations of thelytokous *E. hispida* female wasps occasionally developing on secondary hosts (Hunter, 1999). What we observe in *E. pergandiella*, however, is the oviposition of these unfertilized eggs in primary hosts (Zchori-Fein *et al.*, 2001, this study).

How would this behaviour facilitate an invasion of the bacterium? Based on relatedness and the fact that curing of thelytokous *E. pergandiella* leads to oviposition patterns that resemble those of unmated sexuals, it seems highly likely that the ancestral reproductive biology of the thelytokous population was sexual autoparasitism. To invade this system, infection with *Cardinium* would have to enable an increase in the production of daughters by infected female wasps relative to uninfected female wasps. The behavioural change seen in infected thelytokous female wasps is an increase in the acceptance of primary hosts by unmated female wasps such that

haploid eggs are distributed approximately evenly between host types. In the ancestral sexual population, we expect that *Cardinium* infection would initially be at low frequencies, male wasps would still be present and most female wasps would be mated.

The contribution of these ancestral, mated, *Cardinium*-infected female wasps to the population of infected offspring could follow two different patterns. They might either (a) continue to use hosts evenly, either by indiscriminately laying fertilized and unfertilized eggs in both host types or laying fertilized eggs in primary hosts and unfertilized eggs in secondary hosts; or (b) could lay their fertilized eggs entirely in primary hosts (as fertilization also increases the acceptance of primary hosts; Figs 2b and 3b) and distribute the unfertilized eggs in both host types. In either of the two scenarios described above, these female wasps will produce at least as many infected daughters as uninfected female wasps, with scenario (b) resulting in a greater proportion of infected daughters.

The other important contributors to a successful *Cardinium* invasion in autoparasitoids are unmated, infected female wasps. The importance of this population increases with a scarcity of male wasps. Because of the host-specific developmental requirements, female-biased sex ratios in autoparasitoids are not uncommon, and male wasps may be limiting (especially in seasonal or isolated populations) (Gerling, 1966; Hunter *et al.*, 1993). When male wasps are limiting, female wasps (whether infected or uninfected) may not mate immediately. Unmated, uninfected female wasps will produce only male offspring, while unmated, infected female wasps will produce only female offspring. The change in the behaviour caused by *Cardinium* infection allows the successful development of the latter, increasing the proportion of infected daughters in the population. Whether invasion of *Cardinium* was facilitated by increases in the infected female population caused by offspring of infected, unmated female wasps (mate limitation) alone or in combination with an increase in primary host use by infected mated female wasps, the observed manipulation by *Cardinium* of wasp oviposition behaviour would likely enhance its spread within a population.

The behavioural change observed here implicates *Cardinium* as the causative agent for the addition of primary hosts to the unmated adult wasp's suite of behaviours. That infected thelytokous female wasps can revert to the behaviour of unmated sexual female wasps merely one generation after antibiotic curing indicates that selection on the wasp genome is quite unlikely to be responsible for the change in the behaviour. While there are several documented examples of parasite-mediated change of host behaviour (Moore, 2002), it appears that there are few examples of host behaviour being so directly influenced by inherited intracellular symbionts. One such case involves the *Leptopilina boulardi* Filamentous Virus (LbFV) that is transmitted both vertically and horizontally among *L. boulardi* parasitoid wasps (Varaldi *et al.*, 2005). Infection with the virus appears to increase the likelihood of superparasitism by infected female wasps, and horizontal transmission has also been observed in these superparasitized hosts (Varaldi *et al.*, 2005). Infected wasps are less mobile and are thought to reallocate these resources for increased egg production. Both of these characteristics are likely contributors to the observed increase in superparasitism. The current study suggests that modifying host behaviour is also within the capacity of a strictly vertically transmitted symbiont, especially when the manipulation results in a sizeable increase in fitness for both the symbiont and its host. Because of the reproductive biology of *E. pergandiella*, this causes a visible expansion of the range of acceptable host types, not just a modification of the physiological state of the wasp. A careful examination of other systems might yield more subtle behavioural changes that benefit transmission of symbionts or fitness of infected hosts.

Oviposition in secondary hosts

In thelytokous *E. pergandiella*, including primary hosts as acceptable oviposition sites for unfertilized eggs allows the successful development of offspring. However, thelytokous *E. pergandiella* do not produce offspring in secondary hosts (Zchori-Fein *et al.*, 2001). Therefore, it is curious that they persist in depositing approximately half of their eggs in these hosts. As it would benefit both *Cardinium* and *E. pergandiella* to produce as many progeny as possible, one would predict strong selection to lay all eggs in hosts that result in the successful development of offspring, in this case, primary hosts. The continued use of secondary hosts seems maladaptive, suggesting that there may be constraints because of the mechanism of behavioural change imposed by *Cardinium*.

As infected female wasps continue to accept both host types, we can imagine two possibilities for the mechanism of host manipulation. First, *Cardinium* could induce the physiological effects of being mated, thus making both host types acceptable. Despite an inability to fertilize eggs, attempted selective fertilization would determine where eggs were laid. Only those in primary hosts would develop; so one might predict that, over time, there would be a selection for a shift toward laying eggs only in these hosts. This was not observed, suggesting perhaps that the mechanism of behavioural change is not easily refined by selection. Such a result might be expected if *Cardinium* infection decreased a wasp's ability to discriminate between, or otherwise assess, hosts. There are many examples of loss of function upon parasitism (Moore, 2002), and apparent loss of discrimination may play a role in the *L. boulardi*/LbFV example above (Varaldi *et al.*, 2005). Infection in *L. boulardi* results in levels of superparasitism consistently higher than those of the uninfected population. In the present study, superparasitism levels in the thelytokous population are

consistently higher than in the sexual population (Fig. 4) regardless of infection status.

There are many possible factors involved in the difference in the level of superparasitism between these laboratory populations given their different histories, but a difference because of host limitation is not a plausible explanation. In this study, wasps in the thelytokous-infected treatment and the sexual unmated female wasps laid similar numbers of eggs. The thelytokous-infected treatment attacked both host types and thus had twice as many acceptable hosts as the sexual unmated female wasps that attacked only secondary hosts. Despite this, the thelytokous female wasps superparasitized at a consistently higher rate (2.13 vs. 1.34 eggs/host, Fig. 4), which is the opposite of what would be expected with the greater pool of acceptable hosts available to the thelytokous female wasps. Failure to discriminate between host types may involve processes that are also involved in assessing the parasitized state of the host. Alternatively, superparasitism could be adaptive; a greater degree of superparasitism could be selected for if egg viability or larval ability to overcome host defences was lower because of infection. Finally, lowered mobility (as seen in infected *L. boulardi*; Varaldi *et al*., 2005) could also be partly responsible (we observed clumping of hosts used for oviposition; S.G. Kenyon, unpublished). Previous studies have shown a tendency for superparasitism in other *E. pergandiella* populations (Hunter, 1989; Pedata *et al*., 2002) and that previous oviposition experience may be necessary to make decisions about the state of the host (Hunter, 1989; Ardeh *et al*., 2005). We, however, did not observe any change in behaviour caused by the previous oviposition experience (S.G. Kenyon, unpublished).

The lack of precision in oviposition behaviour (accepting secondary hosts and superparasitism) found in this study causes us to currently favour a 'loss of discrimination' hypothesis for the mechanism of behaviour change. To further test this idea, one might evaluate other aspects of oviposition behaviour, for example, host species and host stage acceptances to determine if these too are broader in the thelytokous population. As well, tests of viability and development of eggs laid in primary hosts or measures of motor activity and behaviours preceding oviposition would allow the evaluation of other possible causes of superparasitism. Finally, when artificial infection of an uninfected *Encarsia* with PI *Cardinium* becomes possible, it would be interesting to follow the course of a new infection. If our hypothesis is correct, we would predict that the loss of discrimination between host types and between parasitized and unparasitized hosts would be perfectly coincident with infection status.

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References

Arakaki, N., Noda, H. & Yamagishi, K. 2000. *Wolbachia*-induced parthenogenesis in the egg parasitoid *Telenomus nawai*. *Entomol. Exp. Appl.* **96**: 177–184.

Ardeh, M.J., de Jong, P.W. & van Lenteren, J.C. 2005. Intra- and interspecific host discrimination in arrhenotokous and thelytokous *Eretmocerus* spp. *Biol. Control* **33**: 74–80.

Bandi, C., Dunn, A.M., Hurst, D.D.H. & Rigaud, T. 2001. Inherited microorganisms, sex-specific virulence and reproductive parasitism. *Trends Parasitol.* **17**: 88–94.

Bográn, C.E. & Heinz, K.M. 2002. Host selection by the heteronomous hyperparasitoid *Encarsia pergandiella*: multiple-choice tests using *Benisia argentifolii* as a primary host. *Entomol. Exp. Appl.* **103**: 11–21.

Bull, J.J. 1983. *Evolution of Sex Determining Mechanisms*. The Benjamin Cummins Pub. Co., London.

DeBarro, P.J. & Hart, P.J. 2001. Antibiotic curing of parthenogenesis in *Eretmocerus mundus* (Australian parthenogenetic form). *Entomol. Exp. Appl.* **99**: 225–230.

Gerling, D. 1966. Studies with whitefly parasites of Southern California. I. *Encarsia pergandiella* Howard (Hymenoptera: Aphelinidae). *Can. Entomol.* **98**: 707–724.

Giorgini, M. 2001. Induction of males in thelytokous populations of *Encarsia meritoria* and *Encarsia protransvena*: a systematic tool. *BioControl.* **46**: 427–438.

Gottlieb, Y. & Zchori-Fein, E. 2001. Irreversible thelytokous reproduction in *Muscidifurax uniraptor*. *Entomol. Exp. Appl.* **100**: 271–278.

Gottlieb, Y., Zachori-Fein, E., Werren, J.H. & Karr, T.L. 2002. Diploidy restoration in *Wolbachia*-infected *Muscidifurax uniraptor* (Hymenoptera: Pteromalidae). *J. Invertebr. Pathol.* **81**: 166–174.

Hunter, M.S. 1989. Sex allocation and egg distribution of an autoparasitoid, *Encarsia pergandiella*, (Hymenoptera: Aphelinidae). *Ecol. Entomol.* **14**: 57–67.

Hunter, M.S. 1999. The influence of parthenogenesis-inducing *Wolbachia* on the oviposition behaviour and sex-specific developmental requirements of autoparasitoid wasps. *J. Evol. Biol.* **12**: 735–741.

Hunter, M.S. & Godfray, H.C.J. 1995. Ecological determinants of sex allocation in an autoparasitoid wasp. *J. Anim. Ecol.* **64**: 95–106.

Hunter, M.S. & Kelly, S.E. 1998. Hyperparasitism by an exotic autoparasitoid: secondary host selection and the window of vulnerability of conspecific and native heterospecific hosts. *Entomol. Exp. Appl.* **89**: 249–259.

Hunter, M.S. & Woolley, J.B. 2001. Evolution and behavioral ecology of heteronomous Aphelinid parasitoids. *Annu. Rev. Entomol.* **46**: 251–290.

Hunter, M.S. & Zchori-Fein, E. 2006. Inherited *Bacteroidetes* symbionts in arthropods. In: Insect Symbiosis, Vol. 2 (K. Bourtzis & T.A. Miller, eds), pp. 39–56. CRC Press, Boca Raton, FL.

Hunter, M.S., Nur, U. & Werren, J.H. 1993. Origin of males by genome loss in an autoparasitoid wasp. *Heredity* **70**: 162–171.

Hunter, M.S., Perlman, S.J. & Kelly, S.E. 2003. A bacterial symbiont in the *Bacteroidetes* induces cytoplasmic incompatibility in the parasitoid wasp *Encarsia pergandiella*. *Proc. R. Soc. Lond. B Biol. Sci.* **270**: 2185–2190.

Jeyaprakash, A. & Hoy, M.A. 2000. Long PCR improves *Wolbachia* DNA amplification: *wsp* sequences found in 76% of sixty-three arthropod species. *Insect Mol. Biol.* **9**: 393–405.

Kajita, H. 1993. Induction of males in the thelytokous wasp *Encarsia formosa* (Gahan) (Hymenoptera: Aphelinidae). *Appl. Entomol. Zool.* **28**: 115–117.

Moore, J. 2002. *Parasites and Behavior of Animals*. Oxford University Press, Oxford.

O'Neill, S.L., Hoffmann, A.A. & Werren, J.H. eds. 1997. *Influential Passengers*. Oxford University Press, Oxford.

Pannebakker, B.A., Pijnacker, L.P., Zwaan, B.J. & Beukeboom, L.W. 2004. Cytology of *Wolbachia*-induced parthenogenesis in *Leptopilina clavipes* (Hymenoptera: Figitidae). *Genome* **47**: 299–303.

Pannebakker, B.A., Schidlo, N.S., Boskamp, G.J.F., Dekker, L., van Dooren, T.J.M., Beukeboom, L.W., Zwaan, B.J., Brakefield, P.M. & van Alphen, J.J.M. 2005. Sexual functionality of *Leptopilina clavipes* (Hymenoptera: Figitidae) after reversing *Wolbachia*-induced parthenogenesis. *J. Evol. Biol.* **18**: 1019–1028.

Pedata, P.A., Giorgini, M. & Guerrieri, E. 2002. Interspecific host discrimination and within-host competition between *Encarsia formosa* and *E. pergandiella* (Hymenoptera: Aphelinidae), two endoparasitoids of whiteflies (Hemiptera: Aleyrodidae). *Bull. Entomol. Res.* **92**: 521–528.

Pijls, J.W.A.M., van Steenbergen, H.J. & van Alphen, J.J.M. 1996. Asexuality cured: the relations and differences between sexual and asexual *Apoanagyrus diversicornis*. *Heredity* **76**: 506–513.

Provencher, L.M., Morse, G.E., Weeks, A.R. & Normark, B.B. 2005. Parthenogenesis in the *Aspidiotus nerii* complex (Hemiptera: Diaspididae): a single origin of a worldwide polyphagous lineage associated with *Cardinium* bacteria. *Ann. Entomol. Soc. Am.* **98**: 629–635.

Russell, J.A. & Moran, N.A. 2006. Costs and benefits of symbiont infection in aphids: variation among symbionts and across temperatures. *Proc. R. Soc. Lond. B Biol. Sci.* **273**: 603–610.

Stouthamer, R. & Kazmer, D.J. 1994. Cytogenetics of microbe-associated parthenogenesis and its consequences for gene flow in *Trichogramma* wasps. *Heredity* **73**: 317–327.

Stouthamer, R. & Mak, F. 2002. Influence of antibiotics on the offspring production of the *Wolbachia*-infected parthenogenetic parasitoid *Encarsia formosa*. *J. Invertebr. Pathol.* **80**: 41–45.

Stouthamer, R., Luck, R.F. & Hamilton, W.D. 1990. Antibiotics cause parthenogenetic *Trichogramma* (Hymenoptera: Trichogrammatidae) to revert to sex. *Proc. Natl Acad. Sci. USA* **87**: 2424–2427.

Stouthamer, R., Breeuwer, J.A.J. & Hurst, G.D.D. 1999. *Wolbachia pipientis*: microbial manipulator of arthropod reproduction. *Annu. Rev. Microbiol.* **53**: 71–102.

Varaldi, J., Boulétreau, M. & Fleury, F. 2005. Cost induced by viral particles manipulating superparasitism behaviour in the parasitoid *Leptopilina boulardi*. *Parasitology* **131**: 161–168.

Walter, G.H. 1983. Differences in host relationships between male and female heteronomous parasitoids (Aphelinidae: Chalcidoidea): a review of host location, oviposition and pre-imaginal physiology and morphology. *J. Entomol. Soc. S. Af.* **46**: 261–282.

Weeks, A.R. & Breeuwer, J.A.J. 2003. A new bacterium from the *Cytophaga-Flavobacterium-Bacteroides* phylum that causes sex-ratio distortion. In: *Insect Symbiosis* (K. Bourtzis & T.A. Miller, eds), pp. 165–176. CRC Press, New York.

Weeks, A.R. & Stouthamer, R. 2003. Increased fecundity associated with infection by a *Cytophaga*-like intracellular bacterium in the predatory mite, *Metaseiulus occidentalis*. *Proc. R. Soc. Lond. B.* (Suppl.)**271**: S193–S195.

Weeks, A.R., Marec, F. & Breeuwer, J.A.J. 2001. A mite species that consists entirely of haploid females. *Science* **292**: 2479–2482.

Weeks, A.R., Velten, R. & Stouthamer, R. 2003. Incidence of a new sex-ratio-distorting endosymbiotic bacterium among arthropods. *Proc. R. Soc. Lond. B Biol. Sci.* **270**: 1857–1865.

Werren, J.H. 1997. Biology of *Wolbachia*. *Annu. Rev. Entomol.* **42**: 587–609.

Werren, J.H., Windsor, D. & Guo, L.R. 1995. Distribution of *Wolbachia* among Neotropical Arthropods. *Proc. R. Soc. Lond. B Biol. Sci.* **262**: 197–204.

Zchori-Fein, E. & Perlman, S.J. 2004. Distribution of the bacterial symbiont *Cardinium* in arthropods. *Mol. Ecol.* **13**: 2009–2016.

Zchori-Fein, E., Roush, R.T. & Hunter, M.S. 1992. Male production induced by antibiotic treatment in *Encarsia formosa* (Hymenoptera: Aphelinidae) and asexual species. *Experientia* **48**: 102–105.

Zchori-Fein, E., Faktor, O., Zeidan, M., Gottlieb, Y., Czosnek, H. & Rosen, D. 1995. Parthenogenesis-inducing microorganisms in *Aphitis* (Hymenoptera: Aphelinidae). *Insect Mol. Biol.* **4**: 173–178.

Zchori-Fein, E., Gottlieb, Y., Kelly, S.E., Brown, J.K., Wilson, J.M., Karr, T.L. & Hunter, M.S. 2001. A newly discovered bacterium associated with parthenogenesis and a change in host selection behavior in parasitoid wasps. *Proc. Natl Acad. Sci. U S A* **98**: 12555–12560.

Zchori-Fein, E., Perlman, S.J., Kelly, S.E., Katzir, N. & Hunter, M.S. 2004. Characterization of a 'Bacteroidetes' symbiont in *Encarsia* wasps (Hymenoptera: Aphelinidae): proposal of '*Candidatus* Cardinium hertigii'. *Int. J. Syst. Evol. Microbiol.* **54**: 961–968.

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