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Cytoplasmic incompatibility and multiple symbiont infection in the ash whitefly parasitoid, *Encarsia inaron*

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Abstract

Terrestrial arthropods are commonly infected with maternally inherited symbionts that cause reproductive incompatibilities between hosts with differing infection status. Such symbionts can have major effects on the efficacy of a biological control program if releases are comprised of mixtures of differentially infected individuals. In this study, the ash whitefly parasitoid, *Encarsia inaron* (Hymenoptera: Aphelinidae) from Arizona was surveyed for the presence of heritable bacterial symbionts; experiments were also performed to test for two phenotypes known to be caused by *Encarsia* symbionts—cytoplasmic incompatibility and changes in oviposition behavior and host use. *E. inaron* has successfully reduced ash whitefly to non-pest status in all three locations it has been released (California, Arizona, and North Carolina) and is also notable as one of the only *Encarsia* species that is not autoparasitic, with both male and female wasps developing as primary parasitoids of whiteflies. We show that *E. inaron* is infected with both *Wolbachia* and *Cardinium*. While there was no effect of the symbionts on oviposition behavior or host use, crosses between doubly infected male wasps and uninfected females resulted in a severe reduction in the number of female offspring; male offspring production was unaffected. This study thus serves as a further warning that ascribing a phenotype to a symbiont with confidence depends on eliminating the possibility of a mixed infection, and establishes *E. inaron* as a useful model for dissecting *Wolbachia–Cardinium* interactions.

Keywords: Biological control; *Cardinium*; Female mortality; Reproductive manipulation; Vertical transmission; *Wolbachia*

1. Introduction

Symbioses between arthropods and maternally inherited microorganisms are ubiquitous and have played a vital role in shaping host ecology and evolution (O'Neill et al., 1997; Stouthamer et al., 1990). Many advances in insect symbiont research involve biological control agents such as parasitic Hymenoptera and predacious mites, or relate to parasitoid–host interactions. Parasitic Hymenoptera are commonly infected with a wide range of maternally inherited symbionts with diverse effects on host fitness, including symbionts that induce parthenogenesis (Stouthamer et al., 1990; Zchori-Fein et al., 2004), cause reproductive incompatibilities between infected and uninfected individuals (Breeuwer and Werren, 1990; Hunter et al., 2003), enable oogenesis (Dedeine et al., 2001; Zchori-Fein et al., 2001), affect parasitoid oviposition behavior (Zchori-Fein et al., 2001; Varaldi et al., 2003), and kill male embryos (Werren et al., 1986). The economically important predacious mite *Metaseiulus occidentalis* was recently discovered to harbor at least four bacterial symbionts, among which *Cardinium* has been implicated in an increase in fecundity, and *Wolbachia* and/or other symbionts may be involved in reproductive incompatibility (Johanowicz and Hoy, 1998; Weeks and Stouthamer, 2004; Hoy and Jeyaprakash, 2005). Also, symbionts in pea aphids appear to be the major source of

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variation in resistance to the dominant parasitoid wasp in North America, perhaps contributing to variability in biological control success of this pest (Oliver et al., 2003, 2005).

In spite of the mounting evidence of the importance of symbionts in natural enemy–pest interactions, relatively few studies have examined the implications of symbiont infection status for biological control efficacy (Stouthamer, 1993; Hunter, 1999a). From what is known already, one might predict both positive and negative effects of infection. For example, bacterial symbionts that induce parthenogenesis may in some situations be useful in mass rearing of parasitic agents for augmentation biological control, when infected wasps produce more daughters than uninfected females (Stouthamer, 1993). Parthenogens may have the further advantage that they do not need to find mates after release (Stouthamer, 1993). Conversely, initial releases of agents may fail if they consist of mixtures of individuals that differ in infection by microorganisms that cause reproductive incompatibilities—incompatible matings will result in high offspring mortality and population decline (Mochiah et al., 2002). Characterization of the symbiont(s) and its role in the biology of a particular natural enemy is thus likely to improve the effectiveness of biological control.

Maternally inherited symbionts are very common in *Encarsia* (Hymenoptera: Aphelinidae), an economically important genus of parasitoids that has been extremely successful for classical and augmentative biological control of whitefly pests (Rose and DeBach, 1981; Gould et al., 1992; Hoddle et al., 1998). Almost all *Encarsia* species have an unusual life history, which is characterized by obligate sex-specific differences in host use (Walter, 1983; Hunter and Woolley, 2001). Whereas female *Encarsia* develop as parasitoids of whiteflies or armored scale insect nymphs, in most cases male *Encarsia* are parasitoids of developing parasitoids, such as conspecific *Encarsia*, as well as other primary parasitoids.This unusual life history has been termed heteronomous hyperparasitism, within which autoparasitism describes the species in which male *Encarsia* can develop on conspecific females (Walter, 1983; Hunter and Woolley, 2001). The origin and maintenance of autoparasitism remains an evolutionary enigma. Autoparasitism has evolved only once in Hymenoptera and appears to be remarkably stable; there are very few cases of reversion from autoparasitism back to a standard parasitoid life history (i.e., males and females developing on the same host), including only two of the approximately 40 species of *Encarsia* for which the biology is known (Viggiani, 1987; Gould et al., 1995; Williams and Polaszek, 1996; Hunter and Woolley, 2001).

Transitions from autoparasitism to parthenogenesis (i.e., all female production), on the other hand, are extremely common in *Encarsia* (Babcock et al., 2001). Thus far, all parthenogenetic lineages that have been sampled have been shown to harbor intracellular endosymbionts (Zchori-Fein et al., 2001). *Encarsia formosa* is infected with *Wolbachia*; males are produced upon treatment with antibiotics (Zchori-Fein et al., 1992), while all other sampled asexual *Encarsia* contain *Cardinium* (Zchori-Fein et al., 2001). *Cardinium*'s involvement in parthenogenesis was recently confirmed when curing of *Cardinium* via antibiotic treatment resulted in male production in *Encarsia hispida* (Zchori-Fein et al., 2004). Indeed, *Cardinium* was the first symbiont other than *Wolbachia* shown to induce parthenogenesis. Microbial parthenogenesis in autoparasitoids is unusual because of the added complexity resulting from the obligate sex-specific differences in host use (Hunter, 1999a; Zchori-Fein et al., 2001). Parthenogenesis-inducing bacteria may need special tricks to succeed in autoparasitic *Encarsia*, because converting male wasps into females may fail if those newly female eggs are laid in a host that is not suitable for female development. Thus, parthenogenetic *Encarsia* must change their oviposition behavior or developmental requirements for the symbiont to invade. A change in behavior involves the oviposition of unfertilized eggs, which develop as females due to bacterial infection, into whitefly hosts (i.e., that are suitable for female development). While this change in oviposition behavior appears to reflect evolution of the oviposition behavior of the wasps in *E. formosa* and *E. hispida*, in parthenogenetic *Encarsia pergandiella*, the change in behavior appears to be directly induced by *Cardinium* (Hunter, 1999b; Zchori-Fein et al., 2001; Kenyon and Hunter, unpubl. data). In this population, wasps that become cured of their infection avoid laying eggs in whitefly hosts, suitable for female development.

In addition to the parthenogenetic lineages, a number of sexual *Encarsia* harbor bacterial endosymbionts, including *Wolbachia* and *Cardinium* (Zchori-Fein et al., 2001; Weeks et al., 2003). In only one sexual *Encarsia* species has the phenotype induced by its bacterial symbiont been determined. *Cardinium* causes cytoplasmic incompatibility (CI) in a sexual population of *E. pergandiella* from Texas (Hunter et al., 2003). Cytoplasmic incompatibility is reproductive incompatibility between infected males and females that are either uninfected, or infected with a different strain or strains of the symbiont. The CI-inducing symbionts spread in a host population because symbionts in the males reduce the fitness of uninfected females through failed matings. In *Cardinium*-infected *E. pergandiella*, uninfected females that mated with infected males produced offspring that died early in development (Hunter et al., 2003). This was the first case of CI shown to be caused by a symbiont other than *Wolbachia*, and further established *Cardinium* as a versatile manipulator of host reproduction. The presence of multiple symbionts that can induce similar phenotypes highlights an important emerging problem in the study of obligate symbioses—one must be very careful in ascribing a certain phenotype to one symbiont without ruling out the presence of others. Indeed, recent surveys of arthropods have shown that multiple infections, such as hosts harboring both *Wolbachia* and *Cardinium*, are common (Weeks et al., 2003; Zchori-Fein and Perlman, 2004).

In this study, we examine symbiont infection and phenotype in *E. inaron*, which has been used successfully to control the ash whitefly in North America (Gould et al., 1992; Pickett et al., 1996; Pickett and Pitcairn, 1999; Pickett and Wall, 2003). *E. inaron* is unusual as it is one of the two

sexual *Encarsia* species that is not autoparasitic—both males and females develop as primary parasitoids of whiteflies. Phylogenetic analyses have shown the absence of autoparasitism to be derived (i.e., evolving from an autoparasitic ancestor) (Babcock et al., 2001). In addition to assessing the infection status of *E. inaron* collected in Arizona, two symbiont-induced phenotypes were tested: (1) cytoplasmic incompatibility, and (2) reversion from autoparasitism to standard parasitism. The latter could occur if symbionts induced changes in oviposition behavior (similar to those occurring in parthenogenetic *E. pergandiella*), but did not induce parthenogenesis.

2. Methods

2.1. Wasp collection and culturing

Approximately 20 *E. inaron* pupae developing in ash whitefly on pomegranate were collected in October, 2002 in Tucson AZ. In Arizona, *E. inaron* was established with the release of wasps sent from California, where the classical biological control program was centered. *E. inaron* was originally collected in Israel and Italy and both populations were released in California (Pickett et al., 1996). In the laboratory, *E. inaron* was maintained on the sweetpotato whitefly, *Bemisia tabaci*, on cowpea (*Vigna unguiculata*) at 25 °C, 16L:8D photoperiod at ambient humidity. Wasps were cured of their bacterial infection by feeding adults 50 mg/ml rifampicin in honey solution for 48 h in each of three consecutive generations. After each treatment, treated and control wasps (fed honey only) were placed in cages containing cowpea seedlings bearing *B. tabaci* nymphs. We confirmed that wasps were uninfected following antibiotic treatment via polymerase chain reaction (PCR; see below). All experiments were performed at least one generation after the last antibiotic treatment.

2.2. Assessing symbiont infection

We used PCR and denaturing gradient gel electrophoresis (DGGE) to assess infection status and frequency. DNA extraction and PCR methods were as described in Hunter et al. (2003), except that the 60 °C lysis step was extended from 15 min to 1 h. The *Wolbachia* strain was identified and characterized using *wsp* primers 81F and 631R (Zhou et al., 1998) and *Wolbachia*-specific 16S primers wolF and wolR (O'Neill et al., 1992). The strain of *Cardinium* was identified and characterized using the *gyrB* primers gyr125F and gyr1023R (Zchori-Fein et al., 2004) and *Cardinium*-specific 16S primers CLOf and CLOr (Weeks et al., 2003). PCR products were sequenced directly in both directions using an ABI sequencer (GATC facility at the University of Arizona). In order to assess infection frequency in the laboratory culture, 30 individual wasps were PCR-screened with the 81F and 631R (for *Wolbachia*) and CLOf and CLOr (for *Cardinium*) primers. We also repeatedly monitored infections in infected and cured lines via PCR.

To verify that *Cardinium* and *Wolbachia* were the only bacteria found in the laboratory population of *E. inaron*, we used the DGGE fingerprinting method. Adults from the infected and uninfected lines, as well as their *B. tabaci* hosts, were placed alive in 96% ethanol and then ground in lysis buffer as described in Frohlich et al. (1999). A 16S rDNA gene fragment (~550 bp) was amplified using PCR and the general bacterial primers 341F and 907R (Muyzer et al., 1993). Reactions were carried out in a 50 µl volume that contained 5 µl of template DNA, 400 mM of each primer, 5 µl of 0.2 mM dNTP 1× ExTaq buffer and 1 U of ExTaq (Takara Bio Inc.). Five microliters of the PCR mix was tested using agarose gel electrophoresis, and the remaining 45 µl containing the amplified DNA fragments were then subjected to a DGGE analysis using the following conditions: separation using a 6% (w/v) acrylamide gel (acrylamide-*N*/*N*′-methylenebisacrylamide 37.5:1) prepared in 1× TAE buffer with a denaturing gradient ranging from 20% to 60%. Polymerization was carried out with TEMED (0.09% v/v) and ammonium persulfate (0.04 w/v). Electrophoresis for separation of PCR fragments was performed at 90 V and 60 °C for 16 h. After electrophoresis, the gels were incubated in ethidium bromide solution (250 ng/ml) for 10 min, rinsed in distilled water and photographed under UV illumination. Bands representing bacteria were eluted, cloned into the pTZ57R/T plasmid vector (Fermentas) and transformed into *E. coli*. For each bacterium, two different clones were sequenced (ABI 3700 DNA analyzer, Macrogen, Korea), and the sequences obtained were compared to known sequences using the BLAST algorithm.

2.3. Testing for cytoplasmic incompatibility

We found female *E. inaron* would only mate in large cages and in the presence of other females. Infected and uninfected wasps were isolated in 1.2 ml glass vials as pupae, and 3–4 days after they emerged, placed in groups in large (3.79 L) ventilated jars in all four possible combinations: infected and uninfected, male and female. Approximately 15 females and 13 males were held in each jar with access to water and honey for 3 days. For each treatment, seven females were then transferred singly to plants infested with mostly third instar *B. tabaci*. All emerging adult offspring were counted and sexed. Sex ratios and the total numbers of daughters and sons (log-transformed) of the four different crosses were compared using logistic regression and one-way ANOVA, respectively.

2.4. Testing for oviposition behavior changes

As mentioned previously, *E. inaron* is unusual as it is one of the only known sexual *Encarsia* that is not autoparasitic—both male and female wasps develop on whitefly hosts. We tested whether symbionts are currently involved with the maintenance of this unusual life history by comparing oviposition behaviors of infected versus cured *E. inaron*. Specifically, we predicted that cured *E. inaron*

might behave like autoparasitoids, by (a) refraining from ovipositing in whitefly hosts when unmated, as was previously demonstrated in an infected and parthenogenetic population of *E. pergandiella* (Zchori-Fein et al., 2001) and/or (b) ovipositing in wasp hosts.

In the whitefly host treatment, we placed 10 infected and 10 uninfected 2- to 3-day-old *E. inaron* virgin females individually in small experimental arenas with a cowpea leaf disc bearing 15 third-early instar *B. tabaci* hosts. Arenas were created by setting leaf discs on top of a ~3 mm layer of 5% cooling water agar. For the wasp host treatment, whiteflies on plants were first infested with sexual *E. pergandiella* and the plants fumigated 3 days later with a dichlorvos-impregnated strip in order to remove all adult *E. pergandiella*. Ten each of infected and uninfected 2- to 3-day-old *E. inaron* virgin females were then placed individually in experimental arenas containing 15 sexual *E. pergandiella* pupae. In all treatments, wasps were removed after 24 h. After removal of experimental *E. inaron* females, arenas were incubated for 24 h and then refrigerated. All hosts were subsequently dissected for the presence of *E. inaron* eggs.

3. Results

3.1. PCR and infection frequency

We identified one strain of *Cardinium* and one of *Wolbachia* in *E. inaron*. The *Cardinium* symbiont is 99% similar at 16S rDNA and 93% similar at gyrB to the *Cardinium* that causes CI in *E. pergandiella* (GenBank Accession Nos.: AY026335, AY332002), and the *Wolbachia* symbiont is in group B and 99% similar at wsp to *Wolbachia* found in *Cadra cautella* (AF020076) and *Tetranychus urticae* (AY785373). The culture in the laboratory appears fixed for both *Cardinium* and *Wolbachia*. Symbiont sequences were deposited in GenBank, under the following accessions: *Wolbachia* 16S and wsp, *Cardinium* 16S and gyrB: DQ317667–DQ317670.

DGGE of non-treated *E. inaron* produced bands corresponding to three different bacteria—*Wolbachia*, *Cardinium*, and an undescribed β-Proteobacterium showing 99% sequence similarity to a bacterium isolated from the environment (AY322153). In contrast, *E. inaron* from the antibiotic-treated line produced bands corresponding to one bacterium, the same β-Proteobacterium found in the infected line. The *B. tabaci* host sample produced a banding pattern that corresponded to four bacteria: *Portiera* (the primary whitefly symbiont), *Hamiltonella*, and *Rickettsia*, as well as the undescribed β-Proteobacterium. The first three symbionts are known to be associated with the B-biotype of *B. tabaci* (Gottlieb et al., 2006). These results confirm that both *Wolbachia* and *Cardinium* are true *Encarsia* symbionts, as these symbionts were absent from the whitefly host. Further, the presence of the β-Proteobacterium in both untreated and antibiotic-treated *E. inaron*, as well as in the *B. tabaci*, suggests that it was acquired horizontally, possibly via feeding, and may be a gut symbiont.

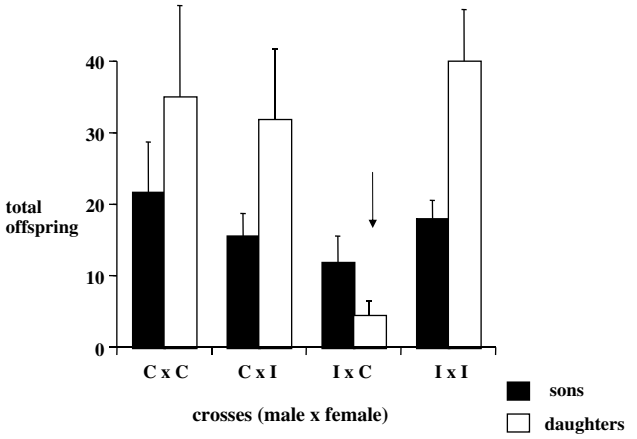

Fig. 1. Mean number of adult *E. inaron* (± SEM) produced from crosses between infected (I) and cured/uninfected and females. Males are listed first in the cross designations. Seven females were used for each cross type. The arrow points to the predicted affected cross if one or both of the symbionts caused cytoplasmic incompatibility.

The absence of both *Cardinium* and *Wolbachia* from the treated wasps suggests that these symbionts were successfully removed by antibiotic treatments. As expected too, the absence of other whitefly secondary symbionts such as *Hamiltonella* and *Rickettsia* in *E. inaron* suggests that these bacteria are not transmitted to the parasitoid in large enough numbers to facilitate detection.

3.2. Cytoplasmic incompatibility

Progeny produced in the cross that was predicted to be incompatible (infected male mated with uninfected female) displayed a significantly different sex ratio (~38% more male-biased) from the other crosses ($\chi^2 = 57.50$, $p < 0.0001$). There was a significant decrease in the number of daughters in the incompatible cross, with females producing an average of 4.29 ± 2.10 daughters, compared to an average number of 35.52 ± 5.65 daughters produced in the compatible crosses ($F_{3,24} = 11.91$, $p < 0.0001$) (Fig. 1). There was no difference in the number of males produced (mean $= 16.68 \pm 2.23$, $F_{3,24} = 0.85$, $p > 0.4$).

3.3. Effect of symbionts on oviposition and autoparasitism

There was no difference in host use between infected and cured *E. inaron* (Table 1). Unmated females of either infection status laid eggs in whitefly hosts, but did not oviposit in *E. pergandiella* wasp pupae.

4. Discussion

We have shown that *E. inaron* is infected with two bacterial endosymbionts, *Wolbachia* and *Cardinium*. Crosses between doubly infected and antibiotic-cured individuals indicated that one or both symbionts cause cytoplasmic incompatibility. The finding of a multiple infection is not in itself unusual, but the study also documents that CI may be

Table 1
Oviposition behavior of *Encarsia*

	Do unmated females oviposit in whiteflies?	Do unmated females oviposit in wasps?
1. Autoparasitic *Encarsia* (sexual)	No	Yes
2. *E. pergandiella* (parthenogenetic)		
Infected w. *Cardinium*	Yes	Yes
Uninfected	Rarely	Yes
3. *E. inaron* (sexual)		
Infected w. *Wolbachia*, *Cardinium*	Yes	No
	8.3 ± 1.1 ($n = 10$)	0 ($n = 10$)
Uninfected	Yes	No
	7.9 ± 1.1 ($n = 10$)	0 ($n = 10$)

Oviposition behavior of parthenogenetic *E. pergandiella* (Zchori-Fein et al., 2001; Kenyon and Hunter, unpubl. data), but not *E. inaron* (this study), is affected by symbionts.

expressed in insects infected with two symbionts that are both known to cause this phenotype independently. As such, it serves as a warning that ascribing a phenotype to a symbiont with confidence depends on eliminating the possibility of a mixed infection. *Wolbachia* and *Cardinium* are estimated to infect ~20% and ~6% of all arthropod species, respectively, and in initial surveys, several species have been found to be doubly infected (Weeks et al., 2003; Zchori-Fein and Perlman, 2004). In one intriguing example, previous studies reported that *Wolbachia* may be associated with incompatibility in the predatory mite *Metaseiulus occidentalis* (Johanowicz and Hoy, 1998); this host was since found to also harbor *Cardinium*, *Rickettsia*, and *Enterobacter* (Weeks and Stouthamer, 2004; Hoy and Jeyaprakash, 2005). Indeed, our study contributes to an emerging pattern of mixed infections of obligate intracellular vertically transmitted symbionts in arthropods in general (e.g., Rousset et al., 1999; Fukatsu and Nikoh, 2000; Sandstrom et al., 2001). A major challenge will be to understand how these symbionts interact with each other in these mixed infections, and how they ensure stable and efficient transmission.

Clearly the next challenge will be to generate singly infected lines, in order to determine which, or both of *Cardinium* and *Wolbachia* cause CI in *E. inaron*. As these two symbionts are unrelated, occurring in different phyla, it should be possible to find antibiotics for which they differ in susceptibility (e.g., Koga et al., 2003; Sakurai et al., 2005). As an alternative approach, it may be possible to use low doses of antibiotics to generate singly infected individuals. This method has been recently used to differentially cure host insects infected with multiple symbiont strains (Koga et al., 2003; Mouton et al., 2003). Previous studies have documented much variation in *Wolbachia* incompatibility strains segregating in natural host populations. For example, hosts may harbor multiple strains that are mutually incompatible (Perrot-Minnot et al., 1996). Strains have also been found that are compatible both with infected and uninfected individuals (Mercot and Poinsot, 1998; Zabalou et al., 2004). Production of singly infected *E. inaron* will allow us to dissect CI strain variation and interactions, and may also offer insights into the mechanism of CI in *Wolbachia* and *Cardinium*. For example, if these distantly related bacteria are mutually compatible, it will suggest that the mechanisms of CI are similar, perhaps due to genes that are shared via lateral transfer.

Thus far, two types of cytoplasmic incompatibility have been described in haplodiploids: (a) female mortality and (b) male conversion (Breeuwer and Werren, 1993; Reed and Werren, 1995; Breeuwer, 1997; Vavre et al., 2000). In the female mortality type, offspring that result from fertilized eggs (i.e., diploid females) die; this type is therefore similar to CI that occurs in conventional diploid species, such as *Drosophila*, and appears to be the most common type of CI in haplodiploid systems. Alternatively, in the male conversion type, paternally inherited (and incompatible) chromosomes are destroyed; as a result, haploid male individuals develop from fertilized eggs. Thus, the male conversion type results in increased production of males, as males are produced both via fertilized and unfertilized eggs. Our experiments show a decline in female progeny in the incompatible cross without a significant increase in male production, suggesting that CI in *E. inaron* is the female mortality type, as has been found in *E. pergandiella*. The CI in *E. pergandiella* appears to differ in one important characteristic from that observed in *E. inaron*. In *E. pergandiella* incompatible crosses, parasitized whiteflies appear developmentally arrested—they never complete development and only die days, and in some cases weeks, later (Hunter et al., 2003). However, we did not observe any developmentally arrested whiteflies in the incompatible *E. inaron* crosses.

Our study finds no support for the role of symbionts in mediating the reversion from autoparasitism in *E. inaron*. The origin and maintenance of autoparasitism in aphelinid chalcidoids remains an enigma. Autoparasitism appears to have evolved once in Hymenoptera, and one model proposes it may have evolved from facultative hyperparasitism (Hunter and Woolley, 2001). Whatever the origin of autoparasitism, a perhaps more interesting question is what makes autoparasitism so evolutionarily stable? One can imagine many environments in which a mutation in a female that allowed her to produce males in whitefly hosts would be favored, for example in early season habitats where only whitefly (and not wasp) hosts are present. Rare males would be strongly selected because of mating opportunities with abundant females. In spite of what appears to be strong potential selection, male development in whitefly or scale insect hosts has not been observed in normal sexual autoparasitoids. The exceptions appear in species in which *Wolbachia* or *Cardinium* cause parthenogenesis, and antibiotic curing yields male primary parasitoids (Hunter, 1999b; Zchori-Fein et al., 2004), or more extraordinarily, when a paternally inherited sex ratio distorter converts fertilized, incipient female eggs into haploid males that develop in whiteflies (Hunter et al., 1993). It is symbiont involvement with male development in primary hosts, as well as with a change of host preference behavior in *E. pergandiella*

(Zchori-Fein et al., 2001) that suggested a possible role of symbionts in the reversion to primary parasitism from autoparasitism in *E. inaron*. Our data do not support the idea that symbionts maintain primary parasitism, but they may have had a role in the initial reversion to primary parasitism. Unfortunately this idea is not testable.

Finally, our study highlights the importance of considering symbiont status in biological control. *E. inaron* was originally sent from two source populations (Israel and Italy) to two different institutions in California, USA (University of California, Riverside and California Department of Food and Agriculture in Sacramento) (Pickett et al., 1996). We do not yet know the infection status of all of the source and established populations, and in this case, it is unclear whether having known this information earlier would have improved biological control. *E. inaron* is a very successful control agent for ash whitefly, and has reduced this exotic pest to non-pest status in all three locations it has been released (California, Arizona, and North Carolina). Nonetheless, one can easily imagine a different outcome. Mixing of two populations with different CI symbiont infection status may cause reproductive failure. It is also common to find that symbiont infection may exact a cost in the fecundity of the infected wasps (Fleury et al., 2000). Exotic natural enemies with undiagnosed symbiont infections may perform suboptimally or perhaps even fail to establish after release, when reproductive failure due to CI occurs, or when their fecundity is greatly reduced relative to uninfected lines. It is likely that some biological control failures are due to problems such as these. In contrast, early diagnosis of symbiont infection status could lead to a symbiont management decision that could improve the effectiveness of the release. Exotic agents with CI symbionts could be simply cured of their symbiont infections in quarantine with antibiotics, before field release makes this procedure impossible.

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