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"Darwin's corollary" and cytoplasmic incompatibility induced by *Cardinium* may contribute to speciation in *Encarsia* wasps (Hymenoptera: Aphelinidae)

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The potential importance of cytoplasmic incompatibility (CI)-inducing bacterial symbionts in speciation of their arthropod hosts has been debated. Theoretical advances have led to a consensus that a role is plausible when CI is combined with other isolating barriers. However, the insect model systems *Nasonia* and *Drosophila* are the only two experimental examples documented. Here, we analyzed the components of reproductive isolation between the parasitoid wasp *Encarsia suzannae*, which is infected by the CI-inducing symbiont *Cardinium*, and its uninfected sibling species *Encarsia gennaroi*. Laboratory crosses demonstrated that: (1) sexual isolation is incomplete; (2) hybrid offspring production is greatly reduced in the interspecific CI cross; (3) viable hybrids may be produced by curing *E. suzannae* males of *Cardinium* with antibiotics; (4) hybrid offspring production in the reciprocal cross is greatly reduced by hybrid inviability due to genetic incompatibilities; (5) hybrid sterility is nearly complete in both directions at the F1 stage. Thus, asymmetrical hybrid incompatibilities and CI act as complementary isolating mechanisms. We propose a new model for contributions of CI symbionts to speciation, with CI reducing gene flow between species in one direction, and in the other, a symbiont sweep resulting in accelerated mtDNA evolution, negative cytonuclear interactions, and hybrid incompatibilities.

KEY WORDS: **Cytonuclear interactions, *Encarsia gennaroi*, *Encarsia suzannae*, haplodiploidy, hybrid incompatibility, reproductive isolation, symbionts.**

The problem of how new species arise puzzled Darwin, and remains a central question in evolutionary biology. Speciation may occur when reproductive isolation arises as the incidental by-product of the gradual, genetic divergence of two populations. In the standard genetic model of speciation, known as the Bateson-Dobzhansky-Muller (BDM) model, when gene flow stops between two populations of a species due to geographical barriers, mutation, genetic drift, and/or natural selection act independently on each population (Orr 1996). Independent changes at different loci in each population cause negative epistatic interactions, known as BDM incompatibilities or hybrid incompatibilities (HI), that cause sterility or inviability of hybrid offspring when the two populations rejoin, and these changes, once established, are usually irreversible (Coyne and Orr 2004). A key element in speciation is therefore the development of mechanisms that prevent gene flow between populations when populations are brought together again, which can act before or after mating. In spite of considerable progress in the understanding of mechanisms of pre- and postzygotic isolation, mostly based on the diploid model organism *Drosophila* (Coyne and Orr 1989a, 1997), and the identification of "speciation genes" (Presgraves 2010; Maheshwari and Barbash 2011) consistent with the BDM model, the evolution of reproductive isolation and speciation has been studied in only a few haplodiploid systems. In haplodiploids, in which males

EVOLUTION
INTERNATIONAL JOURNAL OF ORGANIC EVOLUTION

originate from unfertilized eggs and are haploid, and females originate from fertilized eggs and are diploid, most studies are limited to the parasitic wasps *Nasonia.* In this system, reproductive isolation seems to differ from those of diploid examples in showing stronger postzygotic isolation and weaker prezygotic isolation, although whether this is related to the haplodiploid genetic system awaits a broader comparative study between diploids and haplodiploids (Koevoets and Beukeboom 2009).

For decades, theoretical and empirical studies of speciation have focused almost exclusively on the role of nuclear genes (Orr 2005), but as early as 1959, Laven suggested that the complex pattern of maternally inherited incompatibilities he observed in *Culex pipiens* could be involved in speciation (see also Thompson 1987) even before a microbial agent was known to be behind the pattern of cytoplasmic incompatibility (CI). Maternally inherited, intracellular bacterial CI symbionts *Wolbachia* (α-Proteobacteria) or *Cardinium* (Bacteroidetes) spread in a host population by decreasing the relative fitness of uninfected or differently infected individuals. This is accomplished principally by preventing successful crosses between infected males and uninfected females (unidirectional CI), or between infected males and females infected with a different CI symbiont strain (bidirectional CI) (Turelli 1994). Unidirectional CI may also reduce gene flow to a lesser extent in the opposite (non-CI) direction; when infected females enter populations of uninfected individuals and mate, their infected male offspring cause CI and have low or zero reproductive success. While in diploid systems CI results in embryonic mortality of males and females, in haplodiploids CI crosses result in the death of the (female) progeny developing from fertilized eggs or in the development of male-only progeny, when CI causes fertilized, incipient female eggs to become haploid and develop as functional males (Vavre et al. 2000). CI can play a direct role in postzygotic isolation by causing F1 hybrid inviability among populations with a different infection status. Because CI reduces or eliminates the production of F1 hybrids, it could have a stronger effect on gene-flow reduction than typical recessive genetic incompatibilities (Bengtsson 1985; Gavrilets 1997), which are often expressed in F2 hybrids, backcross hybrids, or in the heterogametic sex (because of Haldane's rule; Bordenstein 2003).

Intracellular bacterial symbionts potentially capable of manipulating reproduction are widespread in arthropods (Zchori-Fein and Perlman 2004; Hilgenboecker et al. 2008; Zug and Hammerstein 2012; Weinert et al. 2015), and CI is the most common host phenotype, hence CI symbionts may play an important role in speciation, even if in a minority of instances (Werren 1998; Bordenstein 2003). A role of CI-*Wolbachia* as a speciation agent has been shown in the insects *Drosophila* and *Nasonia* for unidirectional (Shoemaker et al. 1999) and bidirectional (Bordenstein et al. 2001) CI, respectively. Shoemaker et al. (1999) provided the first experimental evidence for the role of unidirectional CI in the reproductive isolation of two mushroom feeding *Drosophila* species, the *Wolbachia*-infected *Drosophila recens* and the uninfected *D. subquinaria*, in combination with behavioral isolation. Studies on reproductive isolation between *N. vitripennis* and *N. giraulti* (Breeuwer and Werren 1990, 1995; Bordenstein and Werren 1998) hinted at a role of *Wolbachia*. Bordenstein et al. (2001), using the species pair *N. giraulti—N. longicornis*, found that *Wolbachia*-induced bidirectional CI in this hybridization preceded the evolution of other forms of isolation, as no other postmating isolation was found, and premating isolation was weak and asymmetric. As these species pairs were not known to occur in sympatry, these studies then drew some skepticism about the general relevance of the role played by *Wolbachia* in the speciation process (Rokas 2000; Wade 2001; Weeks et al. 2002). That *Wolbachia* can act in concert with genetic and/or geographic isolation mechanisms in nature became clear with the study of sympatric *Drosophila* species by Jaenike et al. (2006), which showed strong unidirectional CI by *Wolbachia* in one of the species, plus asymmetrical premating isolation and hybrid male sterility. In that example CI-*Wolbachia* appears to serve as one of several isolating mechanisms, which together prevent or greatly restrict interspecific gene flow. In another recent example, Miller et al. (2010) showed that, in the *Drosophila paulistorum* complex of semispecies, *Wolbachia* acts to reduce gene flow in two ways. First, it is pathogenic in hybrids, overreplicating, and triggering embryonic lethality and male sterility. Second, *Wolbachia*, which here causes strong bidirectional CI, manipulates sexual behavior via selective mate avoidance in sympatry, which results in premating isolation. These demonstrations of different roles of a CI symbiont in reproductive isolation have helped reframe the question to be debated from: "Can CI symbionts alone induce speciation?" (Turelli 1994; Coyne and Orr 1998; Hurst and Schilthuizen 1998) to: "How do CI symbionts contribute to reproductive isolation and speciation?" (Bordenstein 2003; Engelstädter and Hurst 2009).

Early theoretical models on the role of CI symbionts in speciation showed that when unidirectional CI-*Wolbachia* sweeps to near fixation, there is no population structuring into separate infected and uninfected populations and so no opportunity to restrict gene flow (Turelli 1994; Egas et al. 2002). However, more recent models show that under specific genetic and environmental conditions CI-*Wolbachia* can promote speciation processes in their hosts (Telschow et al. 2005, 2007, 2014; Flor et al. 2007; Engelstädter and Telschow 2009). These theoretical studies use the basic structure of the BDM model, that is, they assume that one ancestral population separates into two populations that, while separated, diverge genetically, and one population acquires a *Wolbachia* infection that causes CI during secondary contact. Brucker and Bordenstein (2012) further hypothesize that, if the BDM model is extended to account for symbionts by replacing one of

the nuclear genes with a microbial symbiont, this would lead to an increase of potential negative epistatic interactions underlying HI in the symbiont model because HI could now arise from incompatible gene–gene, gene–microbe, or microbe–microbe interactions. This scenario awaits theoretical exploration.

In spite of a general consensus that endosymbionts are at least one of the factors that can promote the speciation process (Vavre and Kremer 2014), we are aware of no other experimental examples documented other than in species of *Nasonia* and *Drosophila*. Here, we examine the potential role of the CI-inducing symbiont *Cardinium* in acting as one of several barriers to gene flow between two species of the *Encarsia pergandiella* complex (Hymenoptera: Aphelinidae), parasitoid wasps of whiteflies: *Encarsia suzannae*, which is infected with *Cardinium*, and *Encarsia gennaroi*, which is uninfected (Gebiola et al. 2016). These sibling species can be separated morphologically, and the focal populations are allopatric; the origin of the *E. gennaroi* studied here is Italy, where it was introduced from California (Gebiola et al. 2016), whereas that of *E. suzannae* is Texas. In south Texas the distribution of the two species overlap, and are still able to interbreed and produce hybrid progeny (Johnson 1996). *Cardinium* causes unidirectional CI in *E. suzannae* (= *E. pergandiella* in Johnson 1996, Perlman et al. 2008, 2014; Harris et al. 2010). CI in *E. suzannae* causes female mortality, and the phenotype is a developmentally arrested whitefly host, that is, parasitized whitefly nymphs in which the endoparasitic wasp did not develop beyond the egg or early larval stage (Hunter et al. 2003). *Cardinium* can be eliminated by antibiotic treatment (Perlman et al. 2008), such that control crosses with antibiotic-cured lines can be used to partition sources of offspring mortality relative to the CI crosses. By performing laboratory mating behavior and crossing experiments, we assessed the existence of prezygotic barriers between infected *E. suzannae* and uninfected *E. gennaroi*, and disentangled the effect of CI and HI on postzygotic isolation, respectively. The goal was to evaluate if *Cardinium* could contribute to reproductive isolation and speciation by means of unidirectional CI.

Material and Methods

LABORATORY CULTURES

Sibling species *E. suzannae* and *E. gennaroi* are solitary hymenopteran parasitoid of whiteflies with an unusual "autoparasitic" biology (Hunter and Woolley 2001). Females are primary parasitoids and lay single female eggs in whiteflies, whereas male eggs are laid in developing wasp larvae or pupae enclosed within the whitefly cuticle, either their own species, or other primary parasitoids. An important consequence of this biology for the current study is that *Encarsia* mated females confined on whitefly hosts will produce only female offspring. The *E. suzannae* culture was established from samples collected from *Bemisia tabaci* in the Rio Grande Valley in Texas in 2003, and has been maintained in the laboratory on *B. tabaci* reared on cowpea plants (*Vigna unguiculata*) ever since. This culture is fixed for *Cardinium* infection (Hunter et al. 2003), and the infection is strictly maternally transmitted (Perlman et al. 2008). Hereafter, we refer to this line as iES. An uninfected line was obtained by curing infected adult wasps with 50 mg/ml rifampicin in honey for three generations and then rearing the wasps for several generations after antibiotic treatment before performing experiments; hereafter we refer to this cured strain as cES. The *E. gennaroi* line was obtained from samples collected in Portici, Italy, where this North American species was first introduced from California in 1979 on *Trialeurodes vaporariorum* (EPIT population, Gebiola et al. 2016); hereafter we refer to this culture as EG. The three cultures were reared on *B. tabaci* (the host for female development) on cowpeas (*Vigna unguiculata*), while the host for male development was a primary parasitoid, either *Eretmocerus emiratus* for iES and cES, or *Encarsia formosa* for EG. All cultures were housed in environmental chambers at 27°C in the Hunter laboratory at the University of Arizona, Tucson, AZ, USA.

PREZYGOTIC ISOLATION

To assess the extent of prezygotic isolation, we performed a comprehensive mating experiment, with no-choice (NC), female choice (FC), male choice (MC), and multiple choice (MuC) tests, and at least 20 replicates per cross type (listed in Table 1). Female and male pupae were isolated, and eclosed virgin adults were held and fed with honey for at least 24 hours to allow reproductive maturation. Courtship and mating behavior observations were compared to the series of behaviors that indicated successful mating in both species. When put in a vial, the male approaches the walking female, the female stops upon recognizing the local presence of a male, and, after a short antennation of the female by the male, the male mounts the female, the female lifts the end of her abdomen, and copulation starts, with the male flipping his wings rapidly (Viggiani and Battaglia 1983; our observations). In few seconds copulation is over and the male briefly touches the female abdomen with his legs while detaching from her. After copulation, females are unwilling to mate again, whereas males can remate multiple times.

Matings were observed for a maximum of 10 minutes (usually males approach and mount females almost immediately in a vial) under a microscope, and the occurrence of courtship, copulatory, and postcopulatory behavior was recorded. In NC tests, one female and one heterospecific male were introduced together in a 1.2-ml glass vial. In FC tests, one female was introduced in a 1.2-ml glass vial where one conspecific and one heterospecific male were already present, and upon female introduction the three wasps were tapped toward the bottom of the vial to promote equal encounter rates with the two possible mates. The

observations were stopped after the first successful mating. In MC tests, one male was introduced to a 1.2-ml glass vial where one conspecific and one heterospecific female were already present, the three wasps were tapped toward the bottom of the vial as in the female-choice experiments. Observations were stopped after the first successful mating. For matings between iES and cES, one male (in FC tests) and one female (in MC tests) were marked with a few small grains of a fluorescent powder on their wings. Half of the total females and males of either strain were marked, and no effect of the marking was observed. When the full display of courtship, copulation and postcopulation could not be recorded, females were stored at –80°C before dissecting the female spermatheca to check for sperm transfer as described by White et al. (2009). Upon dissection, females with empty spermathecae were recorded as unmated. MuC tests were performed in a 30-mm diameter circular arena made from an adhesive annular foam pad ("Dr. Scholl's Callus Cushion", www.drscholls.com), with one glass microscope slide serving as the bottom and two on the top that abutted a coverslip that sat vertically in a slice bisecting the pad. Four males and four females (two per species) were introduced into each half of the arena by gently blowing them through a modified Pasteur pipette through a small hole in the side, then the cover slip separation was removed to allow matings. The experiment was video-recorded using a Dino-Lite digital microscope (www.dinolite.us) connected to a computer and was terminated after the first two out of the possible four matings had occurred (Casares et al. 1998). This way we can assume that the mating pairs are true replicates, and not pseudo-replicates affected by the other pairs (Coyne et al. 2005). For the iES-cES treatment, as infected and uninfected individuals are not distinguishable, observations were made under a stereomicroscope, and the mating pairs were aspirated into Pasteur pipettes during or right after postcopulatory behavior. Wasps were then immediately killed in ethanol, and DNA was extracted as in Gebiola et al. (2009). To assess the identity of the mated pairs, we performed diagnostic PCR for *Cardinium* using 16S *Cardinium*-specific primers CLOf and CLOr as in Weeks et al. (2003).

To obtain a general sexual isolation estimate for each interspecific combination, the joint isolation index (*Ipsi*) of Rolán-Alvarez and Caballero (2000), considered the best-performing index under different scenarios and sample sizes (Pérez-Figueroa et al. 2005), was calculated using the software JMATING (Carvajal-Rodríguez and Rolan-Alvarez 2006), which also provides standard deviations and two-tail probabilities of rejecting the null hypothesis of no sexual isolation being true after 10,000 bootstrap resampling. *Ipsi* ranges from –1 to +1 with 0 = random mating. JMATING also estimates *IApsi*, an index of the asymmetry of sexual isolation that results from comparing the Pairwise Sexual Isolation (*PSI*) coefficients for the two directions of each of the three cross types.

POSTZYGOTIC ISOLATION

To test for the effect of CI and HI on reproductive isolation, we executed a full-factorial crossing scheme, resulting in nine cross types, including one known intraspecific CI cross (cES♀ × iES♂), one putative interspecific CI + HI cross (EG♀ × iES♂) and three putative HI crosses (EG♀ × cES♂, iES♀ × EG♂, cES♀ × EG♂). One- or two-day-old virgin females were individually mated to virgin males (all cultured on *E. formosa*) in a 1.2-ml glass vial, then the male was removed and each female was transferred to a 35-mm Petri dish experimental arena. Arenas consisted of a cowpea leaf disk on 1% agar, abaxial side up, and bearing 50–80 3rd-early 4th nymphal instars of *B.tabaci*, and covered with a screen-top lid. Females were allowed to oviposit in whiteflies for 24 h. Following the removal of the female, infested leaves were incubated at 27°C until pupae could be counted (8–10 days). At this point, unparasitized whiteflies were eclosed, wasp pupae represented viable F1 progeny, and developmentally arrested whiteflies (bearing dead parasitoid embryos) represented the fraction of hybrid inviability due to intraspecific CI (Perlman et al. 2008). We expected that also the interspecific CI cross would result in developmentally arrested whiteflies, whereas the fraction of hybrid inviability due to HI may have resulted in either developmentally arrested whiteflies or mortality at the larval stages. Females that did not produce any pupae and no more than two developmentally arrested whiteflies (in the putative CI crosses only) were presumed to be unmated and removed from the analyses. Parasitoid fecundity was estimated by the number of whiteflies parasitized, regardless of whether the parasitism yielded viable offspring. The experiment was set up in three blocks carried out two months apart from each other, with at least eight replicates per cross type per block. We compared the proportions of live parasitoid pupae, arrested whiteflies, and dead parasitoid larvae by fitting a logistic binomial regression to each response variable using the "glm" R function (R Core Team 2016). Three models were thus built, using cross type, and block as predictor variables. The overall significance of each predictor was assessed by Wald statistics, using the function wald.test in the "aod" R package (Lesnoff and Lancelot 2012). To better disentangle the effects of CI and HI, we tested the significance of specific contrasts of interest by Wald statistics.

A subset of F1 hybrid females from all cross types ($n = 57$) was used to produce F2 hybrid male progeny by offering them early pupal stages of either *Encarsia inaron* (in the first block), *Er. emiratus* (in the second block) or *En. formosa* (in the third block) to hyperparasitize. Note that because of haplodiploidy, only F1 female offspring of interspecies crosses are hybrid and receive a complete chromosome complement from both parental species. F1 males develop from unfertilized eggs and receive only the maternal genome. Hybrid males first appear as offspring from F1 hybrid females. Their genome is a recombination of the two

Table 1A. Frequency of matings in different types of trials.

No choice	reps	Success			
EG♀ × EG♂	20	90.0			
EG♀ × iES♂	20	85.0			
EG♀ × cES♂	20	80.0			
iES♀ × iES♂	20	95.0			
iES♀ × EG♂	20	75.0			
iES♀ × cES♂	20	95.0			
cES♀ × cES♂	20	95.0			
cES♀ × EG♂	20	80.0			
cES♀ × iES♂	20	95.0			
Female choice	**reps**	**Con ♂**	**Hetero ♂**	**None**	**Both ♂**
EG♀ × EG♂ × iES♂	40	65.0	27.5	5.0	2.5
EG♀ × EG♂ × cES♂	45	40.0	48.9	6.7	4.4
cES♀ × cES♂ × EG♂	20	75.0	15.0	0.0	10.0
iES♀ × iES♂ × EG♂	20	70.0	10.0	0.0	20.0
iES♀ × iES♂ × cES♂	20	40.0	40.0	5.0	15.0
cES♀ × cES♂ × iES♂	20	55.0	30.0	5.0	10.0
Male choice	**reps**	**Con ♀**	**Hetero ♀**	**None**	
iES♀ × cES♀ × iES♂	20	35.0	65.0	0.0	
EG♀ × iES♀ × iES♂	20	65.0	35.0	0.0	
iES♀ × cES♀ × cES♂	20	60.0	40.0	0.0	
EG♀ × cES♀ × cES♂	20	50.0	45.0	5.0	
EG♀ × cES♀ × EG♂	20	65.0	35.0	0.0	
EG♀ × iES♀ × EG♂	20	35.0	60.0	5.0	
Multiple choice	**reps**	**Con**	**Hetero**		
2 EG♀ × 2 EG ♂ × 2 iES♀ × 2 iES♂	20	62.5	37.5		
2 EG♀ × 2 EG ♂ × 2 cES♀ × 2 cES♂	20	72.5	27.5		
2 iES♀ × 2 iES ♂ × 2 cES♀ × 2 cES♂	20	50.0	50.0		

"Con" refers to conspecific matings and "Hetero" refers to heterospecific matings. All results are reported in percentages.

chromosome complements of their mother and is referred to here as F2 recombinant males. We also examined the occurrence of hybrid sterility by backcrossing F1 females ($n = 160$) in single-pair matings to either of the parental species for 24 h. The number of backcross progeny produced was used as a measure of hybrid fertility. F1 females ($n = 11$) were also crossed with recombinant males to obtain F2 female progeny.

Results

PREZYGOTIC ISOLATION

When not given a choice, matings occurred at high frequencies in all crosses, whereas they were more assortative in FC, MC, and MuC tests (Table 1A). In particular, the joint isolation index (*Ipsi*) was highly significant for all crosses, and significant asymmetry (*IApsi*) was also recorded in female choice tests for the interspecific crosses (Table 1B), with cES and iES females being able to discriminate better against EG males than vice versa. *IApsi* was significant also in the MuC cES × EG cross.

POSTZYGOTIC ISOLATION

The number of successful interspecific matings that resulted in adult females ovipositing in whiteflies ranged from 83.9% (in cES♀ × EG♂) to 93.3% (in EG♀ × cES♂). Females assigned to control (within species) crosses had an average fecundity ranging from 16.4 ± 1.70 SE (in the EG cross) to 29.0 ± 1.09 SE (in the iES cross), and at least 94% of progeny in the four control crosses survived to become healthy pupae. In contrast, a high mortality rate was recorded in the hybrid crosses, with an average fecundity of 11.5 ± 1.15 SE (in EG♀ × iES♂) to 17.4 ± 1.69 SE (in cES♀ × EG♂) (Table 2). The interspecific CI cross resulted in a large proportion of developmentally arrested whiteflies, and a smaller proportion of dead parasitoid larvae (Fig. 1). This latter phenotype was found in all crosses where we had predicted HI (Table 3), hence we associated it with genetic inviability. The intra- and interspecific CI crosses resulted in 72.3% and 64.6% of cytoplasmic inviability, respectively (Fig. 1). In the latter CI cross, 12% of progeny was genetically inviable, a percentage that increased to 16.1% in the same cross when *Cardinium* was

Table 1B. Joint sexual isolation index (*Ipsi*) and asymmetry index (*IApsi*).

	Ipsi			*IApsi*			
Cross type (both directions)	reps	Total	SD	aa/bb	SD	ab/ba	SD
No choice							
iES × cES	40	0.01	0.118	1.00	0.042	1.00	0.043
iES × EG	40	0.07	0.124	1.00	0.046	1.01	0.070
cES × EG	40	0.09	0.122	0.99	0.048	1.00	0.065
Female choice							
iES × cES	40	0.52^{***}	0.099	1.03	0.143	1.27	0.489
iES × EG	60	0.57^{***}	0.086	1.32^{*}	0.117	0.58^{*}	0.149
cES × EG	65	0.53^{***}	0.083	1.26	0.151	0.43^{***}	0.149
Male choice							
iES × cES	40	0.35^{**}	0.122	1.04	0.115	0.85	0.178
iES × EG	40	0.38^{**}	0.123	1.01	0.123	1.32	0.477
cES × EG	40	0.46^{***}	0.118	0.97	0.138	0.92	0.289
Multiple choice							
iES × cES	40	0.45^{***}	0.109	1.28	0.174	1.03	0.279
iES × EG	40	0.46^{***}	0.123	1.01	0.150	1.13	0.431
cES × EG	40	0.70^{***}	0.084	0.71^{*}	0.138	3.11^{*}	1.516

SD = standard deviation; aa/bb = ratio of intraspecific PSI coefficients; ab/ba = ratio of interspecific PSI coefficients; * = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$. Significance indicates difference from the null hypothesis of random mating, which equals 0 for Ipsi and IApsi.

Table 2. Cross types, fecundity, and number of F1 female progeny.

F0 ♀	F0 ♂	Crosses	♀ ovipositing	av. Fecundity ± SE	av. F1 ♀
EG	cES	30	28	13.6 ± 1.18	10.4
EG	iES	27	25	11.5 ± 1.15	3.1
cES	EG	31	26	17.4 ± 1.69	5.5
iES	EG	26	23	15.7 ± 1.94	4.7
cES	iES	28	27	27.2 ± 1.17	7.1
iES	cES	28	28	30.6 ± 0.97	29.4
EG	EG	26	20	16.4 ± 1.70	15.6
cES	cES	28	28	28.0 ± 1.13	26.7
iES	iES	27	26	29.0 ± 1.09	27.4

removed (Fig. 1). In the other direction of these crosses, that is, iES and cES females crossed with EG males, the proportion of genetic inviability dramatically increased to 74.0% and 69.9%, respectively, showing a strong asymmetry with the reciprocal cross (Fig. 1). We note also that a small number of progeny died at the embryonic (resembling the CI phenotype) or larval (resembling the HI phenotype) stage in the control (nonhybrid) crosses; this amount of mortality is consistent with mortality from natural causes that are not related to CI or HI, respectively (Fig. 1). The block effect in the three statistical models was not significant (Wald $\chi^2 = 0.48$, $P = 0.79$ for live pupae, $\chi^2 = 0.29$, $P = 0.87$ for arrested whiteflies, $\chi^2 = 1.5$, $P = 0.47$ for dead parasitoid larvae, d.f. = 2), hence it was removed from the models. Cross type had a significant effect on F1 viable progeny in all interspecific crosses, on cytoplasmic inviability only in the two (intraspecific and interspecific) CI crosses, and on genetic inviability only in the four putative HI crosses. Pairwise comparisons among crosses fully confirmed our predictions, that is, whenever we expected a HI or CI effect, alone or in combination, the effect was statistically significant (Table 3).

Of the 20 F1 hybrid females for each genetic background that were backcrossed with each of the two interspecific parental males (hence 40 per hybrid female type) to produce F2 female progeny, only one F1 female of the EG♀ × iES♂ backcrossed with the cES parental male produced progeny and these were three malformed pupae that failed to emerge as adults. Additionally, at least 14 F1 hybrid females per genetic background were used to produce F2 recombinant males, yet only three females having the

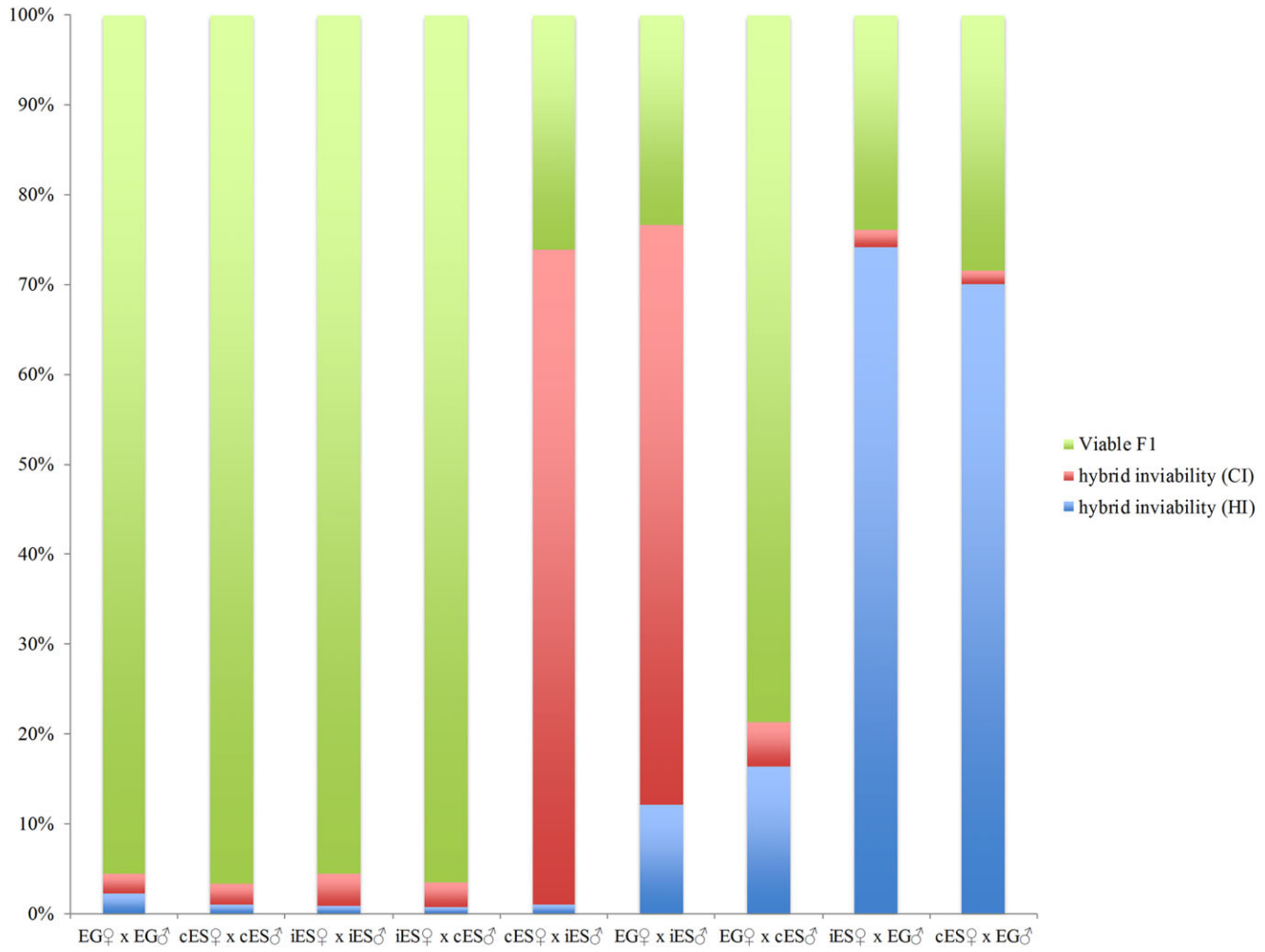

Figure 1. **Stacked histogram summarizing the results of crossing experiments. Cross types are on the *x*-axis, and the outcome of the crosses on the *y*-axis.**

EG♀ × iES♂ genetic background were fertile and produced a total of 13 F2 hybrid males. These males were then crossed with their three fertile mothers and with seven cured females of the same genetic background (EG♀ × iES♂). The cross between mothers and sons produced three F2 hybrid females, which in turn were crossed with their fathers, and used for F2 recombinant male production, but no female or male progeny was obtained. Finally, the ovaries of 20 F1 hybrid females were dissected, showing that they carried from none up to two eggs, while normally these *Encarsia* species carry at birth at least a dozen eggs (Mancini et al. 2013). In sum, hybrid sterility was nearly complete at the F1 stage, and the few hybrid F2 individuals produced were inviable or sterile.

Discussion

The results of the mating assays and crossing tests between *Cardinium*-infected *E. suzannae*, and the sibling uninfected species *E. gennaroi* indicated complete or near-complete postzygotic isolation between species but limited, somewhat asymmetric, prezygotic isolation. In mating trials, there was no heterospecific isolation in no-choice assays, although assortative mating was evident in choice assays. The strongest sexual isolation resulted from female-choice and multiple-choice experiments, whereas no-choice experiments showed no sexual isolation. Male choice experiments also showed more sexual isolation than no choice experiments, implying that male discrimination is also a factor, although female discrimination plays a larger role. In female-choice assays, there appeared to be an asymmetric pattern: *E. suzannae* females, either infected or cured, effectively discriminated against *E. gennaroi* males. On the other hand, uninfected *E. gennaroi* females discriminated against infected *E. suzannae* males in both female and multiple choice tests, whereas they did not discriminate against cured *E. suzannae* males in female choice, but they did in multiple choice. The results emphasize the importance of different types of assays in getting a more complete

Table 3. Pairwise comparisons of crosses based on logistic binomial regression, with predicted and observed outcomes.

Contrast			Inviable F1 progeny			
♀ – ♂	♀ – ♂	Effect on viable F1	CI effect	HI effect	Predicted	Observed
cES-EG	EG-EG	***	ns	***	HI	HI
cES-EG	cES-cES	***	ns	***	HI	HI
EG-cES	cES-cES	***	ns	***	HI	HI
EG-cES	iES-cES	***	ns	***	HI	HI
EG-cES	EG-EG	***	ns	***	HI	HI
EG-iES	EG-EG	***	***	**	CI + HI	CI + HI
EG-iES	EG-cES	***	***	ns	CI	CI
EG-iES	cES-iES	ns	ns	***	HI	HI
cES-iES	cES-cES	***	***	ns	CI	CI
cES-iES	iES-cES	***	***	ns	CI	CI

ns = not significant; * = $P < 0.05$; ** = $P < 0.01$; *** = $P < 0.001$ (Wald test).

picture of the sources of interspecific mating incompatibility in the field. Sexual isolation and its weak asymmetry likely played a reduced role in this study relative to the *D. recens*/*D. subquinaria* example (Shoemaker et al. 1999), where hybrid inviability was absent and F1 hybrids were still fertile, and near complete prezygotic isolation (evident in no choice tests) in one cross direction complemented CI postzygotic isolation in the other. In the current study, there was evidence of only weak prezygotic isolation coupled with complete postzygotic isolation. While F1 hybrid females were easily produced, especially when cured *E. suzannae* males were mated with *E. gennaroi* females, most of them were sterile, and attempts to backcross these females to parental species produced only very few (sterile) females. If we use Cytochrome Oxidase c subunit I (COI) gene substitution rates to estimate divergence time, assuming a COI substitution rate of 1.9% Myr^{-1} as estimated by Machado et al. (2001) for fig-pollinating wasps, the 3.7% uncorrected distance between the two species (Gebiola et al. 2016) may indicate that the pair *E. gennaroi—E. suzannae* began diverging about 2 million years ago, hence earlier than in the *Nasonia* and *D. recens/subquinaria* examples (Shoemaker et al. 1999; Bordenstein et al. 2000). Taken together, all of these results indicate that speciation is complete, yet, remarkably, sexual isolation is still at an incipient stage.

In spite of complete genetic isolation, the willingness of these wasps to mate with heterospecifics, coupled with the serendipitously distinct signature of genetic versus CI mortality in this system, allowed us to examine the nature of the reproductive barriers between these species. The strength of inviability resulting from the hybridization of *E. gennaroi* and *E. suzannae* was strongly asymmetric. Cytoplasmic incompatibility explains over 60% of hybrid lethality in the CI cross (*E. suzannae* male × *E. gennaroi* female), whereas genetic inviability amounts to less than 20% not only in the CI cross but also in the same cross when *Cardinium* is removed by antibiotics (Fig. 1). Here, while genetic inviability remains constant, almost 80% viable F1 female progeny are produced (Fig. 1). This significant difference indicates an additive effect of CI on reproductive isolation. Notably, genetic inviability amounts to over 70% in the cross between *E. suzannae* females and *E. gennaroi* males, complementing CI in the reciprocal direction (Fig. 1). Therefore, in this *Encarsia* species pair, reproductive isolation in the non-CI direction is caused by an asymmetry in the accumulation of BDM incompatibilities, rather than an asymmetry in mating preferences as was found in *Drosophila* (Jaenike et al. 2006).

Postmating isolation asymmetry, that is, reciprocal-cross differences in F1 viability or fertility, is common, and is well documented in *Drosophila*, *Anopheles* mosquitoes, and Lepidoptera. Muller (1942) predicted that the alleles causing postzygotic isolation must act asymmetrically, and Turelli and Moyle (2007) called it ''Darwin's corollary." Darwin's corollary, despite recent naming, joins ''Haldane's rule'' concerning the greater rate of inviability/sterility of the heterogametic sex of interspecific F1 hybrids (Haldane 1922) and the ''large-X effect'' concerning the disproportionate contribution of the X chromosome to heterogametic F1 inviability/sterility (Coyne and Orr 1989b) as one of the three most common features of postzygotic isolation. Virtually every exception to Haldane's rule for inviability in *Drosophila*, for example, involves inviable hybrid females produced in only one direction of a cross. This asymmetry might involve maternally acting nuclear genes, or maternally inherited genes such as cytoplasmic organelles or endosymbionts. Because females from reciprocal crosses have an identical nuclear genotype, this asymmetry suggests a possible role of maternally inherited elements in postzygotic isolation (Coyne and Orr 2004).

The asymmetry of hybrid incompatibilities in the *Encarsia* system that we see now may reflect the history of the

speciation process. We can reasonably suppose that a barrier-separated populations of the ancestor of *E. suzannae* and *E. gennaroi*, and a CI-*Cardinium* infected the *E. suzannae* progenitor population. We can then imagine two scenarios. One is that drift and selection caused an accumulation of BDM incompatibilities, such that, upon secondary contact, the isolation was complete, CI acted primarily in one direction, HI in the other. In this scenario, if the *Cardinium* infection was cured, we would see the underlying symmetric hybrid inviability, CI would be superfluous and have no role in speciation. In the second scenario, cytonuclear interactions led to asymmetric BDM incompatibilities in the direction not isolated by CI. In this situation, CI was critical for causing isolation for separating the nascent species in one direction. This is what we see here, suggesting a role for CI at an early stage of the speciation process. We might further ask whether evidence of Darwin's corollary could be associated with CI-*Cardinium* as well.

Although Darwin's corollary has been observed in various organisms, its molecular bases are still unclear. F1 asymmetries may arise from incompatibilities between mitochondrial and nuclear genes. Negative cytonuclear interactions have been often identified as the genetic basis of both hybrid inviability and infertility (Rand et al. 2004; Burton and Barreto 2012; Burton et al. 2013), thus contributing to reproductive isolation between animal species, including *Nasonia* (Gadau et al. 1999; Ellison et al. 2008; Niehuis et al. 2008) and *Drosophila* (Rand et al. 2001). Turelli and Moyle (2007) suggested that reciprocal cross asymmetries can arise either from stochastic substitutions in mitochondrial genomes, or as a result of differences in mitochondrial relative to nuclear nucleotide substitution rates. In reciprocal crosses between species, the maternal parent with faster mitochondrial evolution will tend to produce less viable F1 hybrids owing to an increased probability of cytonuclear incompatibilities (Bolnick et al. 2008, Chou and Leu 2010; but see Brandvain et al. 2014). High levels of substitution rates in mitochondria are associated with sweeps of a CI-symbiont into a host population, which involves hitchhiking of particular mitochondrial haplotypes. This process may result in the fixation of mildly deleterious mutations followed by compensatory beneficial mutations, all leading to high nucleotide substitution and amino acid replacement rates (Shoemaker et al. 2004; Oliveira et al. 2008). Maternally inherited endosymbionts the hosts requires (e.g., nutritional symbionts) could also contribute to these incompatibilities for the same reasons, that is, sweeps and small effective population sizes. There is evidence that *E. suzannae* has lower mtDNA diversity than *E. gennaroi* (Gebiola et al. 2016), suggestive of a selective sweep of mtDNA. In this and similar situations, then, unidirectional CI symbionts could contribute to maintenance of genetic isolation in both directions after secondary contact; in one direction, directly by CI, and in the other direction elevated nucleotide substitution and amino acid replacement rates in the mitochondria caused by the symbiont sweep could determine asymmetric hybrid inviability, causing highly reduced gene flow in spite of few barriers to mating. This hypothesis is consistent with the evidence for this species pair. Of course, we cannot rule out other maternal effects having a role in asymmetrical isolation, here or in other examples, including genomic imprinting or maternal genetic effects such as proteins or mRNA. This model of how unidirectional CI could lead to speciation is plausible, however, and awaits theoretical exploration and empirical testing in this and other systems.

The model proposed here may explain the contribution of CI to postzygotic barriers, but the pattern observed differs from the *Drosophila recens/subquinaria* system studied by Shoemaker et al. (1999) and Jaenike et al. (2006), where prezygotic barriers evolved rapidly, before genetic incompatibilities were complete. Here, we found no gene flow because of hybrid sterility, suggesting a longer time since separation, but only weak sexual isolation. The current study is similar to previous studies focusing on genetic incompatibilities in haplodiploids that show postzygotic isolation preceding prezygotic isolation. Perrot-Minnot et al. (2004) and Jeong and Stouthamer (2006) crossed different strains of the mite *Tetranychus urticae*, and two species of the parasitic wasp *Trichogramma* (*T. deion* and *T. kaykai*), respectively. While mating occurred, both studies found large hybrid incompatibilities in the F1 females. Since in the haplodiploid genetic system the entire genome is effectively sex-linked (Crozier 1977), rapid accumulation of BDM incompatibilities might be predicted due to a special case of the large-X effect known as "faster-X" (Charlesworth et al. 1987) or "faster-hemizygous-chromosome" (Koevoets and Beukeboom 2009), that is, genes on hemizygous chromosomes may evolve more quickly by enhancing selection on recessive alleles. We thus might predict the speciation model proposed to be most important in systems such as haplodiploids in which postzygotic isolation may precede mating barriers.

Like the *Drosophila* system, the populations of the species pair studied here are allopatric. Yet to be examined is the extent of interspecific gene flow and mating isolation in sympatry. Intriguingly, the species pair *E. gennaroi*/*E. suzannae*, previously known as *E. pergandiella* and *E. tabacivora*, respectively (see Gebiola et al. 2016), overlap in the Rio Grande Valley at the border between Texas and Mexico (Johnson 1996), and limited data hint at strong behavioral isolation in no choice mating tests (Johnson 1996), which would be consistent with evidence of reinforcement.

To conclude, this study fits into the growing consensus that symbiont-induced unidirectional CI plays its most significant role in speciation when it is coupled with additional isolating barriers (Bordenstein 2003). This view is consistent with at least three lines of evidence. First and most obviously, the barrier to gene flow as a result of unidirectional CI is asymmetric, with much greater isolation in one direction than the other. Further, once the

symbiont frequency is above the unstable equilibrium frequency (Turelli 1994), the symbiont spreads to fixation, and no incompatibility remains. Second, between populations that have begun to accumulate genetic differences, the CI symbiont will still spread (and lose its ability to restrict gene flow) as long as CI in one direction is not countered with incompatibilities in the reverse direction. Lastly, even in the one direction CI occurs, and similar to BDM incompatibilities, CI does not completely restrict gene flow, and typically involves incomplete levels of CI, and imperfect bacterial transmission. The view that symbiont-induced CI is more likely to facilitate speciation when it is one of several steps in the evolution of complete reproductive isolation is entirely consistent with how speciation is thought to proceed, through the accumulation of multiple isolating barriers.

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