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Multiple infections and diversity of cytoplasmic incompatibility in a haplodiploid species

L Mouton, H Henri, M Boulétreau and F Vavre
Laboratoire de Biométrie et Biologie Evolutive, Université Claude Bernard, 43 Bd du 11 Novembre 1918, 69622 Villeurbanne Cedex, France

Cytoplasmic incompatibility (CI) is a sperm–egg incompatibility commonly induced by the intracellular endosymbiont bacterium *Wolbachia* that, in diploid species, results in embryo mortality. In haplodiploid species, two types of CI exist depending on whether the incompatible fertilized eggs develop into males (male development (MD)) or abort (female mortality (FM)). CI allows multiple infections to be maintained in host populations, and thus allows interactions to occur between co-infecting strains. In *Leptopilina heterotoma*, three *Wolbachia* strains coexist naturally (*wLhet1*, *wLhet2*, *wLhet3*). When these three strains are all present, they induce a CI of FM type, whereas *wLhet1* alone expresses a CI phenotype intermediate between MD and FM. Here, we compare CI effects in crosses involving insect lines sharing the same nuclear background, but harboring different mixtures of strains. Mating experiments showed that: (i) *wLhet2* and *wLhet3* also induce an intermediate CI when acting alone, and show a bidirectional incompatibility; (ii) there is no interaction between the co-infecting strains in CI expression; (iii) the diversity of *Wolbachia* present within a male host influences the expression of CI: an increase in the number of strains is correlated with a decrease in the proportion of the MD type, which is also correlated with an increase in bacterial density. All these data suggest that the CI of FM type results from a stronger effect than the MD type, which conflicts with the conventional hypotheses used to explain CI diversity in haplodiploids, and could provide some new information about CI mechanisms in insects.
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Introduction

Many endosymbionts cause reproductive abnormalities in their hosts (Hurst, 1993). The most common is *Wolbachia*, a strictly intracellular bacterium present in a wide range of arthropods (Rousset *et al*, 1992; Werren *et al*, 1995; Breeuwer and Jacobs, 1996; Oh *et al*, 2000; Werren and Windsor, 2000), and in filarial nematodes (Bandi *et al*, 1998). This maternally inherited endosymbiont is able to manipulate host reproduction in a variety of ways, which promotes its spread within host populations (Werren, 1997; Stouthamer *et al*, 1999; Stevens *et al*, 2001).

Cytoplasmic incompatibility (CI) is the most common effect of *Wolbachia*: it has been described in crustaceans (Moret *et al*, 2001), arachnids (Breeuwer, 1997), and in a number of insects including mosquitoes, planthoppers, moths, beetles, wasps, and *Drosophila* (for a review, see Stouthamer *et al*, 1999). CI is a sperm–egg incompatibility, which occurs in crosses between males and females with differing *Wolbachia* infection status. It can be either unidirectional when infected males mate with uninfected females, or bidirectional in crosses where both males and females are infected with different CI-inducing *Wolbachia* strains (Hoffmann and Turelli, 1997). Although the molecular mechanism of CI has not yet been identified, a modification/rescue model has been proposed, which assumes the existence of two distinct bacterial functions (Werren, 1997): the modification (mod) function expressed in the male germ line during spermatogenesis, and the rescue (resc) function, expressed in the egg. At karyogamy, the modified paternal chromosomes are improperly condensed, and lost during early embryo development, unless the egg expresses the resc function corresponding to the *Wolbachia* strain present in the male (Breeuwer and Werren, 1993).

In diploid species, CI causes karyogamy failure, resulting in arrested development at an early embryonic stage (Callaini *et al*, 1997; Tram and Sullivan, 2002). In haplodiploid species, only fertilized eggs, which normally develop into diploid females, are subject to CI. When incompatible, they may encounter two types of CI: either they develop into males, like unfertilized eggs (male development (MD) type), or they die (female mortality (FM) type). In the MD type, the paternal set of chromosomes is totally eliminated, leading to haploid eggs that go on to develop into males (Breeuwer and Werren, 1990). The FM type is probably due to partial chromosome fragmentation, resulting in lethal aneuploidy (Breeuwer, 1997; Vavre *et al*, 2001).

One consequence of CI is to confer an advantage on the females containing the highest diversity of *Wolbachia* strains, because they can mate successfully with males of any kind (Frank, 1998). This advantage explains why

Correspondence: L Mouton, Laboratoire de Biométrie et Biologie Evolutive (UMR-CNRS 5558), Université Claude Bernard – Lyon 1, 43 Bd du 11 Novembre 1918, 69622 Villeurbanne Cedex, France.
E-mail: mouton@biomserv.univ-lyon1.fr
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multiple infections are so widespread in nature. Multiple infections provide an opportunity to investigate the phenotypic expression of different bacterial strains within the same host insect, their reciprocal incompatibility relationships, and their possible interactions. Such studies require insect lines with differing *Wolbachia* infection status, and have only been performed in a few species: *Drosophila simulans* (Merçot *et al*, 1995; Sinkins *et al*, 1995), *Aedes albopictus* (Dobson *et al*, 2001), *Nasonia vitripennis* (Perrot-Minnot *et al*, 1996; Bordenstein and Werren, 1998), and *Callosobruchus chinensis* (Kondo *et al*, 2002). These studies have concluded that the CI level increases with the number of strains present in the same male, with additive effects and almost no interaction (Perrot-Minnot *et al*, 1996; Bordenstein and Werren, 1998; Rousset *et al*, 1999; Dobson *et al*, 2001).

The situation may be more complex in haplodiploid species, since the type of CI may also vary as in *Nasonia–Wolbachia* associations, where the type of CI would depend on the host genetic background (Bordenstein *et al*, 2003). Bacterial factors may also be involved in CI type variation, as demonstrated in the *Drosophila* parasitoid *Leptopilina heterotoma*. This parasitic wasp harbors three different *Wolbachia* strains, designated *wLhet1*, *wLhet2*, and *wLhet3* (Vavre *et al*, 1999). If all three strains are present together, they induce a complete CI of FM type, whereas *wLhet1* alone induces an intermediate CI type between MD and FM, that is, some of the fertilized eggs die, whereas others become haploid (Vavre *et al*, 2000, 2001). Thus, the type of CI depends on the diversity of *Wolbachia* co-infecting the individual host, which makes interactions possible between the different strains.

In this paper, we further study the association between *Wolbachia* and *L. heterotoma* using two recently obtained doubly infected lines (*wLhet1*/*wLhet2* or *wLhet1*/*wLhet3*) (Mouton *et al*, 2003), and one singly infected line (*wLhet1*) (Vavre *et al*, 2001). Three main questions addressed were as follows: (i) Do *wLhet2* and *wLhet3* also induce CI? (ii) What are the incompatibility relationships between strains? (iii) How does the diversity of *Wolbachia* strains present influence the expression of CI? Results are discussed with regard to data on bacterial density recently obtained in *L. heterotoma* (Mouton *et al*, 2003).

Materials and methods

Insect strains and rearing

The wasp *L. heterotoma* (Hymenoptera, Figitidae) develops as a solitary larval parasitoid of many *Drosophila* species. In the laboratory, parasitoids were reared on a *Wolbachia*-free strain of *D. melanogaster* originating from S^{te} Foy-les-Lyon, France. Rearing and experiments were carried out at 20°C, LD 12:12 and 70% RH.

We used a line of *Leptopilina heterotoma* originating from Antibes (France), which had become homozygous after 35 generations of sib mating. This A7(123) line harbors three different *Wolbachia* strains: *wLhet1*, *wLhet2*, and *wLhet3* (Vavre *et al*, 1999). An uninfected A7(0), a singly infected A7(1) and two doubly infected sublines, A7(12) and A7(13), were derived from A7(123) a number of generations before the experiments (at least 10 generations), using antibiotic treatments by feeding parasitized *Drosophila* larvae a standard diet containing low concentrations of rifampicin (Vavre *et al*, 2001; Mouton *et al*, 2003). Since all these sublines derived from the same A7(123) inbred line, they shared the same nuclear background and differed solely with regard to their infection status.

Male reproductive capacity

Despite the fact that all the sublines were derived from the highly inbred A7 line, we performed a preliminary experiment to confirm their reciprocal nuclear compatibility and the uniformity of the male reproductive capacity. Isolated virgin A7(123) females (3–4 days old) were individually mated with males (5 days old) of each infection status (A7(0), A7(1), A7(12), A7(13) and A7(123)) to avoid CI (15 couples per cross type). Mating was visually checked, and each female was then provided with a batch of 100 *Drosophila* larvae for 48 h. After development, the number of adult *Drosophila* and male and female wasps emerging from them were counted, and the offspring sex ratio (SR) calculated individually (SR = % males among the emerging wasps). Five control *Drosophila* groups were kept without wasps to estimate the natural egg-to-adult mortality among unparasitized *Drosophila*.

Test for CI

CI was tested in crosses between males (5 days old) and females (4 days old) of various infection statuses. For each combination, 15 pairs of males and virgin females were isolated. After mating had been observed, each female was provided for 48 h with the *Drosophila* larvae emerging from 100 eggs in vials containing sufficient diet for further development (these conditions prevent multiple parasitic infestation of the *Drosophila* larvae). A total of 10 control host groups were kept unparasitized to estimate the natural mortality of *Drosophila* in the absence of parasitoids. After development, the numbers of *Drosophila*, and of male and female wasps emerging from each vial, were recorded (a few crosses where fewer than five wasps had emerged were not taken into consideration in the data analysis). Comparison with compatible crosses involving males and females with the same infection status allowed us to estimate both the CI level and the percentage of MD CI type.

$$\text{CI level} = \left(1 - \frac{F}{F_C}\right) \times 100.$$

The percentage of the MD type is the percentage of fertilized eggs that have been haploidized by *Wolbachia*. It takes into account the excess number of males produced by incompatible crosses ($M - M_C$), and the number of fertilized eggs that suffered CI ($F_C - F$).

$$\text{Percentage of MD type} = \frac{M - M_C}{F_C - F} \times 100.$$

where F_C is the mean number of females emerging from compatible crosses; M_C the mean number of males emerging from compatible crosses; F the number of females emerging from the incompatible cross tested; and M the number of males emerging from the incompatible cross tested.

Results

Male reproductive capacity

There were no statistically significant differences between the numbers of *Drosophila*, or of male and female wasps that emerged from any of the crosses involving A7(123) females and males of various infection statuses (Table 1). This demonstrated that there was no nuclear incompatibility between the different *L. heterotoma* lines, and that the reproductive capacity of the males was the same regardless of their infection status.

Tests for CI

We compared the phenotypic effects of *wLhet2* and *wLhet3*, and tested the influence of the diversity of *Wolbachia* strains on CI expression. Table 2 shows the number of male wasps, the total offspring, the SR, the level of CI, and the percentage of the MD CI type, and Figure 1 shows the number of *Drosophila*, and of male and female wasps emerging from each cross. Cross number 1 is the control cross between A7(0) males and females. In all other crosses, the SR was highly male-biased, with few or no females emerging, indicating a very high CI level. Moreover, the numbers of *Drosophila* were far higher and wasp production lower, which is typical for a CI phenotype of FM type. In some crosses, the increase in the production of males indicates that some incompatible eggs developed into males.

Effects of wLhet2 and wLhet3: Crosses 2 and 4 were used to find out whether *wLhet3* and *wLhet2* are able to induce CI. However, since we failed to obtain lines singly infected with these strains, their effects were in fact tested in the presence of *wLhet1*. A male-biased offspring SR and high reduction in the production of females indicate that both strains induced CI. Moreover, far fewer offspring were produced than from the control cross, and the number of males was higher, indicating that the CIs induced by *wLhet2* and *wLhet3* when present separately were intermediate between MD and FM types, with approximately 30% of the fertilized eggs developing into males.

Crosses 3 and 5 also tested the CI induced by *wLhet2* and *wLhet3*, but in the presence of *wLhet1*, and of *wLhet3*

Table 1 Test for the reproductive capacity of males with various infection statuses

Male (n)	*Number of* Drosophila	*Number of males*	*Number of females*	*SR*
A7(0) (12)	9.58±1.85	18.17±1.25	58.92±2.90	0.310±0.069
A7(1) (15)	11.93±2.49	19.73±1.80	54.53±2.33	0.363±0.134
A7(12) (15)	15.67±3.19	16.00±1.43	51.73±2.03	0.307±0.089
A7(13) (11)	16.00±3.44	15.82±1.38	51.64±2.86	0.305±0.058
A7(123) (10)	15.00±3.40	17.30±1.80	49.40±3.09	0.342±0.065
ANOVA	$F_{4,58}=0.88$ $P=0.4841$	$F_{4,58}=1.14$ $P=0.3472$	$F_{4,58}=2.01$ $P=0.1050$	$F_{4,58}=1.14$ $P=0.3484$

Numbers of *Drosophila* and of male and female wasps emerging, and SR (percentage of male) in crosses between A7(123) females and A7(0), A7(1), A7(12), A7(13) or A7(123) males (mean±standard error). *n* indicates the number of couples tested. One-way ANOVA was carried out after arcsine square root transformation for SR.

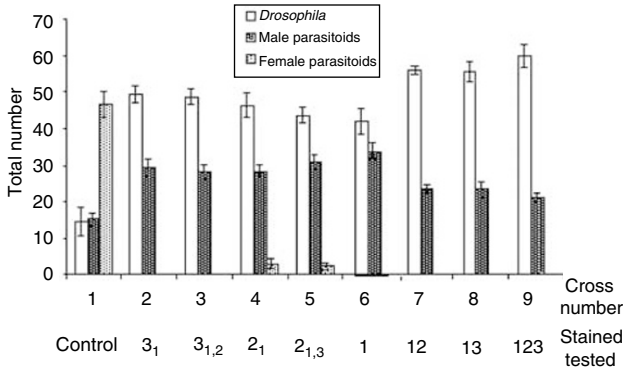

Figure 1 Numbers of *Drosophila*, and of male and female wasps emerging from crosses between males and females with various infection statuses (mean and standard error). Each cross type is identified by the corresponding cross number (see Table 2). The *Wolbachia* strain phenotypic effect tested is indicated for each cross: the bold number corresponds to the *Wolbachia* strain(s) (1 for *wLhet1*, 2 for *wLhet2*, and 3 for *wLhet3*) and the superscript corresponds to the other *Wolbachia* strains also present in the male (for example, 3_1 corresponds to the effect of *wLhet3* in the presence of *wLhet1*).

Table 2 Tests for CI

Cross number (n)	*Cross (female × male)*	*Number of male wasps*	*Total offspring*	*Sex ratio*	*CI level*	*% MD*
1 (8)	A7(0) × A7(0)	15.1±1.7 (a)	61.7±3.8 (a)	0.246±0.029 (a)	0	—
2 (15)	A7(1) × A7(13)	29.5±2.3 (b,c)	29.6±2.3 (b)	0.998±0.002 (b)	99.8±0.1 (a)	31.0±4.9 (a,b)
3 (14)	A7(12) × A7(123)	28.2±1.9 (b,d)	28.3±1.9 (b,d)	0.998±0.002 (b)	99.8±0.2 (a)	28.2±4.0 (b,c)
4 (15)	A7(1) × A7(12)	28.5±1.6 (b)	31.5±2.3 (b)	0.920±0.027 (c)	93.8±2.7 (b)	31.9±4.7 (a,c)
5 (12)	A7(13) × A7(123)	31.1±2.1 (b,c)	33.5±2.4 (b)	0.937±0.024 (c)	94.8±2.0 (b)	36.6±4.9 (a,c)
6 (12)	A7(0) × A7(1)	34.1±2.1 (c)	34.2±2.2 (b)	0.998±0.002 (b)	99.8±0.2 (a)	40.9±4.6 (a)
7 (14)	A7(0) × A7(12)	23.4±1.1 (d,e)	23.4±1.1 (c,d)	1±0 (b)	100.0±0.0 (a)	17.9±2.5 (b,d)
8 (13)	A7(0) × A7(13)	23.3±2.1 (d,e)	23.3±2.1 (c,d)	1±0 (b)	100.0±0.0 (a)	18.8±4.1 (b,d)
9 (12)	A7(0) × A7(123)	21.1±1.3 (a,e)	21.2±1.3 (c)	0.996±0.004 (b)	99.8±0.2 (a)	12.9±2.7 (d)
	Statistical test	$F_{8,106}=7.74$ $P<0.0001$	$F_{8,106}=22.13$ $P<0.0001$	$H_8=71.47$ $P<0.0001$	$H_7=46.02$ $P<0.0001$	$H_7=31.12$ $P<0.0001$

Results of crosses between males and females with various infection statuses. Mean±standard error of numbers of male wasps, total offspring production, SR (percentage of males), CI level, and percentage of MD type. *n* indicates the number of couples tested. Parametric tests were carried out for the number of male wasps and total offspring (one-way ANOVA and least significant difference tests), and nonparametric tests for the three other parameters (Kruskal–Wallis and Mann–Whitney tests). Means marked with the same letter are not significantly different ($P=0.05$).

or *wLhet2*, respectively. Similar results were obtained in crosses 2 and 3 for the effect of *wLhet3*, and in crosses 4 and 5 for the effect of *wLhet2*, indicating that, when co-infecting males, both these strains exert their mod (germline modification) effect on sperm independently. The CI in crosses 3 and 5 suggests that the rescue functions of *wLhet2* and *wLhet3* are distinct, as neither was able to rescue the sperm modified by the other. This was confirmed by the bidirectional incompatibility in reciprocal crosses between the A7(12) and A7(13) lines (Table 3). These crosses also confirm that the CIs induced by *wLhet2* and *wLhet3* are intermediate between the MD and FM types, since the male production was significantly higher in incompatible crosses, even though the percentage of the MD type was lower than previously observed.

Influence of *Wolbachia* diversity on CI: We studied the influence of the diversity of the bacteria on the effect induced in the host by crossing uninfected females with males harboring 0, 1, 2 or 3 *Wolbachia* strains.

When alone, *wLhet1* (cross 6) induced a CI intermediate between the FM and MD types, and an increase in the number of male wasps. We estimate that 40.9% of the fertilized eggs became haploid, and that 59.1% died, which was consistent with a previous observation (Vavre *et al*, 2001).

In crosses involving doubly infected males (crosses 7 and 8), no females emerged, and the number of emerging males was intermediate between crosses 1 and 6. Therefore, when mated with uninfected females, A7(12) and A7(13) males induced a complete CI that was intermediate between the FM and MD types. The percentage of the MD CI type was the same in both these crosses, but was less than 20% of the total CI, which was lower than in cross 6.

When the males were triply infected (cross 9), no females emerged and the number of male wasps was intermediate between those in the control cross and in crosses 7 and 8, although none of the differences was significant. This suggests that even when triply infected males are used, a very few incompatible eggs can develop into males, a result that was not found in previous studies (Vavre *et al*, 2000, 2001). Altogether, these findings indicate that the percentage of males decreases as the diversity of *Wolbachia* strains increases.

Discussion

Specific effects of the three strains (crosses 2–6)

Our results demonstrate that all the three *Wolbachia* strains that infect *L. heterotoma* induce CI. Similar coexistence of CI-inducing strains has been reported in other natural multiple *Wolbachia* associations (Merçot *et al*, 1995; Perrot-Minnot *et al*, 1996; Bordenstein and Werren, 1998; Dobson *et al*, 2001; Kondo *et al*, 2002), and is consistent with theoretical models. The CI effect induced by each strain confers a fitness gain on the multiply infected cytotypes, because multiply infected eggs are compatible with sperm of any infection status, allowing multiple infections to spread and be maintained (Frank, 1998).

Looking in greater detail, it turns out that, when alone, all three strains induce a CI phenotype intermediate between the FM and MD types. Unfortunately, the ability of *wLhet2* and *wLhet3* to induce CI could not be tested in singly infected lines, and the present results must therefore be interpreted with caution. Nevertheless, their specific CI phenotypes were expressed in the same way in doubly and triply infected males, suggesting that the strains, at least *wLhet2* and *wLhet3*, do not interact with regard to their resc or mod functions, and that the CI level induced by each strain does not depend on the presence of the other. This can be related to recent data about *Wolbachia* density in *L. heterotoma*, which show that the density of each strain is specifically regulated, and does not depend on the presence of other strains (Mouton *et al*, 2003). The independence of the density of each strain could account for the stability of CI level induced, regardless of the presence of co-infecting strains.

Effects of multiple infections (crosses 6–9)

When acting alone, each of the three strains induces a CI that is intermediate between the MD and FM types; when associated with *wLhet2* or *wLhet3*, *wLhet1* induces a FM CI with a lower MD component; and when males are triply infected, nearly all incompatible eggs die, indicating an almost complete FM type (Vavre *et al*, 2000; present results). Thus, in *L. heterotoma*, an increase in the number of *Wolbachia* strains co-infecting a male results in a lower proportion of the MD type. At the population level, such increase in mortality may facilitate the spread

Table 3 Test for bidirectional incompatibility between *wLhet2* and *wLhet3*

Cross (female × male) (n)	*Number of* Drosophila	*Number of male wasps*	*Total offspring*	*Sex ratio*	*CI level*	*% MD*
A7(12) × A7(12) (9)	33.0±3.1 (a)	12.3±0.9 (a)	42.4±3.3 (a)	0.298±0.025 (a)	—	—
A7(13) × A7(13) (10)	30.4±3.6 (a)	12.3±1.3 (a)	47.5±3.9 (a)	0.260±0.022 (a)	—	—
A7(12) × A7(13) (12)	57.5±2.4 (b)	18.2±1.6 (b)	18.2±1.6 (b)	1.000±0.000 (b)	100.0±0.0	20.4±4.9
A7(13) × A7(12) (12)	61.4±1.9 (b)	19.2±1.7 (b)	19.3±1.6 (b)	0.989±0.010 (b)	99.5±0.5	20.3±4.4
Statistical test	$F_{3,39}=34.80$ $P<0.0001$	$F_{3,39}=6.146$ $P=0.0016$	$F_{3,39}=34.30$ $P<0.0001$	$H_3=31.43$ $P<0.0001$	$U=78.00$ $P=0.3178$	$U=72.00$ $P<0.9999$

Results of reciprocal crosses between A7(12) and A7(13) males and females. Mean±standard error of the numbers of *Drosophila* and of male wasps, total offspring production, SR (percentage of males), CI level, and percentage of MD type. *n* indicates the number of couples tested. Parametric tests were carried out for the numbers of *Drosophila*, of male wasps, and of the total number of offspring (one-way ANOVA and least significant difference test), and nonparametric tests were used for the three other parameters (Kruskal–Wallis and Mann–Whitney tests). Means marked with the same letter are not significantly different ($P=0.05$).

of multiple infections, since theoretical models have demonstrated that the FM type makes *Wolbachia* more invasive than the MD type (Vavre *et al*, 2000).

This result also indicates that a single host genotype can express different CI types. Conversely, Bordenstein *et al* (2003) have demonstrated in two *Nasonia* species that the host genetic background may also account for variations in the CI type. Therefore, the expression of CI in haplodiploid species is probably under the control of complex nucleo-cytoplasmic interactions.

In the system we are studying here, an increase in *Wolbachia* diversity also leads to an increase in *Wolbachia* density (Mouton *et al*, 2003), making it impossible to distinguish between their relative influences on the CI type. However, previous studies have shown that in multiply infected males the CI level results from the addition of the individual CI effects of each strain (Perrot-Minnot *et al*, 1996; Bordenstein and Werren, 1998; Rousset *et al*, 1999; Dobson *et al*, 2001), leading to an increase of the total *Wolbachia* effect in multiple infections. Likewise, since the proportion of the FM CI type increases with the diversity of *Wolbachia*, the FM type is probably 'harsher' than the MD type. This contrasts with the hypothesis of Breeuwer (1997) and Vavre *et al* (2000), who proposed that the MD type could result from greater modification and the complete loss of paternal chromosomes, so that the fertilized eggs revert back to haploidy and develop as males, whereas the FM type would result from the milder modification and an incomplete loss of paternal chromosomes, resulting in the lethal aneuploidy of incompatible eggs. The results reported here actually suggest the contrary: the FM CI type tends to be associated with the harsher *Wolbachia* effect. This could result either from such an extreme modification of the paternal chromosomes that further embryo development fails for some reason, or from the effects of *Wolbachia* on the cytoskeleton and/or microtubule apparatus of the sperm, which are known to be involved in early embryonic mitosis (Kose and Karr, 1995).

While cytological studies have shown that haploidization does occur in the MD CI type, there is as yet no cytological evidence demonstrating that egg aneuploidy is responsible for inducing FM CI. Clearly, cytogenetic analyses in haplodiploids are required before we can fully understand the diversity of CI they can display, but our results also suggest that analyzing the CI in haplodiploids could be helpful in elucidating the general mechanisms of CI.

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