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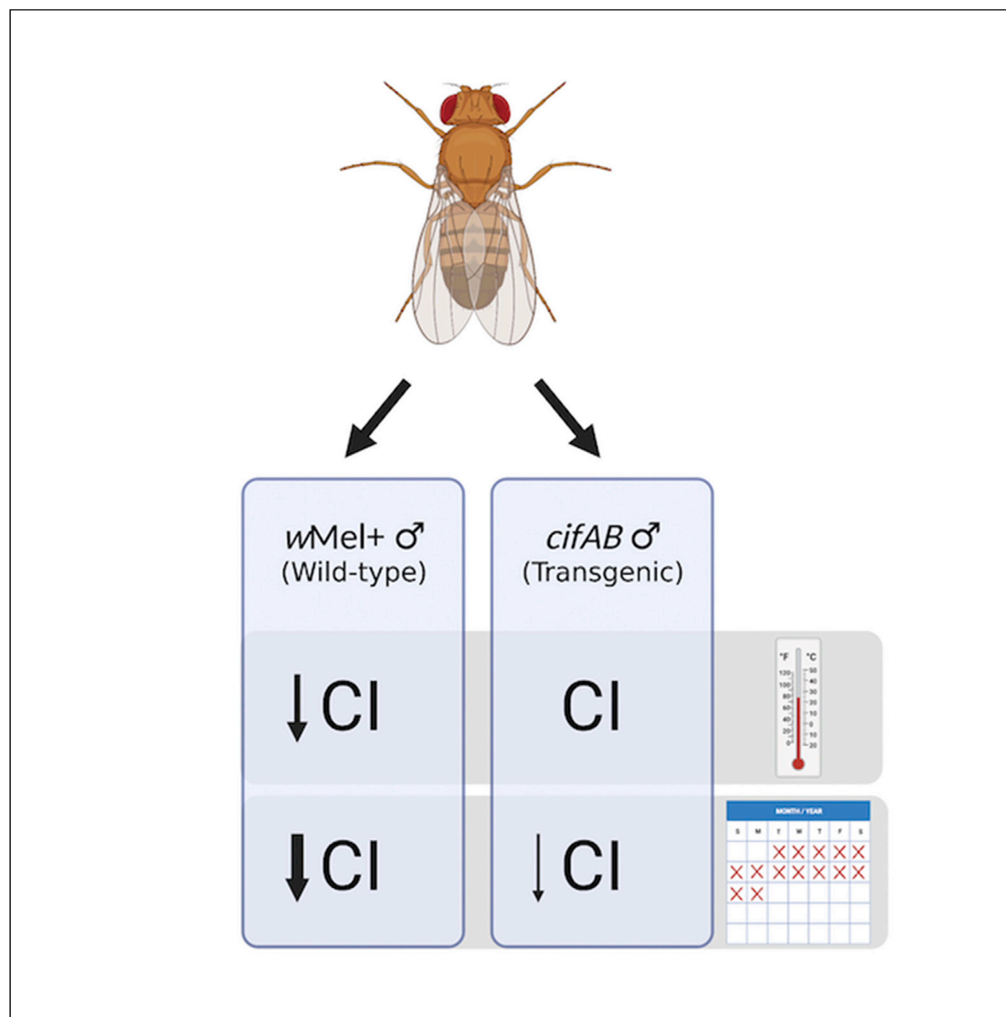

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Highlights

Symbiont adaptations are often weakened by life history and environmental parameters

Cytoplasmic incompatibility (CI) declines with host age and temperature

Transgenic CI is robust to age and temperature variation in *Drosophila melanogaster*

Transgenic CI circumvents pressures that diminish wildtype CI

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Transgenic cytoplasmic incompatibility persists across age and temperature variation in *Drosophila melanogaster*

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SUMMARY

Environmental stressors can impact the basic biology and applications of host-microbe symbioses. For example, *Wolbachia* symbiont densities and cytoplasmic incompatibility (CI) levels can decline in response to extreme temperatures and host aging. To investigate whether transgenic expression of CI-causing *cif* genes overcomes the environmental sensitivity of CI, we exposed transgenic male flies to low and high temperatures as well as aging treatments. Our results indicate that transgenic *cif* expression induces nearly complete CI regardless of temperature and aging, despite severe weakening of *Wolbachia*-based wild-type CI. Strong CI levels correlate with higher levels of *cif* transgene expression in young males. Altogether, our results highlight that transgenic CI persists against common environmental pressures and may be relevant for future control applications involving the *cifA* and *cifB* transgenes.

INTRODUCTION

Wolbachia are maternally transmitted bacteria that occur intracellularly as reproductive endosymbionts in a variety of arthropod species. The genus is estimated to occur in 40–65% of all arthropod species, as well as some nematode species (Charlesworth et al., 2019; Hilgenboecker et al., 2008; Kaur et al., 2021; Lefoulon et al., 2016; Weinert et al., 2015; Zug and Hammerstein, 2012). *Wolbachia* can have a variety of parasitic effects on arthropod reproduction, including parthenogenesis (Huigens et al., 2000), male killing (Duplouy et al., 2010), feminization (Kern et al., 2015), and cytoplasmic incompatibility (CI) (Shropshire et al., 2020). The most common form is CI, which causes embryonic lethality when modified sperm from *Wolbachia*-infected males fertilize the eggs of uninfected females. Infected males and females are compatible with each other owing to *Wolbachia*-mediated rescue of CI-modified sperm (Kaur et al., 2022). As such, infected females that transmit the bacteria exhibit a relative fitness advantage over uninfected ones because of their ability to mate with both infected and uninfected males. This facilitates the selfish spread of *Wolbachia* through host arthropod populations and forms the basis of population suppression and replacement methods used internationally for biological control of mosquitoes (Beebe et al., 2021; Flores and O'Neill, 2018; Hoffmann et al., 2011; Huang et al., 2017; Riegler et al., 2005; Xi et al., 2005).

In insect control strategies, *Wolbachia* inhibit the transmission of arboviruses such as dengue, Zika, and chikungunya viruses, among others (Fraser et al., 2017; Hedges et al., 2008; Jeffries and Walker, 2016; Kean et al., 2015; Moreira et al., 2009; Pais et al., 2018). Because traditional pest control methods use chemical and environmental control tactics with potential off-target effects on beneficial insects and destruction of breeding habitats (Huang et al., 2017; Kay et al., 2000; Subramaniam et al., 2015), *Wolbachia* offer promise as a safer and more sustainable control method in a variety of countries worldwide (Beebe et al., 2021; Jeffries and Walker, 2016; Kean et al., 2015; Ross, 2021; Ross et al., 2020), either for population suppression (Beebe et al., 2021; Bourtzis et al., 2014; Crawford et al., 2020; Laven, 1967; Zhang et al., 2015; Zheng et al., 2019) or population replacement (Hoffmann et al., 2011; Nazni et al., 2019; O'Neill et al., 2018; Utarini et al., 2021; Yen and Failloux, 2020). *Wolbachia*-mediated population suppression occurs when CI-causing male mosquitoes are released and mate with uninfected females, thereby resulting in embryonic lethality and a population size reduction. Although this method is effective, it requires persistent releases of infected males to ensure the target control population does not bounce back in numbers (Beebe et al., 2021). Population replacement occurs when infected male and infected female mosquitoes are released

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together. Crossing them produces infected, viable offspring whereas crosses between infected males and uninfected females results in CI-driven inviability and thus a relative fitness reduction. As such, the uninfected portion of the population will gradually be replaced by infected individuals that suppress arbovirus transmission (Flores and O'Neill, 2018; Huang et al., 2017; Xi et al., 2005; Yen and Failloux, 2020). *Wolbachia* have also been transinfected into the agricultural planthopper pest in a laboratory setting, where it induces CI in the new host, decreases transmission of rice ragged stunt virus, and inhibits viral symptoms in infected rice plants (Gong et al., 2020). Though this has not yet been used in a field setting to control agricultural pests, it shows promise for utilizing *Wolbachia* to control viral transmission in a variety of host-vector systems.

Wolbachia's potential as a biocontrol method currently relies on high CI levels, potentially across wide-ranging environmental conditions within and between days or seasons. Temperature plays a well known role in regulating *Wolbachia* infection (Bordenstein and Bordenstein, 2011; Mouton et al., 2006; Nasehi et al., 2021; Ross et al., 2020; Van Opijnen and Breeuwer, 1999). *Aedes aegypti* mosquitoes, one of the major vectors of arboviruses, experience a decrease in *Wolbachia* load and thereby CI when exposed to warm temperatures in the field (Ross et al., 2019, 2020), though it is possible for infection rates to bounce back on return to cooler temperatures (Ross et al., 2020; Ulrich et al., 2016; Young and Plough, 1926). Host biology may play a role in response to heat stress, as some species only lose *Wolbachia* infection after heat exposure over several generations (Kyei-poku et al., 2003), and some populations are more tolerant of heat stress because of evolution in a warmer geographic cline (David et al., 2005; Rohmer et al., 2004; Young and Plough, 1926). Moreover, low temperatures can reduce *Wolbachia* transmission from mother to offspring in *D. melanogaster* (Hague et al., 2021), and high temperatures impact transmission of native *Wolbachia* in *Culex pipiens quinquefasciatus* (Tokash-Peters et al., 2022). Many infected mosquito releases occur in tropical climates. Therefore, if *Wolbachia* themselves are sensitive to high or low temperatures or host resistance evolution, then other measures for control might be useful to consider in the long-term.

Furthermore, aging can influence *Wolbachia* densities and CI penetrance in select strains. In *Aedes albopictus* mosquitoes, field-reared males with both *w*AlbA and *w*AlbB *Wolbachia* did not experience a decrease in CI levels with age; however, laboratory-reared males with single *w*AlbA exhibited a decrease in CI, perhaps due to lower overall *Wolbachia* densities (Kittayapong et al., 2002; Tortosa et al., 2010). Other mosquito species including *Aedes polynesiensis* infected with a non-native strain of *Wolbachia* (Brelsfoard and Dobson, 2011) and *C. pipiens* infected with a variety of incompatible *Wolbachia* strains (Duron et al., 2007; Rasgon and Scott, 2003) do not demonstrate evidence of CI decreases as male age. To our knowledge, the effect of age on CI strength in *A. aegypti* has not been studied. In *Drosophila simulans* and *D. melanogaster*, CI decreases with male age (Awrahman et al., 2014; Clancy and Hoffmann, 1998; Reynolds and Hoffmann, 2002; Shropshire et al., 2021). Thus, to circumvent these potential environmental sensitivities, synthetically engineering host genomes with the CI-causing genes as well as hypothetical anti-pathogen genes could in theory be developed for future releases that are more robustly penetrant.

At the genetic level, expression of two adjacent *cifA* and *cifB* genes from prophage WO in *Wolbachia* are responsible for causing CI (Beckmann et al., 2017; LePage et al., 2017), whereas *cifA* alone in females sufficiently rescues CI (Shropshire et al., 2018; Shropshire and Bordenstein, 2019). This Two-By-One genetic model of CI is applicable to several *Wolbachia* strains and *cif* gene pairs (Beckmann et al., 2019; Chen et al., 2019; LePage et al., 2017; Shropshire et al., 2018; Shropshire and Bordenstein, 2019). Recent evidence also demonstrates that a One-by-One model is operational in some non-native contexts wherein *cifB* causes CI that is rescued by *cifA* (Adams et al., 2021; Sun et al., 2021). Furthermore, transgenic CI by the *cif* genes can importantly be rescued by wild-type *Wolbachia* infection, and vice versa (Shropshire and Bordenstein, 2019). However, various studies unrelated to CI show that although transgenic organisms can outperform their wild-type counterparts in laboratory experiments, the variation and unpredictability of field settings can often result in equal or poorer performance by transgenic individuals when introduced to their natural habitat (Sundstrom et al., 2007; Zeller et al., 2010). Therefore, investigation into whether transgenic CI is sensitive to natural variables such as temperature and host aging will shed light on the potential utility of *cif* transgenes as complementary tools, if and when *Wolbachia* propagation become ineffective in field releases.

Here we compare the CI levels caused by wild-type *w*Mel-infected males with that of transgenic uninfected males expressing the *cif* genes in the germline at a variety of ages and temperatures. We test the

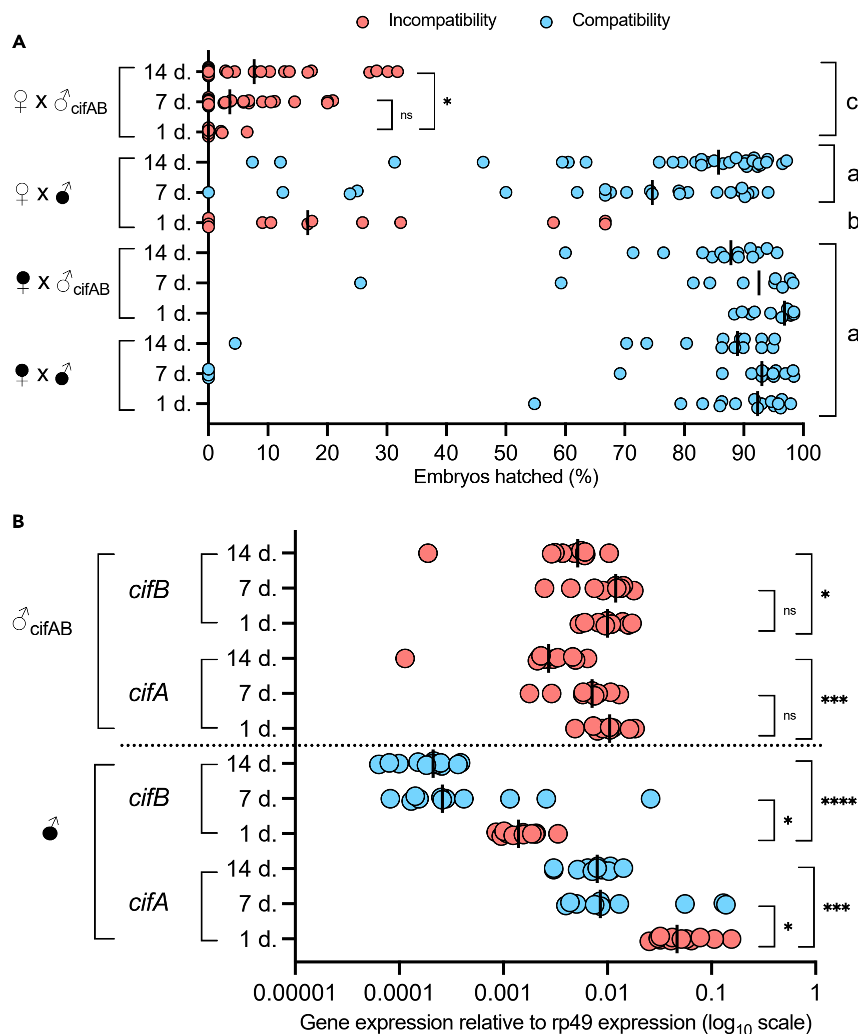

Figure 1. As males age, *Wolbachia*-induced CI is lost, whereas transgenic CI persists in association with strong *cif*transgene expression

(A) Virgin males were aged for 1, 7, or 14 days before mating to wildtype infected or uninfected virgin females to test the effect of male age on CI penetrance compared to the control wild-type rescue cross using 1-day-old males. Filled sex symbols indicate wild-type *Wolbachia* infection, whereas empty sex symbols indicate no infection; transgenic *cif* expression is indicated to the right of unfilled sex symbols. Medians are denoted by a black bar. Letters to the right indicate statistically significant differences between treatments as compared to the control wild-type rescue cross using a Kruskal-Wallis test followed by Dunn's multiple comparison tests (Table S1A; n = 10–30 replicates per genotype). This experiment was conducted twice, and the initial run can be found in Figure S1 with related p values listed in Table S4.

(B) *cifA* and *cifB* gene expression was assessed in siblings of males expressing CI. Stars to the right indicate statistically significant differences between 1-, 7-, and 14-day-old male gene expressions of *cifA* and *cifB*; this was determined by running a Kruskal-Wallis test followed by Dunn's multiple comparison test (Table S1B; * = $p < 0.05$, *** = $p < 0.001$, **** = $p < 0.0001$; n = 9–12). See also Figure S1 and its related Table S4.

hypothesis that transgenic *cif* expression causes stronger CI relative to *w*Mel *Wolbachia* under these stressful physiological or environmental conditions.

RESULTS

High transgenic CI levels are persistent across male age

To test the effects of male age on wild-type and transgenic CI levels in *D. melanogaster*, we measured the percent of (un)hatched CI-embryos from infected males who were 1-day-old, 7-day-old, or 14-day-old at the time of mating (Figures 1 and S1). Males were paired singly with *Wolbachia*-infected (rescue cross)

or uninfected (CI cross) females aged 6–8 days. Among wild-type CI crosses, male age had a significant effect (Kruskal-Wallis test, p<0.001), which is consistent with previous findings (Shropshire et al., 2021). We reconfirm that *Wolbachia* cause strong CI in 1-day-old males (Figure 1A, mdn = 16.7% embryonic viability) and weak to no CI in 7-day-old (Table S1A, Dunn's test, mdn = 74.6%, p = 0.0018) and 14-day-old (Dunn's test, mdn = 85.75%, p<0.0001) males, respectively. As expected, the 7- and 14-day-old wild-type CI crosses showed no significant difference from the 1-day-old wild-type rescue control cross (Table S1A, Dunn's test, p = 0.2084 and p>0.999, respectively). Also consistent with previous findings (LePage et al., 2017; Shropshire and Bordenstein, 2019), uninfected males transgenically expressing *cifA* or *cifB* alone did not cause CI regardless of male age (Figure S1). In contrast to *Wolbachia* infected males, 1-day-old, uninfected, transgenically expressing *cifAB* males caused nearly complete CI (mdn = 0%) that in turn persists as strong CI in 7-day-old males (Table S1A, Dunn's test, mdn = 3.6%, p = 0.1059), and 14-day-old males, the latter of which is however significantly different from the 1-day-old males (Dunn's test, mdn = 7.7%, p = 0.0155). Taken together, these results demonstrate that transgenic CI via *cifAB* dual expression mostly persists as males age whereas wild-type CI rapidly decreases.

The above results specify that transgenic CI is highly penetrant across male age. One hypothesis for this observation is the constitutive and strong expression of the *cifA* and *cifB* transgenes under heterologous expression in adults. We thus used quantitative PCR to measure *cifA* and *cifB* transgene expression levels between male hatch rate siblings of the same genotype at different ages. Wild-type *cifA* and *cifB* expression from 1-day-old males that cause strong CI had a five-to six-fold higher median than that of the corresponding 14-day-old wild-type males that cause weak to no CI (Figure 1B, Table S1B, Dunn's test, *cifA*: p = 0.0002, fold change = 5.82; *cifB*: p <0.0001, fold change = 6.59). For transgenic males, *cifA* and *cifB* expression was not statistically different between 1-day and 7-day-old males that also express similar CI (Figure 1B, Dunn's test, *cifA*: p = 0.2577; *cifB*: p > 0.9999), whereas there was a two-to 4-fold decrease in median gene expression (Table S1B, Dunn's test, *cifA*: p = 0.0004, fold change = 3.86; *cifB*: p = 0.0145, fold change = 1.92) between 1-day-old and 14-day-old males that express statistically weaker CI (Figures 1A and 1B, Dunn's test, CI: p = 0.0155). One reason for this may be fewer number of transgene-expressing sperm cells in older male testes (Sepil et al., 2019). Thus, higher *cif* gene expression is associated with stronger CI among the age groups. Finally, wild-type *cifB* gene expression was significantly lower than *cifA* expression, as expected for *w*Mel (LePage et al., 2017; Lindsey et al., 2018).

High transgenic CI levels are persistent at high and low temperatures

To evaluate variation in the temperature sensitivity of transgenic versus wild-type CI, we implemented CI experiments at the following temperatures, 18, 27, and 29°C, that span the range of high host viability and fertility (David, 2008; Klepsatel et al., 2019; Trotta et al., 2006). Although it has been shown that *D. melanogaster* flies generally do not develop below 10°C (Jean David et al., 1998), exposure to 17 and 29°C constitute a range of viability that can negatively impact fecundity compared to 25°C (Klepsatel et al., 2019). Furthermore, males are sterile at 12°C (Cohet, 1973) and 30°C (David et al., 1971,2005), though eggs exposed to these temperatures during development can still develop into adults (David et al., 1971). Here, we measure CI at 18 and 27°C by the wild-type *w*Mel *Wolbachia* strain and *cif* transgenes as the percentage of inviable embryos relative to the compatible control cross (in which both male and female flies are wild-type *Wolbachia*-infected).

When constantly reared at 18°C, *w*Mel *Wolbachia*-infected males crossed to uninfected females caused significantly reduced hatch rates (Figure 2, mdn = 38.6%) relative to the rescue *w*Mel *Wolbachia* self-control (Table S2, Dunn's test, mdn = 91.7%, p = 0.0051). Consistent with previous work that demonstrated the sensitivity of *Wolbachia* density to low temperature (Hague et al., 2021), these levels of embryonic inviability are roughly half of the *w*Mel CI levels typically obtained at 25°C (LePage et al., 2017; Shropshire and Bordenstein, 2019). Notably at 18°C, dual transgenic *cifAB* expression in males results in complete CI (Figure 2, Table S2, Mann-Whitney U Test, mdn = 0%, p<0.0001) that is rescued by *w*Mel *Wolbachia* in females. Consistent with the Two-by-One genetic model of CI for *w*Mel CI (Shropshire and Bordenstein, 2019), single expression of *cifA* and *cifB* transgenes do not cause CI when transgenically expressed alone as they produce compatible median hatch rates of 95.95 and 94.8%, respectively. These results specify that transgenic CI by dual *cifA* and *cifB* expression is not susceptible to low temperatures and is more effective than wild-type *w*Mel CI under the experimental conditions here.

We also conducted a hatch rate where fathers, mothers, and offspring were reared at 29°C, but found that this resulted in significant sterility across all genotypes (Figure S2). This is consistent with previous literature

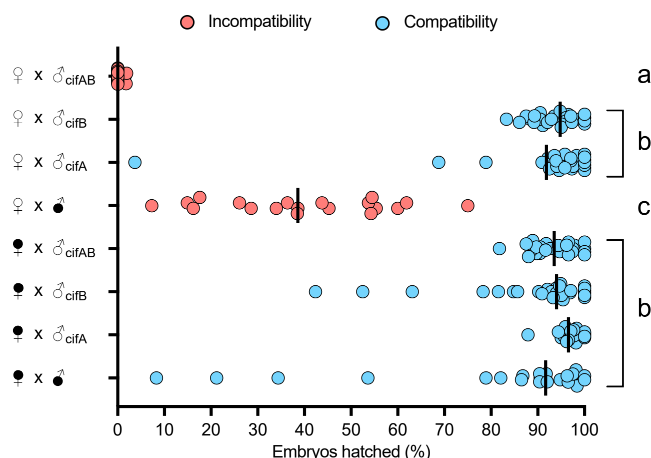

Figure 2. At 18°C, *Wolbachia*-induced CI is intermediate, whereas transgenic CI remains nearly complete

CI assays were conducted at 18°C to determine whether low temperature influenced penetrance as measured by the percentage of hatched embryos. Filled sex symbols indicate wild-type *Wolbachia* infection, whereas unfilled sex symbols are uninfected. *cif* transgene expression is indicated to the right of the corresponding unfilled sex symbol. Medians are shown by a black line, and letters to the right of data points indicate statistical differences as determined by a Kruskal-Wallis test followed by a Dunn's multiple comparison test (Table S2; n = 19–30). All crosses were compared to the control cross of wild-type rescue where both parents were *Wolbachia*-infected.

that *Drosophila* cells deteriorate at this temperature (David et al., 1971; Miquel et al., 1976). To disentangle sex-specific effects of this temperature-induced sterility, we next tested if males reared at 29°C and mated to females reared at 25°C would yield detectable levels of CI. However, results across replicate experiments were highly variable and not interpretatable (Figure S3, Table S5B).

Because of this observation, we reared males and females at 27°C and found that wild-type *w*Mel *Wolbachia* also caused significantly reduced hatch rates (mdn = 31.3%) relative to the rescue *w*Mel *Wolbachia* self-control (Figure 3, Table S3, mdn = 72.7%, p = 0.0002). These levels of CI are also weaker relative to typical *w*Mel CI levels at 25°C (LePage et al., 2017; Shropshire et al., 2018). Notably, the compatible rescue and non-CI crosses at 27°C yielded slightly lower hatch rates (60–70%) versus typical hatch rates at 25°C (90-100%) (LePage et al., 2017; Shropshire and Bordenstein, 2019). Surprisingly, some previous literature has not shown a decrease in male fertility at 27°C (Chakir et al., 2002), though *Drosophila* genetic background (Rohmer et al., 2004; Trotta et al., 2006) or *Wolbachia* infection status could potentially explain such phenotypic plasticity. Thus, complete rearing at 27°C causes some amount of heat stress that lowers reproductive output, but CI levels are still measurable in this context (Figure 3). As expected, the compatible rescue and single transgenic *cifA* and *cifB* crosses do not cause reduced hatch rates. Similar to the low temperature treatment, high temperatures weaken *w*Mel CI (mdn = 31.3%) in comparison to the complete CI maintained by transgenic expression of *cifAB* (Figure 3, Table S3, mdn = 0%, p = 0.0113). These results once again demonstrate that even when exposed to heat stress, transgenic *cifAB* expression persists in causing complete, rescuable CI, whereas wild-type CI is weakened.

DISCUSSION

With the discovery that two genes from prophage WO are responsible for causing and rescuing *w*Mel cytoplasmic incompatibility (CI) in *D. melanogaster* (LePage et al., 2017; Shropshire et al., 2018; Shropshire and Bordenstein, 2019), the question remains as to whether transgenic CI expression by *cifA* and *cifB* is robust across environmental conditions and age. We found that CI remained strong regardless of male age or temperature when *cifA* and *cifB* transgenes were dually expressed.

Wild-type, *w*Mel-induced CI decreases with male age (Shropshire et al., 2021). Our results indicate that *w*Mel and transgenic *cifA* and *cifB* expression decreased in parallel with male age to varying degrees in whole adults (Figure 1), in contrast to age-associated increases in *Wolbachia* density and *cif* gene expression reported in male testes (Shropshire et al., 2021). This difference could be because of sampling differences of RNA from whole bodies versus testes. Previous work in *D. simulans* found that decreasing CI with

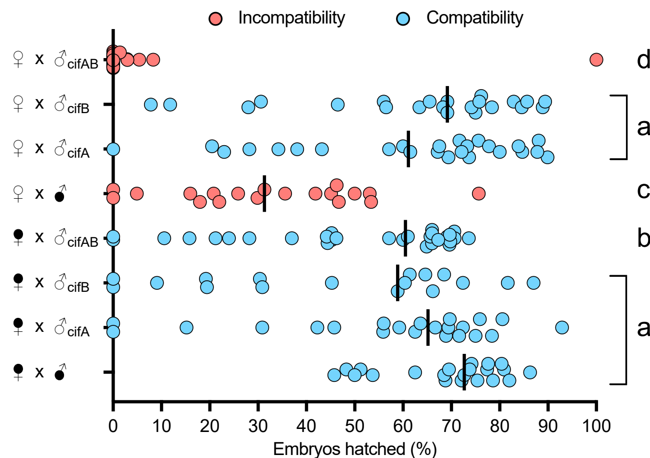

Figure 3. At 27°C, *Wolbachia*-induced CI is intermediate, whereas transgenic CI remains nearly complete

Male and female flies were constantly exposed to 27°C to test the effects of high temperature on transgenic CI penetrance. Wild-type *Wolbachia* infection is denoted by a filled sex symbol and transgene expression in uninfected flies is indicated to the right of unfilled sex symbols. Medians are indicated by a vertical black line, and statistically significant differences between crosses are shown by letters to the right of the data points. These differences were determined by a Kruskal-Wallis test followed by Dunn's multiple comparison tests (Table S3; n = 17–30) comparing all hatch rates to that of the control wild-type rescue cross where both parents are infected. Average hatch percentage from the *cifAB* cross denoted by the letter b was significantly different from the wild-type rescue cross, though it was similar to that of the *cifB* cross that was not significantly different from the rescue cross (*cifB*: p = 0.0546; *cifAB*: p = 0.0357).

increasing age also correlated with lower *Wolbachia* in male testes (Awrahman et al., 2014); however, we did not directly monitor *Wolbachia* densities here, and the impacts of male age on *Wolbachia* in testes may be variable between *Wolbachia strains*. CI strength also varies greatly between hosts (Duron et al., 2007; Nasehi et al., 2021; Van Opijnen and Breeuwer, 1999), so future work will be necessary to determine whether transgenic *cif* expression causes highly penetrant and stable CI in other species, including mosquitoes.

High temperature can also result in the loss of *Wolbachia* infection over time in a variety of arthropod species, and density reductions often correlate with a loss of CI (Bordenstein and Bordenstein, 2011; Kyei-poku et al., 2003; Mouton et al., 2006; Van Opijnen and Breeuwer, 1999). Temperature-dependent CI has cautious implications for control strategies that release *Wolbachia*-infected mosquitoes to curb the spread of mosquito-vectored diseases in high or low temperature environments. Field studies have found that although *w*Mel *Wolbachia* infection frequencies and densities temporarily decrease in *Aedes aegypti* mosquito populations following a heatwave, infection frequencies recover to nearly 100% in more favorable temperatures (Ross et al., 2020). Heat stress also negatively impacts maternal transmission of *Wolbachia* infection to offspring in various insects (Kyei-poku et al., 2003; Ross et al., 2017), and decreases in *Wolbachia* infection rates within a population are typically seen after an increase in temperature (Bordenstein and Bordenstein, 2011; Ross et al., 2017, 2020). Although changes in *Wolbachia* density caused by temperature can vary between *Wolbachia* strains and hosts (Mouton et al., 2006; Ross et al., 2017; Ulrich et al., 2016; Van Opijnen and Breeuwer, 1999), CI strength generally decreases at extreme temperatures (Doremus et al., 2019; Nasehi et al., 2021).

Our results confirm that both high and low temperatures, as well as male aging, result in reduced CI for *Wolbachia*-infected males. Although cyclical heat exposure theoretically allows *Wolbachia* infection to bounce back when populations experience lower temperatures again, it is possible that very extreme temperatures could severely reduce or eradicate *Wolbachia* from a population entirely. For these reasons, transgenic expression of CI-inducing *cif* genes in mosquitoes offers an adjunct or alternative to express CI regardless of infection status in current control efforts. As such, arthropods that are transgenically engineered to express the *cif* genes could enable or reinforce population suppression programs, as transgenic *cifA* and *cifB* expression may induce nearly complete and rescuable CI regardless of male age or temperature. They may also be hypothetically used in population replacement control strategies if the *cif* transgenes can be linked to anti-pathogen transgenes. This remains to be modeled because CI and rescue

would be expressed from the nuclear or mitochondrial genome, instead of the *Wolbachia* genome. Moreover, naive hosts that are vectors of diseases but exhibit imperfect maternal transmission of transinfected *Wolbachia* could in theory be a useful target for transgene-based applications, as transgenic CI remains robust when *Wolbachia*-induced CI is absent.

Our results also reveal highly variable impacts of high temperatures on transgenic CI, including an interesting case in which dual and single transgene expression buffered the heat-induced sterility of male flies (Figures S3A and S3B). Although it is well documented that high temperature stress can induce sterility in male *D. melanogaster* (David et al., 2005; Rohmer et al., 2004; Vollmer et al., 2004), to our knowledge this is the first, tentative evidence that transgenic flies may have greater thermal fertility tolerance than wild-type flies. In a replicate experiment, transgenic expression of the *cif* genes and a control gene (WD0508) unrelated to CI also resulted in buffered susceptibility to heat-induced sterility, suggesting the gene products of the transgenic system itself (e.g., GAL4) or other genetic background effects may play a role in this phenotype (Figure S3). Future research should further investigate the effects of the transgenic system on this phenomenon.

In summary, this work shows the following three results: (1) High and low temperatures markedly weaken *Wolbachia* CI but not transgenic CI. (2) Increasing male age eliminates *Wolbachia*-induced CI in association with decreases in native *cifA* and *cifB* gene expression, whereas transgenic CI is strong but slightly weakened. (3) High temperatures sterilize wild-type flies, and transgenic expression or genetic background effects may partially buffer the heat-induced sterility. Future research may focus on the cellular mechanisms and applications of sustained CI levels in transgenic arthropods.

Limitations of the study

A major limitation of this study is the strict laboratory setting of the work. Extensive research has shown that laboratory results are not necessarily consistent with field observations, particularly for transgenic organisms that may suffer from fitness costs in the wild. For this reason, future research should be conducted to test how robust transgenic CI remains in the field. Furthermore, our study was conducted in *w*Mel's native host, *D. melanogaster*. Because of the variability of host-*Wolbachia* interactions depending on both bacterial strain and host species, we cannot assert that similar CI will be observed in *A. aegypti*, or other mosquito species, transgenically expressing *w*Mel *cifA* and *cifB*.

DATA AVAILABILITY

All the raw data related to main and supplementary figures can be found in Supplementary data file.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.isci.2022.105327.

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AUTHOR CONTRIBUTIONS

Conceptualization, I.T.R., B.A.L., and S.R.B.; Methodology, I.T.R., B.A.L., and S.R.B.; Validation, I.T.R.; Investigation, I.T.R., R.K., and K.N.; Resources, S.R.B.; Data Curation, I.T.R.; Writing – Original Draft, I.T.R. and S.R.B.; Writing – Review and Editing, I.T.R., K.N., B.A.L., R.K., and S.R.B; Visualization, I.T.R. and S.R.B.; Supervision, S.R.B.

DECLARATION OF INTERESTS

S.R.B. is listed on pending patents relevant to this work.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Biological samples		
Whole male *D. melanogaster*	Bloomington Drosophila Stock Center	Experimental animals euthanized for this study
Chemicals, peptides, and recombinant proteins		
TriReagent	Sigma-Aldrich	Cat#: 93289
iTaq Universal SYBR Green Supermix	Bio-Rad	Cat#: 1725124
Critical commercial assays		
Direct-zol MiniPrep Kit	Zymo Research	Cat#: R2050
DNA-Free DNase Treatment and Removal Kit	Invitrogen	Cat#: AM1906
Superscript VILO cDNA Synthesis Kit	Invitrogen	
Deposited data		
Raw and analyzed data	This study	Supplementary Data File
Experimental models: Organisms/strains		
D. melanogaster strain y^1w^*	BDSC	1495
D. melanogaster strain *nos-Gal4;VP16*	BDSC	4937
D. melanogaster transgenic strain singly expressing *cifA*	Developed in (LePage et al., 2017)	https://doi.org/10.1038/nature21391
D. melanogaster transgenic strain singly expressing *cifB*	Developed in (LePage et al., 2017)	https://doi.org/10.1038/nature21391
D. melanogaster transgenic strain dually expressing *cifAB*	Developed in (LePage et al., 2017)	https://doi.org/10.1038/nature21391
D. melanogaster transgenic strain singly expressing *WD0508*	Developed in (LePage et al., 2017)	https://doi.org/10.1038/nature21391
Software and algorithms		
Prism 8	GraphPad	https://www.graphpad.com/
Affinity Designer 1.7	Serif Europe, Nottingham, UK	https://affinity.serif.com/en-us/

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources can be directed to and will be fulfilled by the lead contact, Seth Bordenstein (s.bordenstein@vanderbilt.edu).

Materials availability

This study did not generate any new unique reagents.

Data and code availability

All data used in this study can be found in the supplementary data table files. No new code was generated for this study.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Fly rearing

D. melanogaster flies were maintained in standard food media containing cornmeal, yeast, molasses, tegosept, and propionic acid and incubated at 25°C and 70% RH on a 12-h light/dark cycle following

protocols previously established (LePage et al., 2017), except for experiments wherein the effects of temperature variation were tested. *w*Mel-infected and uninfected variants of y^1w^* (BDSC 1495) were used whereby uninfected lines were generated through three generations of tetracycline treatment as previously described (LePage et al., 2017). Transgenic male flies were created by crossing *nos-Gal4;VP16* (BDSC 4937) driver females with males from established UAS-transgenic *cifA*, *cifB*, *cifAB*, and WD0508 lines; the *nos-GAL4;VP16* driver was used because it has been previously shown to induce *cif* gene expression to cause CI and rescue (Shropshire and Bordenstein, 2019).

CI measurement assays

Since wildtype and transgenic CI results in embryonic lethality, assays rely on measuring the embryo-to-larval hatch rates conducted following protocols previously established (LePage et al., 2017; Layton et al., 2019). To control for the paternal grandmother effect, virgin wildtype *w*Mel-infected females were aged at room temperature for 6–8 days prior to mating to ensure infected grandmothers passed sufficient *Wolbachia* loads to their male and female offspring (Layton et al., 2019). To control for the younger brother effect, virgin males were collected within the first 24 h of emergence and mated in that time period (Yamada et al., 2007), except in the experiments where male age was tested. Virgin female-male pairs were added to 8oz round bottom bottle and topped with a grape juice-agar plate smeared with a small amount of yeast. Grape-juice agar plates were made by first autoclaving a mixture of 12.5 g of agar and 350 mL of de-ionized water. 0.25 g tegosept (methyl 4-hyrdoxybenzoate) dissolved in 10 mL of ethanol and 150 mL of Welch's grape-juice was then added to the autoclaved agar mixture and poured into lids from 35 × 10-mm culture dishes (CytoOne). Bottles were placed in an incubator overnight to allow flies to mate, after which plates were removed (allowing 18-24 h to mate) and replaced with fresh plates. After giving flies 24 h to lay eggs on these plates, they were removed, and initial counts of the total number of eggs laid were conducted. After another 24 h, a final count was conducted to determine the number of hatched eggs. The percentage of embryos that hatched was calculated by dividing the number of hatched embryos by the total number of embryos and multiplying by 100. While plates with fewer than 25 embryos were excluded from analyses in previous studies (LePage et al., 2017), we lowered this threshold to 20 to increase sample size, except for in high temperature experiments (e.g., temperatures above 25°C). In high temperature experiments, the threshold was lowered to include all replicates with 10 or more eggs, to account for decreased egg laying presumably caused by heat stress. Siblings of males were collected, and flash frozen on the day the experiment was set up to be tested for gene expression.

Male age experiments

To test the effects of male age on CI, paternal grandparent crosses were set up in two-week intervals to ensure males could be collected every two weeks (Layton et al., 2019). Virgin males were collected, housed in *Drosophila* vials containing a standard yeast-cornmeal food, and incubated at room temperature. Males were aged 13–15 days (14-day treatment), 6–8 days (7-day treatment), and 0–1 day (1-day treatment). Females mated to each male age group were aged 6–8 days at room temperature.

Temperature experiments

To test the effects of temperature on CI, grandparents of experimental males and females were placed in bottles containing standard yeast-cornmeal food and allowed 4 days to mate and lay eggs at 25°C before being removed, after which bottles were moved to their experimental temperature treatment (18°C or 27°C) to raise the offspring at indicated temperatures. Collected virgin flies were housed at their treatment temperature. Hatch rates were conducted entirely at treatment temperatures, and grape plates were housed at experimental temperatures between the first and second counts.

To test the effect of high temperature (29°C) on transgenic CI, only male flies were exposed to 29°C during their development. We had previously reared both parents and conducted the hatch rate at this temperature, which resulted in egg inviability across all genotypes (see Figure S2); this is consistent with existing literature, which showed that *Drosophila* fertility decreases at 28°C (Clancy and Hoffmann, 1998), and that successive generations can only survive between 13 and 29°C (Cohet, 1973; David et al., 1971; Rohmer et al., 2004). Temperature-induced infertility is often seen in male *Drosophila*, and previous research has shown that heat tolerance is linked to the Y chromosome (David et al., 2005; Rohmer et al., 2004). This led us to test the effects of temporary 29°C exposure on *cif*-induced CI, which may occur in the field when insects experience cyclical heat stress (Rohmer et al., 2004; Ross et al., 2017, 2020; Young and Plough, 1926). Furthermore, it has also been shown that heat-induced sterility in males can be reversed after they

return to favorable temperature (Clancy and Hoffmann, 1998; Rohmer et al., 2004; Vollmer et al., 2004; Young and Plough, 1926). To test this, paternal grandparents were allowed 4 days to lay eggs in bottles housed at 25°C to ensure mating and egg laying behavior were not impacted, after which adult flies were removed, and bottles were moved to 29°C while offspring developed. Virgin males were collected upon emergence, after which they were housed at 29°C until the beginning of the experiment. Females were reared at the standard 25°C, and the remainder of the hatch rate was conducted entirely at 25°C. Hatch rates conducted with males and females both at 18°C, 27°C, and 29°C were not repeated, with the latter temperature producing nearly complete sterility. Hatch rates in which fathers alone were exposed to 29°C (the rest of the hatch rate was conducted at 25°C) were repeated three times, though they were conducted during winter months, where we have consistently seen lower levels of hatching.

METHOD DETAILS

RNA isolation and gene expression

To test the effects of male age on *cif* gene expression, siblings of males used in hatch rates were collected and frozen at −80°C. RNA was extracted from individual, whole flies using Direct-zol MiniPrep kit (Zymo Research) and treated with DNA-Free DNase Treatment and Removal Kit (Invitrogen). Steps involving Trizol were conducted in a chemical fume hood. RNA was then converted to cDNA using the Superscript VILO cDNA Synthesis Kit (Invitrogen) and RT-qPCR was conducted using iTaq Universal SYBR Green Supermix (Bio-Rad). Primers were used as listed in previous literature (LePage et al., 2017; Shropshire et al., 2018), and the conditions were as follows: 95°C 5 min, 40x (95°C 10s, 59°C 1 min), melt curve from 35°C to 95°C, increment 0.5°C. Variation in gene expression across ages was quantified using $2^{-\Delta ct}$ (the difference in ct values between *rp49* and *cif* gene expression levels).

QUANTIFICATION AND STATISTICAL ANALYSIS

Statistical analyses were conducted using GraphPad Prism 8. Kruskal-Wallace tests followed by a Dunnette's (aka, Dunn's) multiple correction test was performed to determine statistical differences between genotypes and testing the effect of male age within the same genotype.

Figures were made using GraphPad Prism 8 and further edited for aesthetics in Affinity Designer 1.7 (Serif Europe, Nottingham, UK). P-values can be found in the Supplementary Tables file, and all the raw data can be found in the Supplementary Data file.