# Symbiont-mediated cytoplasmic incompatibility:
# What have we learned in 50 years?

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**Keywords:** cytoplasmic incompatibility, *Wolbachia*, prophage WO, CifA and CifB, symbiosis

28 **Abstract**

29  Cytoplasmic incompatibility (CI) is the most common symbiont-induced reproductive
30  manipulation. Specifically, symbiont-induced sperm modifications cause catastrophic mitotic
31  defects in the fertilized embryo and ensuing lethality in crosses between symbiotic males and either
32  aposymbiotic females or females harboring a different symbiont strain. However, if the female
33  carries the same symbiont strain, then embryos develop properly, which imparts a relative fitness
34  benefit to symbiont-transmitting mothers. Thus, CI drives maternally transmitted bacteria to high
35  frequencies in arthropod species worldwide. In the past two decades, CI has experienced a boom
36  in interest due in part to its (i) deployment in successful, worldwide efforts to reduce the spread of
37  mosquito-borne diseases, (ii) causation by bacteriophage genes, *cifA* and *cifB*, that modify animal
38  reproductive processes, and (iii) important impacts on incipient speciation. This review serves as
39  a gateway to experimental, conceptual, and quantitative themes of CI and outlines significant gaps
40  in our understanding of CI's mechanism that are ripe for investigation from a diversity of
41  subdisciplines in the life sciences.

## Introduction

From 1938 through the 1960s, an enigmatic, intraspecific incompatibility that caused embryonic death was reported between geographically isolated strains of *Culex pipiens* mosquitoes (Laven, 1951; Marshall, 1938), *Aedes scutellaris* mosquitoes (Smith-White and Woodhill, 1955), and *Nasonia vitripennis* parasitoid wasps (Ryan and Saul, 1968). Crossing experiments in both *Culex* and *Nasonia* surprisingly revealed that the incompatibility was caused by a maternally inherited cytoplasmic factor (Laven, 1951; Ryan and Saul, 1968). This cytoplasmic incompatibility (CI) manifested as embryonic death when males carried the factor, but it was rescued if the female was from the same maternal lineage (Fig. 1A). Intriguingly, *Cu. pipiens* (Laven, 1951) and *N. vitripennis* (Ryan and Saul, 1968) that had this cytoplasmic factor were either compatible, unidirectionally incompatible (Fig. 1A, B), or bidirectionally incompatible (Fig. 1C) with strains of different geographic origin. The underlying cause of these incompatibilities would remain a mystery for several decades.
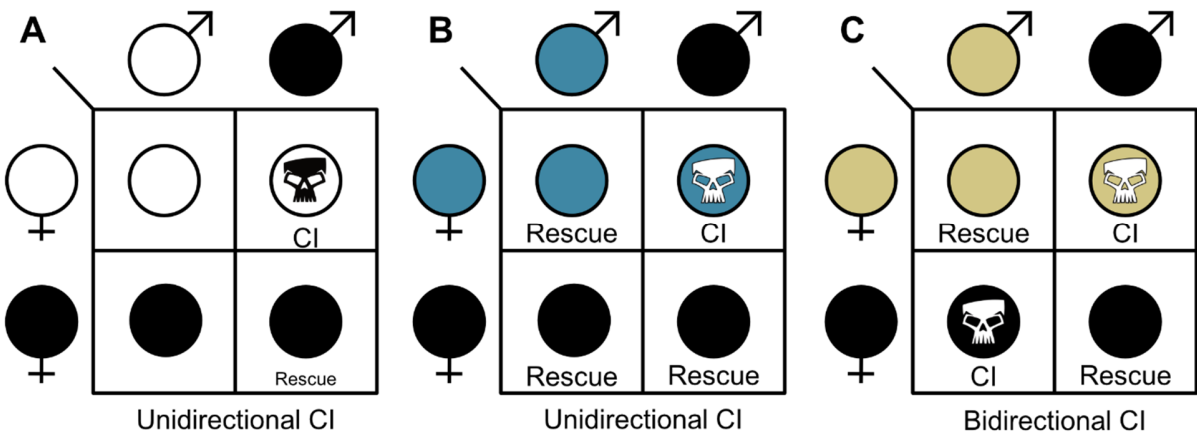
**Figure 1. The three CI crossing relationships.** (A) Unidirectional CI results in embryonic lethality when symbiont-containing males are crossed with aposymbiotic females. Rescue of this embryonic lethality occurs if the female carries a compatible symbiont strain. (B) In some cases, unidirectional CI can emerge when one strain can rescue another strain, but the other strain does not reciprocate the rescue. (C) Bidirectional CI occurs when incompatible strains are present in a population. Rescue occurs if the female likewise harbors the same strain. Filled sex symbols indicate symbiotic hosts. Different colors represent different symbiont strains. Skull symbols represent embryonic death.

Motivated by the finding that Typhus is a Rickettsial disease (Rocha-Lima, 1916), microbiologists Hertig and Wolbach conducted a survey of *Rickettsia*-like bacteria among numerous arthropod orders in and around Boston, Massachusetts in 1924 (Hertig and Wolbach, 1924). The bacteria were classified as *Rickettsia*-like based on size (Rickettsiae tend to be smaller than other bacteria), shape (cocciform or rod), Gram staining (gram-negative), and a Giemsa nucleotide stain (to separate microscopy artifacts from cells with DNA). In the *Cu. pipiens* mosquito, they found tiny rod-like or coccoid, gram-negative, Rickettsial bacteria residing within male and female reproductive cells (Hertig and Wolbach, 1924). The bacteria were absent in

73    various other tissues including oesophageal diverticula, gut tissues, malphighian tubes, fat-body,
74    heart and pericardial cells, salivary glands, and accessory reproductive organs (Hertig and
75    Wolbach, 1924). Intriguingly, when *Cu. pipiens* with this bacteria were reared in the lab, offspring
76    also harbored them in their reproductive tissues as early larva, suggesting that this bacteria was
77    maternally inherited (Hertig and Wolbach, 1924). These bacteria would later be named *Wolbachia*
78    *pipientis* by Dr. Marshall Hertig: *Wolbachia* for Dr. Simeon Burt Wolbach, Hertig's PhD advisor,
79    and *pipientis* for the mosquito it was discovered in (Hertig, 1936). In this review, we will refer to
80    the symbiont as *Wolbachia* since it currently remains a genus of only one recognized species.

81         In 1971, Yen and Barr investigated the effects of CI on embryonic development and
82    discovered a Rickettsia-like bacteria matching the description of *Wolbachia* in the eggs of
83    symbiont-bearing *Cu. pipiens* females (Yen and Barr, 1971). This finding led them to the
84    breakthrough hypothesis that CI is caused by this long-overlooked bacteria (Yen and Barr, 1971).
85    They later tested this hypothesis using crosses with antibiotic-treated and untreated *Cu. pipiens*
86    mosquitoes to reveal that CI is a symbiont derived phenotype caused by *Wolbachia* (Yen and Barr,
87    1973), thus substantiating *Wolbachia* as the etiological agent of CI phenotypes. This initial
88    characterization of *Wolbachia* and CI in *Cu. pipiens* opened the floodgates, with many ensuing
89    studies reporting cases of CI-inducing *Wolbachia* in Diptera (Baton et al., 2013; Bian et al., 2013;
90    Hoffmann et al., 1986; Riegler and Stauffer, 2002), Hymenoptera (Betelman et al., 2017; Dittmer
91    et al., 2016), Coleoptera (Kajtoch and Kotásková, 2018), Hemiptera (Ju et al., 2017; Ramírez-
92    Puebla et al., 2016), Orthoptera (Martinez-Rodriguez and Bella, 2018), Lepidoptera (Arai et al.,
93    2018; Hornett et al., 2008), Thysanoptera (Nguyen et al., 2017), Acari (Gotoh et al., 2007, 2003;
94    Vala et al., 2002), Isopoda (Cordaux et al., 2012; Sicard et al., 2014), and Arachnids (Curry et al.,
95    2015).

96         Among these orders, *Wolbachia* are highly diverse and phylogenetically divided into 17
97    "supergroups" (denoted A-S, excluding G and R), and CI-inducing *Wolbachia* are so far restricted
98    to supergroups A and B (Lefoulon et al., 2020; Lo et al., 2007a, 2007b; Wang et al., 2016).
99    However, despite the considerable diversity between *Wolbachia* strains, the most studied models
100   for CI are the *Wolbachia* of *Culex* (*w*Pip), *Drosophila* (*w*Ri and *w*Mel), *Nasonia* (*w*VitA and
101   *w*VitB), and *Laodelphax* (*w*Str). Aside from *Wolbachia*, the far less common (Weinert et al., 2015;
102   Zchori-Fein and Perlman, 2004) Bacteroidetes bacteria *Cardinium* were found to cause CI nearly
103   three decades later (Hunter et al., 2003; Yen and Barr, 1973). Additionally, unknown symbionts
104   of *Brontispa longissimi* coconut beetles and *Lariophagus distinguendus* parasitoid wasps cause
105   CI, but they are not *Wolbachia* or *Cardinium* (König et al., 2019; Takano et al., 2017). In addition,
106   *Rickettsiella* Gammaproteobacteria cause CI in *Mermessus fradeorum* spiders (Rosenwald et al.,
107   2020). This review will focus primarily on *Wolbachia*-induced CI, but other symbionts will be
108   discussed when information is available.

109         CI has attracted considerable applied interest in the last decade from scientists, companies,
110   and governments because it is at forefront of efforts to reduce the spread of dengue, Zika, and other
111   arboviral infections (Caragata et al., 2016; Crawford et al., 2020; Ford et al., 2019; Hoffmann et
112   al., 2011; O'Connor et al., 2012; O'Neill, 2018; Rasgon, 2007, 2008; Teixeira et al., 2008; WHO,

113  2016; Xi et al., 2005). Two CI-based vector control strategies are deployed worldwide. First, the
114  incompatible insect technique (IIT), also known as population suppression, aims to reduce the
115  population size of disease vectors through release of CI-inducing male insects (Fig. 2A) (Ant et
116  al., 2020; Caputo et al., 2019; Chambers et al., 2011; Crawford et al., 2020; Debug Fresno, 2018;
117  Kyritsis et al., 2019; Laven, 1967; Mains et al., 2016, 2019; O'Connor et al., 2012; Puggioli et al.,
118  2016; X. Zheng et al., 2019). Conversely, the population replacement strategy (PRS) does not
119  reduce population sizes, but instead aims to convert a native population that transmits arboviruses
120  to humans with one that has reduced vectoral capacity (Fig. 2B) (Caragata et al., 2016; Hoffmann
121  et al., 2011; Moreira et al., 2009; O'Neill, 2018; Tantowijoyo et al., 2020; Teixeira et al., 2008;
122  van den Hurk et al., 2012). PRS uses two characteristics of CI-*Wolbachia*: the ability to rapidly
123  spread through populations using CI and the ability of some strains to inhibit replication of
124  arboviruses including dengue, Zika, chikungunya, and yellow fever (Caragata et al., 2016; Moreira
125  et al., 2009; Teixeira et al., 2008; van den Hurk et al., 2012). When male and female mosquitoes
126  bearing pathogen blocking *Wolbachia* are released to sufficiently high frequencies, CI will drive
127  them to frequencies approaching fixation and significantly alleviate the transmission of disease in
128  the region. Both methods have been widely successful in their respective approaches (Crawford et
129  al., 2020; Debug Fresno, 2018; O'Neill et al., 2018; Tantowijoyo et al., 2020).

130        In addition to combatting arthropod-borne diseases, CI has attracted interest from basic
131  scientists because it can cause reproductive isolation and thus be a contributor to speciation (Fig.
132  2C). The Biological Species Concept defines groups of individuals as different species if they
133  cannot interbreed (Dobzhansky, 1937; Mayr, 1963), and species are anticipated to emerge when
134  reproductive isolation between two populations prevents gene flow (Coyne, 2001). Bidirectional
135  and unidirectional CI can both reproductively isolate populations with different infection states,
136  but to different degrees (Brucker and Bordenstein, 2012). Since bidirectional CI restricts gene flow
137  in both cross directions, it can strongly reproductively isolate populations that harbor incompatible
138  symbionts. This is indeed the case between *Nasonia* parasitoid wasps that diverged between ~0.25
139  and 1 million years ago (Bordenstein et al., 2001; Breeuwer and Werren, 1990). Alternatively,
140  unidirectional CI restricts gene flow in one direction and does not appear to contribute to speciation
141  in some host-*Wolbachia* symbioses such as in the *D. yakuba* clade (Cooper et al., 2017). However,
142  North American populations of male symbiont-bearing *D. recens* cause unidirectional CI when
143  mated with aposymbiotic *D. subquinaria* (Jaenike et al., 2006; Shoemaker et al., 1999), reducing
144  gene flow between populations. Intriguingly, *Cardinium* yields similar patterns of asymmetric
145  unidirectional CI between lab populations of *Encarsia suzannae* and its sister species *E. gennaroi*
146  (Gebiola et al., 2017). Together, these studies suggest a role for symbiont-induced CI in
147  reproductive isolation and incipient speciation.

148        In this review, we comprehensively synthesize the CI literature by discussing the impacts
149  of CI research on basic biology and human health, including rapid advances in understanding CI's
150  genetic basis, biochemical properties associated with CI, CI-associated abnormalities, CI strength
151  variation, and host factors that correlate with CI expression. The reviewed works provide a
152  concrete foundation for new testable and robust models, hypotheses, and evidence. Thus, we end

153  with a description of the field's models to explain the mechanistic underpinnings of CI, and place
154  them into the framework of current literature. Under these models, we identify key predictions and
155  questions that motivate future areas of research to continue to build textbook knowledge on one of
156  the most widespread selfish adaptations of symbionts.
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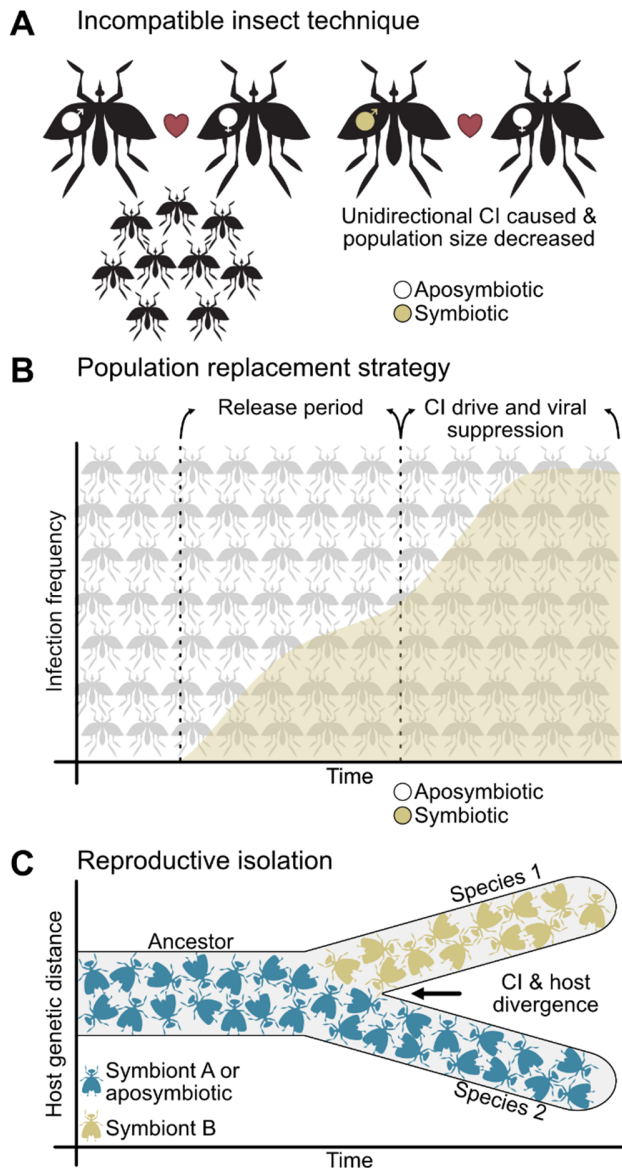
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159  **Figure 2. CI is important to vector control and reproductive isolation between species.** (A) The incompatible
160  insect technique is used to reduce population sizes (Crawford et al., 2020; Laven, 1967). Typically, two aposymbiotic
161  individuals will mate and produce viable offspring (left), but if symbiont-bearing males are released into the population
162  then they will cause unidirectional CI when they mate with aposymbiotic females (right) or bidirectional CI when they
163  mate with females harboring incompatible symbionts (not shown). This yields a reduction in egg hatching and
164  reduction in population size. (B) The population replacement strategy involves the release of both males and females
165  bearing CI-inducing and pathogen blocking symbionts (Hoffmann et al., 2011; O'Neill, 2018). After a period of
166  releases, CI will spread the symbiont to high frequencies where it can block the replication of human diseases. (C) CI-
167  inducing symbionts can cause reproductive isolation through bidirectional or unidirectional CI when different

168  individuals, populations, or species have different incompatible symbiont states (Bordenstein et al., 2001; Breeuwer
169  and Werren, 1990; Gebiola et al., 2017; Jaenike et al., 2006). This reproductive barrier reduces gene flow between
170  hosts with different symbiont states, allowing for their divergence.

171

## What is the genetic basis of CI?

### *Identifying the CI and rescue genes*

174  Until the past decade, the genetic basis of CI has remained elusive. The intangibility of the
175  CI genes was due in no small part to the inability to genetically engineer symbionts such as
176  *Wolbachia* (Iturbe-Ormaetxe et al., 2007; Thiem, 2014). Progress in CI genetics became possible
177  with the genomic sequencing of the *w*Mel *Wolbachia* of *D. melanogaster* in 2004 (Wu et al., 2004).
178  *w*Mel has a streamlined genome with numerous mobile elements including phages (Wu et al.,
179  2004). Notable among these elements was *Wolbachia*'s phage WO, which was first identified in
180  1978 via transmission electron microcopy of viral-like particles in symbiont-bearing *Cu. pipiens*
181  (Wright et al., 1978). Prophage WO encodes a set of proteins termed the Eukaryotic Association
182  Module that share homology to eukaryotic proteins, perhaps due to lateral gene transfer from
183  eukaryotes to the phage, and are predicted to interact with eukaryotic processes (Bordenstein and
184  Bordenstein, 2016). *w*Mel's genome is also enriched with ankyrin proteins that are involved in
185  protein-protein interactions in eukaryotes and are relatively rare in free living bacteria as compared
186  to intracellular bacteria and eukaryotes (Al-Khodor et al., 2010; Jernigan and Bordenstein, 2014).
187  Conversely, genomic sequencing of the mutualistic *w*Bm *Wolbachia* of *Brugia malayia* nematodes
188  revealed it did not contain phage WO nor an enrichment of ankyrins (Foster et al., 2005). These
189  findings suggested a correlation between reproductive parasitism and the presence of phage WO
190  and/or ankyrin genes and motivated hypotheses that they may be involved in CI (Foster et al.,
191  2005; Wu et al., 2004; Yamada et al., 2011).

192  The first attempt to functionally dissect CI's genetic basis generated a list of 12 gene
193  candidates in the *w*Mel genome based on putative host interaction: nine ankyrin genes (WD0294,
194  WD0385, WD0498, WD0514, WD0550, WD0633, WD0636, WD0754, and WD0776), two
195  virulence-related genes (WD0579 and WD0580), and one phage-associated methylase gene
196  (WD0594) (Yamada et al., 2011). Since *Wolbachia* are not genetically tractable (Iturbe-Ormaetxe
197  et al., 2007; Thiem, 2014), *D. melanogaster* transgenic tools were used to test these gene
198  candidates (Duffy, 2002). However, transgenic expression of these genes in aposymbiotic male
199  flies revealed that none recapitulated CI (Yamada et al., 2011). Moreover, neither transcriptional
200  nor genetic variation of *Wolbachia*'s ankyrin genes correlated with a strain's ability to induce CI
201  (Olivier Duron et al., 2007; Papafotiou et al., 2011).

202  Additional 'omic studies would pave the way for identification of new gene candidates.
203  First, mass spectrometry and SDS-page analyses of spermatheca (the female sperm storage organ)
204  extracts from symbiont-bearing *Cu. pipiens* females revealed the prophage WO protein WPIP0282
205  (Beckmann and Fallon, 2013), thus elevating a new candidate for CI and/or rescue and providing
206  additional support to hypotheses that phage WO may contribute to CI. Second, genomic
207  comparisons of the *w*Mel genome against the genome of the non-parasitic *w*Au strain of *D.*

208  *simulans* revealed nine genes absent in the non-parasitic strain that were present in *w*Mel (Sutton
209  et al., 2014). These candidates included numerous genes from *Wolbachia*'s prophage WO
210  including WD0631 which is a *w*Mel homolog of *w*Pip's WPIP0282, WD0632 which is adjacent
211  to WD0631, and a set of transcriptional regulators (Sutton et al., 2014). Thus, evidence continued
212  to build around phage WO genes as CI factors. However, PCR-based assays failed to identify the
213  minor capsid Orf7 of prophage WO in the CI-inducing *w*Rec of *D. recens,* initially lending doubt
214  to the phage WO hypothesis. Though, sequencing of the *w*Rec genome revealed a highly reduced
215  prophage WO with approximately one-quarter the number of genes in a close relative (Metcalf et
216  al., 2014). These gene contained several previously described candidates including the *w*Rec
217  homolog of WPIP0282, four transcriptional regulators also absent in *w*Au, and a *Wolbachia*
218  transcriptional regulator gene *wtrM* linked to regulation of host meiosis in *Cu. molestus*
219  (Beckmann and Fallon, 2013; Pinto et al., 2013; Sutton et al., 2014).

220      The reduced *w*Rec genome would later form the basis of an unbiased, comparative 'omic
221  study assessing the genomes of CI-inducing *Wolbachia*, a genome of a non-parasitic strain and a
222  transcriptome and proteome of *Wolbachia*-carrying ovaries (LePage et al., 2017). This analysis
223  revealed only two phage WO genes in the Eukaryotic Association Module, WD0631 and the
224  adjacent WD0632, as CI candidate genes in the *w*Mel strain of *D. melanogaster* (LePage et al.,
225  2017). These genes would also later be determined to be absent in the parthenogenesis-inducing
226  *w*Tpre strain of *Trichogramma* wasps (Lindsey et al., 2018). WD0631 and WD0632 were named
227  *cytoplasmic incompatibility factors A and B* (*cifA* and *cifB*) respectively (LePage et al., 2017). The
228  gene is referred to in lowercase and italics (*cifA* and *cifB*), the protein is referred to in uppercase
229  with no italics (CifA and CifB), and the strain the specific *cif* gene comes from can be defined with
230  the strain name as a subscript ($cif_{wMel}$ or $cif_{wPip}$). This gene nomenclature is consistent with
231  guidelines from the American Society for Microbiology's Journal of Bacteriology ("Instructions
232  to Authors: The Journal of Bacteriology, Nomenclature," n.d.).

233      With independent 'omic identification of *cifA* and *cifB* as candidates for CI (Beckmann and
234  Fallon, 2013; LePage et al., 2017; Sutton et al., 2014), two studies simultaneously explored the
235  relationship between $cif_{wMel}$ (LePage et al., 2017) and $cif_{wPip}$ (Beckmann et al., 2017) genes and CI
236  using transgenic expression systems in *D. melanogaster*. Singly expressing $cifA_{wMel}$ or $cifB_{wMel}$ in
237  aposymbiotic *D. melanogaster* males failed to induce CI, but dual expression of the genes caused
238  rescuable CI-like hatch rates and cytological embryonic defects (LePage et al., 2017), suggesting
239  that the $cif_{wMel}$ genes cause CI only when expressed together. Similar results were reported when
240  $cifA_{wPip}$ and $cifB_{wPip}$ were dually expressed in aposymbiotic *D. melanogaster* males, but rescue was
241  not achieved (Beckmann et al., 2017), indicating that some biological or technical limitation of the
242  system may have inhibited the ability to rescue transgenic $cif_{wPip}$ CI in a heterologous expression
243  system. Later, similar transgenic experiments revealed that $cifA_{wMel}$ expression in aposymbiotic *D.*
244  *melanogaster* females can rescue CI (Shropshire et al., 2018), motivating a Two-by-One genetic
245  model of CI wherein *cifA* and *cifB* cause CI unless *cifA* is expressed in the ovaries or embryo to
246  rescue it (Fig. 3A). This model was further supported through transgenic expression of $cifA_{wMel}$
247  and $cifB_{wMel}$ in aposymbiotic males to induce transgenic CI, and through crossing them to $cifA_{wMel}$

248    expressing aposymbiotic females to show that transgenic CI can be rescued at levels comparable
249    to a symbiont-bearing female (Shropshire and Bordenstein, 2019).

250        Notably, while the Two-by-One genetic model is most consistent with transgenic
251    expression studies in *D. melanogaster* that achieve rescuable CI (Beckmann et al., 2017; Chen et
252    al., 2019; LePage et al., 2017; Shropshire et al., 2018; Shropshire and Bordenstein, 2019),
253    transgenic expression of $cifB_{wMel}$ and $cifB_{wPip}$ in yeast can cause temperature sensitive lethality that
254    can be inhibited by co-expression with cognate *cifA* (Beckmann et al., 2017). Though, since yeast
255    do not have sperm or eggs, which are the targets of CI, the relevance of phenotypes observed in a
256    heterologous yeast expression system need to be replicated in insect models. However, a divergent
257    *cifB* gene variant from *w*Pip can weakly reduce embryonic hatching when crossed to aposymbiotic
258    females (Chen et al., 2019), but it unknown if this lethality can be rescued. These data lend support
259    to the possibility that some strains may employ a model of CI wherein *cifB* is the CI-causing factor
260    and *cifA* is only the rescue factor. However, interpretation of these results is significantly
261    complicated by the absence of rescue data for any *cifB*-associated reduction in embryonic hatching
262    in insects. Moreover, mutagenesis analyses, described in further detail below, indicate that
263    changing conserved residues across CifA can crucially prevent CI-induction, lending additional
264    support for CifA's important role as a CI-inducing factor (Shropshire et al., 2020). More functional
265    genetic analyses will be necessary to provide evidence for an alternative to the Two-by-One
266    genetic model of CI. Hereafter, we will discuss relevant phenomena in the context of a Two-by-
267    One genetic model.

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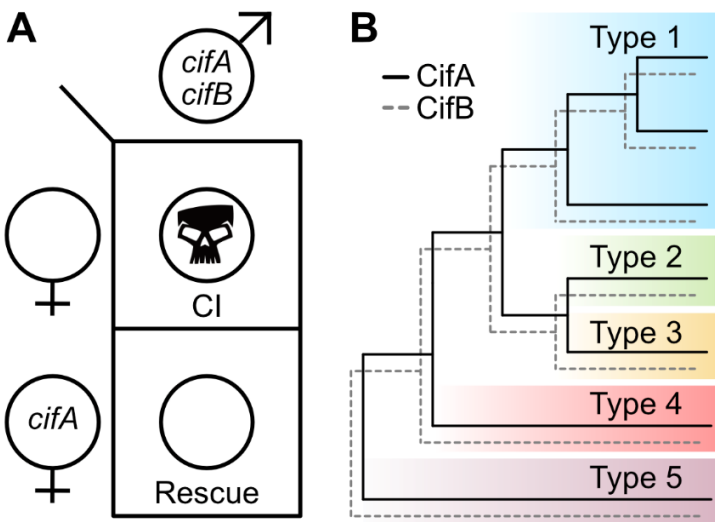
271    **Figure 3. Two-by-One genetic model of Cif-induced CI and Cif phylogeny.** (A) The Two-by-One genetic model
272    of CI surmises that both CifA and CifB must be expressed in males to cause CI, and CifA must be expressed in females
273    to rescue CI (Shropshire and Bordenstein, 2019). (B) CifA and CifB coevolve and are classified into at least five
274    different phylogenetic Types (1-5) (Bing et al., 2019; LePage et al., 2017; Lindsey et al., 2018).

275

*Phylogenetics of the cifA and cifB genes that cause and rescue CI*

277     Initial comparative sequence analysis of Cif proteins revealed that CifA and CifB have
278 concordant phylogenies with considerable divergence across three distinct phylogenetic clades
279 (LePage et al., 2017). Since then, the availability of additional genomes and Cif sequences have
280 exposed at least five clades referred to as Types 1-5 (Fig. 3B) (Bing et al., 2019; Lindsey et al.,
281 2018), and highly divergent Cif-like homologs in *Orientia* and *Rickettsia* bacteria (Gillespie et al.,
282 2018). It is likely that continued genomic sequencing will reveal additional Cif phylogenetic
283 Types. The *w*Mel Cif proteins belong to the Type 1 clade, and *w*Pip has both Type 1 and Type 4
284 Cif proteins. The phylogenetic classification of a *cif* gene can be indicated with a T# in brackets
285 to the right of the gene name (i.e., *cif*$_{wMel[T1]}$ or *cif*$_{wPip[T4]}$).
286     While the *cif* genes are associated with the Eukaryotic Association Module of prophage
287 WO or WO-like islands (Bordenstein and Bordenstein, 2016), Cif phylogeny is not concordant
288 with phage WO or *Wolbachia* phylogeny, potentially reflecting the typically high rates of inter-
289 and intragenic recombination in phage genomes (Bordenstein and Wernegreen, 2004; LePage et
290 al., 2017). Some *cif* genes are flanked by ISWpi1 transposons which may assist horizontal transfer
291 between WO-associated regions or *Wolbachia* strains, but it remains unclear if they alone are
292 responsible for divergence between the phylogeny of the *cif* genes, *Wolbachia*, and phage WO
293 (Cooper et al., 2019; Madhav et al., 2020). To date, only *cif* genes belonging to the Type 1 and 4
294 clades have been experimentally evaluated and confirmed to cause and rescue CI (Beckmann et
295 al., 2017; Chen et al., 2019; LePage et al., 2017; Shropshire et al., 2018; Shropshire and
296 Bordenstein, 2019). These studies suggest that despite considerable divergence in sequence,
297 proteins across the phylogenetic landscape of the Cifs remain capable of causing and rescuing CI.
298 Though, the phenotypic output of the Type 2, Type 3, Type 5, and the *Orientia*/*Rickettsia cif*-like
299 genes have not been experimentally assessed and it remains unknown if they can contribute to CI
300 phenotypes. However, the CI-inducing strains *w*No of *D. simulans* and *w*Stri of *La. striatellus* only
301 have Type 3 or Type 5 genes respectively (Bing et al., 2019; LePage et al., 2017), suggesting that
302 these genes may cause CI and rescue. Since CI has not been reported in *Orientia* or *Rickettsia*
303 species, it less likely these distant *cif*-like homologs contribute to CI (Gillespie et al., 2018).
304 Alternatively, other yet identified gene sets may be CI-capable in CI-causing symbionts. Indeed,
305 genomic and transcriptomic sequencing of CI-inducing *Cardinium* reveals that they do not carry
306 obvious homologs to the CI genes (Mann et al., 2017). More functional genetic studies will be
307 necessary to identify and assess the landscape of *cif*-like genes and to identify alternative CI genes.
308

*Relationships between* cif *sequence diversity and CI phenotypes*

310     There are a number of *Wolbachia* strains that have *cif* genes but do not cause CI. The most
311 common examples of these genotype-phenotype discrepancies are related to obvious disruptions
312 in *cif* gene sequence. For example, the *w*PanMK and *w*PanCI *Wolbachia* strains of *D. pandora*
313 cause male-killing and CI respectively, but the male-killing strain has an early stop codon in CifB

314    that putatively ablates function and may allow for the phenotypic switch from CI to male-killing
315    (Asselin et al., 2018). Additionally, the $w$Mau *Wolbachia* of *D. mauritiana* has Type 3 *cif* genes
316    (LePage et al., 2017; Meany et al., 2019), does not cause CI, but it can rescue CI caused by $w$No
317    (Bourtzis et al., 1998; Rousset and Solignac, 1995; Zabalou et al., 2008). $\text{CifA}_{w\text{Mau}[T3]}$ sequence is
318    highly similar to other CI and rescue-capable *Wolbachia*, but there is a frameshift mutation in
319    $\text{CifB}_{w\text{Mau}[T3]}$ that introduces over ten stop codons. Since CifB is involved only in CI, and CifA in
320    both CI and rescue, these data explain why $w$Mau can rescue but not cause CI (Meany et al., 2019).
321    These observations reveal multiple independent instances of Cif degradation and phenotypic loss,
322    but the pressures that drive these degradation events often remain unknown. Additional
323    comparative genomics coupled with CI measurements will help identify more examples of
324    degradation events, and theoretical analyses of the pressures on *cifA* and *cifB* gene evolution will
325    help explain the circumstances that drive CI loss.

326        While the above examples clearly highlight a relationship between *cif* sequence variation
327    and loss of CI, there are other strains that are more difficult to explain. For example, $w$Suz of *D.*
328    *suzukii* encodes both Type 1 and Type 2 *cif* genes that are highly similar to the strong CI-inducing
329    strain of $w$Ri, but $w$Suz does not cause CI (Cattel et al., 2018; Conner et al., 2017; Hamm et al.,
330    2014; Lindsey et al., 2018). While $\text{CifA}_{w\text{Suz}[T2]}$ has been disrupted by the insertion of a transposase,
331    the $\text{Cif}_{w\text{Suz}[T1]}$ gene pair remains intact and has only 2-4 amino acid substitutions relative to
332    $\text{Cif}_{w\text{Ri}[T1]}$ (Conner et al., 2017; Lindsey et al., 2018). In theory, the Type 1 gene set alone should be
333    CI-capable (Lindsey et al., 2018); though, $\text{Cif}_{w\text{Suz}[T1]}$ mutations may be in key residues for CI
334    expression. Notably, $w$Ri does not cause CI when transinfected into *D. suzukii* (Cattel et al., 2018).
335    Thus, the $\text{Cif}_{w\text{Suz}[T1]}$ proteins may be able to cause CI, but their effects are inhibited by suppressors
336    encoded in the *D. suzukii* genome. In a separate example, the triple-strain infection of $w$AlbA,
337    $w$AlbB, and $w$Mel in *A. albopictus* can cause CI but cannot self-rescue (Ant and Sinkins, 2018).
338    Since each of the individual *Wolbachia* strains in this triple-strain infection can cause CI and are
339    self-compatible in *A. aegypti* (Ant and Sinkins, 2018), neither genetic variation in the *cif* genes
340    alone nor host suppressors can explain the emergence of self-incompatibility. However, it is
341    plausible that some *Wolbachia* may inhibit the reproductive manipulations of other co-infecting
342    strains, but this requires further testing. In summary, the relationships between *Wolbachia* strains
343    and their hosts are likely to have an impact on CI. Additional work is necessary to answer these
344    persistent questions: how does superinfection impact CI expression, how does the host act to
345    suppress CI phenotypes, and what are the evolutionary dynamics that govern these interactions?
346

347    *The genetic basis of bidirectional CI remains unknown*

348        There is strong evidence for the genetic basis of unidirectional CI between *Wolbachia*-
349    bearing and aposymbiotic insects (Fig. 1A) (Beckmann et al., 2017; Chen et al., 2019; LePage et
350    al., 2017; Shropshire et al., 2018; Shropshire and Bordenstein, 2019). However, the genetic basis
351    of bidirectional CI between arthropods with different symbiont strains remains poorly understood
352    (Fig. 1B,C). Sequence divergence in CI-associated factors has long been thought to be a
353    contributing factor to these incompatibilities, namely that divergence in both CI-causing and

354    rescue-causing genes would be required for bidirectional CI to emerge (Charlat et al., 2001).
355    Indeed, phylogenetic analyses of *cif* genes reveal that strains carrying similar alleles tend to be
356    compatible, strains with more distantly related *cif* genes are not, and a single *Wolbachia* strain can
357    have multiple unique *cif* gene pairs (Bonneau et al., 2018a, 2019; LePage et al., 2017). For
358    instance, when *w*Mel is transinfected into a *D. simulans* background, it is unidirectionally
359    incompatible with the native *w*Ri strain, wherein *w*Ri can rescue *w*Mel-induced CI but the
360    reciprocal cross is incompatible (Poinsot et al., 1998). Intriguingly, *w*Ri carries Type 1 *cif* genes
361    closely related to *w*Mel's and a divergent Type 2 gene pair. Thus, it is plausible that *w*Ri can rescue
362    *w*Mel's CI because of CifA$_{w\text{Ri[T1]}}$ whereas *w*Mel cannot rescue *w*Ri's CI because it lacks a rescue
363    gene for the Type 2 gene pair (LePage et al., 2017). Additionally, population genetic analyses of
364    *cif* genes in *w*Pip reveal that there are numerous unique strains, each strain carries multiple closely
365    related *cif* variants that belong to Type 1 and Type 4 *cif* clades, and a single genetic variant of
366    CifB$_{w\text{Pip[T1]}}$ correlates with the inability of one strain of *w*Pip to rescue CI caused by a divergent
367    *w*Pip strain (Atyame et al., 2011b; Bonneau et al., 2018a, 2019). However, while these data suggest
368    that *cif* genetic variation and/or copy number contributes to strain incompatibility, it remains
369    possible that the considerable host genotypic variation between these incompatible populations
370    contributes to these relationships in a way that also correlates with *cif* genotypic diversity (Atyame
371    et al., 2011a). More reductionist functional studies that control for variation in host genotype will
372    be necessary to confirm that *cif* sequence variation alone can explain CI relationships.

373          Historically, CI and rescue were thought to be caused by different genes, and that
374    divergence in both genes would be required for bidirectional CI to evolve relative to an ancestral
375    strain (Charlat et al., 2001). Thus, this model for bidirectional CI requires two steps: one mutation
376    for CI and one for rescue. A major limitation of this model is that the intermediate state, wherein
377    only one of the two phenotypes have shifted, is self-incompatible and represents a 'maladaptive
378    valley' unlikely to persist as a rare variant. Given the abundance of bidirectionally incompatible
379    strains across the arthropod *Wolbachia* (Atyame et al., 2011b; Bordenstein and Werren, 2007;
380    Branca et al., 2009; O'Neill and Karr, 1990; Sicard et al., 2014), and the rarity of so-called
381    'suicidal' self-incompatible strains (Zabalou et al., 2008), crossing this maladaptive valley may be
382    an unlikely evolutionary scenario. In contrast, since CifA is involved in both CI and rescue, it
383    becomes possible for a single mutation that affects CI to also impact rescue. Thus, a single
384    mutation in CifA may shift both CI and rescue phenotypes, yield bidirectional CI relative to an
385    ancestor, and maintain self-compatibility (Shropshire et al., 2018). Indeed, mutagenesis of highly
386    conserved amino acids across CifA reveal that sites within CifA's N-terminal region are crucially
387    important for the expression of both CI and rescue, suggesting that residues in this single region
388    are coopted for both phenotypes (Shropshire et al., 2020). Notably while this one-step model of
389    bidirectional CI avoids the maladaptive valley, it may only spread if transferred into a new
390    aposymbiotic (sub)population since emergence of a new incompatibility type within a symbiont-
391    bearing population would be immediately incompatible with the more common symbiont in the
392    population. More research is needed to fully understand the genetic basis of bidirectional CI and
393    its evolution. For instance, theoretical modeling will be necessary to evaluate additional routes of

394    emergence as it relates to Cif sequence variation, functional genetic assays can be used to unravel
395    the correlation between *cif* sequence variation and (in)compatibility relationships, and population
396    genetic surveys coupled with population dynamics modeling would reveal when a novel variant
397    would be likely to persist in a population.
398

## What is the mechanistic basis of Cif-induced CI?

### *CifA molecular function*

401    Structural homology-based analyses suggest that Type 1 CifA have three putative domains:
402    a catalase-related (catalase-rel) domain involved in the degradation of reactive oxygen species, a
403    domain of unknown function (DUF) 3243 with homology to a Puf-family RNA-binding domain
404    (RBD), and a sterile-like transcriptional regulator (STE) (Fig. 4A) (Lindsey et al., 2018). While
405    the catalase-rel domain is unique to the CifA$_{[T1]}$, the STE is maintained in Type 1-4 genes (Lindsey
406    et al., 2018), and the Puf-family RBD exists in Type 1-5 genes (Bing et al., 2020). Importantly,
407    these annotations are of low predictive value (20-30% probability) and may not withstand
408    experimental testing (Lindsey et al., 2018). On the other hand, sliding window analyses of
409    selection for CifA$_{[T1]}$ suggest that while the full protein is under purifying selection, the catalase-
410    rel domain and the unannotated N-terminal region are under the strongest selection (Shropshire et
411    al., 2018). Indeed, CifA cannot contribute to transgenic CI or rescue when conserved amino acids
412    are mutated within the unannotated N-terminal region or in the putative catalase-rel domain of
413    CifA$_{wMel[T1]}$ (Shropshire et al., 2020). Conversely, when sites are mutated in CifA's DUF domain,
414    it maintains the ability to contribute to rescue, but loses CI-capability (Shropshire et al., 2020).
415    Thus, CifA's N-terminal region is crucially important for both CI and rescue, whereas sites within
416    the DUF domain are only crucial for CI (Fig. 4A). More work will be necessary to determine how
417    and why these mutations impact these phenotypes, but the annotations provided above afford a set
418    of testable hypotheses and questions. For instance, does CifA interact with reactive oxygen species
419    both in the context of CI and rescue, and/or does RNA-binding occur in the context of CI?
420    Biochemical assays testing for these functions will further elucidate how CifA contributes to CI
421    and rescue phenotypes.
422    Additionally, how CifA is involved in both CI and rescue remains largely a mystery. The
423    simplest explanation is that CifA maintains the same function in both CI and rescue. Under this
424    framework, CifA would act on a pathway that can be modified during spermatogenesis and
425    oogenesis to produce opposite affects (Shropshire et al., 2018). If CifA were to drive such a
426    function, then CifB's role in CI would seemingly be auxiliary and perhaps only necessary for
427    localization of CifA to particular targets or, since CifB acts as a deubiquitinase, to protect CifA
428    from degradation by ubiquitin pathways (Beckmann et al., 2017). Alternatively, CifA may be a
429    multi-functional protein that employs one set of functions to cause CI and another to cause rescue
430    (Shropshire et al., 2018). For instance, if CifA targets sex-specific host pathways, CifA can only
431    affect its host in a particular way if that target is available. Additionally, CifA may be modified in
432    some manner that differs between the testes and the ovaries, unlocking unique biochemical

433    functions by posttranslational modification, localization differences, or the expression of different
434    protein conformational isoforms (Shropshire et al., 2018). In summary, little is known about
435    CifA's functional role in CI and rescue, and considerable work is necessary not only to identify its
436    enzymatic capabilities, but also to further elucidate how it can act both to cause and prevent CI.

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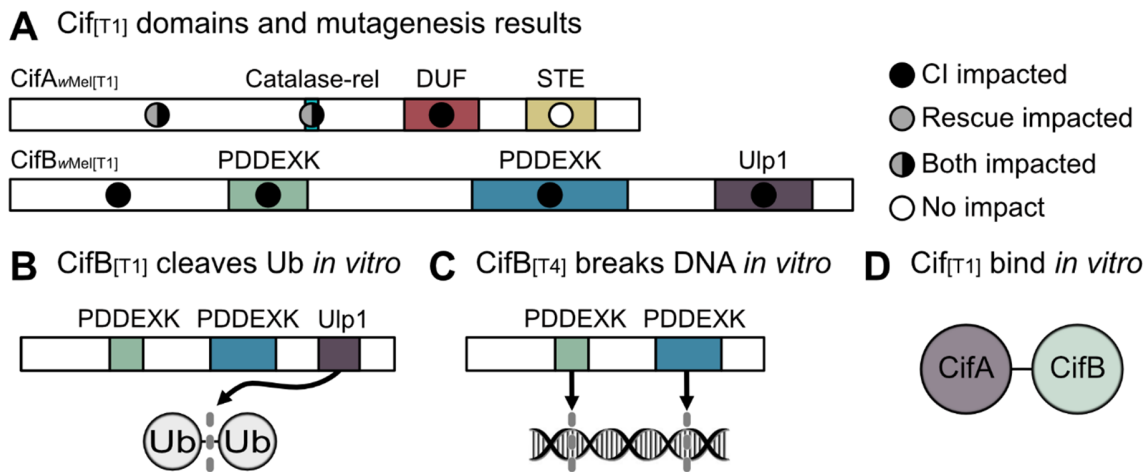

441    **Figure 4. Biochemical characterization of Cif proteins.** (A) Annotated domains in the CifA and CifB proteins and
442    the relative importance of conserved residues in each domain for CI (black circles), rescue (gray circles) or neither
443    phenotype (white circles) as determined by transgenic expression of mutated proteins in aposymbiotic *D.*
444    *melanogaster* (Beckmann et al., 2017; Shropshire et al., 2020). (B) CifB[T1] can cleave ubiquitin chains via its Ulp1
445    deubiquitinase domain *in vitro* (Beckmann et al., 2017). (C) CifB[T4] nuclease domains can cause DNA breaks *in vitro*
446    (Chen et al., 2019). (D) CifA[T1] and CifB[T1] bind each other *in vitro* (Beckmann et al., 2017). Domain architecture is
447    based on homology-based analyses and is of low predictive value (20-30% probability) for CifA (Lindsey et al., 2018),
448    and CifB[T1] PDDEXK nuclease domains lack canonical PD-(D/E)XK motifs (Beckmann et al., 2017), but remain
449    structurally homologous to other PDDEXK nucleases (Lindsey et al., 2018).

450

451    *CifB molecular function*

452          CifB[T1] from both *w*Mel and *w*Pip encode a single putative ubiquitin-like protease (Ulp1)
453    domain (Beckmann et al., 2017; LePage et al., 2017; Lindsey et al., 2018). The Ulp1 domain was
454    later expressed separate from the rest of the protein in *E. coli* and purified for downstream *in vitro*
455    ubiquitin cleavage assays (Beckmann et al., 2017). When exposed to a variety of ubiquitin chains,
456    it was revealed that the Ulp1 cleaves K6-, K11-, K27-, K29-, K33-, K48-, and K63-linked ubiquitin
457    *in vitro*, but with a preference for K63 chains (Fig. 4B) (Beckmann et al., 2017). K63 chains are
458    associated with NF-κB signaling which has diverse functions including innate immunity, DNA
459    transcription, autophagocytosis (Tan et al., 2008; Wertz and Dixit, 2010), and proliferation of cell
460    nuclear antigen (PCNA) (Ripley et al., 2020) that has previously been shown to act abnormally in
461    CI-affected embryos (Landmann et al., 2009). A single amino acid mutation in the catalytic site of
462    the Ulp1 prevents the breakdown of ubiquitin chains *in vitro* (Beckmann et al., 2017). Expressing

the Ulp1 catalytic mutant for CifB$_{w\text{Pip}[T1]}$ and CifB$_{w\text{Mel}[T1]}$ in male *D. melanogaster* alongside CifA did not induce CI, suggesting that deubiquitilase activity is important for CI barring the occurrence of any potential protein structural changes in the mutants (Fig. 4A) (Beckmann et al., 2017; Shropshire et al., 2020). However, some caution is warranted as deubiquitinase assays have not been conducted using the full-length protein, and it is unknown if this activity is maintained *in vivo*. Moreover, it remains unknown what CifB deubiquitinates inside reproductive tissue cells and how this deubiquitination contributes to CI. Future biochemical assays will help answer these persistent questions.

While CifB's Ulp1 is seemingly important for CI, only CifB$_{[T1]}$ have this domain. However, additional mutagenesis assays reveal that other conserved sites across the CifB protein similarly ablate CI function (Fig. 4A) (Shropshire et al., 2020), strongly suggesting other regions of the protein are likewise important for CifB function. For instance, all CifB proteins (Type 1-5) are also annotated with a dimer of PD-(D/E)XK (hereafter PDDEXK) nuclease domains (Bing et al., 2020; Lindsey et al., 2018). Indeed, *in vitro* nuclease assays with CifB$_{w\text{Pip}[T4]}$ confirm that they can nick both double and single stranded DNA (Fig. 4C) (Chen et al., 2019). Moreover, mutating PDDEXK catalytic sites in CifB$_{w\text{Pip}[T4]}$ prevents nuclease activity *in vitro* and CI-inducibility when expressed in *D. melanogaster* (Chen et al., 2019). Unlike the other phylogenetic Types, CifB$_{[T1]}$ proteins do not have the canonical PDDEXK catalytic sites, thus lending doubt to the importance of these domains as nucleases in CifB$_{[T1]}$ (Beckmann et al., 2017). However, these domains remain structurally homologous to other PDDEXK domains (Lindsey et al., 2018) and many functional PDDEXK-like domains lack the canonical PD-(D/E)XK catalytic motifs, opting instead for alternative catalytic residues and structural folds, and some PDDEXK-like domains without catalytic sites are still involved in other DNA-associated processes (Knizewski et al., 2007). Mutating conserved amino acid residues in either of the PDDEXK domains of CifB$_{w\text{Mel}[T1]}$ inhibits its ability to contribute to CI (Shropshire et al., 2020). Additionally, despite *w*Pip containing both Type 1 and 4 genes, there are no notable differences in cytological embryonic defects caused when both genes are expressed as compared to other strains that only have CifB$_{[T1]}$, suggesting that these genes yield similar cytological outcomes (Bonneau et al., 2018b). Biochemical assays will be necessary to evaluate the nuclease activity of a diverse array of CifB proteins including CifB$_{[T1]}$ because the conserved areas in and around the PDDEXK domains across all Cif Types likely persist because of a common function that underpins CifB's involvement in CI.

## *Cif interacting partners*

A list of putative Cif protein binding partners have recently been reported. While CifA$_{[TI]}$ and CifB$_{[T1]}$ bind *in vitro* (Fig. 4D) (Beckmann et al., 2017), it does, however, remain unknown if CifA binds CifB *in vivo* in the testes to cause CI or if maternal CifA binds to paternal CifB in the embryo to cause rescue (Beckmann et al., 2019a, 2019b; Shropshire et al., 2019). More work on the localization, co-localization, and binding profiles of these proteins will elucidate this question. Additionally, Cifs appear to bind to a suite of host proteins that differ based on if CifA and CifB are expressed alone or together. 67 host proteins were identified as Cif binding partners under co-

503  expression of CifA and CifB, whereas 45 proteins were identified with CifB expression alone in
504  pools of male and female *D. melanogaster* (Beckmann et al., 2019c). Of these proteins,
505  karyopherin-α (Kap-α) bound to singly expressing CifB extracts and was notable as
506  overexpression of Kap-α in aposymbiotic females yielded partial rescue (~20% hatch rate
507  improvement) when crossed to transgenic CI males (Beckmann et al., 2019c). Kap-α is a nuclear
508  import receptor and a regulator of p53 which has roles in the protamine-histone exchange process
509  (Beckmann et al., 2019c; Emelyanov et al., 2014). Intriguingly, delayed H3 histone deposition is
510  a hallmark of CI during early embryogenesis (Landmann et al., 2009), suggesting a relationship
511  between CifB, Kap-α, p53 and histone-associated abnormalities in CI. However, it is important to
512  emphasize that, Kap-α was only pulled down when CifB was singly expressed (Beckmann et al.,
513  2019c), suggesting that while Kap-α overexpression may influence rescue-efficiency, it is unclear
514  how it would be part of the rescue mechanism since CifB is not necessary for rescue to occur.
515  More work is essential to determine if CifB's binding to Kap-α contributes to CI and how Cif
516  binding to other host proteins relates to CI and rescue phenotypes.

517

## What is the cytological basis of CI?

519  Decades of research have characterized an in-depth understanding of CI-associated
520  cytological abnormalities. These studies broadly define alterations during spermatogenesis (Fig.
521  5A) and embryogenesis (Fig. 5B), suggesting that CI is associated with a sperm modification prior
522  to fertilization that results in embryonic defects and death. Importantly, the causes of the reported
523  sperm and embryonic abnormalities remain unknown and, in most studies, it is unclear if these
524  observations are directly related to the Cif proteins or are a byproduct of *Wolbachia* infection in
525  the testes. However, these findings provide insight into the ways in which *Wolbachia* and CI
526  influence host reproduction and fertility. Below, we review the cytological changes that occur in
527  spermatogenesis and embryogenesis during CI and rescue, and highlight areas where future
528  research is crucially needed with reductionist assays to disentangle effects of *Wolbachia* infection
529  and CI.

530

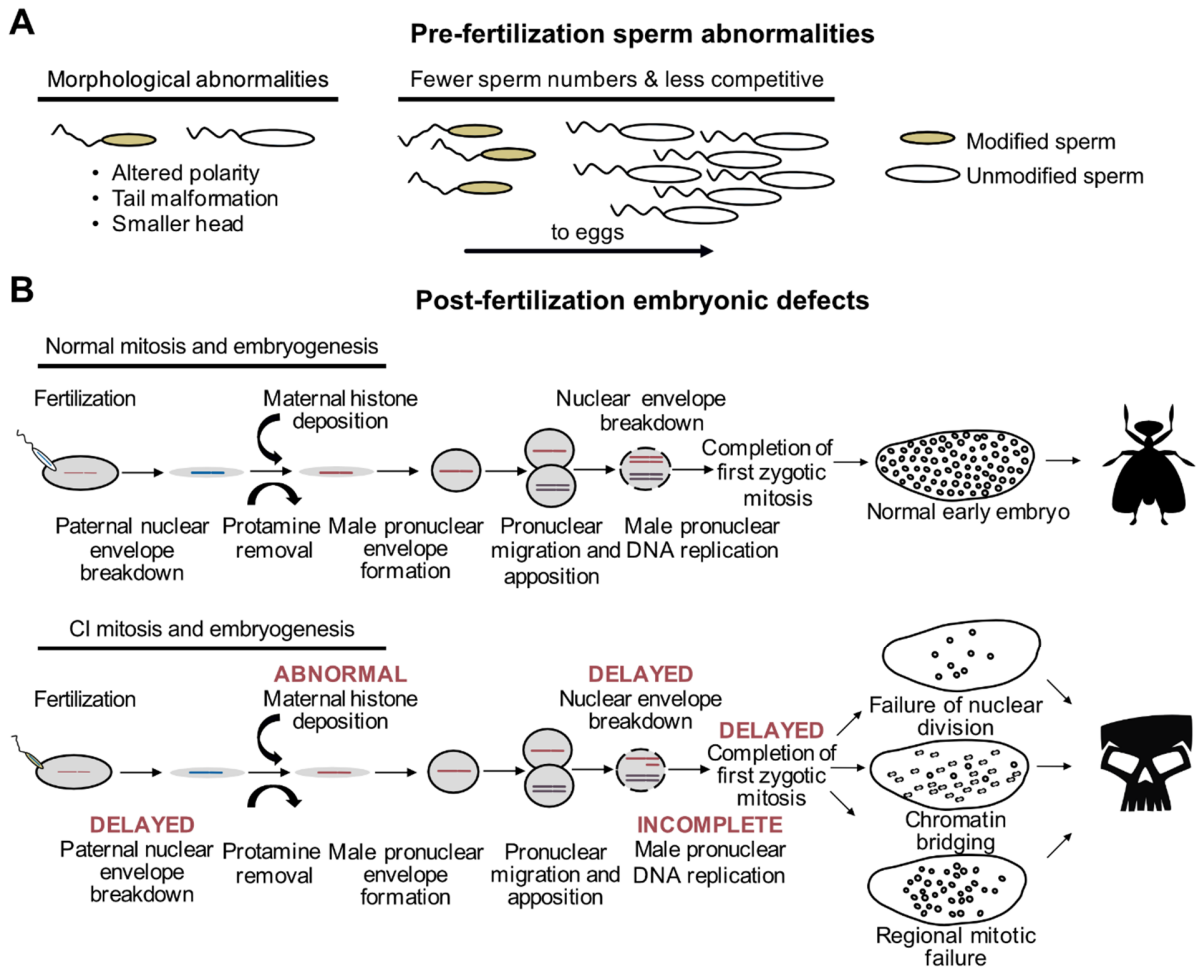



**Figure 5. CI-associated defects occur pre- and post-fertilization.** (A) In males harboring *Wolbachia*, there are several types of sperm abnormalities when compared to their aposymbiotic counterparts. (B) When fertilized with sperm derived from *Wolbachia*-carrying males, embryonic nuclear defects result in the form of delayed paternal nuclear envelope breakdown, abnormal histone deposition and other early mitotic events. These defects then cause embryonic phenotypes observed in CI including chromatin bridging and regional mitotic failures.

## *CI-associated abnormalities prior to fertilization*

Spermatogenesis is a highly regulated process. It begins with cells of the germline stem cell niche (GSCN) replicating into spermatogonia that subsequently undergo mitosis to yield a spermatocyst with 16 spermatocytes (Fuller, 1993; Hackstein, 1987; Lindsley, 1980). Each spermatocyte in the cyst then undergoes two rounds of meiosis to form four spermatids, for a total of 64 spermatids in each cyst. The spermatids then undergo elongation where the sperm tail forms and histones are replaced with protamines for tight packaging of DNA in the nucleus within the sperm head (Rathke et al., 2014). In the final stages of sperm maturation, spermatids undergo individualization to remove excess cytoplasm before becoming mature sperm to enter the seminal vesicle for storage before mating. Impacts of *Wolbachia* on spermatogenesis can result in downstream sperm defects that may be connected to CI (Fig. 5A). For example, symbiont-bearing

549   *D. simulans* flies and *Ephestia* moths produced fewer sperm, and stronger CI was associated with
550   more sperm transfer during copulation in *D. simulans* (Awrahman et al., 2014; Lewis et al., 2011;
551   Snook et al., 2000). When *D. simulans* females mated with *Wolbachia*-bearing and aposymbiotic
552   males, the sperm of aposymbiotic males were more likely to fertilize eggs (Champion de Crespigny
553   and Wedell, 2006), suggesting that *Wolbachia*-modified sperm are less competitive. *Wolbachia*-
554   affected sperm cysts exhibit abnormal morphology with some sperm fused together and other
555   sperm exhibiting randomly-oriented, axoneme-mitochondrial complexes that are responsible for
556   sperm motility (Riparbelli et al., 2007), perhaps explaining fertility defects and variation in sperm
557   competition. However, key questions remain. Are *cif* gene products responsible for the
558   aforementioned sperm abnormalities? If these defects are not caused by the *cif* genes, and are
559   instead a byproduct of other *Wolbachia*-host associations, what is their significance, if any, to
560   reproductive parasitism? In summary, a deeper investigation of these defects as they relate to CI
561   products is needed to confirm their link to CI.

562       Interestingly, *Wolbachia* are not symmetrically distributed in testes, with only some
563   spermatocysts harboring symbionts in the strong CI-inducing *w*Ri strain of *D. simulans* (Clark et
564   al., 2003). Indeed, *w*Ri is almost exclusively localized to the GSCN, and some GSCN remain
565   aposymbiotic, suggesting that the Cif proteins must either act early in spermatogenesis or are
566   diffusible factors that can stably travel into later stages of spermatogenesis (Clark et al., 2003,
567   2002; Riparbelli et al., 2007). *Wolbachia* that persist to infect spermatid tails are stripped during
568   the individuation process and are moved into waste bags where they are presumably degraded
569   (Riparbelli et al., 2007). Not only does this suggest that *Wolbachia* create a diffusible factor that
570   interacts with sperm or spermatogonia to cause CI, but it also helps to explain why paternal
571   *Wolbachia* transfer has not been observed (Yeap et al., 2016) with rare exceptions such as in hybrid
572   *Nasonia* wasps and in transinfected *A. aegypti* (Chafee et al., 2011; Ross et al., 2019a). Future
573   work investigating the localization of CifA and CifB, will determine when and where the Cif
574   proteins act to cause CI and whether they are transferred to the embryo for the potential to directly
575   cause defects during embryogenesis.

576

### *CI-defining abnormalities after fertilization*

578       Abnormalities that define the post-fertilization events underpinning CI are observed during
579   embryogenesis (Fig. 5B). In chronological order, CI-affected embryos experience abnormal
580   maternal H3.3 histone deposition on the male pronucleus, delayed activation of the DNA
581   polymerase cofactors PCNA and cell cycle regulator Cdk1 resulting in incomplete DNA
582   replication, delayed nuclear envelope breakdown prior to the first mitosis, and a delay in the first
583   mitotic event (Landmann et al., 2009; Tram and Sullivan, 2002). These defects often culminate in
584   a chromatin bridging phenotype during the first mitosis, shredding the paternal nuclei and leading
585   to embryonic arrest (Breeuwer and Werren, 1990; Callaini et al., 1996; Lassy and Karr, 1996; Reed
586   and Werren, 1995; Ryan and Saul, 1968; Tram et al., 2006). Notably, it remains unknown what
587   the most proximal event is during CI. It is plausible that abnormal histone deposition is the first
588   CI-causing event during embryogenesis that leads to a cascade of effects culminating in the other

embryonic abnormalities, but there remain open questions. For instance, how do the Cif proteins interact with the host to cause abnormal histone deposition? Are the Cif proteins even transferred with the sperm so that they can cause these defects directly, or are these defects caused by an initial Cif interaction occurring during spermatogenesis? Finally, if Cifs do directly cause abnormal histone deposition, how are these affects rescued by the presence of CifA expressed in the embryo? A combination of cytological, transgenic, and biochemical assays may be necessary to evaluate these questions.

Defects in the first mitotic division are traditionally viewed as the key cytological outcome of CI, but abnormalities later in embryogenesis are also common and are increasingly appreciated (Bonneau et al., 2018b; Callaini et al., 1997; LePage et al., 2017; Ryan and Saul, 1968). There are three distinct phenotypes: early mitotic failures whereby embryonic arrest occurs after several successful rounds of division, regional mitotic failures where some regions of the embryo appear to be dividing without issue, and widespread chromatin bridging in later stages of division (LePage et al., 2017). The cause of these defects remains unknown, but at least two hypotheses can be proposed. First, late stage embryonic defects are caused by the same cascade of abnormalities that often cause arrest during the first mitosis. Under this scenario, the difference in the cytological outcomes of the embryo may be explained by the magnitude of the proximal CI-causing affect. For instance, strong male pronuclear delay can result in complete exclusion of the male pronucleus from early development, yielding an embryo that may attempt to undergo haploid development (Callaini et al., 1997; Tram et al., 2006). In *N. vitripennis* where haploid individuals become males and diploid become females, exclusion of the male pronucleus during CI can manifest in haploidization where even fertilized eggs develop as haploid (Bordenstein et al., 2003; Ryan and Saul, 1968; Tram et al., 2006; Vavre et al., 2001, 2000). Thus, the intensity of pronuclear delay may correspond with the resulting phenotypic profile during embryogenesis, but more work is necessary to determine if these affects translate to late stage embryonic defects. Second, these later stage abnormalities may be independent from the defects preceding the first mitotic failure. Indeed, it has been proposed that different phylogenetic Types of Cif proteins may contribute to different cytological outcomes (Bonneau et al., 2018b). However, *w*Pip, which encodes both Type 1 and 4 genes, display both early and late stage embryonic abnormalities comparable to *w*Mel which encodes only Type 1 genes (Bonneau et al., 2018b; LePage et al., 2017). Despite presumably having different mechanistic bases, *Cardinium* and *Wolbachia* both have converged on similar outcomes during early embryogenesis, including chromatin bridging and abnormal number of chromosomes after the first division (Gebiola et al., 2017). It remains unknown if *Cardinium* CI yields comparable molecular defects and sperm abnormalities to *Wolbachia*-induced CI. Clearly, there are a diverse set of cytological outcomes associated with CI in both *Wolbachia* and *Cardinium*. Additional cytological and reductionistic studies will be necessary to evaluate the cause of this variation and determine how the Cif proteins contribute to these phenotypes.

## What is the host's contribution to CI?

628    It is common that researchers leverage correlations between *Wolbachia* infection state and
629 host expression phenotypes (RNA, protein, etc.) to understand how *Wolbachia* impact their host.
630 When differential expression is correlated with CI phenotypes, these data can yield valuable
631 insights regarding CI's mechanism. Significant correlations between *Wolbachia* infection state and
632 host expression have been measured in *D. melanogaster* (Biwot et al., 2019; He et al., 2019;
633 LePage et al., 2014; Liu et al., 2014; Ote et al., 2016; Xi et al., 2008; Yuan et al., 2015; Y. Zheng
634 et al., 2019; Zheng et al., 2011), *D. simulans* (Brennan et al., 2012; Clark et al., 2006; Xi et al.,
635 2008), *La. striatellus* (Huang et al., 2019; Ju et al., 2017; Liu et al., 2019), *T. urticae* (Bing et al.,
636 2019; Zhang et al., 2015)*, Cu. pipiens* (Pinto et al., 2013)*, and A. albopictus* (Baldridge et al., 2017,
637 2014; Brennan et al., 2012, 2008). Challengingly, as many as 1613 transcripts are differentially
638 expressed between *Wolbachia* infection states (Bing et al., 2019), and as with the cytological
639 abnormalities described above, it is difficult to untangle the effects of *Wolbachia* and CI on host
640 expression profiles.

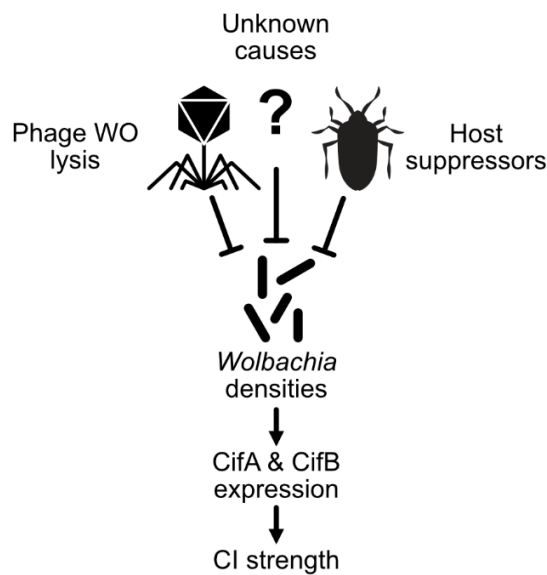
641    However, the most promising candidates associated with CI are those that can be
642 experimentally over- or under-expressed to recapitulate CI-like hatch rates and cytological defects.
643 For example, overexpression of the tumor suppressor gene *lethal giant larvae* [l(2)gl] and myosin
644 II gene *zipper* in aposymbiotic *D. simulans* induces a considerable reduction in hatching that is
645 accompanied with CI-associated cytological defects (Clark et al., 2006). However, CI is not just
646 associated with hatch rate defects, but also the ability to rescue those defects. When l(2)gl and
647 *zipper* over-expressing males were mated to symbiont-bearing females, no change in hatching was
648 observed (Clark et al., 2006), suggesting that hatch rate reductions associated with these factors
649 cannot be rescued and thus are not CI-associated. Nevertheless, there have been numerous studies
650 that have identified host factors that contribute to CI-like embryonic abnormalities and can be
651 rescued by symbiont-bearing females: the aminotransferase *iLve* which mediated branched-chain
652 amino acid biosynthesis in *La. striatellus* (Ju et al., 2017), the sRNA nov-miR-12 which negatively
653 regulates the DNA-binding protein *pipsqueak* (*psq*) in chromatin remodeling in *D. melanogaster*
654 (Y. Zheng et al., 2019), cytosol amino-peptidase-like which are in the sperm acrosome and
655 involved in fertilization in *La. striatellus* (Huang et al., 2019), two seminal fluid proteins (CG9334
656 and CG2668) with unknown function in *D. melanogaster* (Yuan et al., 2015), the histone
657 chaperone *Hira* in *D. melanogaster* and *D. simulans* (Zheng et al., 2011), a Juvenile Hormone
658 protein (JHI-26) involved in development in *D. melanogaster* (Liu et al., 2014), and the immunity-
659 related gene *kenny* (*key*) in *D. melanogaster* (Biwot et al., 2019). Since misexpression of these
660 host products in aposymbiotic males mimic CI-like embryonic defects in a way that can be rescued
661 by symbiont-bearing females, there is support that these products or their pathways are involved
662 in CI, but how these factors relate to cause CI remains unknown, and there is no current evidence
663 that these are binding partners with Cif proteins.

664    In addition to RNA and/or protein expression differences, changes in host physiology and
665 cell biology have also been correlated with CI. For example, *Wolbachia*-bearing *D. melanogaster,*
666 *D. simulans*, *A. albopictus*, *A. polynesiensis*, and *T. urticae* males often have higher reactive
667 oxygen species (ROS) in their testes than aposymbiotic males (Brennan et al., 2012, 2008; Zug

668   and Hammerstein, 2015). It has been hypothesized that this variation in ROS expression patterns
669   is due to an elevated host immune response to *Wolbachia* infection (Zug and Hammerstein, 2015).
670   However, multiple lines of evidence link ROS expression with CI. For example, increased ROS
671   levels are consistently observed among CI-inducing strains (Zug and Hammerstein, 2015), and
672   ROS leads to DNA damage in spermatocytes in *D. simulans* (Brennan et al., 2012). Additionally,
673   lipid hydroperoxide markers of ROS-induced oxidative damage are higher in symbiont-bearing *D.*
674   *melanogaster* (Driver et al., 2004), and PCNA retention is another marker for DNA damage and
675   is observed during the first mitosis of CI-affected embryos (Landmann et al., 2009). Interestingly,
676   overexpression of the *D. melanogaster* gene *key* increases ROS levels and DNA damage in males
677   when mimicking rescuable CI-like hatching and embryonic defects (Biwot et al., 2019). Together,
678   these data support a role for ROS in CI's mechanism, but direct connections remain unclear. One
679   hypothesis is that CifA's putative catalase-related domain does indeed function to interact with
680   ROS (Lindsey et al., 2018). Though, alternatively, ROS may be a byproduct of the host immune
681   response (Zug and Hammerstein, 2015). Biochemical and immunological assays will unravel these
682   relationships.
683

## What causes variation in CI strength?

685   Some *Wolbachia* strains exhibit CI that can vary between 10-100% embryonic death
686   (Awrahman et al., 2014; Clark et al., 2003; Cooper et al., 2017; Hoffmann, 1988; Layton et al.,
687   2019; Reynolds and Hoffmann, 2002; Turelli et al., 2018; Yamada et al., 2007; Zabalou et al.,
688   2004). In fact, a number of *Wolbachia,* including *w*Mel of *D. melanogaster* and *w*Yak of *D.*
689   *yakuba*, were initially characterized as non-parasitic since they had minimal to no impact on
690   embryonic hatching (Charlat et al., 2004; Holden et al., 1993; Zabalou et al., 2004). Later studies
691   would correct these early reports to suggest they can indeed cause CI, but their CI strength is highly
692   dependent on a variety of factors including the age of fathers and grandmothers (Cooper et al.,
693   2017; Layton et al., 2019; Reynolds and Hoffmann, 2002). Generally speaking, the work reviewed
694   below describes a complex relationship between biotic and abiotic factors that influence CI
695   strength. Notably, the bacterial density model of CI, whereby *Wolbachia* densities positively
696   correlate with CI strength, is the major factor driving most of these relationships (Breeuwer and
697   Werren, 1993). Phage WO lysis, host suppressors, and other undescribed interactors may control
698   the variation in *Wolbachia* titers (Fig. 6) (Awrahman et al., 2014; Bordenstein and Bordenstein,
699   2011; Funkhouser-Jones et al., 2018; Layton et al., 2019; Poinsot et al., 1998; Walker et al., 2011).
700   However, there are instances where CI strength variation does not correlate with *Wolbachia*
701   densities (Yamada et al., 2007). Below, we review these works and describe what is known and
702   unknown about the proximal basis of CI strength variation.
703

704

705    **Figure 6. An expanding *Wolbachia* density model of CI strength variation.** The proximal cause of CI is likely
706    CifA and CifB, whose expression level has been connected with intensity in transgenic studies (LePage et al., 2017).
707    *Wolbachia* densities have often correlated with factors that influence CI strength variation (Werren, 1997). In many
708    cases, it remains unknown how these factors influence *Wolbachia* densities. Phage WO lysis (Bordenstein and
709    Bordenstein, 2011) and host suppressors are well documented correlates or causes of density changes (Funkhouser-
710    Jones et al., 2018; Poinsot et al., 1998; Walker et al., 2011).

711

## *Temperature*

713        Temperature is often correlated with CI strength and is likely to contribute to the dynamics
714    that govern *Wolbachia*'s spread (Foo et al., 2019). High temperatures, usually exceeding 27°C,
715    can have a significant negative impact on CI strength in *Wolbachia*-carrying *A. aegypti* (Ross et
716    al., 2020, 2019b), *T. urticae* (van Opijnen and Breeuwer, 1999), *D. simulans* (Hoffmann et al.,
717    1986), *D. melanogaster* (Reynolds and Hoffmann, 2002), *A. scutellaris* (Trpis et al., 1981; Wright
718    and Wang, 1980), *A. albopictus* (Wiwatanaratanabutr and Kittayapong, 2009), and *Nasonia*
719    (Bordenstein and Bordenstein, 2011). There is considerable evidence that high temperature
720    impacts *Wolbachia* densities in various species including *A. albopictus* and *A. aegypti* (Foo et al.,
721    2019; Ross et al., 2020, 2019b), *N. vitripennis* (Bordenstein and Bordenstein, 2011), and *T. urticae*
722    (Lu et al., 2012). High temperatures even cure hosts *of Wolbachia* (Jia et al., 2009). In natural
723    populations of the butterfly *Zizeeria maha*, *Wolbachia* densities vary with season*,* and climate
724    change may be contributing to a decrease in symbiont frequencies in the tropics (Charlesworth et
725    al., 2019; Sumi et al., 2017). Notably in *N. vitripennis* and *T. urticae*, decreased *Wolbachia*
726    densities and CI strength have also been correlated with an increase in phage WO lytic activity
727    with higher temperatures (Bordenstein and Bordenstein, 2011; Lu et al., 2012). Cooler
728    temperatures at or below 19°C have also been associated with decreased CI in *D. simulans* and *N.*
729    *vitripennis* (Bordenstein and Bordenstein, 2011; Reynolds and Hoffmann, 2002). As with warm
730    temperatures, cooler temperatures also yield increased phage WO densities, decreased *Wolbachia*

densities, and decreased CI strength in *N. vitripennis* (Bordenstein and Bordenstein, 2011). These data suggest a phage density model of CI wherein phage WO may respond to temperature extremes by increasing its replication and lysing bacterial cells, thus lowering overall *Wolbachia* densities and resultantly CI levels.

However, while robust support of this model is available in *N. vitripennis* (Bordenstein and Bordenstein, 2011), more work is necessary to test if this is a generalizable phenomenon to other *Wolbachia* strains. For instance, in contrast to the relationships described above, *Wolbachia* in some *D. simulans* lines (Clancy and Hoffmann, 1998) and *Leptopilina heterotoma* wasps (Mouton et al., 2006) replicate more quickly at warmer temperatures, and yet CI strength decreases. Thus, it is plausible that phage WO in these species have a different relationship with temperature than in *N. vitripennis*, and other yet undescribed factors inhibit CI. Moreover, in *E. suzannae* bearing *Ca. hertigii*, high temperatures also yield reduced *Cardinium* densities and lower CI strength (Doremus et al., 2019). However, *Cardinium* do not harbor a phage, and thus phage lysis cannot explain this relationship. Additionally, in this same system, cooler temperatures yield reduced *Cardinium* densities, but an increase in CI strength (Doremus et al., 2019). Thus, here, it seems that bacterial densities alone do not explain the cause of CI strength variation. It is plausible that the factors contributing to CI strength variation in *Wolbachia* and *Cardinium* differ, and comparative phenotypic studies will be necessary to evaluate the differences between these two systems. However, in systems where symbiont density correlates with CI strength, it is plausible that the proximal cause is a shift in CI gene expression that correlates with symbiont densities. Transcript and protein abundance assays of *Wolbachia*'s *cif* genes will help elucidate this relationship when accompanied with measurements of variable CI strength.

*Host behavior and development*

Other correlates of CI strength variation are related to male and paternal grandmother age (Awrahman et al., 2014; Layton et al., 2019; Reynolds and Hoffmann, 2002), male mating rate (Awrahman et al., 2014; De Crespigny et al., 2006), male developmental timing (Yamada et al., 2007), rearing density (Yamada et al., 2007), and nutrition (Clancy and Hoffmann, 1998). All of these factors are significantly impacted by the structure of the population, resource availability, or behavior. Below, we will systematically discuss what, if anything, is known about how each of these factors impact CI strength.

First, male age can be negatively correlated with CI strength. For example, *w*Mel of *D. melanogaster* has nearly no impact on embryonic hatching when males are 3-5 days of age, but can induce significant CI when males are less than 2 days of age (Reynolds and Hoffmann, 2002). Similar results have been observed with *Wolbachia* in *D. simulans* and *N. vitripennis*, but to varying degrees (Breeuwer and Werren, 1993; Karr et al., 1998). Since *Wolbachia* densities decrease with male age in *D. melanogaster, D. simulans,* and *N. vitripennis* hosts (Binnington and Hoffmann, 1989; Breeuwer and Werren, 1993; Bressac and Rousset, 1993; Clark et al., 2002; Karr et al., 1998; Reynolds and Hoffmann, 2002; Riparbelli et al., 2007; Turelli and Hoffmann, 1995; Veneti et al., 2003; Weeks et al., 2007), it is perhaps unsurprising that age also correlates with CI.

771 Moreover, of the factors associated with CI strength, age is also the only one that has been
772 investigated in the context of *cifA* and *cifB* transcription, and does indeed decrease with age
773 alongside *Wolbachia* densities (LePage et al., 2017).

774    Interestingly, while older males have fewer *Wolbachia*, older virgin females have more
775 (Layton et al., 2019). In fact, when females are aged longer prior to mating, their male offspring
776 are laid with higher *Wolbachia* densities and resultantly induce stronger CI (Layton et al., 2019).
777 This phenomena has been termed the paternal grandmother age effect (PGAE) (Layton et al.,
778 2019). It is unclear why age's impact on *Wolbachia* density is sex-specific. However, the
779 relationship between male age, symbiont densities, and CI strength may not be generalizable across
780 all CI-inducing symbionts and their hosts. For instance, *Cardinium* of *E. pergandiella* cause CI
781 that is unaffected by male age (Perlman et al., 2014), and studies disagree about the significance
782 of the impact of age on CI caused by *w*Ri of *D. simulans* (Awrahman et al., 2014; Binnington and
783 Hoffmann, 1989; Bressac and Rousset, 1993). Thus, the impacts of age on symbiont densities and
784 CI may be limited to some *Wolbachia* or alternatively, to particular host backgrounds. More
785 comparative phenotypic work will be needed to understand the broader context of the relationship
786 between age, CI strength, and *cif* expression.

787    Additionally, male mating rate is also negatively correlated with CI strength. For instance,
788 *D. simulans* males mate more frequently than aposymbiotic males, and the increased mating rate
789 yields weaker CI in later matings (Awrahman et al., 2014; De Crespigny et al., 2006). Symbiont-
790 bearing males also transfer more sperm during copulation than aposymbiotic males during the first
791 mating encounter, and decreased sperm transfer in subsequent matings corresponds with weaker
792 CI (Awrahman et al., 2014). As such, the increased mating frequency may be a behavioral
793 adaptation employed by some hosts to restore reproductive compatibility between symbiont-
794 bearing males and aposymbiotic females (Awrahman et al., 2014). As with temperature and age
795 (Bordenstein and Bordenstein, 2011; Layton et al., 2019; Reynolds and Hoffmann, 2002), it has
796 likewise been hypothesized that *Wolbachia* densities may decrease upon remating (Awrahman et
797 al., 2014), but this hypothesis has not been tested. Alternatively, it has also been hypothesized that
798 the amount of time that sperm remains in contact with *Wolbachia* corresponds with how strong CI
799 can be (Karr et al., 1998), thus remating may contribute to high sperm turnover that limits
800 *Wolbachia*-sperm exposure. These hypotheses can be tested via *Wolbachia* density assays and
801 microscopy of reproductive tissues upon remating.

802    Male development time is likewise correlated with CI strength. Here, *w*Mel-bearing male
803 *D. melanogaster* induce stronger CI when they are the first emerging males of a clutch (Yamada
804 et al., 2007). The younger brothers, which take longer to develop but are approximately the same
805 age, cause weaker CI. This phenotype has been termed the younger brother effect (YBE) (Yamada
806 et al., 2007). The YBE is an outlier in phenotypes associated with CI strength variation in that
807 younger and older brothers have comparable adult *Wolbachia* densities (Yamada et al., 2007),
808 suggesting an alternative mechanism for the relationship between developmental timing and CI
809 strength. However, it is also plausible that while younger and older brothers have similar bacterial
810 densities, their localization may shift such that cells more important to CI expression have higher

811  densities than other cells in the testes (Clark et al., 2002). Alternatively, *Wolbachia* densities of
812  the adult male may be less informative than density differences during embryonic or larval
813  development. For instance, the PGAE, as described above, revealed that sons of older females
814  caused stronger CI and while their sons did not have higher *Wolbachia* densities as adults, they
815  did have higher densities during embryogenesis (Layton et al., 2019). Intriguingly, *Wolbachia*
816  densities rapidly declined in aged females after mating and embryo laying, suggesting that many
817  *Wolbachia* were transferred from the ovaries to the developing egg and ultimately embryo. Thus,
818  it is plausible that *Wolbachia* densities would correlate with deposition order such that first laid
819  older brothers would have higher densities than younger brothers laid soon after (Layton et al.,
820  2019). While these hypotheses remain associated and to be formally tested, it is also notable that
821  the YBE does not appear to apply to *w*Ri of *D. simulans* (Yamada et al., 2007)*,* other studies have
822  failed to replicate these results in other *w*Mel-bearing *D. melanogaster* lines (LePage et al., 2017),
823  and the opposite phenotype is observed with *Cardinium* of *Encarsia* where older brothers cause
824  weaker CI (Perlman et al., 2014). Thus, additional work is necessary to replicate the YBE in *D.*
825  *melanogaster* and other symbiont-host combinations and to evaluate its cause via longitudinal
826  developmental studies of *Wolbachia* densities. Moreover, understanding why *Cardinium* and
827  *Wolbachia* CI are differentially impacted by these factors is important in determining how
828  infection dynamics relate to reproductive manipulation.

829        Finally, rearing density and nutrition can also impact CI strength relationships. For
830  instance, when *w*Mel-bearing *D. melanogaster* are reared in high densities, CI strength is lower
831  than if they are reared in low densities (Yamada et al., 2007). The initial hypothesis behind this
832  correlation was that high density rearing led to nutritional stress which translated to less *Wolbachia*
833  (Yamada et al., 2007). While this hypothesis has not been explicitly tested, there is a reasonable
834  logical framework behind it. Indeed, multiple studies have shown that *D. simulans* males exposed
835  to nutritional stress have weaker CI than males with abundant resources (Clancy and Hoffmann,
836  1998; Sinkins et al., 1995). Notably, nutritional stress is also correlated with reduced *Wolbachia*
837  densities, supporting models, as above, where *Wolbachia* densities and *cif* expression are the
838  proximal factors driving the relationship to CI strength. That said, it is important to note that recent
839  microscopy studies have shown that standard qPCR-based measures of *Wolbachia* densities may
840  not be adequate under nutritional stress since host ploidy is subject to variation based on diet
841  (Christensen et al., 2019). Thus, it is plausible that qPCR-based variation in *Wolbachia* densities
842  under nutritional stress may in fact be driven by variation in host ploidy and not *Wolbachia* density.
843  Replication of these studies will be necessary to confidently link nutrition, rearing density, and
844  *Wolbachia* densities to CI strength.

845        The factors described above do not work on CI in isolation but instead seem to be mingled
846  in a state of perpetual complexity. For instance, the impact of temperature on CI strength in *D.*
847  *melanogaster* is dependent on male age, where 1-day old males reared at 25°C induce stronger CI
848  than those reared at 19°C, but the inverse is true with 3 and 5-day old males (Reynolds and
849  Hoffmann, 2002). Moreover, age has a variable impact on CI strength in different host
850  backgrounds, suggesting that genotypic variation in either the host or *Wolbachia* strain may impact

851   these relationships (O. Duron et al., 2007; Reynolds and Hoffmann, 2002). These studies highlight
852   the complexity of *Wolbachia*-host-environment interactions and should motivate additional
853   investigation to resolve the factors that underpin these variations and the host genetic loci that
854   influence how impactful each factor might be in each host.

855

856   *Host genetics*

857      Relationships between *Wolbachia* phenotypes and host genotypes are frequently
858   investigated through transinfections of a *Wolbachia* strain into a non-native background via
859   injection (Hughes and Rasgon, 2014) or introgression of one species cytoplasm into another host's
860   background via repeated backcrossing (Chafee et al., 2011). For example, *w*Mel *Wolbachia* of *D.*
861   *melanogaster* traditionally cause weak CI (Holden et al., 1993), but induce consistently strong CI
862   when transinfected into either *D. simulans* or *A. aegypti* (Poinsot et al., 1998; Walker et al., 2011).
863   Similar results have also been observed when *w*Yak, *w*Tei, and *w*San, which induce weak CI in
864   the *D. yakuba* complex (Charlat et al., 2004; Cooper et al., 2017; Zabalou et al., 2004), are
865   transferred into *D. simulans* (Zabalou et al., 2008). Moreover, despite seemingly carrying the same
866   *Wolbachia*, different genetic lineages of the wasp *N. longicornis* express different compatibility
867   relationships with other strains (Raychoudhury and Werren, 2012), and *w*VitA of *N. vitripennis*
868   causes weak CI in its native host but strong CI when introgressed into *N. giraulti* (Chafee et al.,
869   2011). Intriguingly, this affect is only observed with *w*VitA and does not apply to *w*VitB, which
870   also causes CI, suggesting that both host and *Wolbachia* genetics play a role in CI phenotypes.
871   These studies support models that predict hosts will be selected to develop resistance against CI-
872   induction (Prout, 1994; Turelli, 1994), and raise many questions about an evolutionary arms race
873   between *Wolbachia* and its host to control reproductive parasitism.

874      There are at least two broad models for the mechanisms of host suppression of CI:
875   divergence of host products that are targets for CI (defensive model) or evolution of host products
876   that inhibit and suppress *Wolbachia* or CI products (offensive model). First, a defensive model
877   would predict that the pathway(s) that CI act(s) on in the host must be conserved enough for CI to
878   be transferable between species, but also malleable enough for the pathway(s) to become resistant
879   to CI-induction. The host genes, transcripts, and proteins described earlier in this review are
880   excellent candidates since they can mimic CI phenotypes (Biwot et al., 2019; Huang et al., 2019;
881   Ju et al., 2017; Yuan et al., 2015; Zheng et al., 2011). However, studies are necessary to investigate
882   genetic variation in these host products to assess the possibility that they are under selection to
883   suppress CI. Other candidates would be direct binding partners of CifA and CifB (Beckmann et
884   al., 2019c), but nothing is known about how natural variation in these products may relate to CI
885   suppression. Conversely, an offensive model could yield the evolution of host genes involved in
886   *Wolbachia* density regulation or some other target with indirect effects on CI strength. Notably,
887   since these products may not be involved in the CI mechanism, they would not necessarily be
888   expected to be conserved. For instance, the *Wolbachia* density suppressor (*Wds*) gene of *Nasonia*
889   acts to suppress densities of *w*VitA, is taxon restricted to bees and wasps, and is under positive
890   selection as would be expected for a suppressor acting in an evolutionary arms race with

891    *Wolbachia* (Funkhouser-Jones et al., 2018). Since *Wds* is only present in Hymenoptera, it clearly
892    is not generalizable as a standard mechanism of *Wolbachia* and CI suppression. However, it is
893    plausible that other hosts have converged on comparable mechanisms of CI suppression.
894    Additional research will be needed to reveal the diversity of mechanisms surrounding CI
895    suppression and to understand the dynamics controlling their evolution.
896

## What are the models for *Wolbachia*-induced CI and rescue?

897

898    Numerous models have been proposed to explain CI and rescue mechanisms. First, we
899    discuss the utility of the classical phenotype-based modification/rescue (*mod/resc*) model in a post-
900    genomic world (Shropshire and Bordenstein, 2019; Werren, 1997). Additionally, despite
901    considerable advances in the genetics and biochemistry of CI, numerous mechanistic models are
902    used to describe CI and rescue (Beckmann et al., 2019a; Poinsot et al., 2003; Shropshire et al.,
903    2019). These models divide into two discrete categories: host-modification (HM) and toxin-
904    antidote (TA) (Beckmann et al., 2019a; Shropshire et al., 2019). HM-based models assume that
905    the CI-inducing factors act directly to modify host male products and that rescue occurs through
906    either removal of these modifications or otherwise reversing the effects through a separate host-
907    modification in the female. TA-based models assume that the CI-inducing factors are transported
908    into the embryo via the sperm and are toxic after fertilization unless the rescue factor is present,
909    binds to the CI toxin, and inhibits its toxicity. We discuss each of these models and their variants
910    in the context of a Two-by-One genetic framework (Shropshire and Bordenstein, 2019), but it is
911    crucial to reemphasize that while both CifA and CifB proteins are required to induce CI, the
912    specific biochemical mechanism underlying each protein's contribution to CI and rescue remains
913    unknown and there remains insufficient data to confirm any of these models.

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## A          Host-Modification Model

Key predictions:
- CifA and/or CifB modify male host products
- Modification causes CI in the embryo
- CifA in the egg/ovary reverses/inhibits the modification
- CifA and/or CifB transfer with sperm is not required

## B          Toxin-Antidote Model

Key predictions:
- CifB (toxin) travels with sperm to the egg
- CifA (antidote) degrades quickly and does not travel with the sperm to the egg
- CifA in eggs inactivates CifB from sperm

**Figure 7. The Host-Modification and Toxin-Antidote models of CI mechanism**. (A) The Host-Modification (HM) model predicts that the Cif proteins impart a modification on male-derived products that result in CI unless CifA is available in the embryo to reverse or otherwise inhibit the male-derived modification (Shropshire et al., 2019; Werren, 1997). (B) The Toxin-Antidote (TA) model predicts that CifB is the primary toxin that is transferred to the embryo via the sperm, and that rescue occurs when CifA binds CifB in the embryo and inhibits its toxicity (Beckmann et al., 2019a; Hurst, 1991; Shropshire et al., 2019; Werren, 1997).

*The mod/resc model.*

927    The *mod*/*resc* model defines a *mod* factor as a CI-inducing product produced in males and
928    a *resc* factor as a rescue-inducing product produced in females (Werren, 1997). The *mod*/*resc*
929    model is agnostic to the genetic, biochemical, enzymatic, or cytological basis of CI. Instead, the
930    *mod*/*resc* model provides a framework for describing the phenotypic expression of different
931    *Wolbachia* strains. For example, a standard CI-inducing strain that can self-rescue would be
932    denoted as *mod+*/*resc+*. Less common phenotypes include so-called suicidal *Wolbachia*
933    (*mod+*/*resc-*) and *Wolbachia* that do not cause CI but can rescue CI induced by other strains (*mod-*
934    /*resc+*) (Ant and Sinkins, 2018; Meany et al., 2019; Zabalou et al., 2008). *Wolbachia* that do not
935    cause CI or rescue are designated *mod-*/*resc-*.

936    The *mod*/*resc* model assumes that for bidirectional CI to occur, the *mod* and *resc* factors
937    would differ in such a way that they remain functional but are incompatible with each other
938    (Charlat et al., 2001; Werren, 1997). As such, a strain can carry multiple *mod* or *resc* factors that
939    determine the compatibility relationships with other strains, and the *mod*/*resc* model can be used
940    to estimate the number of *mod* and *resc* factors within a host (Zabalou et al., 2008). To do this,
941    *Wolbachia* strains are transinfected into the same genetic background and then crossed to
942    determine the incompatibility relationships between strains or against aposymbiotic flies. A strain
943    that causes CI against an aposymbiotic female is considered to have at least one mod factor. If it
944    can rescue itself then it has at least one *resc* factor. If two CI-inducing and self-compatible strains
945    are bidirectionally incompatible, then it is assumed that each carry at least one set of *mod* and *resc*
946    factors but that they are not the same. Indeed, crossing experiments between various *Wolbachia*
947    strains have revealed unidirectional and bidirectional incompatibilities which have led to
948    agreement that *Wolbachia* frequently carry multiple *mod* and *resc* factors (Poinsot et al., 1998;
949    Zabalou et al., 2008).

950    With the identification of the CI and rescue genes (Beckmann et al., 2017; Chen et al.,
951    2019; LePage et al., 2017; Shropshire et al., 2018; Shropshire and Bordenstein, 2019), it is
952    compelling to abandon the *mod*/*resc* model in favor of a purely genetic description of CI
953    relationships. With the ever-growing availability of genomic datasets, acceptance of a gene-centric
954    analysis of CI may be the simplest way to predict CI-capability to a symbiont. However, while
955    sequence information can indeed yield informed hypotheses about a strain's CI-capability, some
956    hosts suppress their symbiont's CI (Chafee et al., 2011; Poinsot et al., 1998; Walker et al., 2011),
957    and some symbiont strains exhibit different forms of reproductive parasitism based on their host
958    background (Fujii et al., 2001; Jaenike, 2007; Sakamoto et al., 2005; Sasaki et al., 2002; Zabalou
959    et al., 2008). Thus, we propose that a modern framework for describing CI relationships should
960    involve both phenotypic data described under the *mod*/*resc* model and genetic data described under
961    the Two-by-One model. The areas where genetic and phenotypic information disagree will be
962    exciting avenues for future investigation since it will likely reveal novel mechanisms of CI
963    suppression and conflict between *Wolbachia* and host (Funkhouser-Jones et al., 2018). Both
964    models will serve as a useful framework to describe these systems.

965

966    *HM-based mechanistic models.*

967        HM models make at least two key predictions. First, male host products are modified by
968   Cifs (Shropshire et al., 2019). There are numerous pre-fertilization defects associated with
969   infection by CI-inducing *Wolbachia* including changes in sperm morphology and competitive
970   ability (Champion de Crespigny and Wedell, 2006; Riparbelli et al., 2007), supporting that the host
971   is modified prior to fertilization. It is unknown, however, whether these outcomes are due to pre-
972   fertilization defects causatively related to CI or general responses to a testes *Wolbachia* infection.
973   Second, and most crucially, the proximal CI modifications causing death of the fertilized embryo
974   is rescued (e.g., replaced or otherwise negated) by CifA in the embryo (Shropshire et al., 2018;
975   Shropshire and Bordenstein, 2019). Here, CifA cannot bind with male-transferred CifB products
976   since no Cif protein is transferred with the sperm to the embryo and the modification occurs prior
977   to fertilization. Instead, CifA may interact with host processes to reverse or otherwise stop the
978   effects of CI caused by CifA and CifB protein expression in males. As such, assessment of the
979   location of CifA and CifB binding (testes or embryo), the transfer of Cif products, if any, with the
980   sperm, and the interactions that Cif have with the host will further inform this model. We discuss
981   three additional non-exclusive HM-based models below: titration-restitution, mistiming, and
982   goalkeeper.

983        The titration-restitution model (a.k.a. the "sink" hypothesis) was originally proposed by
984   Werren in 1997 (Werren, 1997) and posits that CI is induced by over- or under-expression of host
985   products or pathways in the testes/sperm and rescue occurs when the same products are
986   misregulated in the opposite direction in the ovaries/embryo (Fig. 8A) (Kose and Karr, 1995;
987   Poinsot et al., 2003; Werren, 1997). Indeed, *Wolbachia* has a considerable impact on expression
988   profiles, and some genes are differentially expressed in male and female reproductive tissues, and
989   numerous host factors meet these criterion (Baldridge et al., 2017, 2014; Bing et al., 2019; Yuan
990   et al., 2015), as described in section above. There are at least two ways in which CifA and CifB
991   proteins can underpin the titration-restitution model. First, since CI and rescue would occur
992   through titration of the same host product or pathway, it is feasible that CifA, which acts on both
993   sides of the phenotype (Shropshire and Bordenstein, 2019), may drive these expression changes.
994   Under such a model, CifB may act as an 'accessory protein' that enables CifA to target a paternally
995   derived product that it would otherwise not be able to reach on its own. Second, CifA may act on
996   its own to up- or down-regulate host products but has the opposite impact on that product when
997   CifB is present. As such, rescue would occur through CifA's lone action which counteracts the
998   misregulation caused by CifA and CifB dual expression.

999        Notably, titration-restitution models can explain bidirectional CI if Cif products from
1000  different strains have variable impacts on multiple host expression pathways. Thus, rescue would
1001  not be possible from a second strain since it could be targeting the wrong host factor or pathway.
1002  Indeed, divergent CI genes may differentially impact host pathways. For example, only the CifB[T1]
1003  sequences maintain a functional Ulp1 domain while the other four CifB clades have a dimer of
1004  PDDEXK nucleases that is also present in CifB[T1] (Beckmann et al., 2017; Bing et al., 2020;
1005  Lindsey et al., 2018). It is feasible that CifB with different domains impact different host pathways.
1006  Alternatively, Cif proteins may have differential impacts on the level of misregulation instead of

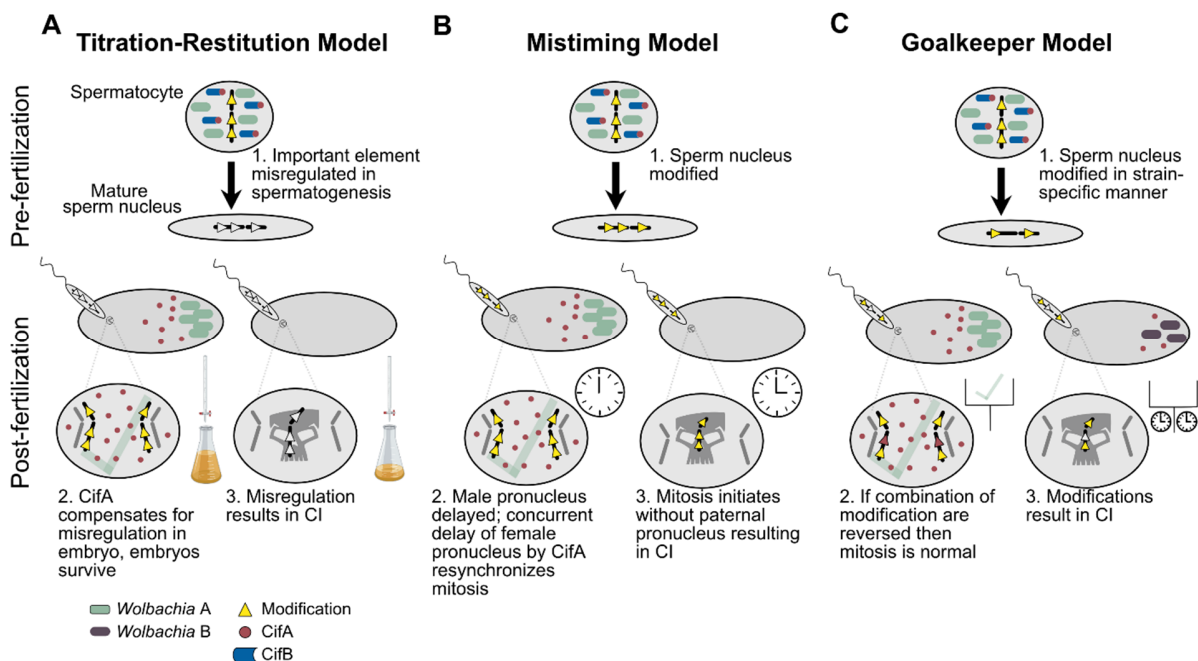
1007    or in addition to impacting multiple host pathways which may influence incompatibility
1008    relationships. More work will be necessary to understand if *cif* expression influences
1009    transcriptional and translational variation and how that variation corresponds to CI.

1010    The mistiming model (a.k.a. the "slow motion" hypothesis) was first explicitly proposed
1011    by Tram and Sullivan in 2002 (Tram and Sullivan, 2002) and is based on the observation that the
1012    paternal pronucleus has slowed development relative to the female pronucleus in CI crosses, the
1013    rescue cross has normal cell cycle timing, and the female pronucleus continues development
1014    despite the slowdown in both *Drosophila* and *Nasonia* species (Fig. 8B) (Callaini et al., 1996;
1015    Ferree and Sullivan, 2006; Ryan and Saul, 1968; Tram and Sullivan, 2002). This established the
1016    hypotheses that delayed male pronuclear development is responsible for emergent defects in early
1017    embryogenesis, and that resynchronization of the development may occur by comparably slowing
1018    down the development of the female pronucleus or slowing the cell cycle in rescue. Since the cell
1019    cycle timing of the female pronucleus is what establishes the timing for the first mitosis (Bossan
1020    et al., 2011), symbiont-bearing females do not induce CI since the male pronucleus reach
1021    apposition prior to the female pronucleus. Though, the reciprocal cross would be incompatible
1022    because the female pronucleus will have finished development prior to the male and the first
1023    mitosis would have initiated before the male pronucleus arrives. Importantly, this model predicts
1024    that CI crosses are subject to haploidization of diploid offspring since the male pronucleus could
1025    be completely excluded from mitosis if it was significantly slowed. This is indeed the case in *N.*
1026    *vitripennis* where CI often manifests as only male offspring since haploid offspring are viable in
1027    this species but develop as males (Bordenstein et al., 2003).

1028    The mistiming model proposes that CI and rescue have comparable impacts on the
1029    development of male and female gametes respectively. As such, a single gene could in theory be
1030    responsible for both CI and rescue (Poinsot et al., 2003). Under this paradigm, CifA may enact a
1031    slowdown in both tissues since it is involved in both phenotypes (Shropshire and Bordenstein,
1032    2019). However, if this were the case, then what would be the purpose of CifB? It is possible that
1033    CifB is responsible for localizing CifA to a male specific target where it imposes the same
1034    outcomes on its host. Since this hypothetical male product would not be available in the embryo,
1035    CifB would not have a role in rescue. However, an alternative model for mistiming is that rescue
1036    may not occur through slowing down the female pronucleus but may instead work by removing
1037    the slowdown agents from the male pronucleus. Together, these models would help to explain the
1038    proximal cause (misregulation) and culminating effects (mistiming) of CI. More work will be
1039    necessary to understand if rescue occurs via slowdown of the female pronucleus or from speeding-
1040    up the male pronucleus.

1041    A major limitation of the mistiming model is that it cannot explain bidirectional CI. Since
1042    mistiming proposes that rescue happens through delaying the female pronucleus as much as or
1043    greater than the male pronucleus, a sufficiently strong delay should yield compatibility with any
1044    strain that has a weaker male delay. As such, only unidirectional CI should manifest between
1045    strains where the strain inducing the stronger delay is capable of rescue. The goalkeeper model
1046    was proposed in 2011 as a way to address this limitation (Fig. 8C) (Bossan et al., 2011). In addition

1047   to the expectations of the mistiming model, goalkeeper suggests that a secondary factor
1048   unassociated with this mistiming may also be involved in CI. The combined contribution of these
1049   two mod factors leads to CI. Under this paradigm, CifA and CifB may contribute to different kinds
1050   of defects during spermatogenesis, each contributing in somewhat independent ways to CI-
1051   induction. Rescue must then negate the impact of both factors. Thus, for CifA to rescue CI it would
1052   not only need to contribute to a delay in the pronuclear development but also reverse the impacts
1053   of a secondary source of modification. Notably, since the titration-restitution model does not make
1054   predictions about the developmental timing of the male and female pronuclei, it is compatible with
1055   both mistiming and goalkeeper models, and can help explain mistiming through misregulation of
1056   host factors in a manner that leads to slowed development. More functional genetic, biochemical,
1057   and cytological studies are necessary to understand how a goalkeeper model and/or a combination
1058   of these HM-based models may contribute to CI.

1059

1060
1061   **Figure 8. Extensions of the Host-Modification model**. (A)The Titration-Restitution Model posits that an element
1062   within mature sperm is either over- or under-expressed in males due to Cif protein expression, but this alteration is
1063   then remedied in the female as a result of CifA through a reconstitution of the required element (Werren, 1997). (B)
1064   The Mistiming Model posits that a modification in the male sperm causes a delay in the formation of the male
1065   pronucleus that results in CI if CifA does not cause a concurrent delay in the maternal pronucleus, resynchronizing
1066   mitosis between the two pronuclei (Tram and Sullivan, 2002). (C) The Goalkeeper Model expands on the Mistiming
1067   Model and posits that the male product modification occurs in a strain-specific quantity, and may involve multiple
1068   modifications that need to be remedied to rescue the lethality (Bossan et al., 2011).

1069

1070   *TA-based mechanistic models*

1071          Since *Wolbachia* are not paternally inherited, Hurst proposed in 1991 that *Wolbachia* make
1072   a CI-inducing toxin that diffuses into the sperm cytoplasm and is transferred to the egg during
1073   fertilization and causes death (Hurst, 1991). Rescue then occurs when *Wolbachia* in the egg

1074    produce an antidote that binds to the toxin and prevents it from killing the embryo (Hurst, 1991).
1075    This TA model makes two key predictions (Beckmann et al., 2019a; Shropshire et al., 2019). First,
1076    the Cif proteins are transferred to the embryo. Mass spectrometry of spermatheca from symbiont-
1077    bearing *Cu. pipiens* females mated with symbiont-bearing males reveal fragments of CifA
1078    (Beckmann and Fallon, 2013). These later data have been used to support this prediction, but since
1079    these females were *Wolbachia*-infected (Beckmann and Fallon, 2013) and CifA is also the rescue
1080    protein (Chen et al., 2019; Shropshire et al., 2018), the most parsimonious explanation for CifA's
1081    presence in symbiont-bearing spermatheca is related to *Wolbachia* infection in females and
1082    potentially rescue, not CI-induction. It remains possible that Cif proteins are transferred, but this
1083    is not the simplest interpretation of currently available data. Second, if the proteins are transferred,
1084    then maternal CifA must bind to the CI toxin to prevent function. *In vitro* biochemical assays
1085    reveal that CifA and CifB are capable of binding (Beckmann et al., 2017), but it remains unknown
1086    if they bind as a toxin complex to induce CI or if CifA binds to CifB in the embryo to rescue CI.
1087    Moreover, while CifB's Ulp1 domain is an *in vitro* deubiquitinase, CifA's binding to CifB does
1088    not inhibit deubiquitinase activity, suggesting that if binding is for the purpose of rescue it is not
1089    inhibiting one of CifB's biochemical functions (Beckmann et al., 2017). As such, assays
1090    investigating if the Cif products are transferred to the embryo at all and where the Cif proteins bind
1091    each other in reproductive tissue cells will inform the foundation of this hypothesis.

1092        The TA model traditionally states that the toxin and antidote are separate factors (Poinsot
1093    et al., 2003). However, our genetic understanding is that CifA is involved in both CI and rescue.
1094    There are two ways to update the model to a Two-by-One genetic model (Shropshire and
1095    Bordenstein, 2019) while maintaining the key assumptions of the TA model (Hurst, 1991). First,
1096    CifB may be the sole toxin but requires CifA as an antidote even during spermatogenesis to prevent
1097    overly defective sperm (Beckmann et al., 2019a). For this to work, CifA is expected to degrade
1098    faster than CifB, leaving CifB alone to enter the egg as a toxin unless it binds to maternally derived
1099    CifA (Beckmann et al., 2019a). Alternatively, CifA and CifB could work together as a toxin
1100    complex that enters the embryo and is then rescued by maternally derived CifA. Binding assays
1101    coupled with microscopy and localization studies will reveal when and where CifA acts relative
1102    to CifB.

1103        As described above, the TA model aims to explain unidirectional CI between symbiont-
1104    bearing and aposymbiotic individuals. A modification of the TA model, called lock-and-key,
1105    expands the TA model to explain incompatibilities between *Wolbachia* strains. The lock-and-key
1106    model, like TA, proposes that a toxin is transferred from symbiont-bearing males to the embryo
1107    and will cause embryonic death unless an antidote is supplied. Toxins in this case are called locks,
1108    and antidotes are keys. The toxin lock is proposed to bind to or otherwise interfere with factors
1109    associated with proper embryonic development unless the antidote key is available to remove the
1110    lock. Bidirectional CI can then be explained by one strain carrying a set of locks and keys that are
1111    not compatible with the other strains' locks and keys because of differences in binding affinity.
1112    This model leveraged predictions of the *mod*/*resc* model that (i) strains can have multiple sets of
1113    *mod*/lock and *resc*/key factors and that (ii) a key is more likely to bind to its associated lock than

1114    to a divergent lock. Indeed, *Wolbachia* exhibit considerable *cif* polymorphism (Bing et al., 2020;
1115    Bonneau et al., 2019; LePage et al., 2017; Lindsey et al., 2018) and binding of CifA and CifB is
1116    strongest between cognate partners (Beckmann et al., 2017). However, the lingering questions with
1117    the TA model also apply with the lock-and-key model. Additionally, functional validation that
1118    divergent Cif proteins are functional, that they have differential impacts on the host, and contribute
1119    summatively to incompatibilities are lacking.

1120

## Conclusion

1122        *Wolbachia* were first discovered in *Cu. pipiens* mosquitoes in 1924 and were later linked
1123    to CI in 1973 (Hertig and Wolbach, 1924; Yen and Barr, 1973). Since then, a century of advances
1124    have significantly expanded our recognition of *Wolbachia*'s incredible and complex toolset. In
1125    particular, biologists now appreciate CI as a common form of reproductive parasitism that
1126    symbionts including *Wolbachia* and *Cardinium* use to rapidly spread through populations (Hunter
1127    et al., 2003; Rosenwald et al., 2020; Takano et al., 2017; Turelli, 1994; Weinert et al., 2015; Zug
1128    and Hammerstein, 2012). CI is associated with reproductive isolation (Bordenstein et al., 2001;
1129    Gebiola et al., 2017; Jaenike et al., 2006) and is leveraged as a successful tool in the prevention of
1130    arboviral diseases that infect humans (Crawford et al., 2020; O'Neill, 2018; Tantowijoyo et al.,
1131    2020). The last decade has seen a rapid expansion in our understanding of phage WO's role in CI
1132    genetics (Beckmann et al., 2019c; Chen et al., 2019; LePage et al., 2017; Shropshire et al., 2018;
1133    Shropshire and Bordenstein, 2019), phylogenetics (Bing et al., 2020; LePage et al., 2017; Lindsey
1134    et al., 2018), and mechanism (Beckmann et al., 2019c, 2017; Chen et al., 2019; Shropshire et al.,
1135    2020). Moreover, considerable effort has been made to describe CI-defining cytological defects
1136    (Ferree and Sullivan, 2006; Landmann et al., 2009), link variation in host expression with CI
1137    phenotypes (Biwot et al., 2019; Liu et al., 2014; Zheng et al., 2011), and to untangle factors that
1138    influence CI strength such as *Wolbachia* densities and phage WO lytic activity (Bordenstein and
1139    Bordenstein, 2011; Layton et al., 2019; Reynolds and Hoffmann, 2002; Yamada et al., 2007).
1140    Together, this significant body of literature has motivated models to explain how CI works
1141    (Beckmann et al., 2019a; Bossan et al., 2011; Poinsot et al., 2003; Shropshire et al., 2019). These
1142    works have stone-by-stone erected a steady foundation that will serve as a launching point for
1143    exciting new discoveries that help us fully appreciate the complexity of this powerful form of
1144    reproductive manipulation.

1145

1146

## Acknowledgments

This work was supported by National Institutes of Health awards R01 AI132581 and R01 AI143725, National Science Foundation award IOS 1456778, and the Vanderbilt Microbiome Initiative to S.R.B., a National Science Foundation Graduate Research Fellowship DGE-144519 to J.D.S., and a National Institutes of Health Ruth Kirschstein Postdoctoral Fellowship to B.A.L. Any opinion, findings, and conclusions or recommendations expressed in this material are those of the authors(s) and do not necessarily reflect the views of the National Institutes of Health, the National Science Foundation, or Vanderbilt University.

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