Symbiont-mediated Cytoplasmic Incompatibility:
What Have We Learned in 50 years?

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Abstract

Cytoplasmic incompatibility (CI) is the most common symbiont-induced reproductive manipulation. Specifically, symbiont-induced sperm modifications cause catastrophic mitotic defects in the fertilized embryo and ensuing lethality in crosses between symbiotic males and either aposymbiotic females or females harboring a different symbiont strain. However, if the female carries the same symbiont strain, then embryos develop properly, which imparts a relative fitness benefit to symbiont-transmitting mothers. Thus, CI drives maternally transmitted bacteria to high frequencies in arthropod species worldwide. In the past two decades, CI has experienced a boom in interest due in part to its (i) deployment in successful, worldwide efforts to reduce the spread of mosquito-borne diseases, (ii) causation by bacteriophage genes, *cifA* and *cifB*, that modify animal reproductive processes, and (iii) important impacts on incipient speciation. This review serves as a gateway to experimental, conceptual, and quantitative themes of CI and outlines significant gaps in our understanding of CI's mechanism that are ripe for investigation from a diversity of 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.
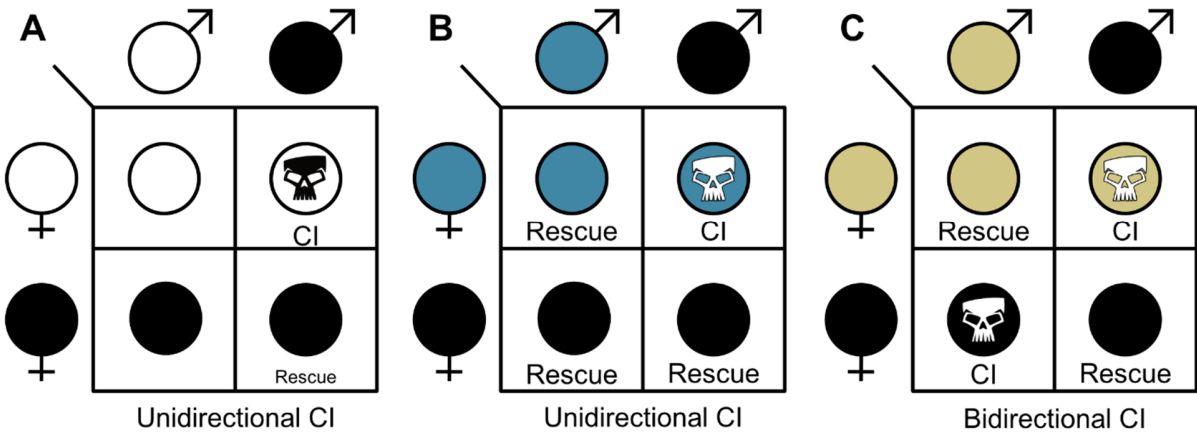
Figure 1. The three CI crossing relationships. (A) Unidirectional CI results in embryonic lethality when symbiont-containing males are crossed with aposymbiotic females. Rescue of this embryonic lethality occurs if the female carries a compatible symbiont strain. (B) In some cases, unidirectional CI 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 Rickettsia-like bacteria matching the description of *Wolbachia* in the eggs of symbiont-
83   bearing *Cu. pipiens* females (Yen and Barr, 1971). This finding led them to the breakthrough
84   hypothesis that CI is caused by this long-overlooked bacteria (Yen and Barr, 1971). They later
85   tested this hypothesis using crosses with antibiotic-treated and untreated *Cu. pipiens* mosquitoes
86   to reveal that CI is a symbiont derived phenotype caused by *Wolbachia* (Yen and Barr, 1973), thus
87   substantiating *Wolbachia* as the etiological agent of CI phenotypes. This initial characterization of
88   *Wolbachia* and CI in *Cu. pipiens* opened the floodgates, with many ensuing studies reporting cases
89   of CI-inducing *Wolbachia* in Diptera (Baton et al., 2013; Bian et al., 2013; Hoffmann et al., 1986;
90   Riegler and Stauffer, 2002), Hymenoptera (Betelman et al., 2017; Dittmer et al., 2016), Coleoptera
91   (Kajtoch and Kotásková, 2018), Hemiptera (Ju et al., 2017; Ramírez-Puebla et al., 2016),
92   Orthoptera (Martinez-Rodriguez and Bella, 2018), Lepidoptera (Arai et al., 2018; Hornett et al.,
93   2008), Thysanoptera (Nguyen et al., 2017), Acari (Gotoh et al., 2007, 2003; Vala et al., 2002),
94   Isopoda (Cordaux et al., 2012; Sicard et al., 2014), and Arachnids (Curry et al., 2015).

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

108   CI has attracted considerable applied interest in the last decade from scientists, companies,
109 and governments because it is at forefront of efforts to reduce the spread of dengue, Zika, and other
110 arboviral infections (Caragata et al., 2016; Crawford et al., 2020; Ford et al., 2019; Hoffmann et
111 al., 2011; O'Connor et al., 2012; O'Neill, 2018; Rasgon, 2007, 2008; Teixeira et al., 2008; WHO,
112 2016; Xi et al., 2005). Two CI-based vector control strategies are deployed worldwide. First, the

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

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

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

153 them into the framework of current literature. Under these models, we identify key predictions and
154 questions that motivate future areas of research to continue to build textbook knowledge on one of
155 the most widespread selfish adaptations of symbionts.
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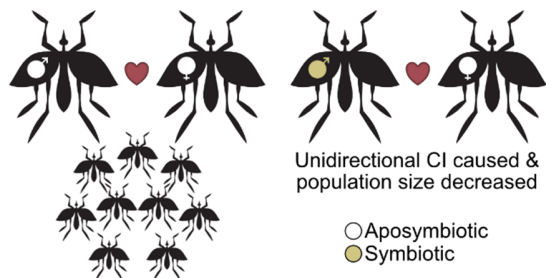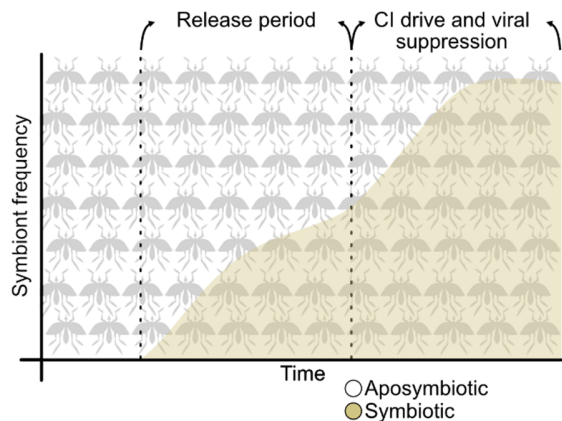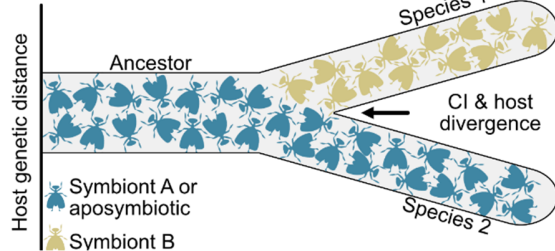
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158 **Figure 2. CI is important to vector control and reproductive isolation between species.** (A) The incompatible
159 insect technique is used to reduce population sizes (Crawford et al., 2020; Laven, 1967). Typically, two aposymbiotic
160 individuals will mate and produce viable offspring (left), but if symbiont-bearing males are released into the population
161 then they will cause unidirectional CI when they mate with aposymbiotic females (right) or bidirectional CI when they
162 mate with females harboring incompatible symbionts (not shown). This yields a reduction in egg hatching and
163 reduction in population size. (B) The population replacement strategy involves the release of both males and females
164 bearing CI-inducing and pathogen blocking symbionts (Hoffmann et al., 2011; O'Neill, 2018). After a period of
165 releases, CI will spread the symbiont to high frequencies where it can block the replication of human diseases. (C) CI-
166 inducing symbionts can cause reproductive isolation through bidirectional or unidirectional CI when different
167 individuals, populations, or species have different incompatible symbiont states (Bordenstein et al., 2001; Breeuwer
168 and Werren, 1990; Gebiola et al., 2017; Jaenike et al., 2006). This reproductive barrier reduces gene flow between
169 hosts with different symbiont states, allowing for their divergence.

170

What is the genetic basis of CI?

Identifying the CI and rescue genes

173 Until the past decade, the genetic basis of CI has remained elusive. The intangibility of the
174 CI genes was due in no small part to the inability to genetically engineer symbionts such as
175 *Wolbachia* (Iturbe-Ormaetxe et al., 2007; Thiem, 2014). Progress in CI genetics became possible
176 with the genomic sequencing of the *w*Mel *Wolbachia* of *D. melanogaster* in 2004 (Wu et al., 2004).
177 *w*Mel has a streamlined genome with numerous mobile elements including phages (Wu et al.,
178 2004). Notable among these elements was *Wolbachia*'s phage WO, which was first identified in
179 1978 via transmission electron microcopy of viral-like particles in symbiont-bearing *Cu. pipiens*
180 (Wright et al., 1978). Prophage WO encodes a set of proteins termed the Eukaryotic Association
181 Module that share homology to eukaryotic proteins, perhaps due to lateral gene transfer from
182 eukaryotes to the phage, and are predicted to interact with eukaryotic processes (Bordenstein and
183 Bordenstein, 2016). *w*Mel's genome is also enriched with ankyrin proteins that are involved in
184 protein-protein interactions in eukaryotes and are relatively rare in free living bacteria as compared
185 to intracellular bacteria and eukaryotes (Al-Khodor et al., 2010; Jernigan and Bordenstein, 2014).
186 Conversely, genomic sequencing of the mutualistic *w*Bm *Wolbachia* of *Brugia malayia* nematodes
187 revealed it did not contain phage WO nor an enrichment of ankyrins (Foster et al., 2005). These
188 findings suggested a correlation between reproductive parasitism and the presence of phage WO
189 and/or ankyrin genes and motivated hypotheses that they may be involved in CI (Foster et al.,
190 2005; Wu et al., 2004; Yamada et al., 2011). Numerous additional genome sequencing projects
191 would also be integral to identifying candidate genes for CI and rescue, including *w*Pip, *w*Au, *w*Ri,
192 *w*Ha, and *w*Rec (Klasson et al., 2009, 2008; Metcalf et al., 2014; Salzberg et al., 2009; Sutton et
193 al., 2014).

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

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

209 comparisons of the *w*Mel genome against the genome of the non-parasitic *w*Au strain of *D.*
210 *simulans* revealed nine genes absent in the non-parasitic strain that were present in *w*Mel (Sutton
211 et al., 2014). These candidates included numerous genes from *Wolbachia*'s prophage WO
212 including WD0631 which is a *w*Mel homolog of *w*Pip's WPIP0282, WD0632 which is adjacent
213 to WD0631, and a set of transcriptional regulators (Sutton et al., 2014). Thus, evidence continued
214 to build around phage WO genes as CI factors. Finally, sequencing of the *w*Rec genome revealed
215 a highly reduced prophage WO with approximately one-quarter the number of genes in a close
216 relative (Metcalf et al., 2014). These gene contained several previously described candidates
217 including the *w*Rec homolog of WPIP0282, four transcriptional regulators also absent in *w*Au, and
218 a *Wolbachia* transcriptional regulator gene *wtrM* linked to regulation of host meiosis in *Cu.*
219 *molestus* (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 is unknown if this lethality can be rescued. These data lend
259   support to the possibility that some strains may employ a model of CI wherein *cifB* is the CI-
260   causing factor and *cifA* is only the rescue factor. However, interpretation of these results is
261   significantly complicated by the absence of rescue data for any *cifB*-associated reduction in
262   embryonic hatching in insects. Moreover, mutagenesis analyses, described in further detail below,
263   indicate that changing conserved residues across CifA can crucially prevent CI-induction, lending
264   additional support for CifA's important role as a CI-inducing factor (Shropshire et al., 2020). More
265   functional genetic analyses will be necessary to provide evidence for an alternative to the Two-by-
266   One genetic model of CI. Hereafter, we will discuss relevant phenomena in the context of a Two-
267   by-One genetic model.
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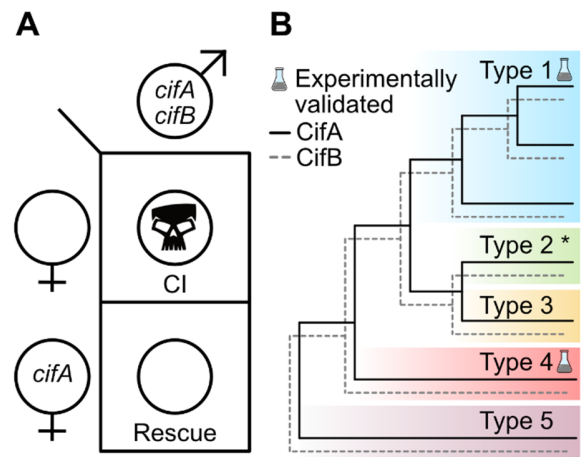
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270   **Figure 3. Two-by-One genetic model of Cif-induced CI and Cif phylogeny.** (A) The Two-by-One genetic model
271   of CI surmises that both CifA and CifB must be expressed in males to cause CI, and CifA must be expressed in females
272   to rescue CI (Shropshire and Bordenstein, 2019). (B) CifA and CifB coevolve and are classified into at least five
273   different phylogenetic Types (1-5) (Bing et al., 2020; LePage et al., 2017; Lindsey et al., 2018; Martinez et al., 2020).
274   To date, only Type 1 *cifs* from *w*Mel and *w*Pip, and Type 4 *cifs* from *w*Pip, have been experimentally confirmed to
275   cause and rescue CI (Beckmann et al., 2017; Chen et al., 2019; LePage et al., 2017; Shropshire et al., 2018; Shropshire
276   and Bordenstein, 2019). Moreover, unpublished results from JDS and SRB suggest that Type 2 *cifs* from *w*Ri are CI
277   and rescue-capable (denoted with an asterisk).

278

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

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280   Initial comparative sequence analysis of Cif proteins revealed that CifA and CifB have
281   concordant phylogenies with considerable divergence across three distinct phylogenetic clades
282   (LePage et al., 2017). Since then, the availability of additional genomes and Cif sequences have
283   exposed at least five clades referred to as Types 1-5 (Fig. 3B) (Bing et al., 2020; Lindsey et al.,
284   2018; Martinez et al., 2020), and highly divergent Cif-like homologs in *Orientia* and *Rickettsia*
285   bacteria (Gillespie et al., 2018). It is likely that continued genomic sequencing will reveal
286   additional Cif phylogenetic Types. The *w*Mel Cif proteins belong to the Type 1 clade, and *w*Pip
287   has both Type 1 and Type 4 Cif proteins. The phylogenetic classification of a *cif* gene can be
288   indicated with a T# in brackets to the right of the gene name (i.e., *cif*$_{wMel[T1]}$ or *cif*$_{wPip[T4]}$).

289   While the *cif* genes are associated with the Eukaryotic Association Module of prophage
290   WO or WO-like islands (Bordenstein and Bordenstein, 2016), Cif phylogeny is not concordant
291   with phage WO or *Wolbachia* phylogeny, potentially reflecting the typically high rates of inter-
292   and intragenic recombination in phage genomes (Bordenstein and Wernegreen, 2004; LePage et
293   al., 2017). Some *cif* genes are flanked by ISWpi1 transposons which may assist horizontal transfer
294   between WO-associated regions or *Wolbachia* strains, but it remains unclear if they alone are
295   responsible for divergence between the phylogeny of the *cif* genes, *Wolbachia*, and phage WO
296   (Cooper et al., 2019; Madhav et al., 2020). To date, only *cif* genes belonging to the Type 1 and 4
297   clades have been experimentally evaluated and confirmed to cause and rescue CI (Beckmann et
298   al., 2017; Chen et al., 2019; LePage et al., 2017; Shropshire et al., 2018; Shropshire and
299   Bordenstein, 2019). However, unpublished data suggests *cif* genes in the Type 2 clade can cause
300   and rescue CI (JDS and SRB, unpublished data). These studies suggest that despite considerable
301   divergence in sequence, proteins across the phylogenetic landscape of the Cifs remain capable of
302   causing and rescuing CI. Though, the phenotypic output of the Type 3, Type 5, and the
303   *Orientia*/*Rickettsia cif*-like genes have not been experimentally assessed in published works and it
304   remains unknown if they can contribute to CI phenotypes. However, the CI-inducing strains *w*No
305   of *D. simulans* and *w*Stri of *La. striatellus* only have Type 3 or Type 5 genes respectively (Bing et
306   al., 2020; LePage et al., 2017), suggesting that these genes may cause CI and rescue. Since CI has
307   not been reported in *Orientia* or *Rickettsia* species, it less likely these distant *cif*-like homologs
308   contribute to CI (Gillespie et al., 2018). Alternatively, other yet identified gene sets may be CI-
309   capable in CI-causing symbionts. Indeed, genomic and transcriptomic sequencing of CI-inducing
310   *Cardinium* reveals that they do not carry obvious homologs to the CI genes (Mann et al., 2017).
311   More functional genetic studies will be necessary to identify and assess the landscape of *cif*-like
312   genes and to identify alternative CI genes.

313

Relationships between cif *sequence diversity and CI phenotypes*

314

315   Theory predicts that selection should favor the maintenance of rescue, but not CI, when
316   *Wolbachia* are at equilibrium in the population since CI would no longer increase *Wolbachia*'s
317   prevalence in the population (Turelli, 1994). In this context, and under the Two-by-One genetic
318   model of CI in which *cifA* is involved in both CI and rescue while *cifB* is involved in CI, putative

319 loss-of-function mutations in CI will accumulate preferentially in *cifB*, though not universally,
320 relative to *cifA*. Consistent with this hypothesis, there are approximately twice as many putative
321 loss-of-function mutations in *cifB* relative to *cifA* (Martinez et al., 2020), suggesting that CI is
322 ablated more frequently than rescue. For example, the *w*PanMK and *w*PanCI *Wolbachia* strains of
323 *D. pandora* cause male-killing and CI respectively, but the male-killing strain has an early stop
324 codon in CifB that putatively inhibits function and may allow for the phenotypic switch from CI
325 to male-killing (Asselin et al., 2018). Additionally, the *w*Mau *Wolbachia* of *D. mauritiana* encodes
326 Type 3 Cif proteins (LePage et al., 2017; Meany et al., 2019) and does not cause CI, but it can
327 rescue CI caused by the closely-related *w*No of *D. simulans* (Bourtzis et al., 1998; Rousset and
328 Solignac, 1995; Zabalou et al., 2008). CifA$_{w\text{Mau}[T3]}$ sequence is identical to the CI and rescue-
329 capable *w*No *Wolbachia* strain, but CifB$_{w\text{Mau}[T3]}$ has a frameshift that introduces over ten stop
330 codons that in turn associates with the loss of CI (Meany et al., 2019). However, it is important to
331 note that two CI-capable *Wolbachia*, *w*Yak of *D. yakuba* and *w*Rec of *D. recens* (Cooper et al.,
332 2017; Shoemaker et al., 1999), have putative loss-of-function mutations in the form of truncations
333 in all of their *cifB* genes (Martinez et al., 2020). These results suggest that while the homologs
334 appear pseudogene-like, they may be functional. Indeed, dual expression of *cifA;B$_{w\text{Rec}[T1]}$*
335 transgenes in uninfected males yields a rescuable hatch rate reduction (JDS and SRB, unpublished
336 data). In sum, loss-of-function mutations are common in *cifB*, resulting in ablation of CI while
337 maintaining rescue. Moreover, evolution-guided mutagenesis assays across the Cif proteins reveal
338 that conserved residues in the CifA C-terminal domain of unknown function are crucial for CI-
339 induction. Thus, it is plausible that seemingly innocuous amino acid changes within this CifA
340 region may ablate CI and yet maintain rescue. Higher resolution comparative genomic analyses
341 coupled with phenotypic data will be necessary to evaluate this hypothesis. More work will also
342 be necessary to functionally assess the impacts of putative loss-of-function mutations on CI and
343 rescue capabilities.

344   While the above examples clearly highlight a relationship between *cif* sequence variation
345 and loss of CI, there are other strains that are more difficult to explain. For example, *w*Suz of *D.*
346 *suzukii* encodes both Type 1 and Type 2 *cif* genes that are highly similar to the strong CI-inducing
347 strain of *w*Ri, but *w*Suz does not cause CI (Cattel et al., 2018; Conner et al., 2017; Hamm et al.,
348 2014; Lindsey et al., 2018). While CifA$_{w\text{Suz}[T2]}$ has been disrupted by the insertion of a transposase,
349 the Cif$_{w\text{Suz}[T1]}$ gene pair remains intact and has only 2-4 amino acid substitutions relative to
350 Cif$_{w\text{Ri}[T1]}$ (Conner et al., 2017; Lindsey et al., 2018). In theory, the Type 1 gene set alone should be
351 CI-capable (Lindsey et al., 2018); though, Cif$_{w\text{Suz}[T1]}$ mutations may be in key residues for CI
352 expression. Notably, *w*Ri does not cause CI when transinfected into *D. suzukii* (Cattel et al., 2018).
353 Thus, the Cif$_{w\text{Suz}[T1]}$ proteins may be able to cause CI, but their effects are inhibited by suppressors
354 encoded in the *D. suzukii* genome. In a separate example, the triple-strain infection of *w*AlbA,
355 *w*AlbB, and *w*Mel in *A. albopictus* can cause CI but cannot self-rescue (Ant and Sinkins, 2018).
356 Since each of the individual *Wolbachia* strains in this triple-strain infection can cause CI and are
357 self-compatible in *A. aegypti* (Ant and Sinkins, 2018), neither genetic variation in the *cif* genes
358 alone nor host suppressors can explain the emergence of self-incompatibility. However, it is

359   plausible that some *Wolbachia* may inhibit the reproductive manipulations of other co-infecting
360   strains, but this requires further testing. In summary, the relationships between *Wolbachia* strains
361   and their hosts are likely to have an impact on CI. Additional work is necessary to answer these
362   persistent questions: how does superinfection impact CI expression, how does the host act to
363   suppress CI phenotypes, and what are the evolutionary dynamics that govern these interactions?
364

The genetic basis of bidirectional CI remains unknown

366   There is strong evidence for the genetic basis of unidirectional CI between *Wolbachia*-
367   bearing and aposymbiotic insects (Fig. 1A) (Beckmann et al., 2017; Chen et al., 2019; LePage et
368   al., 2017; Shropshire et al., 2018; Shropshire and Bordenstein, 2019). However, the genetic basis
369   of bidirectional CI between arthropods with different symbiont strains remains poorly understood
370   (Fig. 1B,C). Sequence divergence in CI-associated factors has long been thought to be a
371   contributing factor to these incompatibilities, namely that divergence in both CI-causing and
372   rescue-causing genes would be required for bidirectional CI to emerge (Charlat et al., 2001).
373   Indeed, phylogenetic analyses of *cif* genes reveal that strains carrying similar alleles tend to be
374   compatible, strains with more distantly related *cif* genes are not, and a single *Wolbachia* strain can
375   have multiple unique *cif* gene pairs (Bonneau et al., 2018a, 2019; LePage et al., 2017). For
376   instance, when *w*Mel is transinfected into a *D. simulans* background, it is unidirectionally
377   incompatible with the native *w*Ri strain, wherein *w*Ri can rescue *w*Mel-induced CI but the
378   reciprocal cross is incompatible (Poinsot et al., 1998). Intriguingly, *w*Ri carries Type 1 *cif* genes
379   closely related to *w*Mel's and a divergent Type 2 gene pair. Thus, it is plausible that *w*Ri can rescue
380   *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
381   gene for the Type 2 gene pair (LePage et al., 2017). Additionally, population genetic analyses of
382   *cif* genes in *w*Pip reveal that there are numerous unique strains, each strain carries multiple closely
383   related *cif* variants that belong to Type 1 and Type 4 *cif* clades, and a single genetic variant of
384   CifB$_{w\text{Pip[T1]}}$ correlates with the inability of one strain of *w*Pip to rescue CI caused by a divergent
385   *w*Pip strain (Atyame et al., 2011b; Bonneau et al., 2018a, 2019). However, while these data suggest
386   that *cif* genetic variation and/or copy number contributes to strain incompatibility, it remains
387   possible that the considerable host genotypic variation between these incompatible populations
388   contributes to these relationships in a way that also correlates with *cif* genotypic diversity (Atyame
389   et al., 2011a). More reductionist functional studies that control for variation in host genotype will
390   be necessary to confirm that *cif* sequence variation alone can explain CI relationships.

391   Historically, CI and rescue were thought to be caused by different genes, and that
392   divergence in both genes would be required for bidirectional CI to evolve relative to an ancestral
393   strain (Charlat et al., 2001). Thus, this model for bidirectional CI requires two steps: one mutation
394   for CI and one for rescue. A major limitation of this model is that the intermediate state, wherein
395   only one of the two phenotypes have shifted, is self-incompatible and represents a 'maladaptive
396   valley' unlikely to persist as a rare variant. Given the abundance of bidirectionally incompatible
397   strains across the arthropod *Wolbachia* (Atyame et al., 2011b; Bordenstein and Werren, 2007;
398   Branca et al., 2009; O'Neill and Karr, 1990; Sicard et al., 2014), and the rarity of so-called

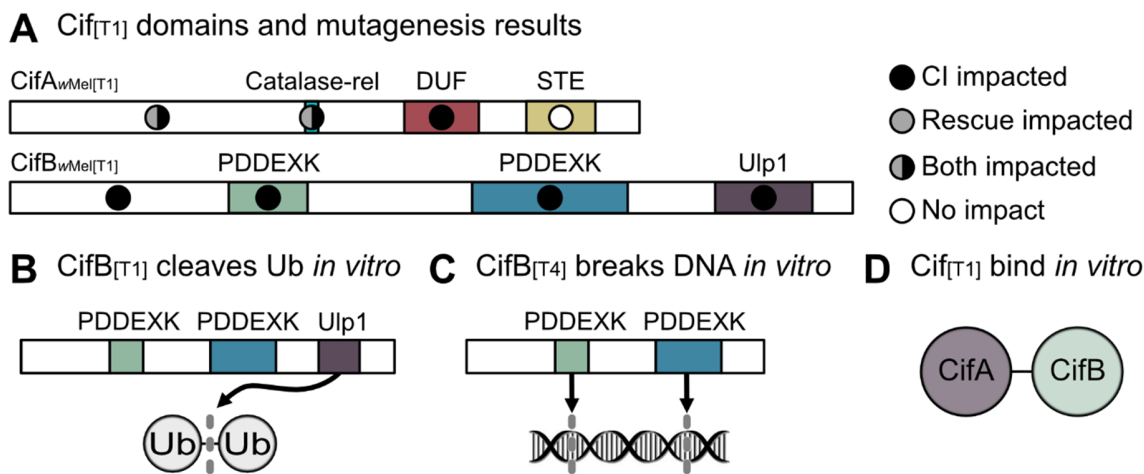
399   'suicidal' self-incompatible strains (Zabalou et al., 2008), crossing this maladaptive valley may be
400   an unlikely evolutionary scenario. In contrast, since CifA is involved in both CI and rescue, it
401   becomes possible for a single mutation that affects CI to also impact rescue. Thus, a single
402   mutation in CifA may shift both CI and rescue phenotypes, yield bidirectional CI relative to an
403   ancestor, and maintain self-compatibility (Shropshire et al., 2018). Indeed, mutagenesis of highly
404   conserved amino acids across CifA reveal that sites within CifA's N-terminal region are crucially
405   important for the expression of both CI and rescue, suggesting that residues in this single region
406   are coopted for both phenotypes (Shropshire et al., 2020). Notably while this one-step model of
407   bidirectional CI avoids the maladaptive valley, it may only spread if transferred into a new
408   aposymbiotic (sub)population since emergence of a new incompatibility type within a symbiont-
409   bearing population would be immediately incompatible with the more common symbiont in the
410   population. More research is needed to fully understand the genetic basis of bidirectional CI and
411   its evolution. For instance, theoretical modeling will be necessary to evaluate additional routes of
412   emergence as it relates to Cif sequence variation, functional genetic assays can be used to unravel
413   the correlation between *cif* sequence variation and (in)compatibility relationships, and population
414   genetic surveys coupled with population dynamics modeling would reveal when a novel variant
415   would be likely to persist in a population.

416

What is the mechanistic basis of Cif-induced CI?

CifA molecular function

419   Structural homology-based analyses suggest that Type 1 CifA have three putative domains:
420   a catalase-related (catalase-rel) domain involved in the degradation of reactive oxygen species, a
421   domain of unknown function (DUF) 3243 with homology to a Puf-family RNA-binding domain
422   (RBD), and a sterile-like transcriptional regulator (STE) (Fig. 4A) (Lindsey et al., 2018). While
423   the catalase-rel domain is unique to the $CifA_{[T1]}$, the STE is maintained in Type 1-4 genes (Lindsey
424   et al., 2018), and the Puf-family RBD exists in Type 1-5 genes (Bing et al., 2020; Martinez et al.,
425   2020). Importantly, these annotations are of low predictive value (20-30% probability) and may
426   not withstand experimental testing (Lindsey et al., 2018). On the other hand, sliding window
427   analyses of selection for $CifA_{[T1]}$ suggest that while the full protein is under purifying selection,
428   the catalase-rel domain and the unannotated N-terminal region are under the strongest selection
429   (Shropshire et al., 2018). Indeed, CifA cannot contribute to transgenic CI or rescue when
430   conserved amino acids are mutated within the unannotated N-terminal region or in the putative
431   catalase-rel domain of $CifA_{wMel[T1]}$ (Shropshire et al., 2020). Conversely, when sites are mutated
432   in CifA's DUF domain, it maintains the ability to contribute to rescue, but loses CI-capability
433   (Shropshire et al., 2020). Thus, CifA's N-terminal region is crucially important for both CI and
434   rescue, whereas sites within the DUF domain are only crucial for CI (Fig. 4A). More work will be
435   necessary to determine how and why these mutations impact these phenotypes, but the annotations
436   provided above afford a set of testable hypotheses and questions. For instance, does CifA interact
437   with reactive oxygen species both in the context of CI and rescue, and/or does RNA-binding occur

438   in the context of CI? Biochemical assays testing for these functions will further elucidate how
439   CifA contributes to CI and rescue phenotypes.
440   Additionally, how CifA is involved in both CI and rescue remains largely a mystery. The
441   simplest explanation is that CifA maintains the same function in both CI and rescue. Under this
442   framework, CifA would act on a pathway that can be modified during spermatogenesis and
443   oogenesis to produce opposite affects (Shropshire et al., 2018). If CifA were to drive such a
444   function, then CifB's role in CI would seemingly be auxiliary and perhaps only necessary for
445   localization of CifA to particular targets or, since CifB acts as a deubiquitinase, to protect CifA
446   from degradation by ubiquitin pathways (Beckmann et al., 2017). Alternatively, CifA may be a
447   multi-functional protein that employs one set of functions to cause CI and another to cause rescue
448   (Shropshire et al., 2018). For instance, if CifA targets sex-specific host pathways, CifA can only
449   affect its host in a particular way if that target is available. Additionally, CifA may be modified in
450   some manner that differs between the testes and the ovaries, unlocking unique biochemical
451   functions by posttranslational modification, localization differences, or the expression of different
452   protein conformational isoforms (Shropshire et al., 2018). In summary, little is known about
453   CifA's functional role in CI and rescue, and considerable work is necessary not only to identify its
454   enzymatic capabilities, but also to further elucidate how it can act both to cause and prevent CI.
455
456

457
458
459   **Figure 4. Biochemical characterization of Cif proteins.** (A) Annotated domains in the CifA and CifB proteins and
460   the relative importance of conserved residues in each domain for CI (black circles), rescue (gray circles) or neither
461   phenotype (white circles) as determined by transgenic expression of mutated proteins in aposymbiotic *D.
462   melanogaster* (Beckmann et al., 2017; Shropshire et al., 2020). (B) CifB[T1] can cleave ubiquitin chains via its Ulp1
463   deubiquitinase domain *in vitro* (Beckmann et al., 2017). (C) CifB[T4] nuclease domains can cause DNA breaks *in vitro*
464   (Chen et al., 2019). (D) CifA[T1] and CifB[T1] bind each other *in vitro* (Beckmann et al., 2017). Domain architecture is
465   based on homology-based analyses and is of low predictive value (20-30% probability) for CifA (Lindsey et al., 2018),
466   and CifB[T1] PDDEXK nuclease domains lack canonical PD-(D/E)XK motifs (Beckmann et al., 2017), but remain
467   structurally homologous to other PDDEXK nucleases (Lindsey et al., 2018).

468

CifB molecular function

CifB[T1] from both *w*Mel and *w*Pip encode a single putative ubiquitin-like protease (Ulp1) domain (Beckmann et al., 2017; LePage et al., 2017; Lindsey et al., 2018). The Ulp1 domain was later expressed separate from the rest of the protein in *E. coli* and purified for downstream *in vitro* ubiquitin cleavage assays (Beckmann et al., 2017). When exposed to a variety of ubiquitin chains, it was revealed that the Ulp1 cleaves K6-, K11-, K27-, K29-, K33-, K48-, and K63-linked ubiquitin *in vitro*, but with a preference for K63 chains (Fig. 4B) (Beckmann et al., 2017). K63 chains are associated with NF-κB signaling which has diverse functions including innate immunity, DNA transcription, autophagocytosis (Tan et al., 2008; Wertz and Dixit, 2010), and proliferation of cell nuclear antigen (PCNA) (Ripley et al., 2020) that has previously been shown to act abnormally in CI-affected embryos (Landmann et al., 2009). A single amino acid mutation in the catalytic site of 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, if anything, inside reproductive tissue cells and how this deubiquitination contributes to CI. It is also interesting that nuclear localization of the male-killing protein Spaid in the endosymbiont *Spiroplasma poulsoni*i is impacted by a domain annotated as a deubiquitinase, suggesting that host ubiquitin regulation or localization to host nuclei is important for reproductive manipulation (Harumoto and Lemaitre, 2018). Future biochemical assays will help answer these persistent questions.

While CifB's Ulp1 is seemingly important for CI, only CifB[T1] have this domain. Moreover, 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; Martinez et al., 2020). 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).

509   Additionally, despite *w*Pip containing both Type 1 and 4 genes, there are no notable differences in
510   cytological embryonic defects caused when both genes are expressed as compared to other strains
511   that only have CifB$_{[T1]}$, suggesting that these genes yield similar cytological outcomes (Bonneau
512   et al., 2018b). Biochemical assays will be necessary to evaluate the nuclease activity of a diverse
513   array of CifB proteins including CifB$_{[T1]}$ because the conserved areas in and around the PDDEXK
514   domains across all Cif Types likely persist because of a common function that underpins CifB's
515   involvement in CI.

516

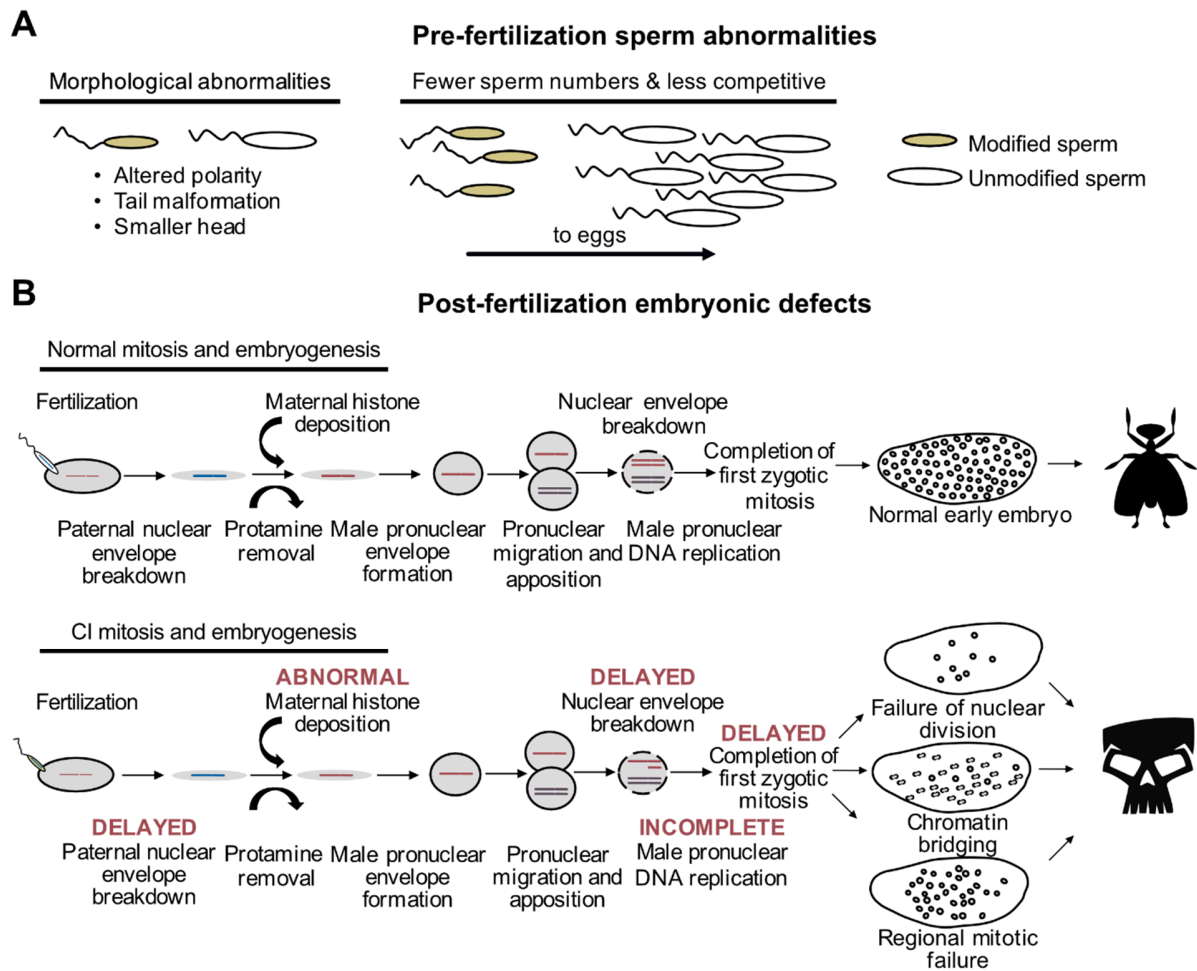
517   *Cif interacting partners*

518   A list of putative Cif protein binding partners have recently been reported. While CifA$_{[TI]}$
519   and CifB$_{[T1]}$ bind *in vitro* (Fig. 4D) (Beckmann et al., 2017), it does, however, remain unknown if
520   CifA binds CifB *in vivo* in the testes to cause CI or if maternal CifA binds to paternal CifB in the
521   embryo to cause rescue (Beckmann et al., 2019a, 2019b; Shropshire et al., 2019). More work on
522   the localization, co-localization, and binding profiles of these proteins will elucidate this question.
523   Additionally, Cifs appear to bind to a suite of host proteins that differ based on if CifA and CifB
524   are expressed alone or together. 67 host proteins were identified as Cif binding partners under co-
525   expression of CifA and CifB, whereas 45 proteins were identified with CifB expression alone in
526   pools of male and female *D. melanogaster* (Beckmann et al., 2019c). Karyopherin-α (Kap-α) is
527   notable among these proteins. It bound to singly expressing CifB extracts, and its overexpression
528   in aposymbiotic females yielded partial rescue (~20% hatch rate improvement) when crossed to
529   transgenic CI males (Beckmann et al., 2019c). Kap-α is a nuclear import receptor and a regulator
530   of p53 which has roles in the protamine-histone exchange process (Beckmann et al., 2019c;
531   Emelyanov et al., 2014). Intriguingly, delayed H3 histone deposition is a hallmark of CI during
532   early embryogenesis (Landmann et al., 2009), suggesting a relationship between CifB, Kap-α, p53
533   and histone-associated abnormalities in CI. However, it is important to emphasize that, Kap-α was
534   only pulled down when CifB was singly expressed (Beckmann et al., 2019c), suggesting that while
535   Kap-α overexpression may influence rescue-efficiency, it is unclear how it would be part of the
536   rescue mechanism since CifB is not necessary for rescue to occur. More work is essential to
537   determine if CifB's binding to Kap-α contributes to CI and how Cif binding to other host proteins
538   relates to CI and rescue phenotypes.

539

540   # What is the cytological basis of CI?

541   Decades of research have characterized an in-depth understanding of CI-associated
542   cytological abnormalities. These studies broadly define alterations during spermatogenesis (Fig.
543   5A) and embryogenesis (Fig. 5B), suggesting that CI is associated with a sperm modification prior
544   to fertilization that results in embryonic defects and death. Importantly, the causes of the reported
545   sperm and embryonic abnormalities remain unknown and, in most studies, it is unclear if these
546   observations are directly related to the Cif proteins or are a byproduct of *Wolbachia* symbionts in
547   the testes. However, these findings provide insight into the ways in which *Wolbachia* and CI

548 influence host reproduction and fertility. Below, we review the cytological changes that occur in
549 spermatogenesis and embryogenesis during CI and rescue, and highlight areas where future
550 research is crucially needed with reductionist assays to disentangle effects of *Wolbachia* symbiosis
551 and CI.
552

553
554 **Figure 5. CI-associated defects occur pre- and post-fertilization.** (A) In males harboring *Wolbachia*, there are
555 several types of sperm abnormalities when compared to their aposymbiotic counterparts. (B) When fertilized with
556 sperm derived from *Wolbachia*-carrying males, embryonic nuclear defects result in the form of delayed paternal
557 nuclear envelope breakdown, abnormal histone deposition and other early mitotic events. These defects then cause
558 embryonic phenotypes observed in CI including chromatin bridging and regional mitotic failures.

559

CI-associated abnormalities prior to fertilization

561   Spermatogenesis is a highly regulated process. It begins with cells of the germline stem
562 cell niche (GSCN) replicating into spermatogonia that subsequently undergo mitosis to yield a
563 spermatocyst with 16 spermatocytes (Fuller, 1993; Hackstein, 1987; Lindsley, 1980). Each
564 spermatocyte in the cyst then undergoes two rounds of meiosis to form four spermatids, for a total
565 of 64 spermatids in each cyst. The spermatids then undergo elongation where the sperm tail forms

566   and histones are replaced with protamines for tight packaging of DNA in the nucleus within the
567   sperm head (Rathke et al., 2014). In the final stages of sperm maturation, spermatids undergo
568   individualization to remove excess cytoplasm before becoming mature sperm to enter the seminal
569   vesicle for storage before mating. Impacts of *Wolbachia* on spermatogenesis can result in
570   downstream sperm defects that may be connected to CI (Fig. 5A). For example, symbiont-bearing
571   *D. simulans* flies and *Ephestia* moths produced fewer sperm, and stronger CI was associated with
572   more sperm transfer during copulation in *D. simulans* (Awrahman et al., 2014; Lewis et al., 2011;
573   Snook et al., 2000). When *D. simulans* females mated with *Wolbachia*-bearing and aposymbiotic
574   males, the sperm of aposymbiotic males were more likely to fertilize eggs (Champion de Crespigny
575   and Wedell, 2006), suggesting that *Wolbachia*-modified sperm are less competitive. *Wolbachia*-
576   affected sperm cysts exhibit abnormal morphology with some sperm fused together and other
577   sperm exhibiting randomly-oriented, axoneme-mitochondrial complexes that are responsible for
578   sperm motility (Riparbelli et al., 2007), perhaps explaining fertility defects and variation in sperm
579   competition. However, key questions remain. Are *cif* gene products responsible for the
580   aforementioned sperm abnormalities? If these defects are not caused by the *cif* genes, and are
581   instead a byproduct of other *Wolbachia*-host associations, what is their significance, if any, to
582   reproductive parasitism? In summary, a deeper investigation of these defects as they relate to CI
583   products is needed to confirm their link to CI.

584   Interestingly, *Wolbachia* are not symmetrically distributed in testes, with only some
585   spermatocysts harboring symbionts in the strong CI-inducing *w*Ri strain of *D. simulans* (Clark et
586   al., 2003). Indeed, *w*Ri is almost exclusively localized to the GSCN, and some GSCN remain
587   aposymbiotic, suggesting that the Cif proteins must either act early in spermatogenesis or are
588   diffusible factors that can stably travel into later stages of spermatogenesis (Clark et al., 2003,
589   2002; Riparbelli et al., 2007). *Wolbachia* are stripped during the individuation process and moved
590   into waste bags where they are presumably degraded (Riparbelli et al., 2007). Not only does this
591   suggest that *Wolbachia* create a diffusible factor that interacts with sperm or spermatogonia to
592   cause CI, but it also helps to explain why paternal *Wolbachia* transfer has not been observed (Yeap
593   et al., 2016) with rare exceptions such as in hybrid *Nasonia* wasps and in transinfected *A. aegypti*
594   (Chafee et al., 2011; Ross et al., 2019a). Future work investigating the localization of CifA and
595   CifB, will determine when and where the Cif proteins act to cause CI and whether they are
596   transferred to the embryo for the potential to directly cause defects during embryogenesis.

597

598   *CI-defining abnormalities after fertilization*

599   Abnormalities that define the post-fertilization events underpinning CI are observed during
600   embryogenesis (Fig. 5B). In chronological order, CI-affected embryos experience abnormal
601   maternal H3.3 histone deposition on the male pronucleus, delayed activation of the DNA
602   polymerase cofactors PCNA and cell cycle regulator Cdk1 resulting in incomplete DNA
603   replication, delayed nuclear envelope breakdown prior to the first mitosis, and a delay in the first
604   mitotic event (Landmann et al., 2009; Tram and Sullivan, 2002). These defects often culminate in
605   a chromatin bridging phenotype during the first mitosis, shredding the paternal nuclei and leading

606   to embryonic arrest (Breeuwer and Werren, 1990; Callaini et al., 1996; Lassy and Karr, 1996; Reed
607   and Werren, 1995; Ryan and Saul, 1968; Tram et al., 2006). Notably, it remains unknown what
608   the most proximal event is during CI. It is plausible that abnormal histone deposition is the first
609   CI-causing event during embryogenesis that leads to a cascade of effects culminating in the other
610   embryonic abnormalities, but there remain open questions. For instance, how do the Cif proteins
611   interact with the host to cause abnormal histone deposition? Are the Cif proteins even transferred
612   with the sperm so that they can cause these defects directly, or are these defects caused by an initial
613   Cif interaction occurring during spermatogenesis? Finally, if Cifs do directly cause abnormal
614   histone deposition, how are these affects rescued by the presence of CifA expressed in the embryo?
615   A combination of cytological, transgenic, and biochemical assays may be necessary to evaluate
616   these questions.

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

645   *Cardinium*. Additional cytological and reductionistic studies will be necessary to evaluate the
646   cause of this variation and determine how the Cif proteins contribute to these phenotypes.
647

What is the host's contribution to CI?

649   It is common that researchers leverage correlations between *Wolbachia* symbiont state and
650   host expression phenotypes (RNA, protein, etc.) to understand how *Wolbachia* impact their host.
651   When differential expression is correlated with CI phenotypes, these data can yield valuable
652   insights regarding CI's mechanism. Significant correlations between *Wolbachia* symbiont state
653   and host expression have been measured in *D. melanogaster* (Biwot et al., 2019; He et al., 2019;
654   LePage et al., 2014; Liu et al., 2014; Ote et al., 2016; Xi et al., 2008; Yuan et al., 2015; Y. Zheng
655   et al., 2019; Zheng et al., 2011), *D. simulans* (Brennan et al., 2012; Clark et al., 2006; Xi et al.,
656   2008), *La. striatellus* (Huang et al., 2019; Ju et al., 2017; Liu et al., 2019), *T. urticae* (Bing et al.,
657   2019; Zhang et al., 2015)*, Cu. pipiens* (Pinto et al., 2013)*, and A. albopictus* (Baldridge et al., 2017,
658   2014; Brennan et al., 2012, 2008). Challengingly, as many as 1613 transcripts are differentially
659   expressed between *Wolbachia* symbiont states (Bing et al., 2019), and as with the cytological
660   abnormalities described above, it is difficult to untangle the effects of *Wolbachia* and CI on host
661   expression profiles.

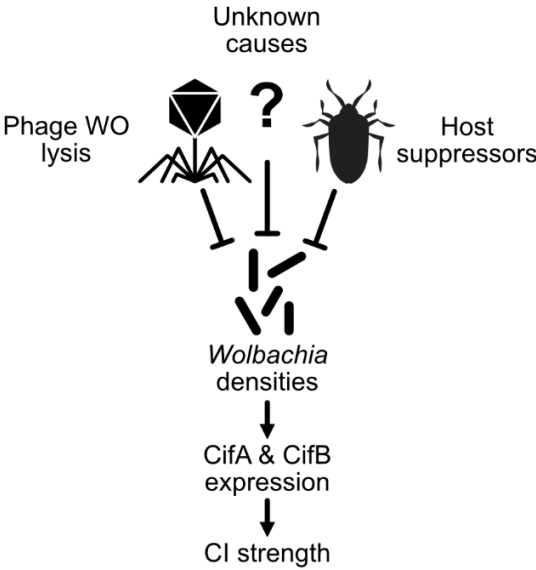
662   However, the most promising candidates associated with CI are those that can be
663   experimentally over- or under-expressed to recapitulate CI-like hatch rates and cytological defects.
664   For example, overexpression of the tumor suppressor gene *lethal giant larvae* [l(2)gl] and myosin
665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

685   In addition to RNA and/or protein expression differences, changes in host physiology and
686 cell biology have also been correlated with CI. For example, *Wolbachia*-bearing *D. melanogaster,*
687 *D. simulans*, *A. albopictus*, *A. polynesiensis*, and *T. urticae* males often have higher reactive
688 oxygen species (ROS) in their testes than aposymbiotic males (Brennan et al., 2012, 2008; Zug
689 and Hammerstein, 2015). It has been hypothesized that this variation in ROS expression patterns
690 is due to an elevated host immune response to *Wolbachia* symbiosis (Zug and Hammerstein, 2015).
691 However, multiple lines of evidence link ROS expression with CI. For example, increased ROS
692 levels are consistently observed among CI-inducing strains (Zug and Hammerstein, 2015), and
693 ROS leads to DNA damage in spermatocytes in *D. simulans* (Brennan et al., 2012). Additionally,
694 lipid hydroperoxide markers of ROS-induced oxidative damage are higher in symbiont-bearing *D.*
695 *melanogaster* (Driver et al., 2004), and PCNA retention is another marker for DNA damage and
696 is observed during the first mitosis of CI-affected embryos (Landmann et al., 2009). Interestingly,
697 overexpression of the *D. melanogaster* gene *key* increases ROS levels and DNA damage in males
698 when mimicking rescuable CI-like hatching and embryonic defects (Biwot et al., 2019). Together,
699 these data support a role for ROS in CI's mechanism, but direct connections remain unclear. One
700 hypothesis is that CifA's putative catalase-related domain does indeed function to interact with
701 ROS (Lindsey et al., 2018). Though, alternatively, ROS may be a byproduct of the host immune
702 response (Zug and Hammerstein, 2015). Biochemical and immunological assays will unravel these
703 relationships.

704

What causes variation in CI strength?

706   Some *Wolbachia* strains exhibit CI that can vary between 10-100% embryonic death
707 (Awrahman et al., 2014; Clark et al., 2003; Cooper et al., 2017; Hoffmann, 1988; Layton et al.,
708 2019; Reynolds and Hoffmann, 2002; Turelli et al., 2018; Yamada et al., 2007; Zabalou et al.,
709 2004). In fact, a number of *Wolbachia,* including *w*Mel of *D. melanogaster* and *w*Yak of *D.*
710 *yakuba*, were initially characterized as non-parasitic since they had minimal to no impact on
711 embryonic hatching (Charlat et al., 2004; Holden et al., 1993; Zabalou et al., 2004). Later studies
712 would correct these early reports to suggest they can indeed cause CI, but their CI strength is highly
713 dependent on a variety of factors including the age of fathers and grandmothers (Cooper et al.,
714 2017; Layton et al., 2019; Reynolds and Hoffmann, 2002). Generally speaking, the work reviewed
715 below describes a complex relationship between biotic and abiotic factors that influence CI
716 strength. Notably, the bacterial density model of CI, whereby *Wolbachia* densities positively
717 correlate with CI strength, is the major factor driving most of these relationships (Breeuwer and
718 Werren, 1993). Phage WO lysis, host suppressors, and other undescribed interactors may control
719 the variation in *Wolbachia* titers (Fig. 6) (Awrahman et al., 2014; Bordenstein and Bordenstein,
720 2011; Funkhouser-Jones et al., 2018; Layton et al., 2019; Poinsot et al., 1998; Walker et al., 2011).
721 However, there are instances where CI strength variation does not correlate with *Wolbachia*
722 densities (Yamada et al., 2007). Below, we review these works and describe what is known and
723 unknown about the proximal basis of CI strength variation.

724

725
726   **Figure 6. An expanding *Wolbachia* density model of CI strength variation.** The proximal cause of CI is likely
727   CifA and CifB, whose expression level has been connected with intensity in transgenic studies (LePage et al., 2017).
728   *Wolbachia* densities have often correlated with factors that influence CI strength variation (Werren, 1997). In many
729   cases, it remains unknown how these factors influence *Wolbachia* densities. Phage WO lysis (Bordenstein and
730   Bordenstein, 2011) and host suppressors are well documented correlates or causes of density changes (Funkhouser-
731   Jones et al., 2018; Poinsot et al., 1998; Walker et al., 2011).

732

733   *Temperature*

734   Temperature is often correlated with CI strength and is likely to contribute to the dynamics
735   that govern *Wolbachia*'s spread (Foo et al., 2019). High temperatures, usually exceeding 27°C,
736   can have a significant negative impact on CI strength in *Wolbachia*-carrying *A. aegypti* (Ross et
737   al., 2020, 2019b), *T. urticae* (van Opijnen and Breeuwer, 1999), *D. simulans* (Hoffmann et al.,
738   1986), *D. melanogaster* (Reynolds and Hoffmann, 2002), *A. scutellaris* (Trpis et al., 1981; Wright
739   and Wang, 1980), *A. albopictus* (Wiwatanaratanabutr and Kittayapong, 2009), and *Nasonia*
740   (Bordenstein and Bordenstein, 2011). There is considerable evidence that high temperature
741   impacts *Wolbachia* densities in various species including *A. albopictus* and *A. aegypti* (Foo et al.,
742   2019; Ross et al., 2020, 2019b), *N. vitripennis* (Bordenstein and Bordenstein, 2011), and *T. urticae*
743   (Lu et al., 2012). High temperatures even cure hosts *of Wolbachia* (Jia et al., 2009). In natural
744   populations of the butterfly *Zizeeria maha*, *Wolbachia* densities vary with season*,* and climate
745   change may be contributing to a decrease in symbiont frequencies in the tropics (Charlesworth et
746   al., 2019; Sumi et al., 2017). Notably in *N. vitripennis* and *T. urticae*, decreased *Wolbachia*
747   densities and CI strength have also been correlated with an increase in phage WO lytic activity
748   with higher temperatures (Bordenstein and Bordenstein, 2011; Lu et al., 2012). Cooler
749   temperatures at or below 19°C have also been associated with decreased CI in *D. simulans* and *N.*
750   *vitripennis* (Bordenstein and Bordenstein, 2011; Reynolds and Hoffmann, 2002). As with warm
751   temperatures, cooler temperatures also yield increased phage WO densities, decreased *Wolbachia*

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

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

774

Host behavior and development

776   Other correlates of CI strength variation are related to male and paternal grandmother age
777   (Awrahman et al., 2014; Layton et al., 2019; Reynolds and Hoffmann, 2002), male mating rate
778   (Awrahman et al., 2014; De Crespigny et al., 2006), male developmental timing (Yamada et al.,
779   2007), rearing density (Yamada et al., 2007), and nutrition (Clancy and Hoffmann, 1998). All of
780   these factors are significantly impacted by the structure of the population, resource availability, or
781   behavior. Below, we will systematically discuss what, if anything, is known about how each of
782   these factors impact CI strength.

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

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

795   Interestingly, while older males have fewer *Wolbachia*, older virgin females have more
796   (Layton et al., 2019). In fact, when females are aged longer prior to mating, their male offspring
797   are laid with higher *Wolbachia* densities and resultantly induce stronger CI (Layton et al., 2019).
798   This phenomena has been termed the paternal grandmother age effect (PGAE) (Layton et al.,
799   2019). It is unclear why age's impact on *Wolbachia* density is sex-specific. However, the
800   relationship between male age, symbiont densities, and CI strength may not be generalizable across
801   all CI-inducing symbionts and their hosts. For instance, *Cardinium* of *E. pergandiella* cause CI
802   that is unaffected by male age (Perlman et al., 2014), and studies disagree about the significance
803   of the impact of age on CI caused by *w*Ri of *D. simulans* (Awrahman et al., 2014; Binnington and
804   Hoffmann, 1989; Bressac and Rousset, 1993). Thus, the impacts of age on symbiont densities and
805   CI may be limited to some *Wolbachia* or alternatively, to particular host backgrounds. More
806   comparative phenotypic work will be needed to understand the broader context of the relationship
807   between age, CI strength, and *cif* expression.

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

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

832   densities than other cells in the testes (Clark et al., 2002). Alternatively, *Wolbachia* densities of
833   the adult male may be less informative than density differences during embryonic or larval
834   development. For instance, the PGAE, as described above, revealed that sons of older females
835   caused stronger CI and while their sons did not have higher *Wolbachia* densities as adults, they
836   did have higher densities during embryogenesis (Layton et al., 2019). Intriguingly, *Wolbachia*
837   densities rapidly declined in aged females after mating and embryo laying, suggesting that many
838   *Wolbachia* were transferred from the ovaries to the developing egg and ultimately embryo. Thus,
839   it is plausible that *Wolbachia* densities would correlate with deposition order such that first laid
840   older brothers would have higher densities than younger brothers laid soon after (Layton et al.,
841   2019). While these hypotheses remain associated and to be formally tested, it is also notable that
842   the YBE does not appear to apply to *w*Ri of *D. simulans* (Yamada et al., 2007)*,* other studies have
843   failed to replicate these results in other *w*Mel-bearing *D. melanogaster* lines (LePage et al., 2017),
844   and the opposite phenotype is observed with *Cardinium* of *Encarsia* where older brothers cause
845   weaker CI (Perlman et al., 2014). Thus, additional work is necessary to replicate the YBE in *D.*
846   *melanogaster* and other symbiont-host combinations and to evaluate its cause via longitudinal
847   developmental studies of *Wolbachia* densities. Moreover, understanding why *Cardinium* and
848   *Wolbachia* CI are differentially impacted by these factors is important in determining how
849   symbiont dynamics relate to reproductive manipulation.

850   Finally, rearing density and nutrition can also impact CI strength relationships. For
851   instance, when *w*Mel-bearing *D. melanogaster* are reared in high densities, CI strength is lower
852   than if they are reared in low densities (Yamada et al., 2007). The initial hypothesis behind this
853   correlation was that high density rearing led to nutritional stress which translated to less *Wolbachia*
854   (Yamada et al., 2007). While this hypothesis has not been explicitly tested, there is a reasonable
855   logical framework behind it. Indeed, multiple studies have shown that *D. simulans* males exposed
856   to nutritional stress have weaker CI than males with abundant resources (Clancy and Hoffmann,
857   1998; Sinkins et al., 1995). Notably, nutritional stress is also correlated with reduced *Wolbachia*
858   densities, supporting models, as above, where *Wolbachia* densities and *cif* expression are the
859   proximal factors driving the relationship to CI strength. That said, it is important to note that recent
860   microscopy studies have shown that standard qPCR-based measures of *Wolbachia* densities may
861   not be adequate under nutritional stress since host ploidy is subject to variation based on diet
862   (Christensen et al., 2019). Thus, it is plausible that qPCR-based variation in *Wolbachia* densities
863   under nutritional stress may in fact be driven by variation in host ploidy and not *Wolbachia* density.
864   Replication of these studies will be necessary to confidently link nutrition, rearing density, and
865   *Wolbachia* densities to CI strength. Importantly, rearing density does not influence CI strength in
866   *w*AlbA and *w*AlbB *A. albopictus* (Dutton and Sinkins, 2004), suggesting that even if *w*Mel CI is
867   impacted by rearing-density, this effect is perhaps not generalizable across *Wolbachia*-host
868   combinations.

869   The factors described above do not work on CI in isolation but instead seem to be mingled
870   in a state of perpetual complexity. For instance, the impact of temperature on CI strength in *D.*
871   *melanogaster* is dependent on male age, where 1-day old males reared at 25°C induce stronger CI

872 than those reared at 19ºC, but the inverse is true with 3 and 5-day old males (Reynolds and
873 Hoffmann, 2002). Moreover, age has a variable impact on CI strength in different host
874 backgrounds, suggesting that genotypic variation in either the host or *Wolbachia* strain may impact
875 these relationships (O. Duron et al., 2007; Reynolds and Hoffmann, 2002). These studies highlight
876 the complexity of *Wolbachia*-host-environment interactions and should motivate additional
877 investigation to resolve the factors that underpin these variations and the host genetic loci that
878 influence how impactful each factor might be in each host.

879

880 *Host genetics*

881   Relationships between *Wolbachia* phenotypes and host genotypes are frequently
882 investigated through transinfections of a *Wolbachia* strain into a non-native background via
883 injection (Hughes and Rasgon, 2014) or introgression of one species cytoplasm into another host's
884 background via repeated backcrossing (Chafee et al., 2011). For example, *w*Mel *Wolbachia* of *D.*
885 *melanogaster* traditionally cause weak CI (Holden et al., 1993), but induce consistently strong CI
886 when transinfected into either *D. simulans* or *A. aegypti* (Poinsot et al., 1998; Walker et al., 2011).
887 Similar results were also observed when *w*Tei which induce weak or no CI in the *D. yakuba*
888 complex (Charlat et al., 2004; Cooper et al., 2017; Martinez et al., 2020; Zabalou et al., 2004), are
889 transferred into *D. simulans* (Zabalou et al., 2008). Moreover, despite seemingly carrying the same
890 *Wolbachia*, different genetic lineages of the wasp *N. longicornis* express different compatibility
891 relationships with other strains (Raychoudhury and Werren, 2012), and *w*VitA of *N. vitripennis*
892 causes weak CI in its native host but strong CI when introgressed into *N. giraulti* (Chafee et al.,
893 2011). Intriguingly, this affect is only observed with *w*VitA and does not apply to *w*VitB, which
894 also causes CI, suggesting that both host and *Wolbachia* genetics play a role in CI phenotypes.
895 These studies support models that predict hosts will be selected to develop resistance against CI-
896 induction (Prout, 1994; Turelli, 1994), and raise many questions about an evolutionary arms race
897 between *Wolbachia* and its host to control reproductive parasitism.

898   There are at least two broad models for the mechanisms of host suppression of CI:
899 divergence of host products that are targets for CI (defensive model) or evolution of host products
900 that inhibit and suppress *Wolbachia* or CI products (offensive model). First, a defensive model
901 would predict that the pathway(s) that CI act(s) on in the host must be conserved enough for CI to
902 be transferable between species, but also malleable enough for the pathway(s) to become resistant
903 to CI-induction. The host genes, transcripts, and proteins described earlier in this review are
904 excellent candidates since they can mimic CI phenotypes (Biwot et al., 2019; Huang et al., 2019;
905 Ju et al., 2017; Yuan et al., 2015; Zheng et al., 2011). However, studies are necessary to investigate
906 genetic variation in these host products to assess the possibility that they are under selection to
907 suppress CI. Other candidates would be direct binding partners of CifA and CifB (Beckmann et
908 al., 2019c), but nothing is known about how natural variation in these products may relate to CI
909 suppression. Conversely, an offensive model could yield the evolution of host genes involved in
910 *Wolbachia* density regulation or some other target with indirect effects on CI strength. Notably,
911 since these products may not be involved in the CI mechanism, they would not necessarily be

912   expected to be conserved. For instance, the *Wolbachia* density suppressor (*Wds*) gene of *Nasonia*
913   acts to suppress densities of *w*VitA, is taxon restricted to bees and wasps, and is under positive
914   selection as would be expected for a suppressor acting in an evolutionary arms race with
915   *Wolbachia* (Funkhouser-Jones et al., 2018). Since *Wds* is only present in Hymenoptera, it clearly
916   is not generalizable as a standard mechanism of *Wolbachia* and CI suppression. However, it is
917   plausible that other hosts have converged on comparable mechanisms of CI suppression.
918   Additional research will be needed to reveal the diversity of mechanisms surrounding CI
919   suppression and to understand the dynamics controlling their evolution.
920

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

922   Numerous models have been proposed to explain CI and rescue mechanisms. First, we
923   discuss the utility of the classical phenotype-based modification/rescue (*mod*/*resc*) model in a post-
924   genomic world (Shropshire and Bordenstein, 2019; Werren, 1997). Additionally, despite
925   considerable advances in the genetics and biochemistry of CI, numerous mechanistic models are
926   used to describe CI and rescue (Beckmann et al., 2019a; Poinsot et al., 2003; Shropshire et al.,
927   2019). These models divide into two discrete categories: host-modification (HM) and toxin-
928   antidote (TA) (Beckmann et al., 2019a; Shropshire et al., 2019). HM-based models assume that
929   the CI-inducing factors act directly to modify host male products and that rescue occurs through
930   either removal of these modifications or otherwise reversing the effects through a separate host-
931   modification in the female. TA-based models assume that the CI-inducing factors are transported
932   into the embryo via the sperm and are toxic after fertilization unless the rescue factor is present,
933   binds to the CI toxin, and inhibits its toxicity. We discuss each of these models and their variants
934   in the context of a Two-by-One genetic framework (Shropshire and Bordenstein, 2019), but it is
935   crucial to reemphasize that while both CifA and CifB proteins are required to induce CI, the
936   specific biochemical mechanism underlying each protein's contribution to CI and rescue remains
937   unknown and there remains insufficient data to confirm any of these models.
938
939
940
941

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

942
943 **Figure 7. The Host-Modification and Toxin-Antidote models of CI mechanism**. (A) The Host-Modification (HM)
944 model predicts that the Cif proteins impart a modification on male-derived products that result in CI unless CifA is
945 available in the embryo to reverse or otherwise inhibit the male-derived modification (Shropshire et al., 2019; Werren,
946 1997). (B) The Toxin-Antidote (TA) model predicts that CifB is the primary toxin that is transferred to the embryo
947 via the sperm, and that rescue occurs when CifA binds CifB in the embryo and inhibits its toxicity (Beckmann et al.,
948 2019a; Hurst, 1991; Shropshire et al., 2019; Werren, 1997).

949

950 *The mod/resc model.*

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

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

974   With the identification of the CI and rescue genes (Beckmann et al., 2017; Chen et al.,
975 2019; LePage et al., 2017; Shropshire et al., 2018; Shropshire and Bordenstein, 2019), it is
976 compelling to abandon the *mod*/*resc* model in favor of a purely genetic description of CI
977 relationships. With the ever-growing availability of genomic datasets, acceptance of a gene-centric
978 analysis of CI may be the simplest way to predict CI-capability to a symbiont. However, while
979 sequence information can indeed yield informed hypotheses about a strain's CI-capability, some
980 hosts suppress their symbiont's CI (Chafee et al., 2011; Poinsot et al., 1998; Walker et al., 2011),
981 and some symbiont strains exhibit different forms of reproductive parasitism based on their host
982 background (Fujii et al., 2001; Jaenike, 2007; Sakamoto et al., 2005; Sasaki et al., 2002; Zabalou
983 et al., 2008). Thus, we propose that a modern framework for describing CI relationships should
984 involve both phenotypic data described under the *mod*/*resc* model and genetic data described under
985 the Two-by-One model. For example, if genomic sequencing of a novel *Wolbachia* precedes
986 phenotypic observations, then a genetic analysis could reveal *cifA* and *cifB* homologs that are either
987 intact and fully coding relative to those in CI-inducing strains or contain putative loss-of-function
988 mutations. Phenotypic data is of course necessary to confirm the hypothesis. Indeed, *w*Yak of *D.*
989 *yakuba* and *w*Rec of *D. recens* cause CI, but they have *cifB* genes with stop codons that truncate
990 the proteins relative to the *w*Mel and *w*Pip *cifB* (Cooper et al., 2017; Martinez et al., 2020;

991   Shoemaker et al., 1999). As such, a genetic description of these strains alone could result in
992   mischaracterization of *w*Yak and *w*Rec as non-parasitic strains with putative *cifB* pseudogenes. It
993   is only with knowledge of both *cif* gene sequence and phenotypic data that a complete
994   understanding of the basis of CI in these strains is possible. Thus, both the Two-by-One and
995   *mod*/*resc* models will serve as a useful framework to describe these systems.
996

997   *HM-based mechanistic models.*

998   HM models make at least two key predictions. First, male host products are modified by
999   Cifs (Shropshire et al., 2019). There are numerous pre-fertilization defects associated with CI-
1000   inducing *Wolbachia* including changes in sperm morphology and competitive ability (Champion
1001   de Crespigny and Wedell, 2006; Riparbelli et al., 2007), supporting that the host is modified prior
1002   to fertilization. It is unknown, however, whether these outcomes are due to pre-fertilization defects
1003   causatively related to CI or general responses to *Wolbachia* in the testes . Second, and most
1004   crucially, the proximal CI modifications causing death of the fertilized embryo is rescued (e.g.,
1005   replaced or otherwise negated) by CifA in the embryo (Shropshire et al., 2018; Shropshire and
1006   Bordenstein, 2019). Here, CifA cannot bind with male-transferred CifB products since no Cif
1007   protein is transferred with the sperm to the embryo and the modification occurs prior to
1008   fertilization. Instead, CifA may interact with host processes to reverse or otherwise stop the effects
1009   of CI caused by CifA and CifB protein expression in males. As such, assessment of the location
1010   of CifA and CifB binding (testes or embryo), the transfer of Cif products, if any, with the sperm,
1011   and the interactions that Cif have with the host will further inform this model. We discuss three
1012   additional non-exclusive HM-based models below: titration-restitution, mistiming, and
1013   goalkeeper.

1014   The titration-restitution model (a.k.a. the "sink" hypothesis) was originally proposed by
1015   Werren in 1997 (Werren, 1997) and posits that CI is induced by over- or under-expression of host
1016   products or pathways in the testes/sperm and rescue occurs when the same products are
1017   misregulated in the opposite direction in the ovaries/embryo (Fig. 8A) (Kose and Karr, 1995;
1018   Poinsot et al., 2003; Werren, 1997). Indeed, *Wolbachia* has a considerable impact on expression
1019   profiles, and some genes are differentially expressed in male and female reproductive tissues, and
1020   numerous host factors meet these criterion (Baldridge et al., 2017, 2014; Bing et al., 2019; Yuan
1021   et al., 2015), as described in section above. There are at least two ways in which CifA and CifB
1022   proteins can underpin the titration-restitution model. First, since CI and rescue would occur
1023   through titration of the same host product or pathway, it is feasible that CifA, which acts on both
1024   sides of the phenotype (Shropshire and Bordenstein, 2019), may drive these expression changes.
1025   Under such a model, CifB may act as an 'accessory protein' that enables CifA to target a paternally
1026   derived product that it would otherwise not be able to reach on its own. Second, CifA may act on
1027   its own to up- or down-regulate host products but has the opposite impact on that product when
1028   CifB is present. As such, rescue would occur through CifA's lone action which counteracts the
1029   misregulation caused by CifA and CifB dual expression.

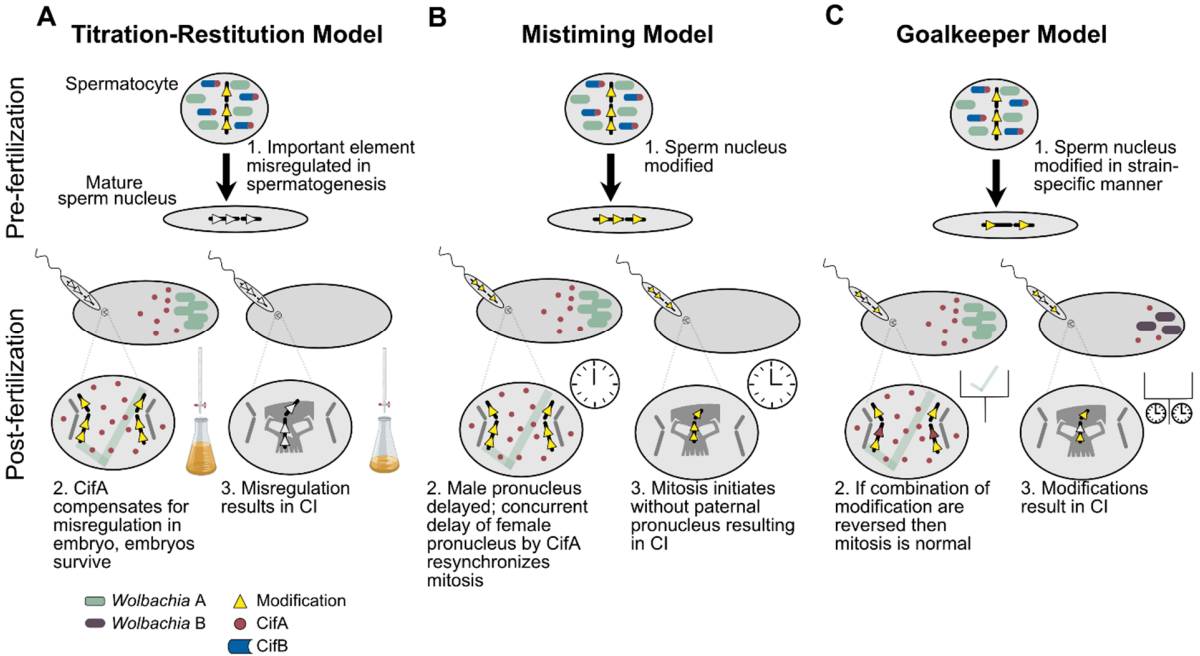
1030   Notably, titration-restitution models can explain bidirectional CI if Cif products from
1031 different strains have variable impacts on multiple host expression pathways. Thus, rescue would
1032 not be possible from a second strain since it could be targeting the wrong host factor or pathway.
1033 Indeed, divergent CI genes may differentially impact host pathways. For example, only the CifB$_{[T1]}$
1034 sequences maintain a functional Ulp1 domain while the other four CifB clades have a dimer of
1035 PDDEXK nucleases that is also present in CifB$_{[T1]}$ (Beckmann et al., 2017; Bing et al., 2020;
1036 Lindsey et al., 2018; Martinez et al., 2020). It is feasible that CifB with different domains impact
1037 different host pathways. Alternatively, Cif proteins may have differential impacts on the level of
1038 misregulation instead of or in addition to impacting multiple host pathways which may influence
1039 incompatibility relationships. More work will be necessary to understand if *cif* expression
1040 influences transcriptional and translational variation and how that variation corresponds to CI.

1041   The mistiming model (a.k.a. the "slow motion" hypothesis) was first explicitly proposed
1042 by Tram and Sullivan in 2002 (Tram and Sullivan, 2002) and is based on the observation that the
1043 paternal pronucleus has slowed development relative to the female pronucleus in CI crosses, the
1044 rescue cross has normal cell cycle timing, and the female pronucleus continues development
1045 despite the slowdown in both *Drosophila* and *Nasonia* species (Fig. 8B) (Callaini et al., 1996;
1046 Ferree and Sullivan, 2006; Ryan and Saul, 1968; Tram and Sullivan, 2002). This established the
1047 hypotheses that delayed male pronuclear development is responsible for emergent defects in early
1048 embryogenesis, and that resynchronization of the development may occur by comparably slowing
1049 down the development of the female pronucleus or slowing the cell cycle in rescue. Since the cell
1050 cycle timing of the female pronucleus is what establishes the timing for the first mitosis (Bossan
1051 et al., 2011), symbiont-bearing females do not induce CI since the male pronucleus reach
1052 apposition prior to the female pronucleus. Though, the reciprocal cross would be incompatible
1053 because the female pronucleus will have finished development prior to the male and the first
1054 mitosis would have initiated before the male pronucleus arrives. Importantly, this model predicts
1055 that CI crosses are subject to haploidization of diploid offspring since the male pronucleus could
1056 be completely excluded from mitosis if it was significantly slowed. This is indeed the case in *N.*
1057 *vitripennis* where CI often manifests as only male offspring since haploid offspring are viable in
1058 this species but develop as males (Bordenstein et al., 2003).

1059   The mistiming model proposes that CI and rescue have comparable impacts on the
1060 development of male and female gametes respectively. As such, a single gene could in theory be
1061 responsible for both CI and rescue (Poinsot et al., 2003). Under this paradigm, CifA may enact a
1062 slowdown in both tissues since it is involved in both phenotypes (Shropshire and Bordenstein,
1063 2019). However, if this were the case, then what would be the purpose of CifB? It is possible that
1064 CifB is responsible for localizing CifA to a male specific target where it imposes the same
1065 outcomes on its host. Since this hypothetical male product would not be available in the embryo,
1066 CifB would not have a role in rescue. However, an alternative model for mistiming is that rescue
1067 may not occur through slowing down the female pronucleus but may instead work by removing
1068 the slowdown agents from the male pronucleus. Together, these models would help to explain the
1069 proximal cause (misregulation) and culminating effects (mistiming) of CI. More work will be

1070   necessary to understand if rescue occurs via slowdown of the female pronucleus or from speeding-
1071   up the male pronucleus.

1072   A major limitation of the mistiming model is that it cannot explain bidirectional CI. Since
1073   mistiming proposes that rescue happens through delaying the female pronucleus as much as or
1074   greater than the male pronucleus, a sufficiently strong delay should yield compatibility with any
1075   strain that has a weaker male delay. As such, only unidirectional CI should manifest between
1076   strains where the strain inducing the stronger delay is capable of rescue. The goalkeeper model
1077   was proposed in 2011 as a way to address this limitation (Fig. 8C) (Bossan et al., 2011). In addition
1078   to the expectations of the mistiming model, goalkeeper suggests that a secondary factor
1079   unassociated with this mistiming may also be involved in CI. The combined contribution of these
1080   two mod factors leads to CI. Under this paradigm, CifA and CifB may contribute to different kinds
1081   of defects during spermatogenesis, each contributing in somewhat independent ways to CI-
1082   induction. Rescue must then negate the impact of both factors. Thus, for CifA to rescue CI it would
1083   not only need to contribute to a delay in the pronuclear development but also reverse the impacts
1084   of a secondary source of modification. Notably, since the titration-restitution model does not make
1085   predictions about the developmental timing of the male and female pronuclei, it is compatible with
1086   both mistiming and goalkeeper models, and can help explain mistiming through misregulation of
1087   host factors in a manner that leads to slowed development. More functional genetic, biochemical,
1088   and cytological studies are necessary to understand how a goalkeeper model and/or a combination
1089   of these HM-based models may contribute to CI.

1090

1091
1092   **Figure 8. Extensions of the Host-Modification model**. (A)The Titration-Restitution Model posits that an element
1093   within mature sperm is either over- or under-expressed in males due to Cif protein expression, but this alteration is
1094   then remedied in the female as a result of CifA through a reconstitution of the required element (Werren, 1997). (B)
1095   The Mistiming Model posits that a modification in the male sperm causes a delay in the formation of the male

1096   pronucleus that results in CI if CifA does not cause a concurrent delay in the maternal pronucleus, resynchronizing
1097   mitosis between the two pronuclei (Tram and Sullivan, 2002). (C) The Goalkeeper Model expands on the Mistiming
1098   Model and posits that the male product modification occurs in a strain-specific quantity, and may involve multiple
1099   modifications that need to be remedied to rescue the lethality (Bossan et al., 2011).
1100

1101   *TA-based mechanistic models*

1102   Since *Wolbachia* are not paternally inherited, Hurst proposed in 1991 that *Wolbachia* make
1103   a CI-inducing toxin that diffuses into the sperm cytoplasm and is transferred to the egg during
1104   fertilization and causes death (Hurst, 1991). Rescue then occurs when *Wolbachia* in the egg
1105   produce an antidote that binds to the toxin and prevents it from killing the embryo (Hurst, 1991).
1106   This TA model makes two key predictions (Beckmann et al., 2019a; Shropshire et al., 2019). First,
1107   the Cif proteins are transferred to the embryo. Mass spectrometry of spermatheca from symbiont-
1108   bearing *Cu. pipiens* females mated with symbiont-bearing males reveal fragments of CifA
1109   (Beckmann and Fallon, 2013). These later data have been used to support this prediction, but since
1110   these females harbor *Wolbachia* (Beckmann and Fallon, 2013) and CifA is also the rescue protein
1111   (Chen et al., 2019; Shropshire et al., 2018), the most parsimonious explanation for CifA's presence
1112   in symbiont-bearing spermatheca is related to *Wolbachia* in females and potentially rescue, not
1113   CI-induction. It remains possible that Cif proteins are transferred, but this is not the simplest
1114   interpretation of currently available data. Second, if the proteins are transferred, then maternal
1115   CifA must bind to the CI toxin to prevent function. *In vitro* biochemical assays reveal that CifA
1116   and CifB are capable of binding (Beckmann et al., 2017), but it remains unknown if they bind as
1117   a toxin complex to induce CI or if CifA binds to CifB in the embryo to rescue CI. Moreover, while
1118   CifB's Ulp1 domain is an *in vitro* deubiquitinase, CifA's binding to CifB does not inhibit
1119   deubiquitinase activity, suggesting that if binding is for the purpose of rescue it is not inhibiting
1120   one of CifB's biochemical functions (Beckmann et al., 2017). As such, assays investigating if the
1121   Cif products are transferred to the embryo at all and where the Cif proteins bind each other in
1122   reproductive tissue cells will inform the foundation of this hypothesis.

1123   The TA model traditionally states that the toxin and antidote are separate factors (Poinsot
1124   et al., 2003). However, our genetic understanding is that CifA is involved in both CI and rescue.
1125   There are two ways to update the model to a Two-by-One genetic model (Shropshire and
1126   Bordenstein, 2019) while maintaining the key assumptions of the TA model (Hurst, 1991). First,
1127   CifB may be the sole toxin but requires CifA as an antidote even during spermatogenesis to prevent
1128   overly defective sperm (Beckmann et al., 2019a). For this to work, CifA is expected to degrade
1129   faster than CifB, leaving CifB alone to enter the egg as a toxin unless it binds to maternally derived
1130   CifA (Beckmann et al., 2019a). Alternatively, CifA and CifB could work together as a toxin
1131   complex that enters the embryo and is then rescued by maternally derived CifA. Binding assays
1132   coupled with microscopy and localization studies will reveal when and where CifA acts relative
1133   to CifB.

1134   As described above, the TA model aims to explain unidirectional CI between symbiont-
1135   bearing and aposymbiotic individuals. A modification of the TA model, called lock-and-key,
1136   expands the TA model to explain incompatibilities between *Wolbachia* strains. The lock-and-key

1137 model, like TA, proposes that a toxin is transferred from symbiont-bearing males to the embryo
1138 and will cause embryonic death unless an antidote is supplied. Toxins in this case are called locks,
1139 and antidotes are keys. The toxin lock is proposed to bind to or otherwise interfere with factors
1140 associated with proper embryonic development unless the antidote key is available to remove the
1141 lock. Bidirectional CI can then be explained by one strain carrying a set of locks and keys that are
1142 not compatible with the other strains' locks and keys because of differences in binding affinity.
1143 This model leveraged predictions of the *mod*/*resc* model that (i) strains can have multiple sets of
1144 *mod*/lock and *resc*/key factors and that (ii) a key is more likely to bind to its associated lock than
1145 to a divergent lock. Indeed, *Wolbachia* exhibit considerable *cif* polymorphism (Bing et al., 2020;
1146 Bonneau et al., 2019; LePage et al., 2017; Lindsey et al., 2018; Martinez et al., 2020) and binding
1147 of CifA and CifB is strongest between cognate partners (Beckmann et al., 2017). However, the
1148 lingering questions with the TA model also apply with the lock-and-key model. Additionally,
1149 functional validation that divergent Cif proteins are functional, that they have differential impacts
1150 on the host, and contribute summatively to incompatibilities are lacking.

1151

Conclusion

1153   *Wolbachia* were first discovered in *Cu. pipiens* mosquitoes in 1924 and were later linked
1154 to CI in 1973 (Hertig and Wolbach, 1924; Yen and Barr, 1973). Since then, a century of advances
1155 have significantly expanded our recognition of *Wolbachia*'s incredible and complex toolset. In
1156 particular, biologists now appreciate CI as a common form of reproductive parasitism that
1157 symbionts including *Wolbachia* and *Cardinium* use to rapidly spread through populations (Hunter
1158 et al., 2003; Rosenwald et al., 2020; Takano et al., 2017; Turelli, 1994; Weinert et al., 2015; Zug
1159 and Hammerstein, 2012). CI is associated with reproductive isolation (Bordenstein et al., 2001;
1160 Gebiola et al., 2017; Jaenike et al., 2006) and is leveraged as a successful tool in the prevention of
1161 arboviral diseases that infect humans (Crawford et al., 2020; O'Neill, 2018; Tantowijoyo et al.,
1162 2020). The last decade has seen a rapid expansion in our understanding of phage WO's role in CI
1163 genetics (Beckmann et al., 2019c; Chen et al., 2019; LePage et al., 2017; Shropshire et al., 2018;
1164 Shropshire and Bordenstein, 2019), phylogenetics (Bing et al., 2020; LePage et al., 2017; Lindsey
1165 et al., 2018; Martinez et al., 2020), and mechanism (Beckmann et al., 2019c, 2017; Chen et al.,
1166 2019; Shropshire et al., 2020). Moreover, considerable effort has been made to describe CI-
1167 defining cytological defects (Ferree and Sullivan, 2006; Landmann et al., 2009), link variation in
1168 host expression with CI phenotypes (Biwot et al., 2019; Liu et al., 2014; Zheng et al., 2011), and
1169 to untangle factors that influence CI strength such as *Wolbachia* densities and phage WO lytic
1170 activity (Bordenstein and Bordenstein, 2011; Layton et al., 2019; Reynolds and Hoffmann, 2002;
1171 Yamada et al., 2007). Together, this significant body of literature has motivated models to explain
1172 how CI works (Beckmann et al., 2019a; Bossan et al., 2011; Poinsot et al., 2003; Shropshire et al.,
1173 2019). These works have stone-by-stone erected a steady foundation that will serve as a launching
1174 point for exciting new discoveries that help us fully appreciate the complexity of this powerful
1175 form of reproductive manipulation. Looking forward, key areas of investigation will involve the
1176 relative roles of CifA and CifB in induction of CI, the cell biology of the Cif proteins, the genetic

1177   basis of bidirectional CI, the cytogenetic basis of CI strength variation, linkage of Cif expression

1178   with cytological abnormalities pre- and post-fertilization, Cif-induced CI's molecular and

1179   biochemical basis, and mechanisms of host suppression of CI.

1180

1181

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References

Al-Khodor S, Price CT, Kalia A, Abu Kwaik Y. 2010. Functional diversity of ankyrin repeats in microbial proteins. *Trends in Microbiology* **18**:132–139. doi:10.1016/j.tim.2009.11.004

Ant TH, Herd C, Louis F, Failloux AB, Sinkins SP. 2020. *Wolbachia* transinfections in *Culex quinquefasciatus* generate cytoplasmic incompatibility. *Insect Mol Biol* **29**:1–8. doi:10.1111/imb.12604

Ant TH, Sinkins SP. 2018. A *Wolbachia* triple-strain infection generates self-incompatibility in *Aedes albopictus* and transmission instability in *Aedes aegypti*. *Parasit Vectors* **11**:295. doi:10.1186/s13071-018-2870-0

Arai H, Hirano T, Akizuki N, Abe A, Nakai M, Kunimi Y, Inoue MN. 2018. Multiple Infection and Reproductive Manipulations of *Wolbachia* in *Homona magnanima* (Lepidoptera: Tortricidae). *Microb Ecol*. doi:10.1007/s00248-018-1210-4

Asselin AK, Villegas-Ospina S, Hoffmann AA, Brownlie JC, Johnson KN. 2018. Contrasting patterns of virus protection and functional incompatibility genes in two conspecific *Wolbachia* strains from *Drosophila pandora*. *Appl Environ Microbiol* AEM.02290-18. doi:10.1128/AEM.02290-18

Atyame CM, Delsuc F, Pasteur N, Weill M, Duron O. 2011a. Diversification of *Wolbachia* endosymbiont in the *Culex pipiens* mosquito. *Mol Biol Evol* **28**:2761–2772. doi:10.1093/molbev/msr083

Atyame CM, Duron O, Tortosa P, Pasteur N, Fort P, Weill M. 2011b. Multiple *Wolbachia* determinants control the evolution of cytoplasmic incompatibilities in *Culex pipiens* mosquito populations. *Mol Ecol* **20**:286–298. doi:10.1111/j.1365-294X.2010.04937.x

Awrahman ZA, Champion de Crespigny F, Wedell N. 2014. The impact of *Wolbachia*, male age and mating history on cytoplasmic incompatibility and sperm transfer in *Drosophila simulans*. *J Evol Biol* **27**:1–10. doi:10.1111/jeb.12270

Baldridge G, Higgins L, Witthuhn B, Markowski T, Baldridge A, Armien A, Fallon A. 2017. Proteomic analysis of a mosquito host cell response to persistent *Wolbachia* infection. *Res Microbiol* **168**:609–625. doi:10.1016/j.resmic.2017.04.005

Baldridge GD, Baldridge AS, Witthuhn BA, Higgins L, Markowski TW, Fallon AM. 2014. Proteomic profiling of a robust *Wolbachia* infection in an *Aedes albopictus* mosquito cell line. *Mol Microbiol* **94**:537–556. doi:10.1111/mmi.12768

Baton LA, Pacidônio EC, Gonçalves D da S, Moreira LA. 2013. *w*Flu: characterization and evaluation of a native *Wolbachia* from the mosquito *Aedes fluviatilis* as a potential vector control agent. *PLoS ONE* **8**:e59619. doi:10.1371/journal.pone.0059619

Beckmann J, Bonneau M, Chen H, Hochstrasser M, Poinsot D, Merçot H, Weill M, Sicard M, Charlat S. 2019a. The Toxin–Antidote Model of Cytoplasmic Incompatibility: Genetics and Evolutionary Implications. *Trends in Genetics*. doi:10.1016/j.tig.2018.12.004

Beckmann J, Bonneau M, Chen H, Hochstrasser M, Poinsot D, Merçot H, Weill M, Sicard M, Charlat S. 2019b. Caution does not preclude predictive and testable models of cytoplasmic incompatibility: a reply to Shropshire et al. *Trends in Genetics* **0**. doi:10.1016/j.tig.2019.03.002

Beckmann J, Fallon AM. 2013. Detection of the *Wolbachia* protein WPIP0282 in mosquito spermathecae: implications for cytoplasmic incompatibility. *Insect Biochem Mol Biol* **43**:867–878. doi:10.1016/j.ibmb.2013.07.002

Beckmann J, Ronau JA, Hochstrasser M. 2017. A *Wolbachia* deubiquitylating enzyme induces cytoplasmic incompatibility. *Nature Microbiology* **2**:17007. doi:10.1038/nmicrobiol.2017.7

Beckmann J, Sharma GD, Mendez L, Chen H, Hochstrasser M. 2019c. The *Wolbachia* cytoplasmic incompatibility enzyme CidB targets nuclear import and protamine-histone exchange factors. *eLife* **8**:e50026. doi:10.7554/eLife.50026

Betelman K, Caspi-Fluger A, Shamir M, Chiel E. 2017. Identification and characterization of bacterial symbionts in three species of filth fly parasitoids. *FEMS Microbiol Ecol* **93**. doi:10.1093/femsec/fix107

Bian G, Joshi D, Dong Y, Lu P, Zhou G, Pan X, Xu Y, Dimopoulos G, Xi Z. 2013. *Wolbachia* invades *Anopheles stephensi* populations and induces refractoriness to Plasmodium infection. *Science* **340**:748–751. doi:10.1126/science.1236192

Bing X-L, Lu Y-J, Xia C-B, Xia X, Hong X-Y. 2019. Transcriptome of *Tetranychus urticae* embryos reveals insights into *Wolbachia*-induced cytoplasmic incompatibility. *Insect Mol Biol*. doi:10.1111/imb.12620

Bing X-L, Zhao D-S, Sun J-T, Zhang K-J, Hong X-Y. 2020. Genomic analysis of *Wolbachia* from *Laodelphax striatellus* (Delphacidae, Hemiptera) reveals insights into its "Jekyll and Hyde" mode of infection pattern. *Genome Biol Evol*. doi:10.1093/gbe/evaa006

Binnington KC, Hoffmann AA. 1989. *Wolbachia*-like organisms and cytoplasmic incompatibility in *Drosophila simulans*. *Journal of Invertebrate Pathology* **54**:344–352. doi:10.1016/0022-2011(89)90118-3

Biwot JC, Zhang H-B, Liu C, Qiao J-X, Yu X-Q, Wang Y-F. 2019. *Wolbachia*-induced expression of kenny gene in testes affects male fertility in *Drosophila melanogaster*. *Insect Sci*. doi:10.1111/1744-7917.12730

Bonneau M, Atyame C, Beji M, Justy F, Cohen-Gonsaud M, Sicard M, Weill M. 2018a. *Culex pipiens* crossing type diversity is governed by an amplified and polymorphic operon of *Wolbachia*. *Nature Communications* **9**. doi:10.1038/s41467-017-02749-w

Bonneau M, Caputo B, Ligier A, Caparros R, Unal S, Perriat-Sanguinet M, Arnoldi D, Sicard M, Weill M. 2019. Variation in *Wolbachia cidB* gene, but not *cidA*, is associated with cytoplasmic incompatibility mod phenotype diversity in *Culex pipiens*. *Molecular Ecology* **28**:4725–4736. doi:10.1111/mec.15252

Bonneau M, Landmann F, Labbé P, Justy F, Weill M, Sicard M. 2018b. The cellular phenotype of cytoplasmic incompatibility in *Culex pipiens* in the light of *cidB* diversity. *PLOS Pathogens* **14**:e1007364. doi:10.1371/journal.ppat.1007364

Bordenstein Sarah R., Bordenstein Seth R. 2016. Eukaryotic association module in phage WO genomes from *Wolbachia*. *Nature Communications* **7**:13155. doi:10.1038/ncomms13155

Bordenstein Sarah R., Bordenstein Seth R. 2011. Temperature affects the tripartite interactions between bacteriophage WO, *Wolbachia*, and cytoplasmic incompatibility. *PLoS One* **6**:e29106. doi:10.1371/journal.pone.0029106

Bordenstein SR, O'Hara FP, Werren JH. 2001. *Wolbachia*-induced incompatibility precedes other hybrid incompatibilities in *Nasonia*. *Nature* **409**:707–710. doi:10.1038/35055543

Bordenstein SR, Uy JJ, Werren JH. 2003. Host genotype determines cytoplasmic incompatibility type in the haplodiploid genus *Nasonia*. *Genetics* **164**:223–233.

Bordenstein SR, Wernegreen JJ. 2004. Bacteriophage flux in endosymbionts (*Wolbachia*): Infection frequency, lateral transfer, and recombination rates. *Molecular Biology and Evolution* **21**:1981–1991. doi:10.1093/molbev/msh211

1282 Bordenstein SR, Werren JH. 2007. Bidirectional incompatibility among divergent *Wolbachia*
1283   and incompatibility level differences among closely related *Wolbachia* in *Nasonia*.
1284   *Heredity (Edinb)* **99**:278–287. doi:10.1038/sj.hdy.6800994
1285 Bossan B, Koehncke A, Hammerstein P. 2011. A New Model and Method for Understanding
1286   *Wolbachia*-Induced Cytoplasmic Incompatibility. *PLOS ONE* **6**:e19757.
1287   doi:10.1371/journal.pone.0019757
1288 Bourtzis K, Dobson SL, Braig HR, O'Neill SL. 1998. Rescuing *Wolbachia* have been
1289   overlooked. *Nature* **391**:852–853. doi:10.1038/36017
1290 Branca A, Vavre F, Silvain J-F, Dupas S. 2009. Maintenance of adaptive differentiation by
1291   *Wolbachia* induced bidirectional cytoplasmic incompatibility: the importance of sib-
1292   mating and genetic systems. *BMC Evol Biol* **9**:185. doi:10.1186/1471-2148-9-185
1293 Breeuwer J, Werren J. 1990. Microorganisms associated with chromosome destruction and
1294   reproductive isolation between 2 insect species. *Nature* **346**:558–560.
1295   doi:10.1038/346558a0
1296 Breeuwer JA, Werren JH. 1993. Cytoplasmic incompatibility and bacterial density in Nasonia
1297   vitripennis. *Genetics* **135**:565–574.
1298 Brennan LJ, Haukedal JA, Earle JC, Keddie B, Harris HL. 2012. Disruption of redox
1299   homeostasis leads to oxidative DNA damage in spermatocytes of *Wolbachia*-infected
1300   *Drosophila simulans*. *Insect Mol Biol* **21**:510–520. doi:10.1111/j.1365-
1301   2583.2012.01155.x
1302 Brennan LJ, Keddie BA, Braig HR, Harris HL. 2008. The endosymbiont *Wolbachia pipientis*
1303   induces the expression of host antioxidant proteins in an *Aedes albopictus* cell line. *PLoS*
1304   *One* **3**:e2083. doi:10.1371/journal.pone.0002083
1305 Bressac C, Rousset F. 1993. The Reproductive Incompatibility System in *Drosophila simulans*:
1306   Dapi-Staining Analysis of the *Wolbachia* Symbionts in Sperm Cysts. *Journal of*
1307   *Invertebrate Pathology* **61**:226–230. doi:10.1006/jipa.1993.1044
1308 Brucker RM, Bordenstein SR. 2012. Speciation by symbiosis. *Trends in Ecology & Evolution*
1309   **27**:443–451. doi:10.1016/j.tree.2012.03.011
1310 Callaini G, Dallai R, Riparbelli MG. 1997. *Wolbachia*-induced delay of paternal chromatin
1311   condensation does not prevent maternal chromosomes from entering anaphase in
1312   incompatible crosses of *Drosophila simulans*. *Journal of Cell Science* **110**:271–280.
1313 Callaini G, Riparbelli MG, Giordano R, Dallai R. 1996. Mitotic Defects Associated with
1314   Cytoplasmic Incompatibility in *Drosophila simulans*. *Journal of Invertebrate Pathology*
1315   **67**:55–64. doi:10.1006/jipa.1996.0009
1316 Caputo B, Moretti R, Manica M, Serini P, Lampazzi E, Bonanni M, Fabbri G, Pichler V, Della
1317   Torre A, Calvitti M. 2019. A bacterium against the tiger: preliminary evidence of fertility
1318   reduction after release of *Aedes albopictus* males with manipulated *Wolbachia* infection
1319   in an Italian urban area. *Pest Manag Sci*. doi:10.1002/ps.5643
1320 Caragata EP, Dutra HLC, Moreira LA. 2016. Inhibition of Zika virus by *Wolbachia* in *Aedes*
1321   *aegypti*. *Microb Cell* **3**:293–295. doi:10.15698/mic2016.07.513
1322 Cattel J, Nikolouli K, Andrieux T, Martinez J, Jiggins F, Charlat S, Vavre F, Lejon D, Gibert P,
1323   Mouton L. 2018. Back and forth *Wolbachia* transfers reveal efficient strains to control
1324   spotted wing *Drosophila* populations. *Journal of Applied Ecology* **55**:2408–2418.
1325   doi:10.1111/1365-2664.13101

1326 Chafee ME, Zecher CN, Gourley ML, Schmidt VT, Chen JH, Bordenstein Sarah R., Clark ME,
1327   Bordenstein Seth R. 2011. Decoupling of host-symbiont-phage coadaptations following
1328   transfer between insect species. *Genetics* **187**:203–215. doi:10.1534/genetics.110.120675
1329 Chambers EW, Hapairai L, Peel BA, Bossin H, Dobson SL. 2011. Male mating competitiveness
1330   of a *Wolbachia*-introgressed *Aedes polynesiensis* strain under semi-field conditions. *PLoS*
1331   *Negl Trop Dis* **5**:e1271. doi:10.1371/journal.pntd.0001271
1332 Champion de Crespigny FE, Wedell N. 2006. *Wolbachia* infection reduces sperm competitive
1333   ability in an insect. *Proc Biol Sci* **273**:1455–1458. doi:10.1098/rspb.2006.3478
1334 Charlat S, Ballard JWO, Mercot H. 2004. What maintains noncytoplasmic incompatibility
1335   inducing *Wolbachia* in their hosts: a case study from a natural *Drosophila yakuba*
1336   population. *J Evol Biol* **17**:322–330.
1337 Charlat S, Calmet C, Merçot H. 2001. On the mod resc Model and the Evolution of *Wolbachia*
1338   Compatibility Types. *Genetics* **159**:1415–1422.
1339 Charlesworth J, Weinert LA, Araujo EV, Welch JJ. 2019. *Wolbachia*, *Cardinium* and climate: an
1340   analysis of global data. *Biol Lett* **15**:20190273. doi:10.1098/rsbl.2019.0273
1341 Chen H, Ronau JA, Beckmann J, Hochstrasser M. 2019. A *Wolbachia* nuclease and its binding
1342   partner provide a distinct mechanism for cytoplasmic incompatibility. *PNAS* **116**:22314–
1343   22321. doi:10.1073/pnas.1914571116
1344 Christensen S, Camacho M, Sharmin Z, Momtaz AJMZ, Perez L, Navarro G, Triana J, Samarah
1345   H, Turelli M, Serbus LR. 2019. Quantitative methods for assessing local and bodywide
1346   contributions to *Wolbachia* titer in maternal germline cells of *Drosophila*. *BMC*
1347   *Microbiology* **19**:206. doi:10.1186/s12866-019-1579-3
1348 Clancy DJ, Hoffmann AA. 1998. Environmental effects on cytoplasmic incompatibility and
1349   bacterial load in *Wolbachia*-infected *Drosophila simulans*. *Entomologia Experimentalis*
1350   *et Applicata* **86**:13–24. doi:10.1046/j.1570-7458.1998.00261.x
1351 Clark ME, Heath BD, Anderson CL, Karr TL. 2006. Induced paternal effects mimic cytoplasmic
1352   incompatibility in *Drosophila*. *Genetics* **173**:727–734. doi:10.1534/genetics.105.052431
1353 Clark ME, Veneti Z, Bourtzis K, Karr TL. 2003. *Wolbachia* distribution and cytoplasmic
1354   incompatibility during sperm development: the cyst as the basic cellular unit of CI
1355   expression. *Mechanisms of Development* **120**:185–198. doi:10.1016/S0925-
1356   4773(02)00424-0
1357 Clark ME, Veneti Z, Bourtzis K, Karr TL. 2002. The distribution and proliferation of the
1358   intracellular bacteria *Wolbachia* during spermatogenesis in *Drosophila*. *Mechanisms of*
1359   *Development* **111**:3–15. doi:10.1016/S0925-4773(01)00594-9
1360 Conner WR, Blaxter ML, Anfora G, Ometto L, Rota-Stabelli O, Turelli M. 2017. Genome
1361   comparisons indicate recent transfer of *w*Ri-like *Wolbachia* between sister species
1362   *Drosophila suzukii* and *D. subpulchrella*. *Ecol Evol* **7**:9391–9404. doi:10.1002/ece3.3449
1363 Cooper BS, Ginsberg PS, Turelli M, Matute DR. 2017. *Wolbachia* in the *Drosophila yakuba*
1364   complex: pervasive frequency variation and weak cytoplasmic incompatibility, but no
1365   apparent effect on reproductive isolation. *Genetics* **205**:333–351.
1366   doi:10.1534/genetics.116.196238
1367 Cooper BS, Vanderpool D, Conner WR, Matute DR, Turelli M. 2019. *Wolbachia* acquisition by
1368   *Drosophila yakuba*-clade hosts and transfer of incompatibility loci between distantly
1369   related *Wolbachia*. *Genetics*. doi:10.1534/genetics.119.302349
1370 Cordaux R, Pichon S, Hatira HBA, Doublet V, Greve P, Marcade I, Braquart-Varnier C, Souty-
1371   Grosset C, Charfi-Cheikhrouha F, Bouchon D. 2012. Widespread *Wolbachia* infection in

terrestrial isopods and other crustaceans. *Zookeys* 123–131.
doi:10.3897/zookeys.176.2284

Coyne J. 2001. Reproductive IsolationEncyclopedia of Genetics. Elsevier. pp. 1679–1686.
doi:10.1006/rwgn.2001.1442

Crawford JE, Clarke DW, Criswell V, Desnoyer M, Cornel D, Deegan B, Gong K, Hopkins KC,
Howell P, Hyde JS, Livni J, Behling C, Benza R, Chen W, Dobson KL, Eldershaw C,
Greeley D, Han Y, Hughes B, Kakani E, Karbowski J, Kitchell A, Lee E, Lin T, Liu J,
Lozano M, MacDonald W, Mains JW, Metlitz M, Mitchell SN, Moore D, Ohm JR,
Parkes K, Porshnikoff A, Robuck C, Sheridan M, Sobecki R, Smith P, Stevenson J,
Sullivan J, Wasson B, Weakley AM, Wilhelm M, Won J, Yasunaga A, Chan WC,
Holeman J, Snoad N, Upson L, Zha T, Dobson SL, Mulligan FS, Massaro P, White BJ.
2020. Efficient production of male Wolbachia-infected Aedes aegypti mosquitoes enables
large-scale suppression of wild populations. *Nat Biotechnol* **38**:482–492.
doi:10.1038/s41587-020-0471-x

Curry MM, Paliulis LV, Welch KD, Harwood JD, White JA. 2015. Multiple endosymbiont
infections and reproductive manipulations in a linyphiid spider population. *Heredity
(Edinb)* **115**:146–152. doi:10.1038/hdy.2015.2

De Crespigny FEC, Pitt TD, Wedell N. 2006. Increased male mating rate in *Drosophila* is
associated with *Wolbachia* infection. *Journal of Evolutionary Biology* **19**:1964–1972.
doi:10.1111/j.1420-9101.2006.01143.x

Debug Fresno. 2018. Debug Fresno 2018 results in 95% suppression! *Debug Project*.

Dittmer J, van Opstal EJ, Shropshire JD, Bordenstein SR, Hurst GDD, Brucker RM. 2016.
Disentangling a holobiont - recent advances and perspectives in *Nasonia* wasps. *Front
Microbiol* **7**:1478. doi:10.3389/fmicb.2016.01478

Dobzhansky T. 1937. Genetics and the Origin of Species. Columbia University Press.

Doremus MR, Kelly SE, Hunter MS. 2019. Exposure to opposing temperature extremes causes
comparable effects on *Cardinium* density but contrasting effects on *Cardinium*-induced
cytoplasmic incompatibility. *PLOS Pathogens* **15**:e1008022.
doi:10.1371/journal.ppat.1008022

Driver C, Georgiou A, Georgiou G. 2004. The contribution by mitochondrially induced oxidative
damage to aging in *Drosophila melanogaster*. *Biogerontology* **5**:185–192.
doi:10.1023/B:BGEN.0000031156.75376.e3

Duffy JB. 2002. GAL4 system in *Drosophila*: a fly geneticist's Swiss army knife. *Genesis* **34**:1–
15. doi:10.1002/gene.10150

Duron Olivier, Boureux A, Echaubard P, Berthomieu A, Berticat C, Fort P, Weill M. 2007.
Variability and expression of ankyrin domain genes in *Wolbachia* variants infecting the
mosquito *Culex pipiens*. *Journal of Bacteriology* **189**:4442–4448. doi:10.1128/JB.00142-
07

Duron O., Fort P, Weill M. 2007. Influence of aging on cytoplasmic incompatibility, sperm
modification and *Wolbachia* density in *Culex pipiens* mosquitoes. *Heredity* **98**:368–374.
doi:10.1038/sj.hdy.6800948

Dutton TJ, Sinkins SP. 2004. Strain-specific quantification of *Wolbachia* density in *Aedes
albopictus* and effects of larval rearing conditions. *Insect Molecular Biology* **13**:317–322.
doi:10.1111/j.0962-1075.2004.00490.x

Emelyanov AV, Rabbani J, Mehta M, Vershilova E, Keogh MC, Fyodorov DV. 2014.
Drosophila TAP/p32 is a core histone chaperone that cooperates with NAP-1, NLP, and

1418   nucleophosmin in sperm chromatin remodeling during fertilization. *Genes Dev* **28**:2027–
1419   2040. doi:10.1101/gad.248583.114

1420 Ferree PM, Sullivan W. 2006. A genetic test of the role of the maternal pronucleus in
1421   *Wolbachia*-induced cytoplasmic incompatibility in *Drosophila melanogaster*. *Genetics*
1422   **173**:839–847. doi:10.1534/genetics.105.053272

1423 Foo IJ-H, Hoffmann AA, Ross PA. 2019. Cross-Generational Effects of Heat Stress on Fitness
1424   and *Wolbachia* Density in *Aedes aegypti* Mosquitoes. *Trop Med Infect Dis* **4**.
1425   doi:10.3390/tropicalmed4010013

1426 Ford SA, Allen SL, Ohm JR, Sigle LT, Sebastian A, Albert I, Chenoweth SF, McGraw EA.
1427   2019. Selection on *Aedes aegypti* alters *Wolbachia*-mediated dengue virus blocking and
1428   fitness. *Nat Microbiol* 1–8. doi:10.1038/s41564-019-0533-3

1429 Foster J, Ganatra M, Kamal I, Ware J, Makarova K, Ivanova N, Bhattacharyya A, Kapatral V,
1430   Kumar S, Posfai J, Vincze T, Ingram J, Moran L, Lapidus A, Omelchenko M, Kyrpides
1431   N, Ghedin E, Wang S, Goltsman E, Joukov V, Ostrovskaya O, Tsukerman K, Mazur M,
1432   Comb D, Koonin E, Slatko B. 2005. The *Wolbachia* genome of *Brugia malayi*:
1433   Endosymbiont evolution within a human pathogenic nematode. *Plos Biology* **3**:599–614.
1434   doi:10.1371/journal.pbio.0030121

1435 Fujii Y, Kageyama D, Hoshizaki S, Ishikawa H, Sasaki T. 2001. Transfection of *Wolbachia* in
1436   Lepidoptera: the feminizer of the adzuki bean borer *Ostrinia scapulalis* causes male
1437   killing in the Mediterranean flour moth *Ephestia kuehniella*. *Proc Biol Sci* **268**:855–859.
1438   doi:10.1098/rspb.2001.1593

1439 Fuller MT. 1993. Spermatogenesis.

1440 Funkhouser-Jones LJ, Opstal EJ van, Sharma A, Bordenstein SR. 2018. The Maternal Effect
1441   Gene Wds Controls *Wolbachia* Titer in *Nasonia*. *Current Biology* **0**. doi:10/gdg6rg

1442 Gebiola M, Giorgini M, Kelly SE, Doremus MR, Ferree PM, Hunter MS. 2017. Cytological
1443   analysis of cytoplasmic incompatibility induced by *Cardinium* suggests convergent
1444   evolution with its distant cousin *Wolbachia*. *Proc Biol Sci* **284**.
1445   doi:10.1098/rspb.2017.1433

1446 Gillespie JJ, Driscoll TP, Verhoeve VI, Rahman MS, Macaluso KR, Azad AF. 2018. A Tangled
1447   Web: Origins of Reproductive Parasitism. *Genome Biol Evol* **10**:2292–2309.
1448   doi:10.1093/gbe/evy159

1449 Gotoh T, Noda H, Hong X-Y. 2003. *Wolbachia* distribution and cytoplasmic incompatibility
1450   based on a survey of 42 spider mite species (Acari: Tetranychidae) in Japan. *Heredity*
1451   *(Edinb)* **91**:208–216. doi:10.1038/sj.hdy.6800329

1452 Gotoh T, Sugasawa J, Noda H, Kitashima Y. 2007. *Wolbachia*-induced cytoplasmic
1453   incompatibility in Japanese populations of *Tetranychus urticae* (Acari: Tetranychidae).
1454   *Exp Appl Acarol* **42**:1–16. doi:10.1007/s10493-007-9072-3

1455 Hackstein JH. 1987. Spermatogenesis in Drosophila. *Results Probl Cell Differ* **15**:63–116.
1456   doi:10.1007/978-3-540-47184-4_2

1457 Hamm CA, Begun DJ, Vo A, Smith CCR, Saelao P, Shaver AO, Jaenike J, Turelli M. 2014.
1458   *Wolbachia* do not live by reproductive manipulation alone: infection polymorphism in
1459   *Drosophila suzukii* and *D. subpulchrella*. *Mol Ecol* **23**:4871–4885.
1460   doi:10.1111/mec.12901

1461 Harumoto T, Lemaitre B. 2018. Male-killing toxin in a bacterial symbiont of *Drosophila*. *Nature*
1462   1. doi:10/gddr6s

He Z, Zheng Y, Yu W-J, Fang Y, Mao B, Wang Y-F. 2019. How do *Wolbachia* modify the *Drosophila* ovary? New evidences support the "titration-restitution" model for the mechanisms of *Wolbachia*-induced CI. *BMC Genomics* **20**:608. doi:10.1186/s12864-019-5977-6

Hertig M. 1936. The Rickettsia, *Wolbachia pipientis* (gen. et sp.n.) and Associated Inclusions of the Mosquito, *Culex pipiens*. *Parasitology* **28**:453–486. doi:10.1017/S0031182000022666

Hertig M, Wolbach SB. 1924. Studies on Rickettsia-Like Micro-Organisms in Insects. *J Med Res* **44**:329-374.7.

Hoffmann AA. 1988. Partial cytoplasmic incompatibility between two Australian populations of *Drosophila melanogaster*. *Entomologia Experimentalis et Applicata* **48**:61–67. doi:10.1111/j.1570-7458.1988.tb02299.x

Hoffmann AA, Montgomery BL, Popovici J, Iturbe-Ormaetxe I, Johnson PH, Muzzi F, Greenfield M, Durkan M, Leong YS, Dong Y, Cook H, Axford J, Callahan AG, Kenny N, Omodei C, McGraw EA, Ryan PA, Ritchie SA, Turelli M, O'Neill SL. 2011. Successful establishment of *Wolbachia* in *Aedes* populations to suppress dengue transmission. *Nature* **476**:454–457. doi:10.1038/nature10356

Hoffmann AA, Turelli M, Simmons GM. 1986. Unidirectional incompatibility between populations of *Drosophila simulans*. *Evolution* **40**:692–701. doi:10.1111/j.1558-5646.1986.tb00531.x

Holden PR, Jones P, Brookfield JF. 1993. Evidence for a *Wolbachia* symbiont in *Drosophila melanogaster*. *Genet Res* **62**:23–29.

Hornett EA, Duplouy AMR, Davies N, Roderick GK, Wedell N, Hurst GDD, Charlat S. 2008. You can't keep a good parasite down: evolution of a male-killer suppressor uncovers cytoplasmic incompatibility. *Evolution* **62**:1258–1263. doi:10.1111/j.1558-5646.2008.00353.x

Huang H-J, Cui J-R, Chen J, Bing X-L, Hong X-Y. 2019. Proteomic analysis of *Laodelphax striatellus* gonads reveals proteins that may manipulate host reproduction by *Wolbachia*. *Insect Biochemistry and Molecular Biology* 103211. doi:10.1016/j.ibmb.2019.103211

Hughes GL, Rasgon JL. 2014. Transinfection: a method to investigate *Wolbachia*–host interactions and control arthropod-borne disease. *Insect Molecular Biology* **23**:141–151. doi:10/f5t46g

Hunter MS, Perlman SJ, Kelly SE. 2003. A bacterial symbiont in the Bacteroidetes induces cytoplasmic incompatibility in the parasitoid wasp *Encarsia pergandiella*. *Proc Biol Sci* **270**:2185–2190. doi:10.1098/rspb.2003.2475

Hurst LD. 1991. The evolution of cytoplasmic incompatibility or when spite can be successful. *J Theor Biol* **148**:269–277. doi:10.1016/s0022-5193(05)80344-3

Instructions to Authors: The Journal of Bacteriology, Nomenclature. n.d. http://jb.asm.org/site/misc/journal-ita_nom.xhtml

Iturbe-Ormaetxe I, Howie J, O'Neill S. 2007. Development of *Wolbachia* transformation by homologous recombination. Heron Island, Queensland, Australia: Progress report meeting for the Grand Challenges in Human Health Grant.

Jaenike J. 2007. Spontaneous emergence of a new *Wolbachia* phenotype. *Evolution* **61**:2244–2252. doi:10.1111/j.1558-5646.2007.00180.x

Jaenike J, Dyer KA, Cornish C, Minhas MS. 2006. Asymmetrical reinforcement and *Wolbachia* infection in *Drosophila*. *PLoS Biol* **4**:e325. doi:10.1371/journal.pbio.0040325

1509   Jernigan KK, Bordenstein SR. 2014. Ankyrin domains across the Tree of Life. *PeerJ* **2**:e264.
1510   doi:10.7717/peerj.264
1511   Jia F-X, Yang M-S, Yang W-J, Wang J-J. 2009. Influence of continuous high temperature
1512   conditions on *Wolbachia* infection frequency and the fitness of *Liposcelis tricolor*
1513   (Psocoptera: Liposcelididae). *Environ Entomol* **38**:1365–1372. doi:10.1603/022.038.0503
1514   Ju J-F, Hoffmann AA, Zhang Y-K, Duan X-Z, Guo Y, Gong J-T, Zhu W-C, Hong X-Y. 2017.
1515   *Wolbachia*-induced loss of male fertility is likely related to branch chain amino acid
1516   biosynthesis and iLvE in *Laodelphax striatellus*. *Insect Biochem Mol Biol* **85**:11–20.
1517   doi:10.1016/j.ibmb.2017.04.002
1518   Kajtoch Ł, Kotásková N. 2018. Current state of knowledge on *Wolbachia* infection among
1519   Coleoptera: a systematic review. *PeerJ* **6**:e4471. doi:10.7717/peerj.4471
1520   Karr TL, Yang W, Feder ME. 1998. Overcoming cytoplasmic incompatibility in *Drosophila*.
1521   *Proc Biol Sci* **265**:391–395. doi:10.1098/rspb.1998.0307
1522   Klasson L, Walker T, Sebaihia M, Sanders MJ, Quail MA, Lord A, Sanders S, Earl J, O'Neill
1523   SL, Thomson N, Sinkins SP, Parkhill J. 2008. Genome evolution of *Wolbachia* strain
1524   *w*Pip from the *Culex pipiens* group. *Mol Biol Evol* **25**:1877–1887.
1525   doi:10.1093/molbev/msn133
1526   Klasson L, Westberg J, Sapountzis P, Naslund K, Lutnaes Y, Darby AC, Veneti Z, Chen L,
1527   Braig HR, Garrett R, Bourtzis K, Andersson SGE. 2009. The mosaic genome structure of
1528   the *Wolbachia w*Ri strain infecting *Drosophila simulans*. *Proc Natl Acad Sci U S A*
1529   **106**:5725–5730. doi:10.1073/pnas.0810753106
1530   Knizewski L, Kinch LN, Grishin NV, Rychlewski L, Ginalski K. 2007. Realm of PD-(D/E)XK
1531   nuclease superfamily revisited: detection of novel families with modified transitive meta
1532   profile searches. *BMC Struct Biol* **7**:40. doi:10.1186/1472-6807-7-40
1533   König K, Zundel P, Krimmer E, König C, Pollmann M, Gottlieb Y, Steidle JLM. 2019.
1534   Reproductive isolation due to prezygotic isolation and postzygotic cytoplasmic
1535   incompatibility in parasitoid wasps. *Ecol Evol* **9**:10694–10706. doi:10.1002/ece3.5588
1536   Kose H, Karr TL. 1995. Organization of *Wolbachia pipientis* in the *Drosophila* fertilized egg and
1537   embryo revealed by an anti-*Wolbachia* monoclonal antibody. *Mech Dev* **51**:275–288.
1538   Kyritsis GA, Augustinos AA, Livadaras I, Cáceres C, Bourtzis K, Papadopoulos NT. 2019.
1539   Medfly-*Wolbachia* symbiosis: genotype x genotype interactions determine host's life
1540   history traits under mass rearing conditions. *BMC Biotechnol* **19**:96. doi:10.1186/s12896-
1541   019-0586-7
1542   Landmann F, Orsi GA, Loppin B, Sullivan W. 2009. *Wolbachia*-Mediated Cytoplasmic
1543   Incompatibility Is Associated with Impaired Histone Deposition in the Male Pronucleus.
1544   *PLOS Pathogens* **5**:e1000343. doi:10.1371/journal.ppat.1000343
1545   Lassy CW, Karr TL. 1996. Cytological analysis of fertilization and early embryonic
1546   development in incompatible crosses of *Drosophila simulans*. *Mechanisms of*
1547   *Development* **57**:47–58. doi:10.1016/0925-4773(96)00527-8
1548   Laven H. 1967. Eradication of *Culex pipiens fatigans* through Cytoplasmic Incompatibility.
1549   *Nature* **216**:383–384. doi:10.1038/216383a0
1550   Laven H. 1951. Crossing Experiments with *Culex* Strains. *Evolution* **5**:370–375.
1551   doi:10.1111/j.1558-5646.1951.tb02795.x
1552   Layton EM, On J, Perlmutter JI, Bordenstein SR, Shropshire JD. 2019. Paternal grandmother age
1553   affects the strength of *Wolbachia*-induced cytoplasmic incompatibility in *Drosophila*
1554   *melanogaster*. *mBio* **10**. doi:10.1128/mBio.01879-19

1555 Lefoulon E T, Clark T, Borveto F, Perriat-Sanguinet M, Moulia C, Slatko B, Gavotte L. 2020.
1556   Pseudoscorpion Wolbachia symbionts: Diversity and Evidence for a New Supergroup S.
1557   *BMC Microbiology*. doi:10.21203/rs.2.22118/v1

1558 LePage DP, Jernigan KK, Bordenstein SR. 2014. The relative importance of DNA methylation
1559   and Dnmt2-mediated epigenetic regulation on *Wolbachia* densities and cytoplasmic
1560   incompatibility. *PeerJ* **2**:e678. doi:10.7717/peerj.678

1561 LePage DP, Metcalf JA, Bordenstein Sarah R., On J, Perlmutter JI, Shropshire JD, Layton EM,
1562   Funkhouser-Jones LJ, Beckmann J, Bordenstein Seth R. 2017. Prophage WO genes
1563   recapitulate and enhance *Wolbachi*a-induced cytoplasmic incompatibility. *Nature*
1564   **543**:243–247. doi:10.1038/nature21391

1565 Lewis Z, Champion de Crespigny FE, Sait SM, Tregenza T, Wedell N. 2011. *Wolbachia*
1566   infection lowers fertile sperm transfer in a moth. *Biol Lett* **7**:187–189.
1567   doi:10.1098/rsbl.2010.0605

1568 Lindsey A, Rice DW, Bordenstein Sarah R., Brooks AW, Bordenstein Seth R., Newton ILG.
1569   2018. Evolutionary genetics of cytoplasmic incompatibility genes *cifA* and *cifB* in
1570   prophage WO of *Wolbachia*. *Genome Biol Evol* **10**:434–451. doi:10/gcvmkm

1571 Lindsley DL. 1980. Spermatogenesis. *The Genetics and Biology of Drosophila* **2**:225–294.

1572 Liu C, Wang J-L, Zheng Y, Xiong E-J, Li J-J, Yuan L-L, Yu X-Q, Wang Y-F. 2014. *Wolbachia*-
1573   induced paternal defect in *Drosophila* is likely by interaction with the juvenile hormone
1574   pathway. *Insect Biochem Mol Biol* **49**:49–58. doi:10.1016/j.ibmb.2014.03.014

1575 Liu L, Zhang K-J, Rong X, Li Y-Y, Liu H. 2019. Identification of *Wolbachia*-Responsive
1576   miRNAs in the Small Brown Planthopper, *Laodelphax striatellus*. *Front Physiol* **10**:928.
1577   doi:10.3389/fphys.2019.00928

1578 Lo N, Paraskevopoulos C, Bourtzis K, O'Neill SL, Werren JH, Bordenstein SR, Bandi C. 2007a.
1579   Taxonomic status of the intracellular bacterium *Wolbachia pipientis*. *International*
1580   *Journal of Systematic and Evolutionary Microbiology,* **57**:654–657.
1581   doi:10.1099/ijs.0.64515-0

1582 Lo N, Paraskevopoulos C, Bourtzis K, O'Neill SL, Werren JH, Bordenstein SR, Bandi C. 2007b.
1583   Taxonomic status of the intracellular bacterium *Wolbachia pipientis*. *Int J Syst Evol*
1584   *Microbiol* **57**:654–657. doi:10.1099/ijs.0.64515-0

1585 Lu M-H, Zhang K-J, Hong X-Y. 2012. Tripartite associations among bacteriophage WO,
1586   *Wolbachia*, and host affected by temperature and age in *Tetranychus urticae*. *Exp Appl*
1587   *Acarol* **58**:207–220. doi:10.1007/s10493-012-9578-1

1588 Madhav M, Parry R, Morgan JAT, James P, Asgari S. 2020. *Wolbachia* endosymbiont of the
1589   horn fly *Haematobia irritans irritans* : a supergroup A strain with multiple horizontally
1590   acquired cytoplasmic incompatibility genes. *Applied and Environmental Microbiology*.
1591   doi:10.1128/AEM.02589-19

1592 Mains JW, Brelsfoard CL, Rose RI, Dobson SL. 2016. Female Adult *Aedes albopictus*
1593   Suppression by *Wolbachia*-Infected Male Mosquitoes. *Scientific Reports* **6**:33846.
1594   doi:10.1038/srep33846

1595 Mains JW, Kelly PH, Dobson KL, Petrie WD, Dobson SL. 2019. Localized Control of *Aedes*
1596   *aegypti* (Diptera: Culicidae) in Miami, FL, via Inundative Releases of *Wolbachia*-
1597   Infected Male Mosquitoes. *J Med Entomol* **56**:1296–1303. doi:10.1093/jme/tjz051

1598 Mann E, Stouthamer CM, Kelly SE, Dzieciol M, Hunter MS, Schmitz-Esser S. 2017.
1599   Transcriptome Sequencing Reveals Novel Candidate Genes for *Cardinium hertigii*-

1600   Caused Cytoplasmic Incompatibility and Host-Cell Interaction. *mSystems* **2**.
1601   doi:10.1128/mSystems.00141-17
1602 Marshall JF. 1938. The British mosquitoes. London: British Museum.
1603 Martinez J, Klasson L, Welch JJ, Jiggins FM. 2020. Life and death of selfish genes: comparative
1604   genomics reveals the dynamic evolution of cytoplasmic incompatibility. *Mol Biol Evol*.
1605   doi:10.1093/molbev/msaa209
1606 Martinez-Rodriguez P, Bella JL. 2018. *Chorthippus parallelus* and *Wolbachia*: Overlapping
1607   Orthopteroid and Bacterial Hybrid Zones. *Front Genet* **9**:604.
1608   doi:10.3389/fgene.2018.00604
1609 Mayr E. 1963. Animal species and evolution. Belknap Press of Harvard University Press.
1610 Meany MK, Conner WR, Richter SV, Bailey JA, Turelli M, Cooper BS. 2019. Loss of
1611   cytoplasmic incompatibility and minimal fecundity effects explain relatively low
1612   *Wolbachia* frequencies in *Drosophila mauritiana*. *Evolution*. doi:10.1111/evo.13745
1613 Metcalf JA, Jo M, Bordenstein Sarah R., Jaenike J, Bordenstein Seth R. 2014. Recent genome
1614   reduction of *Wolbachia* in *Drosophila recens* targets phage WO and narrows candidates
1615   for reproductive parasitism. *PeerJ* **2**:e529. doi:10.7717/peerj.529
1616 Moreira LA, Iturbe-Ormaetxe I, Jeffery JA, Lu G, Pyke AT, Hedges LM, Rocha BC, Hall-
1617   Mendelin S, Day A, Riegler M, Hugo LE, Johnson KN, Kay BH, McGraw EA, van den
1618   Hurk AF, Ryan PA, O'Neill SL. 2009. A *Wolbachia* symbiont in *Aedes aegypti* limits
1619   infection with dengue, Chikungunya, and Plasmodium. *Cell* **139**:1268–1278.
1620   doi:10.1016/j.cell.2009.11.042
1621 Mouton L, Henri H, Bouletreau M, Vavre F. 2006. Effect of temperature on *Wolbachia* density
1622   and impact on cytoplasmic incompatibility. *Parasitology* **132**:49–56.
1623   doi:10.1017/S0031182005008723
1624 Nguyen DT, Morrow JL, Spooner-Hart RN, Riegler M. 2017. Independent cytoplasmic
1625   incompatibility induced by *Cardinium* and *Wolbachia* maintains endosymbiont
1626   coinfections in haplodiploid thrips populations. *Evolution* **71**:995–1008.
1627   doi:10.1111/evo.13197
1628 O'Connor L, Plichart C, Sang AC, Brelsfoard CL, Bossin HC, Dobson SL. 2012. Open release of
1629   male mosquitoes infected with a *Wolbachia* biopesticide: field performance and infection
1630   containment. *PLoS Negl Trop Dis* **6**:e1797. doi:10.1371/journal.pntd.0001797
1631 O'Neill SL. 2018. The use of *Wolbachia* by the World Mosquito Program to interrupt
1632   transmission of *Aedes aegypti* transmitted viruses. *Adv Exp Med Biol* **1062**:355–360.
1633   doi:10.1007/978-981-10-8727-1_24
1634 O'Neill SL, Karr TL. 1990. Bidirectional incompatibility between conspecific populations of
1635   *Drosophila simulans*. *Nature* **348**:178–180. doi:10.1038/348178a0
1636 O'Neill SL, Ryan PA, Turley AP, Wilson G, Retzki K, Iturbe-Ormaetxe I, Dong Y, Kenny N,
1637   Paton CJ, Ritchie SA, Brown-Kenyon J, Stanford D, Wittmeier N, Anders KL, Simmons
1638   CP. 2018. Scaled deployment of *Wolbachia* to protect the community from dengue and
1639   other *Aedes* transmitted arboviruses. *Gates Open Res* **2**:36.
1640   doi:10.12688/gatesopenres.12844.2
1641 Ote M, Ueyama M, Yamamoto D. 2016. *Wolbachia* Protein TomO Targets nanos mRNA and
1642   Restores Germ Stem Cells in *Drosophila* Sex-lethal Mutants. *Curr Biol* **26**:2223–2232.
1643   doi:10.1016/j.cub.2016.06.054

Papafotiou G, Oehler S, Savakis C, Bourtzis K. 2011. Regulation of *Wolbachia* ankyrin domain encoding genes in *Drosophila* gonads. *Res Microbiol* **162**:764–772. doi:10.1016/j.resmic.2011.06.012

Perlman SJ, Dowdy NJ, Harris LR, Khalid M, Kelly SE, Hunter MS. 2014. Factors affecting the strength of *Cardinium*-induced cytoplasmic incompatibility in the parasitic wasp *Encarsia pergandiella* (Hymenoptera: Aphelinidae). *Microb Ecol* **67**:671–678. doi:10.1007/s00248-013-0359-0

Pinto SB, Stainton K, Harris S, Kambris Z, Sutton ER, Bonsall MB, Parkhill J, Sinkins SP. 2013. Transcriptional regulation of *Culex pipiens* mosquitoes by *Wolbachia* influences cytoplasmic incompatibility. *PLoS Pathog* **9**:e1003647. doi:10.1371/journal.ppat.1003647

Poinsot D, Bourtzis K, Markakis G, Savakis C, Mercot H. 1998. *Wolbachia* transfer from *Drosophila melanogaster* into *D. simulans*: Host effect and cytoplasmic incompatibility relationships. *Genetics* **150**:227–237.

Poinsot D, Charlat S, Mercot H. 2003. On the mechanism of *Wolbachia*-induced cytoplasmic incompatibility: confronting the models with the facts. *Bioessays* **25**:259–265. doi:10.1002/bies.10234

Prout T. 1994. Some Evolutionary Possibilities for a Microbe that Causes Incompatibility in its Host. *Evolution* **48**:909–911. doi:10.2307/2410496

Puggioli A, Calvitti M, Moretti R, Bellini R. 2016. *w*Pip *Wolbachia* contribution to *Aedes albopictus* SIT performance: Advantages under intensive rearing. *Acta Trop* **164**:473–481. doi:10.1016/j.actatropica.2016.10.014

Ramírez-Puebla ST, Ormeño-Orrillo E, Vera-Ponce de León A, Lozano L, Sanchez-Flores A, Rosenblueth M, Martínez-Romero E. 2016. Genomes of *Candidatus Wolbachia bourtzisii* *w*DacA and *Candidatus Wolbachia pipientis* *w*DacB from the Cochineal Insect *Dactylopius coccus* (Hemiptera: Dactylopiidae). *G3 (Bethesda)* **6**:3343–3349. doi:10.1534/g3.116.031237

Rasgon J. 2007. Population replacement strategies for controlling vector populations and the use of Wolbachia pipientis for genetic drive. *J Vis Exp* 225. doi:10.3791/225

Rasgon JL. 2008. Using Predictive Models to Optimize *Wolbachia*-Based Strategies for Vector-Borne Disease Control In: Aksoy S, editor. Transgenesis and the Management of Vector-Borne Disease. New York, NY: Springer New York. pp. 114–125. doi:10.1007/978-0-387-78225-6_10

Rathke C, Baarends WM, Awe S, Renkawitz-Pohl R. 2014. Chromatin dynamics during spermiogenesis. *Biochimica et Biophysica Acta (BBA) - Gene Regulatory Mechanisms*, Chromatin and epigenetic regulation of animal development **1839**:155–168. doi:10.1016/j.bbagrm.2013.08.004

Raychoudhury R, Werren JH. 2012. Host genotype changes bidirectional to unidirectional cytoplasmic incompatibility in *Nasonia longicornis*. *Heredity (Edinb)* **108**:105–114. doi:10.1038/hdy.2011.53

Reed K, Werren J. 1995. Induction of paternal genome loss by the paternal-sex-ratio chromosome and cytoplasmic incompatibility bacteria (*Wolbachia*) - A comparative-study of early embryonic events. *Molecular Reproduction and Development* **40**:408–418. doi:10.1002/mrd.1080400404

Reynolds KT, Hoffmann AA. 2002. Male age, host effects and the weak expression or non-expression of cytoplasmic incompatibility in *Drosophila* strains infected by maternally transmitted *Wolbachia. Genet Res* **80**:79–87.

Riegler M, Stauffer C. 2002. *Wolbachia* infections and superinfections in cytoplasmically incompatible populations of the European cherry fruit fly *Rhagoletis cerasi* (Diptera, Tephritidae). *Mol Ecol* **11**:2425–2434.

Riparbelli MG, Giordano R, Callaini G. 2007. Effects of Wolbachia on sperm maturation and architecture in *Drosophila simulans* Riverside. *Mechanisms of Development* **124**:699–714. doi:10.1016/j.mod.2007.07.001

Ripley BM, Gildenberg MS, Washington MT. 2020. Control of DNA Damage Bypass by Ubiquitylation of PCNA. *Genes (Basel)* **11**. doi:10.3390/genes11020138

Rocha-Lima H da. 1916. Zur Aetiologie des Fleckfiebers. *Dtsch Med Wochenschr* **42**:1353–1354. doi:10.1055/s-0028-1135469

Rosenwald LC, Sitvarin MI, White JA. 2020. Endosymbiotic Rickettsiella causes cytoplasmic incompatibility in a spider host. *bioRxiv* 2020.03.03.975581. doi:10.1101/2020.03.03.975581

Ross PA, Axford JK, Callahan AG, Richardson KM, Hoffmann AA. 2019a. Stable phenotypic effects of an unstable deleterious Wolbachia infection. *bioRxiv* 853473. doi:10.1101/853473

Ross PA, Axford JK, Yang Q, Staunton KM, Ritchie SA, Richardson KM, Hoffmann AA. 2020. Heatwaves cause fluctuations in *w*Mel *Wolbachia* densities and frequencies in *Aedes aegypti. PLoS Negl Trop Dis* **14**:e0007958. doi:10.1371/journal.pntd.0007958

Ross PA, Ritchie SA, Axford JK, Hoffmann AA. 2019b. Loss of cytoplasmic incompatibility in *Wolbachia*-infected *Aedes aegypti* under field conditions. *PLOS Neglected Tropical Diseases* **13**:e0007357. doi:10.1371/journal.pntd.0007357

Rousset F, Solignac M. 1995. Evolution of single and double *Wolbachia* symbioses during speciation in the *Drosophila simulans* complex. *Proc Natl Acad Sci U S A* **92**:6389–6393.

Ryan S, Saul G. 1968. Post-fertilization effect of incompatibility factors in *Mormoniella. Molecular and General Genetics* **103**:29-. doi:10.1007/BF00271154

Sakamoto H, Ishikawa Y, Sasaki T, Kikuyama S, Tatsuki S, Hoshizaki S. 2005. Transinfection reveals the crucial importance of *Wolbachia* genotypes in determining the type of reproductive alteration in the host. *Genet Res* **85**:205–210. doi:10.1017/S0016672305007573

Salzberg SL, Puiu D, Sommer DD, Nene V, Lee NH. 2009. Genome sequence of the *Wolbachia* endosymbiont of *Culex quinquefasciatus* JHB. *J Bacteriol* **191**:1725. doi:10.1128/JB.01731-08

Sasaki T, Kubo T, Ishikawa H. 2002. Interspecific transfer of *Wolbachia* between two lepidopteran insects expressing cytoplasmic incompatibility: a *Wolbachia* variant naturally infecting *Cadra cautella* causes male killing in *Ephestia kuehniella. Genetics* **162**:1313–1319.

Shoemaker DD, Katju V, Jaenike J. 1999. *Wolbachia* and the Evolution of Reproductive Isolation Between *Drosophila recens* and *Drosophila subquinaria. Evolution* **53**:1157–1164. doi:10.1111/j.1558-5646.1999.tb04529.x

Shropshire JD, Bordenstein SR. 2019. Two-by-one model of cytoplasmic incompatibility: synthetic recapitulation by transgenic expression of *cifA* and *cifB* in *Drosophila. PLOS Genetics* **15**:e1008221. doi:10.1371/journal.pgen.1008221

1734 Shropshire JD, Kalra M, Bordenstein SR. 2020. Evolution-guided mutagenesis of the
1735   cytoplasmic incompatibility proteins: Identifying CifA's complex functional repertoire
1736   and new essential regions in CifB. *bioRxiv* 2020.05.13.093815.
1737   doi:10.1101/2020.05.13.093815

1738 Shropshire JD, Leigh B, Bordenstein Sarah R., Duplouy A, Riegler M, Brownlie JC, Bordenstein
1739   Seth R. 2019. Models and nomenclature for cytoplasmic incompatibility: caution over
1740   premature conclusions – a response to Beckmann et al. *Trends in Genetics* **0**.
1741   doi:10.1016/j.tig.2019.03.004

1742 Shropshire JD, On J, Layton EM, Zhou H, Bordenstein SR. 2018. One prophage WO gene
1743   rescues cytoplasmic incompatibility in *Drosophila melanogaster*. *Proc Natl Acad Sci U S*
1744   *A* **115**:4987–4991. doi:10.1073/pnas.1800650115

1745 Sicard M, Bouchon D, Ceyrac L, Raimond R, Thierry M, Le Clec'h W, Marcadé I, Caubet Y,
1746   Grève P. 2014. Bidirectional cytoplasmic incompatibility caused by *Wolbachia* in the
1747   terrestrial isopod *Porcellio dilatatus*. *J Invertebr Pathol* **121**:28–36.
1748   doi:10.1016/j.jip.2014.06.007

1749 Sinkins SP, Braig HR, O'Neill SL. 1995. *Wolbachia* superinfections and the expression of
1750   cytoplasmic incompatibility. *Proc Biol Sci* **261**:325–330. doi:10.1098/rspb.1995.0154

1751 Smith-White S, Woodhill AR. 1955. The Nature and Significance of non-reciprocal Fertility in
1752   *Aedes scutellaris* and other Mosquitoes. *Proceedings of the Linnean Society of New South*
1753   *Wales* **79**.

1754 Snook RR, Cleland SY, Wolfner MF, Karr TL. 2000. Offsetting effects of *Wolbachia* infection
1755   and heat shock on sperm production in *Drosophila simulans*: Analyses of fecundity,
1756   fertility and accessory gland proteins. *Genetics* **155**:167–178.

1757 Sumi T, Miura K, Miyatake T. 2017. *Wolbachia* density changes seasonally amongst populations
1758   of the pale grass blue butterfly, *Zizeeria maha* (Lepidoptera: Lycaenidae). *PLoS ONE*
1759   **12**:e0175373. doi:10.1371/journal.pone.0175373

1760 Sutton ER, Harris SR, Parkhill J, Sinkins SP. 2014. Comparative genome analysis of *Wolbachia*
1761   strain *w*Au. *BMC Genomics* **15**:928. doi:10.1186/1471-2164-15-928

1762 Takano S, Tuda M, Takasu K, Furuya N, Imamura Y, Kim S, Tashiro K, Iiyama K, Tavares M,
1763   Amaral AC. 2017. Unique clade of alphaproteobacterial endosymbionts induces complete
1764   cytoplasmic incompatibility in the coconut beetle. *PNAS* **114**:6110–6115.
1765   doi:10.1073/pnas.1618094114

1766 Tan JMM, Wong ESP, Kirkpatrick DS, Pletnikova O, Ko HS, Tay S-P, Ho MWL, Troncoso J,
1767   Gygi SP, Lee MK, Dawson VL, Dawson TM, Lim K-L. 2008. Lysine 63-linked
1768   ubiquitination promotes the formation and autophagic clearance of protein inclusions
1769   associated with neurodegenerative diseases. *Hum Mol Genet* **17**:431–439.
1770   doi:10.1093/hmg/ddm320

1771 Tantowijoyo W, Andari B, Arguni E, Budiwati N, Nurhayati I, Fitriana I, Ernesia I, Daniwijaya
1772   EW, Supriyati E, Yusdiana DH, Victorius M, Wardana DS, Ardiansyah H, Ahmad RA,
1773   Ryan PA, Simmons CP, Hoffmann AA, Rancès E, Turley AP, Johnson P, Utarini A,
1774   O'Neill SL. 2020. Stable establishment of wMel Wolbachia in Aedes aegypti populations
1775   in Yogyakarta, Indonesia. *PLoS Negl Trop Dis* **14**:e0008157.
1776   doi:10.1371/journal.pntd.0008157

1777 Teixeira L, Ferreira A, Ashburner M. 2008. The bacterial symbiont *Wolbachia* induces resistance
1778   to RNA viral infections in *Drosophila melanogaster*. *PLoS Biol* **6**:e2.
1779   doi:10.1371/journal.pbio.1000002

1780 Thiem S. 2014. A genetic manipulation system for *Wolbachia* in mosquitoes. Michigan State
1781   University: USDA.
1782 Tram U, Fredrick K, Werren JH, Sullivan W. 2006. Paternal chromosome segregation during the
1783   first mitotic division determines *Wolbachia*-induced cytoplasmic incompatibility
1784   phenotype. *Journal of Cell Science* **119**:3655–3663. doi:10.1242/jcs.03095
1785 Tram U, Sullivan W. 2002. Role of delayed nuclear envelope breakdown and mitosis in
1786   *Wolbachia*-induced cytoplasmic incompatibility. *Science* **296**:1124–1126.
1787   doi:10.1126/science.1070536
1788 Trpis M, Perrone JB, Reissig M, Parker KL. 1981. Control of cytoplasmic incompatibility in the
1789   *Aedes scutellaris* complex Incompatible crosses become compatible by treatment of
1790   larvae with heat or antibiotics. *J Hered* **72**:313–317.
1791   doi:10.1093/oxfordjournals.jhered.a109513
1792 Turelli M. 1994. Evolution of incompatibility-inducing microbes and their hosts. *Evolution*
1793   **48**:1500–1513. doi:10.1111/j.1558-5646.1994.tb02192.x
1794 Turelli M, Cooper BS, Richardson KM, Ginsberg PS, Peckenpaugh B, Antelope CX, Kim KJ,
1795   May MR, Abrieux A, Wilson DA, Bronski MJ, Moore BR, Gao J-J, Eisen MB, Chiu JC,
1796   Conner WR, Hoffmann AA. 2018. Rapid global spread of *w*Ri-like *Wolbachia* across
1797   multiple *Drosophila*. *Current Biology* **28**:963-971.e8. doi:10.1016/j.cub.2018.02.015
1798 Turelli M, Hoffmann AA. 1995. Cytoplasmic incompatibility in *Drosophila simulans*: dynamics
1799   and parameter estimates from natural populations. *Genetics* **140**:1319–1338.
1800 Vala F, Weeks A, Claessen D, Breeuwer J a. J, Sabelis MW. 2002. Within- and between-
1801   population variation for *Wolbachia*-induced reproductive incompatibility in a
1802   haplodiploid mite. *Evolution* **56**:1331–1339.
1803 van den Hurk AF, Hall-Mendelin S, Pyke AT, Frentiu FD, McElroy K, Day A, Higgs S, O'Neill
1804   SL. 2012. Impact of *Wolbachia* on infection with chikungunya and yellow fever viruses
1805   in the mosquito vector *Aedes aegypti*. *PLoS Negl Trop Dis* **6**:e1892.
1806   doi:10.1371/journal.pntd.0001892
1807 van Opijnen T, Breeuwer JA. 1999. High temperatures eliminate *Wolbachia*, a cytoplasmic
1808   incompatibility inducing endosymbiont, from the two-spotted spider mite. *Exp Appl*
1809   *Acarol* **23**:871–881.
1810 Vavre F, Dedeine F, Quillon M, Fouillet P, Fleury F, Bouletreau M. 2001. Within-species
1811   diversity of *Wolbachia*-induced cytoplasmic incompatibility in haplodiploid insects.
1812   *Evolution* **55**:1710–1714.
1813 Vavre F, Fleury F, Varaldi J, Fouillet P, Bouletreau M. 2000. Evidence for female mortality in
1814   *Wolbachia*-mediated cytoplasmic incompatibility in haplodiploid insects: epidemiologic
1815   and evolutionary consequences. *Evolution* **54**:191–200.
1816 Veneti Z, Clark ME, Zabalou S, Karr TL, Savakis C, Bourtzis K. 2003. Cytoplasmic
1817   incompatibility and sperm cyst infection in different *Drosophila-Wolbachia* associations.
1818   *Genetics* **164**:545–552.
1819 Walker T, Johnson PH, Moreira LA, Iturbe-Ormaetxe I, Frentiu FD, McMeniman CJ, Leong YS,
1820   Dong Y, Axford J, Kriesner P, Lloyd AL, Ritchie SA, O'Neill SL, Hoffmann AA. 2011.
1821   The *w*Mel *Wolbachia* strain blocks dengue and invades caged *Aedes aegypti* populations.
1822   *Nature* **476**:450–453. doi:10.1038/nature10355
1823 Wang GH, Jia L-Y, Xiao J-H, Huang D-W. 2016. Discovery of a new *Wolbachia* supergroup in
1824   cave spider species and the lateral transfer of phage WO among distant hosts. *Infect*
1825   *Genet Evol* **41**:1–7. doi:10.1016/j.meegid.2016.03.015

Weeks AR, Turelli M, Harcombe WR, Reynolds KT, Hoffmann AA. 2007. From parasite to mutualist: Rapid evolution of *Wolbachia* in natural populations of *Drosophila*. *Plos Biology* **5**:997–1005. doi:10.1371/journal.pbio.0050114

Weinert LA, Araujo-Jnr EV, Ahmed MZ, Welch JJ. 2015. The incidence of bacterial endosymbionts in terrestrial arthropods. *Proc R Soc B* **282**:20150249. doi:10.1098/rspb.2015.0249

Werren JH. 1997. Biology of *Wolbachia*. *Annual Review of Entomology* **42**:587–609. doi:10.1146/annurev.ento.42.1.587

Wertz IE, Dixit VM. 2010. Signaling to NF-kappaB: regulation by ubiquitination. *Cold Spring Harb Perspect Biol* **2**:a003350. doi:10.1101/cshperspect.a003350

WHO. 2016. Mosquito (vector) control emergency response and preparedness for Zika virus. *WHO*. http://www.who.int/neglected_diseases/news/mosquito_vector_control_response/en/

Wiwatanaratanabutr I, Kittayapong P. 2009. Effects of crowding and temperature on *Wolbachia* infection density among life cycle stages of *Aedes albopictus*. *J Invertebr Pathol* **102**:220–224. doi:10.1016/j.jip.2009.08.009

Wright J, Sjostrand F, Portaro J, Barr A. 1978. Ultrastructure of Rickettsia-like microorganism *Wolbachia pipientis* and associated virus-like bodies in mosquito *Culex pipiens*. *Journal of Ultrastructure Research* **63**:79–85. doi:10.1016/S0022-5320(78)80046-X

Wright JD, Wang B-T. 1980. Observations on *Wolbachiae* in mosquitoes. *Journal of Invertebrate Pathology* **35**:200–208. doi:10.1016/0022-2011(80)90185-8

Wu M, Sun LV, Vamathevan J, Riegler M, Deboy R, Brownlie JC, McGraw EA, Martin W, Esser C, Ahmadinejad N, Wiegand C, Madupu R, Beanan MJ, Brinkac LM, Daugherty SC, Durkin AS, Kolonay JF, Nelson WC, Mohamoud Y, Lee P, Berry K, Young MB, Utterback T, Weidman J, Nierman WC, Paulsen IT, Nelson KE, Tettelin H, O'Neill SL, Eisen JA. 2004. Phylogenomics of the reproductive parasite *Wolbachia pipientis w*Mel: A streamlined genome overrun by mobile genetic elements. *Plos Biology* **2**:327–341. doi:10.1371/journal.pbio.0020069

Xi Z, Gavotte L, Xie Y, Dobson SL. 2008. Genome-wide analysis of the interaction between the endosymbiotic bacterium *Wolbachia* and its *Drosophila* host. *BMC Genomics* **9**:1. doi:10.1186/1471-2164-9-1

Xi Z, Khoo CCH, Dobson SL. 2005. *Wolbachia* establishment and invasion in an *Aedes aegypti* laboratory population. *Science* **310**:326–328. doi:10.1126/science.1117607

Yamada R, Floate KD, Riegler M, O'Neill SL. 2007. Male development time influences the strength of *Wolbachia*-induced cytoplasmic incompatibility expression in *Drosophila melanogaster*. *Genetics* **177**:801–808. doi:10.1534/genetics.106.068486

Yamada R, Iturbe-Ormaetxe I, Brownlie JC, O'Neill SL. 2011. Functional test of the influence of *Wolbachia* genes on cytoplasmic incompatibility expression in *Drosophila melanogaster*. *Insect Mol Biol* **20**:75–85. doi:10.1111/j.1365-2583.2010.01042.x

Yeap HL, Rasic G, Endersby-Harshman NM, Lee SF, Arguni E, Le Nguyen H, Hoffmann AA. 2016. Mitochondrial DNA variants help monitor the dynamics of *Wolbachia* invasion into host populations. *Heredity (Edinb)* **116**:265–276. doi:10.1038/hdy.2015.97

Yen JH, Barr AR. 1973. The etiological agent of cytoplasmic incompatibility in *Culex pipiens*. *Journal of Invertebrate Pathology* **22**:242–250. doi:10.1016/0022-2011(73)90141-9

Yen JH, Barr AR. 1971. New Hypothesis of the Cause of Cytoplasmic Incompatibility in *Culex pipiens* L. *Nature* **232**:657–658. doi:10.1038/232657a0

Yuan L-L, Chen X, Zong Q, Zhao T, Wang J-L, Zheng Y, Zhang M, Wang Z, Brownlie JC, Yang F, Wang Y-F. 2015. Quantitative Proteomic Analyses of Molecular Mechanisms Associated with Cytoplasmic Incompatibility in *Drosophila melanogaster* Induced by *Wolbachia*. *J Proteome Res* **14**:3835–3847. doi:10.1021/acs.jproteome.5b00191

Zabalou S, Apostolaki A, Pattas S, Veneti Z, Paraskevopoulos C, Livadaras I, Markakis G, Brissac T, Mercot H, Bourtzis K. 2008. Multiple rescue factors within a *Wolbachia* strain. *Genetics* **178**:2145–2160. doi:10.1534/genetics.107.086488

Zabalou S, Charlat S, Nirgianaki A, Lachaise D, Mercot H, Bourtzis K. 2004. Natural *Wolbachia* infections in the *Drosophila yakuba* species complex do not induce cytoplasmic incompatibility but fully rescue the *w*Ri modification. *Genetics* **167**:827–834. doi:10.1534/genetics.103.015990

Zchori-Fein E, Perlman SJ. 2004. Distribution of the bacterial symbiont *Cardinium* in arthropods. *Mol Ecol* **13**:2009–2016. doi:10.1111/j.1365-294X.2004.02203.x

Zhang Y-K, Ding X-L, Rong X, Hong X-Y. 2015. How do hosts react to endosymbionts? A new insight into the molecular mechanisms underlying the *Wolbachia*-host association. *Insect Mol Biol* **24**:1–12. doi:10.1111/imb.12128

Zheng X, Zhang D, Li Y, Yang C, Wu Y, Liang X, Liang Y, Pan X, Hu L, Sun Q, Wang X, Wei Y, Zhu J, Qian W, Yan Z, Parker AG, Gilles JRL, Bourtzis K, Bouyer J, Tang M, Zheng B, Yu J, Liu J, Zhuang J, Hu Zhigang, Zhang M, Gong J-T, Hong X-Y, Zhang Z, Lin L, Liu Q, Hu Zhiyong, Wu Z, Baton LA, Hoffmann AA, Xi Z. 2019. Incompatible and sterile insect techniques combined eliminate mosquitoes. *Nature* **572**:56–61. doi:10.1038/s41586-019-1407-9

Zheng Y, Ren P-P, Wang J-L, Wang Y-F. 2011. *Wolbachia*-induced cytoplasmic incompatibility is associated with decreased Hira expression in male *Drosophila*. *PLoS ONE* **6**:e19512. doi:10.1371/journal.pone.0019512

Zheng Y, Shen W, Bi J, Chen M-Y, Wang R-F, Ai H, Wang Y-F. 2019. Small RNA analysis provides new insights into cytoplasmic incompatibility in *Drosophila melanogaster* induced by *Wolbachia*. *J Insect Physiol* **118**:103938. doi:10.1016/j.jinsphys.2019.103938

Zug R, Hammerstein P. 2015. *Wolbachia* and the insect immune system: what reactive oxygen species can tell us about the mechanisms of *Wolbachia*–host interactions. *Front Microbiol* **6**. doi:10.3389/fmicb.2015.01201

Zug R, Hammerstein P. 2012. Still a host of hosts for *Wolbachia*: analysis of recent data suggests that 40% of terrestrial arthropod species are infected. *PLOS ONE* **7**:e38544. doi:10.1371/journal.pone.0038544