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1 **From *Wolbachia* genomics to phenotype: molecular models of**
2 **cytoplasmic incompatibility must account for the multiplicity of**
3 **compatibility types.**

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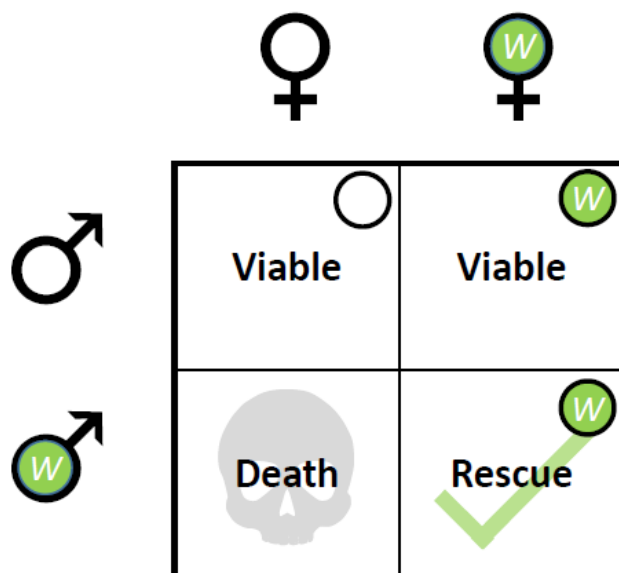
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11 **Abstract**

12 *Wolbachia* endosymbionts commonly induce cytoplasmic incompatibility, making infected
13 males' sperm lethal to the embryos unless these are rescued by the same bacterium,
14 inherited from their mother. Causal genes were recently identified but two families of
15 mechanistic models are still opposed. In the toxin-antidote model, interaction between the
16 toxin and the antidote is required for rescuing the embryos. In host modification models, a
17 host factor is misregulated in sperm and rescue occurs through compensation or withdrawal
18 of this modification. While these models have been thoroughly discussed, the multiplicity of
19 compatibility types, i.e., the existence of many mutually incompatible strains, as seen in
20 *Culex* mosquitoes, has not received sufficient attention. To explain such a fact, host
21 modification models must posit that the same embryonic defects can be induced and
22 rescued through a large variety of host targets. Conversely, the toxin-antidote model simply
23 accommodates this pattern in a lock-key fashion, through variations in the toxin-antidote
24 interaction sites.

25
26
27 Keywords: *Wolbachia*, toxin-antidote, symbiosis, *Culex*, *Drosophila*

28 **Introduction**

29 Cytoplasmic incompatibility (CI) denotes a phenomenon of conditional sterility induced by
30 various endosymbiotic bacteria, of which *Wolbachia* is the most renowned. In its simplest
31 form (Figure 1), CI occurs when males carrying *Wolbachia* mate with uninfected females. In
32 such crosses, fertilization takes place normally but most or all embryos die before hatching
33 [1–3]. In contrast, development proceeds if the female is infected, regardless of the male’s
34 infection status. This means that *Wolbachia* makes infected females *more* fertile than
35 uninfected ones on average and on the contrary renders infected males *less* fertile than
36 uninfected ones. However, the male side does not matter as far as *Wolbachia* is concerned
37 since only females transmit the infection to their offspring, through the egg cytoplasm. The
38 infected lineage frequency thus tends to increase, and more effectively so if the infection
39 has no negative side effect and is perfectly transmitted across generations [4]. In this
40 perspective, CI is understood as a selfish adaptive feature of the symbiont, increasing its
41 chances of invading new species following rare events of horizontal transmission [5].



42

43 **Figure 1: CI in its simplest form: compatibility relationships between infected and**

44 **uninfected individuals.** Uninfected females engender fewer viable offspring than infected

45 ones, because they are not immune to the sperm produced by infected males. The upper-
46 right circle shows the infection state of the progeny: empty (uninfected) or filled (infected).

47 “W” stands for *Wolbachia*.

48

49 Cytological studies indicate that CI induces similar cellular phenotypes in a wide range of
50 hosts [6–11]. During the first embryonic division, paternal chromosomes exhibit defects in
51 condensation and improperly segregate during anaphase. The resulting embryos are
52 aneuploid or haploid and thus inviable, except in haplodiploid species where they may
53 survive as males [12]. While these developmental failures were described a while ago, the
54 underlying genetics have remained elusive until recent years. Because embryonic viability is
55 impeded by the sperm of infected males and rescued by the presence of *Wolbachia* in the
56 eggs, it has long been formalized that CI is a two-sided phenomenon, implying some kind of
57 “modification” in mature sperm and some kind of “rescue” taking place in the eggs [13,14].
58 This modification/rescue or *mod/resc* model is generally acknowledged as a flexible
59 framework that could accommodate any underlying molecular mechanism (box 1).

60

61 Two causal genes were recently identified concomitantly in the *Wolbachia* genomes of
62 major CI model systems: the *wPip* strain from *Culex pipiens* mosquitoes and the *wMel* strain
63 from *Drosophila melanogaster* flies [15,16] (although other genes may also be involved, e.g.
64 Beckmann, eLife 2019, Scholz Nat Com 2021). In both study systems, these genes, generally
65 referred to as “CI factors” (*cif*) form an operon-like structure in the prophage WO region
66 [16]. Transgenic expression of the upstream gene (*cifA*) is necessary and sufficient to prevent
67 CI onset in fly embryos, that is, to recapitulate the *resc* function. Transgenic expression of
68 *cifB* is toxic in yeasts and this effect is rescued by its coexpression with *cifA*, making *CifB* a

69 good “*mod* factor” candidate. Yet only when *cifA* and *cifB* are coexpressed in male flies is CI
70 induction effectively recapitulated. Intriguingly, transgenic expression of *cifB* from another
71 strain (*wRec*, naturally present in *Drosophila recens*) is sufficient to induce strong CI-like
72 embryonic mortality, but this effect is not rescuable, casting doubt on the hypothesis that it
73 induces the same molecular defects as the dual *cifA/B* expression [17]. Putting aside this
74 particular case, transgenic expression of the CifA protein thus seems paradoxically required
75 for both CI rescue and induction. This may indicate that CifA displays two distinct and
76 somewhat opposite molecular activities depending on the context, making it a *mod* co-factor
77 in maturing sperm and a *resc* factor in embryos, as proposed in the so-called “two-by-one
78 model” [18]. Alternatively CifA may just act as a rescue factor, required not only for the
79 normal development of embryos but also for preventing the inherent toxicity of CifB during
80 spermatogenesis [19]. A recent study in *Anopheles gambiae* mosquitoes appears to fit this
81 view: transgenic expression in males of a single *cifB* gene from the *wPip* was sufficient to
82 induce CI [20]. Besides, rather than being a necessary *mod* factor in this system, high
83 expression of *cifA* in males attenuates the CI penetrance. Within the *cif* genes family, at least
84 two active operons have been identified, that can be specifically referred to as *cid* and *cin*,
85 based on the enzymatic activity, deubiquitinase or nuclease, of their downstream protein.
86 Yet, comparative genomics studies led to the description of additional homologs distributed
87 into five clades [16,21–23]. Thus far, CI induction has been experimentally associated with
88 *cid* and *cin* operons, respectively falling into clades I and IV, and also with one operon from
89 group II, but putative functional domains have been identified in all five groups
90 [15,17,18,24].

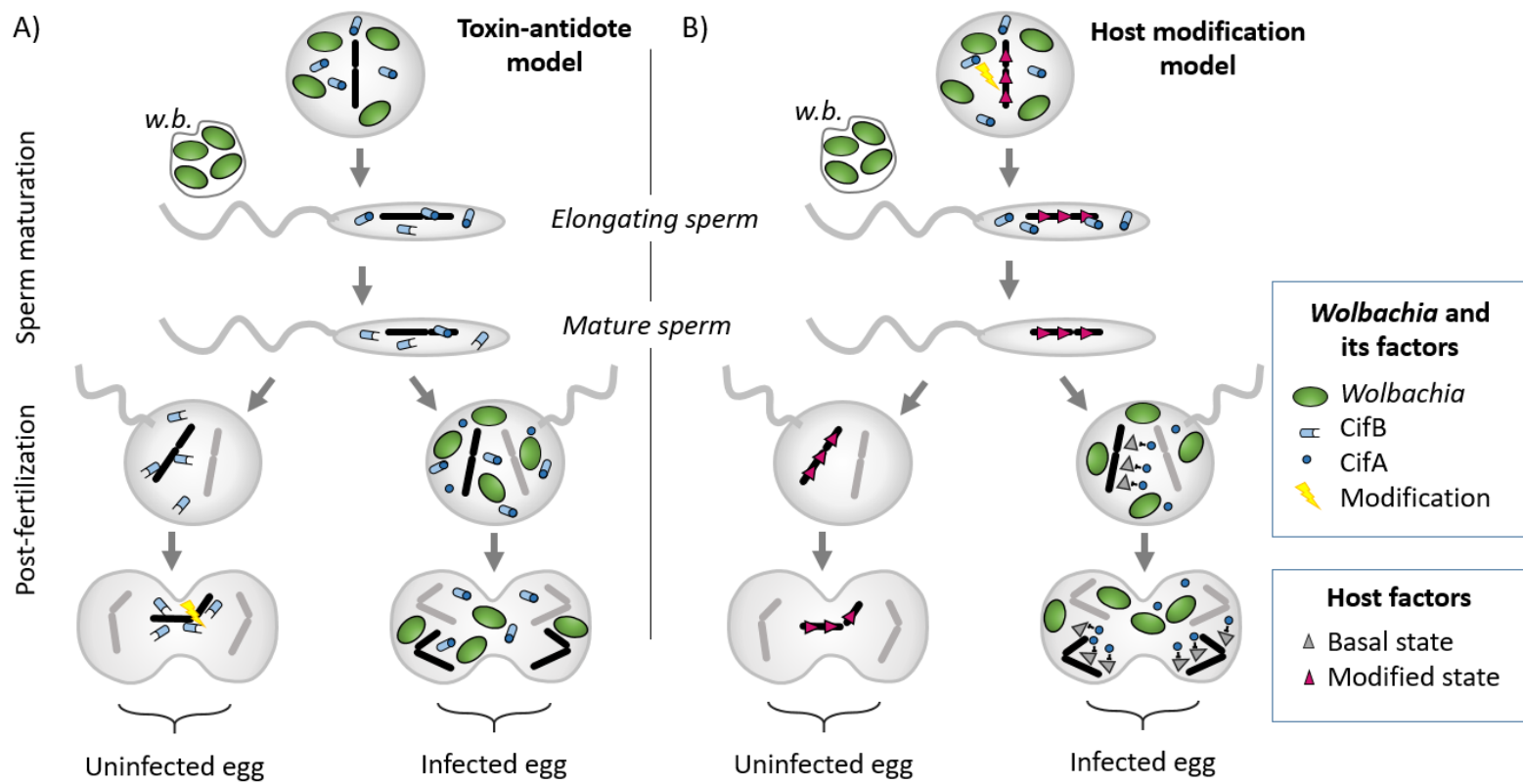
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92 Now that the key CI effectors have been identified, understanding their molecular
93 mechanism will require deciphering what these proteins actually do. The scope of
94 possibilities remains very large, making it relevant to evaluate the various plausible
95 mechanistic models in light of the current data. Several recent review papers thoroughly
96 discussed these models [19,25–28] but perhaps did not put enough emphasis on one specific
97 question that may help guiding future research: based on current knowledge, should we
98 expect the CI rescue to stem from a direct interaction between CifA and CifB in the
99 embryos? Answering this question will essentially distinguish two families of mechanistic
100 models that are still opposed, namely host-modification (HM) versus toxin-antidote (TA)
101 models (Figure 2) [17,19,28,29].

102

103 As recently summarized (e.g. [17,19]), under TA models, the *mod* and *resc* factors act like a
104 lock and key: the toxin (*mod*) affecting the paternal chromosomes is transported through
105 sperm into the embryo where its interaction with the antidote (*resc*) is needed to prevent
106 the modification and thereby let the first mitosis proceed (Figure 2A). On the contrary, HM
107 models propose that the *mod/resc* interaction is only indirect and happens through host
108 effectors: the *mod* factor(s) induces paternal DNA defects but is not transferred into the
109 embryo so that rescue happens by reversing its effects (Figure 2B). In fact, in one particular
110 HM version, called the “mistiming model”, the *mod* and *resc* sides of CI stem from a single
111 process: slowing down the mitotic dynamics; under this model, embryos are only viable if
112 the paternal and maternal pronuclei are synchronous. A second, perhaps more abstract HM
113 version, called the “goalkeeper model”, assumes modification takes place in males in a
114 strain-specific quantity, needing to be precisely remedied to rescue embryos. Finally, the

115 “titration-restitution” model posits that *Wolbachia* alters the concentration of a crucial host
 116 effector in sperm and thus induces CI unless this is counter-balanced in the embryo.



117 **Figure 2: Schematic views of the two main families of CI mechanistic models.** In both
 118 frameworks, *Wolbachia* is removed from maturing sperm into waste bags (w.b.). a) The
 119 toxin-antidote (TA) model predicts that a toxin produced by the paternal *Wolbachia* is
 120 transported with paternal DNA into the eggs and causes mitotic defects unless it is directly
 121 inhibited by an antidote produced by the maternal *Wolbachia*. The paternal antidote may
 122 also be required during sperm development but is presumably less stable than the toxin and
 123 would thus be degraded prior to fertilization. b) In Host Modification (HM) models, a host
 124 factor is modified in the sperm. This modification causes developmental defects unless CifA
 125 in the egg inhibits/reverses its effects. In both frameworks, the maternal CifA proteins would
 126 constitute the rescue factor, while paternal CifB (with a debated contribution of paternal
 127 CifA) would be responsible for CI induction.

128

129 Which of these two families of models is most likely correct? Binding assays provide some
130 elements of answer by showing that the CidA and CidB proteins, as well as CinA and CinB,
131 tightly bind *in vitro*, while CidA only binds to few other elements ([30], resp. [24]). While not
132 ruling out HM models, this pattern indicates that a central requirement of TA models is likely
133 fulfilled although it still awaits *in vivo* confirmation. Notably, another central requirement of
134 the TA model, namely, the production of the CidB protein in sperm and its conveyance into
135 the eggs has not been documented yet, neither in *Culex* nor in *Drosophila*. However, such
136 absence of evidence should not be taken as evidence of absence, because this protein has
137 not been specifically searched for in these tissues. In any case, we now turn to consider a
138 different line of arguments, building on the multiplicity of *Wolbachia* compatibility types.

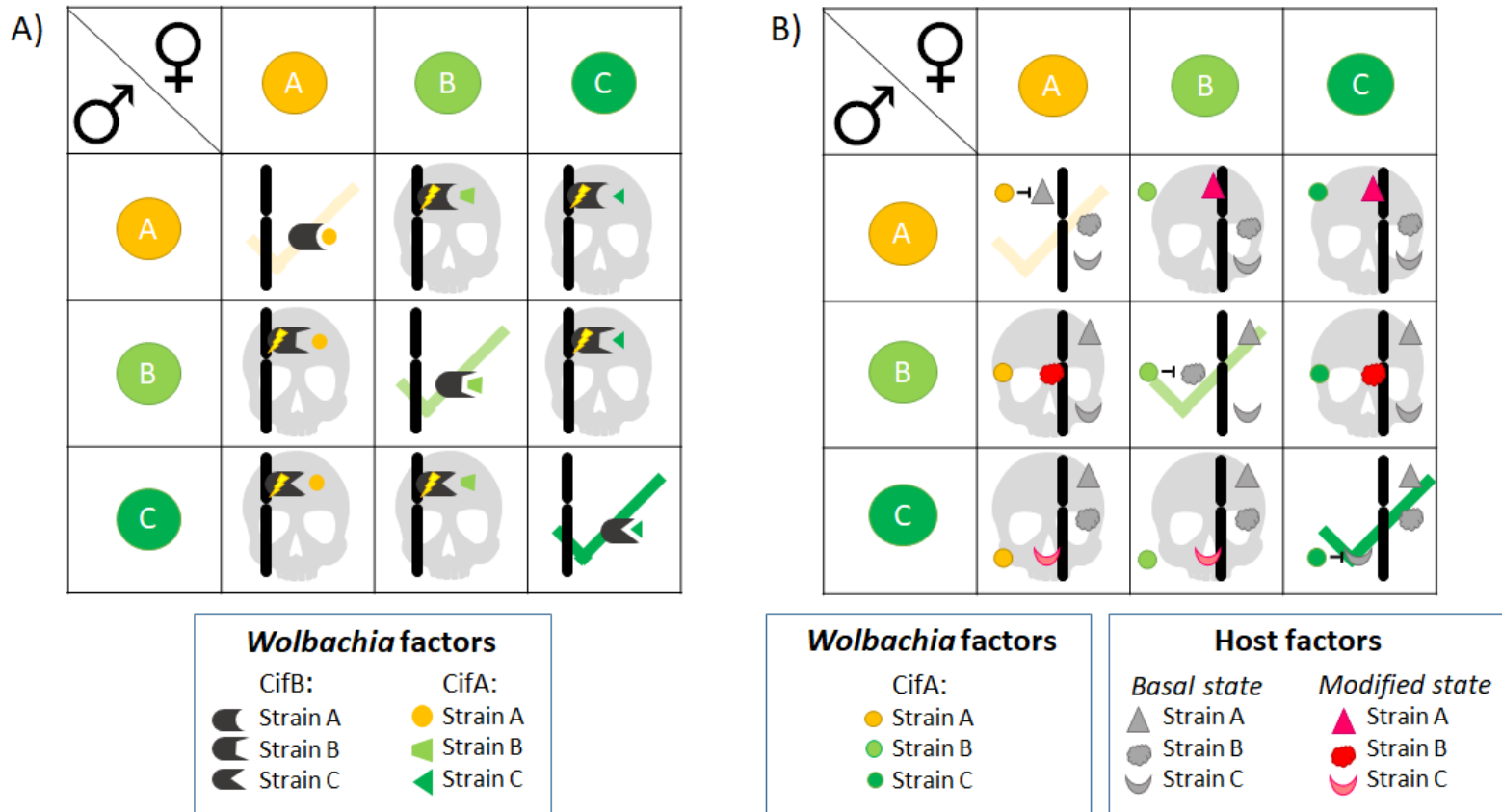
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140 **Compatibility types: a simple case study**

141 We have so far described CI in its simplest form, that occurring between infected males and
142 uninfected females, a pattern often referred to as unidirectional CI, because the reverse
143 cross produces viable progeny (Figure 1). Yet it has long been known that CI may also occur
144 between males and females that are both infected, but by distinct *Wolbachia* strains (Fig. 3).
145 The *Drosophila simulans* system provides a rather simple situation to apprehend this
146 phenomenon (reviewed in [31]). This species naturally hosts several *Wolbachia* lineages,
147 usually called “strains”, easily distinguished on the basis of standard molecular markers such
148 as 16S rRNA. Some of these strains happen to have lost the ability to induce CI [32,33] but
149 three of them are still capable of both CI induction and rescue: *wRi*, *wHa*, and *wNo*, originally
150 described in lines from Riverside (California), Hawaii and Noumea (New Caledonia),
151 respectively. These three strains display quantitative differences with regard to CI

152 penitance but most importantly, they are all bidirectionally incompatible. In that sense,
 153 they are said to harbor distinct “compatibility types”.

154



155 **Figure 3: A simple case of mutual incompatibilities explained under the TA and HM**
 156 **frameworks.** a) In TA models, compatibility patterns can be explained by a lock-and-key
 157 analogy: rescue occurs only if the antidote (the key) can appropriately bind the toxin (the
 158 lock), preventing or reversing modifications of the paternal chromatin. Bidirectionally
 159 incompatible *Wolbachia* strains are predicted to carry incompatible locks and keys (as
 160 represented here by non-complementary shapes). Various toxins and antidotes would thus
 161 differ in their protein-protein interaction regions, but not necessarily in their functional
 162 domains (represented here with a constant shape). b) In the HM framework, mutually
 163 incompatible *Wolbachia* produce *mod* factors that necessarily target different host effectors

164 and are specifically matched by their *resc* counterpart. These various pathways would lead
165 to the same paternal chromatin defects.

166 To what extent can the TA and HM frameworks account for such mutual incompatibilities
167 (Fig. 3)? Within the TA model, the multiplicity of compatibility types is readily explained by
168 the possibility that distinct *mod/resc* pairs could differ, not in their targets, but in their
169 interaction sites, as the Lock-Key analogy makes obvious (Fig. 3A). Within the HM family, the
170 mistiming model, while elegant in proposing that CI induction and rescue may stem from a
171 single phenomenon, just fails to explain the existence of more than one compatibility type,
172 as previously noted [29]. In contrast, other HM models may account for mutual
173 incompatibilities, but this would imply that the *mod* factor(s) of incompatible *Wolbachia*
174 strains achieve the same cytological defects through the activation of different host effectors
175 (Figure 3B). Furthermore, the *resc* factors should similarly vary in their targets to specifically
176 inhibit the consequences of their *mod* counterpart (Figure 3B). The above-mentioned
177 existence of more than one functional family within *cifB* genes (*cidB*, *cinB* and possibly other
178 yet uncharacterized enzymes) could form the basis of such a diversity. Transgenic
179 expressions in yeast provide indirect evidence that the *cid* and *cin* operons from *wPip* may
180 be mutually incompatible: CidA and CinA can only rescue the toxicity respectively caused by
181 CidB and CinB [15]. Yet the same approach suggested that compatibility types do not simply
182 come down to enzymatic families: while the toxicity associated with *cidB* from *wHa* was fully
183 rescued by its cognate *cidA*, expression of the *cidA* gene from *wPip* only lead to imperfect
184 rescue [30]. The existence of incompatibilities within functional families is further suggested
185 by pull down assays indicating that CidB from *wPip* only interacts with its cognate CidA, and
186 not with either CinA from *wPip* or CidA from the *wMel* operon [15]. Recent transgenesis
187 experiments in *Drosophila* provide mixed results regarding the degree of specificity

188 associated with different Cif factors from distinct strains carrying clade I and/or clade II
189 operons, presumably differing in their enzymatic activities [17]. The CI induced by two
190 distinct clade I operons was interchangeably rescued by their CifA factors, suggesting
191 compatibility would be preserved within the deubiquitinase functional family, at least
192 between the two *Wolbachia* strains tested. Yet, surprisingly, a clade I CifA protein was found
193 able to rescue the CI induced by a clade II operon, although the reciprocal was not true.
194 Coming back to the aforementioned *Wolbachia* strains from *D. simulans*, it is noticeable that
195 the *wRi* genome carries both a type I (*cid*) and a type II operon, while *wHa* harbors only a
196 type I operon and *wNo* only a type III operon [16,21]. Assuming compatibility is preserved
197 within functional families, embryos carrying the *wRi* strain should be immune to the CI
198 induced by *wHa*. Yet they are not, which further highlights that incompatibility patterns
199 cannot be readily reduced to enzymatic families.

200

201 In brief, the TA model can rather simply explain bidirectional incompatibilities through
202 changes in the CifA-CifB interaction sites. Notably, this model does not rule out the
203 possibility that different *Wolbachia* strains may induce the same cellular defects through
204 different effectors (as suggested by the fact that both nuclease and deubiquitinase activities
205 produce similar cytological phenotypes) but it does not rely on this enzymatic diversity to
206 explain mutual incompatibilities (Figure 3A). The HM framework also remains compatible
207 with the existence of more than one compatibility type. Mutual incompatibilities would then
208 stem from differences in the host targets of the distinct *mod* factor(s), appropriately
209 matched by their *resc* counterparts (Figure 3B). The existence of more than one functional
210 family offers at best a partial explanation for the observed compatibility patterns. While still
211 plausible, the HM framework then faces the challenge of explaining that a variety of distinct

212 pathways would lead to the same cytological phenotype. As we shall now discuss, a further
213 challenge is to explain that compatibility types are substantially more diverse than suggested
214 by the *Drosophila* case.

215

216

217 **Compatibility types: a bigger picture**

218 While the *D. simulans* case is sufficient to demonstrate that different *Wolbachia* strains may
219 be mutually incompatible, the scale of this phenomenon is better apprehended by
220 considering data from *Culex pipiens*. Even before *Wolbachia* was identified as the causal
221 agent, this species was known for displaying complex patterns of incompatibilities among
222 distinct geographic populations [34–36]. Following the observation that, in contrast with *D.*
223 *simulans*, *C. pipiens* only hosts one *Wolbachia* “strain” (named *wPip*) as far as standard
224 molecular markers such as 16S rRNA can tell, it has been hypothesized that host genetic
225 variation likely contributed to this complexity [37]. Yet, more discriminant molecular
226 markers soon revealed that the *wPip* clade is in fact composed of several lineages [38] and
227 more importantly, experiments controlling for the host genetic background repeatedly
228 indicated that *Wolbachia* alone is responsible for the observed incompatibilities [35,36,39].

229

230 Once acknowledged, this feature provides important insights with regard to *Wolbachia*
231 compatibility types. First, they are numerous: the number of mutually incompatible strains
232 in *C. pipiens* exceeds by far those reported in *D. simulans*. For instance, by reciprocally
233 crossing 19 different *C. pipiens* lines, 15 distinct compatibility profiles have been revealed
234 [35,40]. Second, they can diverge very rapidly, as indicated by the study of intra-population
235 CI variations [41–44]. Third, they can be asymmetrical: strain A may rescue the CI induced by

236 strain B without the reciprocal being true. Fourth, they can be non-transitive: strains A and B
237 may be mutually compatible and yet display distinct compatibility patterns with strain C
238 [36,45]. On the basis of these properties, it was possible to predict not only that the CI genes
239 should display an important diversity in *C. pipiens*, but also that a single *Wolbachia* genome
240 should carry more than one *mod/resc* pair [36,40].

241 The recently uncovered diversity of the *cif* genes in *wPip* matched these predictions
242 rather neatly [43]. Both *cin* and *cid* genes are found in *wPip* genomes but all strains carry
243 these two enzymatic types of CI effectors, ruling out the hypothesis that incompatibilities
244 could result from carrying either one or the other. Most critically, while the *cin* genes are
245 monomorphic in all *wPip* genomes studied so far, the *cid* genes are amplified and diversified,
246 with up to six different copies of a given *cid* gene within a single genome [43]. Furthermore,
247 *cid* diversity strongly correlates with CI patterns [43,44]. Notably, mutual incompatibilities
248 have nothing to do with differences in the Cid deubiquitinase functional domain, which is
249 monomorphic, but rather with polymorphism in CidA/CidB interaction sites [43,44],
250 Hochstrasser 2021.

251 It may be obvious already to the reader that these latter results better fit a TA than
252 an HM framework. The large diversity of compatibility types constitutes a serious difficulty
253 for the HM models, because it unreasonably multiplies the number of distinct host targets,
254 both on the male and female sides, through which *Wolbachia* strains would produce the
255 same cellular phenotype. The goalkeeper model [46] may in theory offer a partial solution to
256 this problem in suggesting that within an HM framework, the high number of targets
257 required could be attenuated by quantitative differences in *cif* genes expressions. Yet, such
258 quantitative variations would fail to explain the non-transitive CI relationships observed in
259 *Culex pipiens*, unless one assumes host genetic variation is also involved, an hypothesis that,

260 once again, was repeatedly ruled out in *Culex pipiens*, e.g. lastly in [39]. Furthermore, if
261 changing the gene expression is indeed presumably “*easier than evolving a new functional*
262 *protein*” [46], the existence of multiple polymorphic copies of *cid* genes is demonstrated in
263 *Culex* [43]. Much more parsimoniously, within the TA framework, mutual incompatibilities
264 can simply result from differences in CidA/CidB interaction sites. Intermediate affinities
265 among Cid proteins and quantitative variations in their production could also readily explain
266 the intermediate levels of rescue recently described [39].

267 **Conclusion**

268 It is arguably premature to conclude that the HM versus TA debate is over. A better
269 understanding of what the Cif proteins actually do will be required to reach that point.
270 However, unless one assumes that the *Culex* case represents an oddity relying on peculiar
271 mechanisms, the multiplicity of *Wolbachia* compatibility types weighs heavy in the balance
272 and has perhaps received too limited attention in previous discussions of CI mechanistic
273 models [17,19,28]. Among other approaches, further studies focusing on the binding
274 affinities between different versions of the wPip Cid proteins along with a deeper
275 assessment of *cid* genes diversity in this system will certainly contribute to deciphering the
276 mechanisms and evolution of CI.

277

278 **Box 1: A quick guide to the CI models jungle**

279 Mod/resc, two-by-one, lock/key, toxin/antidote, host-modification, mistiming, goalkeeper,
280 titration-restitution... Many terms are used in the current literature to describe molecular
281 models of CI, from which a certain confusion may arise to the newcomer. To complement
282 the main text, we provide here brief definitions of these various terms.

- 283 • **Mod/resc** stands for “modification / rescue”. The mod/resc model is currently not
284 considered as a specific molecular model, but rather a summary formulation of the
285 general idea that CI has both a paternal and maternal side: it is induced by some
286 “modification” occurring in infected males, and it is “rescued” by something occurring in
287 the egg. All the following models can fit in this general framework.
- 288 • Similarly, the **two-by-one** model should not be considered as a specific molecular model,
289 but rather a synthetic reminder that, at least in the *Drosophila* transgenic system,
290 expressing cifA alone is sufficient to rescue the eggs, while both cifA and cifB are
291 required in males to recapitulate the induction of CI. Debates are taking place with
292 regard to the generality of this pattern, or its explanation, which may or not involve cifA
293 being considered as a dual factor, involved not only in the rescue of CI, but also its
294 induction.
- 295 • The **toxin-antidote** (TA) and **host-modification** (HM) terms are currently used to describe
296 and distinguish two main families of explanations.
- 297 • In TA models, a toxin is deposited in sperm and transmitted to the egg upon fertilization,
298 leading to embryonic defaults unless an antidote is there, transmitted from the maternal
299 *Wolbachia*, that binds the toxin, which somehow prevents its effects. Compatibility
300 patterns among strains then result from the (in)ability of male-borne toxins and female-
301 borne antidotes to bind. In that sense, the TA model, in the current usage of this term, is
302 strictly equivalent to a **lock-key** model, although in principle the toxin-antidote wording
303 could be seen as more general.
- 304 • In HM models, CI is due to some modifications of host factors, ultimately affecting the
305 paternal DNA and leading to embryonic defaults unless they are either compensated or
306 reversed in the egg by the maternal *Wolbachia*. HM models thus do not posit any direct

307 interaction between the *cif* proteins. (In)compatibility between strains may then arise
308 through qualitative and/or quantitative differences in the targeted host factors. Several
309 more specific models fit within the HM family. The **mistiming** model proposes that CI
310 stems from asynchrony in the timing of events taking place in the paternal and maternal
311 pronuclei after fertilization. The **titration-restitution** model posits that some host factor
312 is removed (titrated) in sperm and conversely made more abundant (restituted) in the
313 egg. Finally, the **goalkeeper** model is slightly more abstract. Its name metaphorically
314 refers to the idea that, just as a goalkeeper moves both horizontally and vertically, CI
315 induction may imply a qualitative and a quantitative dimension: not only the host-
316 modification but also its degree would matter.

317

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320

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