From Wolbachia genomics to phenotype: molecular models of cytoplasmic incompatibility must account for the multiplicity of compatibility types

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Abstract

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

Keywords: *Wolbachia*, toxin-antidote, symbiosis, *Culex, Drosophila*

Introduction

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

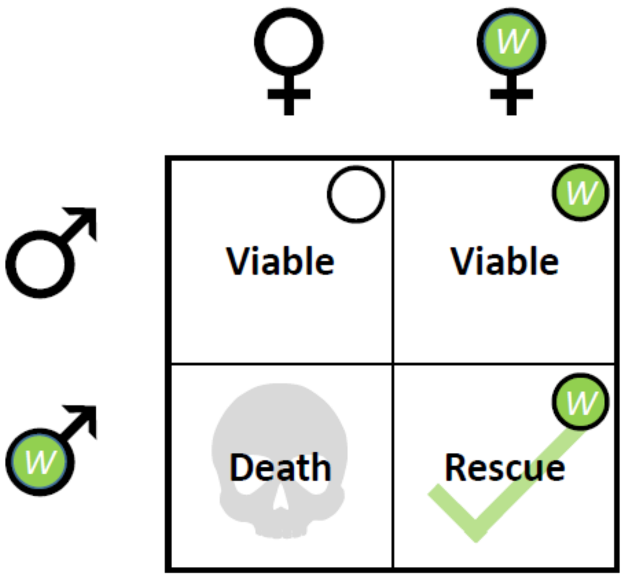

Figure 1: CI in its simplest form: compatibility relationships between infected and uninfected individuals. Uninfected females engender fewer viable offspring than infected

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

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

Two causal genes were recently identified concomitantly in the *Wolbachia* genomes of major CI model systems: the *w*Pip strain from *Culex pipiens* mosquitoes and the *w*Mel strain from *Drosophila melanogaster* flies [15,16] (although other genes may also be involved, e.g. Beckmann, eLife 2019, Scholz Nat Com 2021). In both study systems, these genes, generally referred to as "CI factors" (*cif*) form an operon-like structure in the prophage WO region [16]. Transgenic expression of the upstream gene (*cifA*) is necessary and sufficient to prevent CI onset in fly embryos, that is, to recapitulate the *resc* function. Transgenic expression of *cifB* is toxic in yeasts and this effect is rescued by its coexpression with *cifA*, making CifB a good "*mod* factor" candidate. Yet only when *cifA* and *cifB* are coexpressed in male flies is CI induction effectively recapitulated. Intriguingly, transgenic expression of *cifB* from another strain (*w*Rec, naturally present in *Drosophila recens*) is sufficient to induce strong CI-like embryonic mortality, but this effect is not rescuable, casting doubt on the hypothesis that it induces the same molecular defects as the dual *cifA/B* expression [17]. Putting aside this particular case, transgenic expression of the CifA protein thus seems paradoxically required for both CI rescue and induction. This may indicate that CifA displays two distinct and somewhat opposite molecular activities depending on the context, making it a *mod* co-factor in maturing sperm and a *resc* factor in embryos, as proposed in the so-called "two-by-one model" [18]. Alternatively CifA may just act as a rescue factor, required not only for the normal development of embryos but also for preventing the inherent toxicity of CifB during spermatogenesis [19]. A recent study in *Anopheles gambiae* mosquitoes appears to fit this view: transgenic expression in males of a single *cifB* gene from the *w*Pip was sufficient to induce CI [20]. Besides, rather than being a necessary *mod* factor in this system, high expression of *cifA* in males attenuates the CI penetrance. Within the *cif* genes family, at least two active operons have been identified, that can be specifically referred to as *cid* and *cin*, based on the enzymatic activity, deubiquitinase or nuclease, of their downstream protein. Yet, comparative genomics studies led to the description of additional homologs distributed into five clades [16,21–23]. Thus far, CI induction has been experimentally associated with *cid* and *cin* operons, respectively falling into clades I and IV, and also with one operon from group II, but putative functional domains have been identified in all five groups [15,17,18,24].

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

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

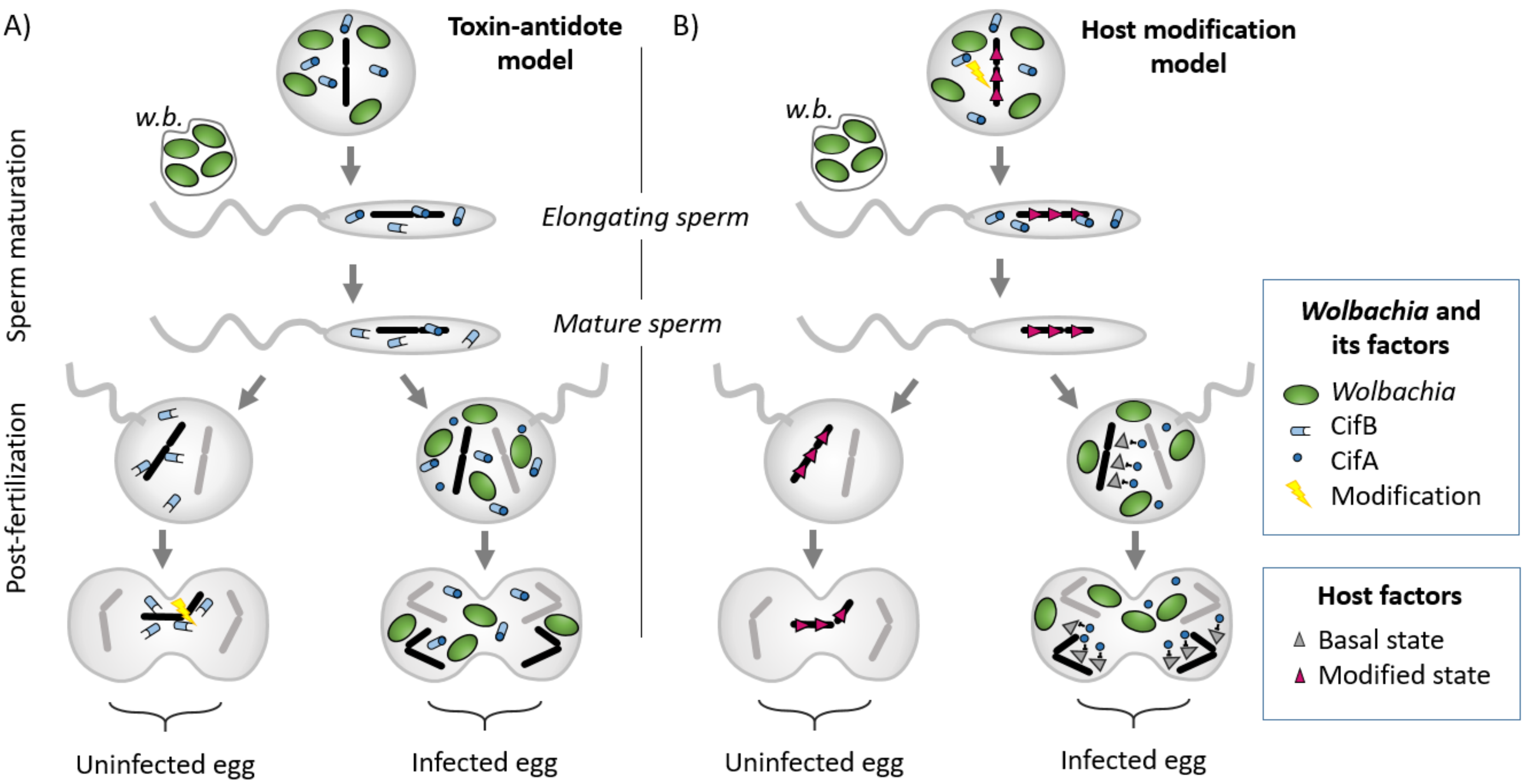

Figure 2: Schematic views of the two main families of CI mechanistic models. In both frameworks, Wolbachia is removed from maturing sperm into waste bags (w.b.). a) The toxin-antidote (TA) model predicts that a toxin produced by the paternal *Wolbachia* is transported with paternal DNA into the eggs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

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

Compatibility types: a simple case study

We have so far described CI in its simplest form, that occurring between infected males and uninfected females, a pattern often referred to as unidirectional CI, because the reverse cross produces viable progeny (Figure 1). Yet it has long been known that CI may also occur between males and females that are both infected, but by distinct *Wolbachia* strains (Fig. 3). The *Drosophila simulans* system provides a rather simple situation to apprehend this phenomenon (reviewed in [31]). This species naturally hosts several *Wolbachia* lineages, usually called “strains”, easily distinguished on the basis of standard molecular markers such as 16S rRNA. Some of these strains happen to have lost the ability to induce CI [32,33] but three of them are still capable of both CI induction and rescue: *w*Ri, *w*Ha, and *w*No, originally described in lines from Riverside (California), Hawaii and Noumea (New Caledonia), respectively. These three strains display quantitative differences with regard to CI penetrance but most importantly, they are all bidirectionally incompatible. In that sense, they are said to harbor distinct “compatibility types”.

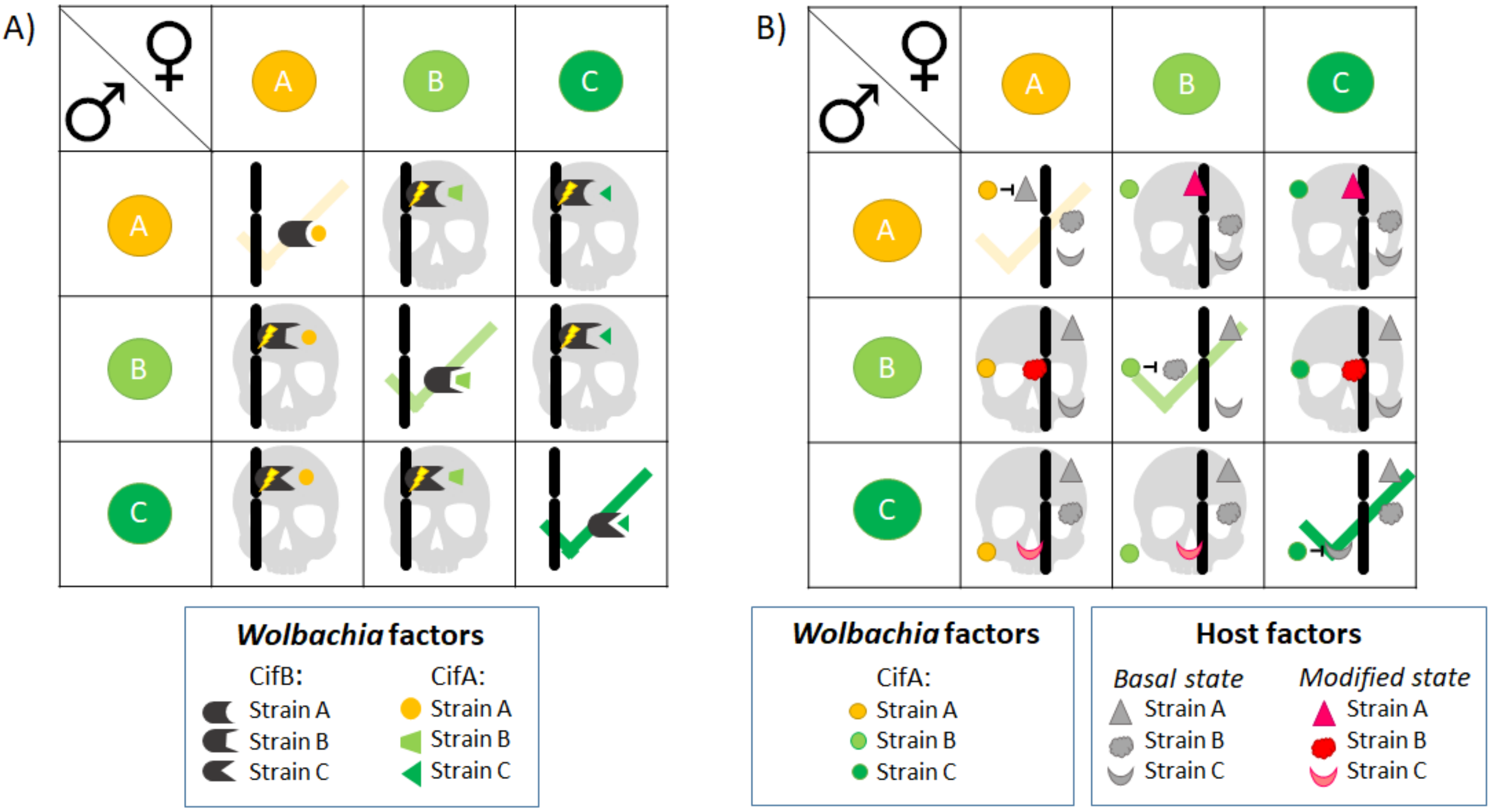

Figure 3: A simple case of mutual incompatibilities explained under the TA and HM frameworks. a) In TA models, compatibility patterns can be explained by a lock-and-key analogy: rescue occurs only if the antidote (the key) can appropriately bind the toxin (the lock), preventing or reversing modifications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

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

To what extent can the TA and HM frameworks account for such mutual incompatibilities (Fig. 3)? Within the TA model, the multiplicity of compatibility types is readily explained by the possibility that distinct *mod/resc* pairs could differ, not in their targets, but in their interaction sites, as the Lock-Key analogy makes obvious (Fig. 3A). Within the HM family, the mistiming model, while elegant in proposing that CI induction and rescue may stem from a single phenomenon, just fails to explain the existence of more than one compatibility type, as previously noted [29]. In contrast, other HM models may account for mutual incompatibilities, but this would imply that the *mod* factor(s) of incompatible *Wolbachia* strains achieve the same cytological defects through the activation of different host effectors (Figure 3B). Furthermore, the *resc* factors should similarly vary in their targets to specifically inhibit the consequences of their *mod* counterpart (Figure 3B). The above-mentioned existence of more than one functional family within *cifB* genes (*cidB*, *cinB* and possibly other yet uncharacterized enzymes) could form the basis of such a diversity. Transgenic expressions in yeast provide indirect evidence that the *cid* and *cin* operons from *w*Pip may be mutually incompatible: CidA and CinA can only rescue the toxicity respectively caused by CidB and CinB [15]. Yet the same approach suggested that compatibility types do not simply come down to enzymatic families: while the toxicity associated with *cidB* from *w*Ha was fully rescued by its cognate *cidA*, expression of the *cidA* gene from *w*Pip only lead to imperfect rescue [30]. The existence of incompatibilities within functional families is further suggested by pull down assays indicating that CidB from *w*Pip only interacts with its cognate CidA, and not with either CinA from *w*Pip or CidA from the *w*Mel operon [15]. Recent transgenesis experiments in *Drosophila* provide mixed results regarding the degree of specificity associated with different Cif factors from distinct strains carrying clade I and/or clade II operons, presumably differing in their enzymatic activities [17]. The CI induced by two distinct clade I operons was interchangeably rescued by their CifA factors, suggesting compatibility would be preserved within the deubiquitinase functional family, at least between the two *Wolbachia* strains tested. Yet, surprisingly, a clade I CifA protein was found able to rescue the CI induced by a clade II operon, although the reciprocal was not true. Coming back to the aforementioned *Wolbachia* strains from *D. simulans*, it is noticeable that the *w*Ri genome carries both a type I (*cid*) and a type II operon, while *w*Ha harbors only a type I operon and *w*No only a type III operon [16,21]. Assuming compatibility is preserved within functional families, embryos carrying the *w*Ri strain should be immune to the CI induced by *w*Ha. Yet they are not, which further highlights that incompatibility patterns cannot be readily reduced to enzymatic families.

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

Compatibility types: a bigger picture

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

Once acknowledged, this feature provides important insights with regard to *Wolbachia* compatibility types. First, they are numerous: the number of mutually incompatible strains in *C. pipiens* exceeds by far those reported in *D. simulans*. For instance, by reciprocally crossing 19 different *C. pipiens* lines, 15 distinct compatibility profiles have been revealed [35,40]. Second, they can diverge very rapidly, as indicated by the study of intra-population CI variations [41–44]. Third, they can be asymmetrical: strain A may rescue the CI induced by strain B without the reciprocal being true. Fourth, they can be non-transitive: strains A and B may be mutually compatible and yet display distinct compatibility patterns with strain C [36,45]. On the basis of these properties, it was possible to predict not only that the CI genes should display an important diversity in *C. pipiens*, but also that a single *Wolbachia* genome should carry more than one *mod/resc* pair [36,40].

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

It may be obvious already to the reader that these latter results better fit a TA than an HM framework. The large diversity of compatibility types constitutes a serious difficulty for the HM models, because it unreasonably multiplies the number of distinct host targets, both on the male and female sides, through which *Wolbachia* strains would produce the same cellular phenotype. The goalkeeper model [46] may in theory offer a partial solution to this problem in suggesting that within an HM framework, the high number of targets required could be attenuated by quantitative differences in *cif* genes expressions. Yet, such quantitative variations would fail to explain the non-transitive CI relationships observed in *Culex pipiens,* unless one assumes host genetic variation is also involved, an hypothesis that, once again, was repeatedly ruled out in *Culex pipiens*, e.g. lastly in [39]. Furthermore, if changing the gene expression is indeed presumably *"easier than evolving a new functional protein"* [46], the existence of multiple polymorphic copies of *cid* genes is demonstrated in *Culex* [43]. Much more parsimoniously, within the TA framework, mutual incompatibilities can simply result from differences in CidA/CidB interaction sites. Intermediate affinities among Cid proteins and quantitative variations in their production could also readily explain the intermediate levels of rescue recently described [39].

Conclusion

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

Box 1: A quick guide to the CI models jungle

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

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

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

Acknowledgments

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