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PERSPECTIVE

Mechanistically comparing reproductive manipulations caused by selfish chromosomes and bacterial symbionts

Elena Dalla Benetta[1,2] · Omar S. Akbari[2] · Patrick M. Ferree[1]

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Abstract

Insects naturally harbor a broad range of selfish agents that can manipulate their reproduction and development, often leading to host sex ratio distortion. Such effects directly benefit the spread of the selfish agents. These agents include two broad groups: bacterial symbionts and selfish chromosomes. Recent studies have made steady progress in uncovering the cellular targets of these agents and their effector genes. Here we highlight what is known about the targeted developmental processes, developmental timing, and effector genes expressed by several selfish agents. It is now becoming apparent that: (1) the genetic toolkits used by these agents to induce a given reproductive manipulation are simple, (2) these agents target sex-specific cellular processes very early in development, and (3) in some cases, similar processes are targeted. Knowledge of the molecular underpinnings of these systems will help to solve long-standing puzzles and provide new tools for controlling insect pests.

Opening section

Decades ago, science writers like Richard Dawkins and Robert Trivers helped popularize the term selfish genes (Dawkins 1977; Burt and Trivers 2006). It is an especially appropriate descriptor for genetic elements like transposons (a.k.a., "jumping genes") and viruses, which hijack certain cellular processes in order to replicate and spread themselves. And in doing so, they can impose ill effects on the organism (Orgel and Crick 1980; Hurst et al. 1996; Hurst and Werren 2001; Werren 2011; McLaughlin and Malik 2017). The selfish genes term also applies to more esoteric cases, like certain "housekeeping" genes with essential, though not-so-captivating cellular functions, but which, through duplication and mutational change, have obtained the selfish property of being transmitted at extraordinarily high frequencies (Powers and Ganetzky 1991; Larracuente and Presgraves 2012). Some non-coding sequences have alternative forms, or alleles, that are selfish, such that they can block the transmission of non-selfish alleles in order to outcompete them (Bengtsson 1977; Bengtsson and Uyenoyama 1990; Hurst et al. 1996; Fishman and Kelly 2015). To imagine that the individual elements of the genome can behave in these bullish ways when their actions are at odds with the wellbeing of the organism underscores why they have commanded such strong scientific interest.

Another less commonly known group of selfish agents has similarly captivated biologists. This group includes a number of bacterial symbionts—those that spend their entire life cycles within the cell cytoplasm or extracellular fluids of their eukaryotic hosts (Werren et al. 2008; Weinert et al. 2015)—and, oddly, whole chromosomes (Bell and Burt 1990; Camacho 2005; Borisov and Myshliavkina 2019).

What unites these two very different types of selfish agents and makes them so intriguing? First, they are pervasive within the arthropods, especially among insects (Zug and Hammerstein 2012; Weinert et al. 2015; D'Ambrosio et al. 2017). Certain selfish bacterial symbionts are estimated to infect more than 70 percent of all insect species (Hilgenboecker et al. 2008). Second, in comparison to selfish genes, these agents are much larger, on the scale of

Associate editor: Aurora Ruiz-Herrera

✉ Patrick M. Ferree
pferree@kecksci.claremont.edu

[1] W. M. Keck Science Department, Claremont McKenna, Pitzer, and Scripps Colleges, Claremont , CA 91711, USA

[2] Division of Biological Sciences, Section of Cell and Developmental Biology, University of California, San Diego 9500 Gilman Drive, CA 92093, USA

organelle size. This characteristic is helpful for experimentation; both bacteria and chromosomes can be visualized using standard fluorescence microscopy, providing researchers the ability to directly observe their dynamics in host cells and tissues.

The third and most important unifying characteristic of these selfish agents is what makes them selfish: the profound effects they have on the reproduction of their hosts. The majority of these agents were discovered because they strongly influence host population dynamics, in many cases leading to severely distorted sex ratios (Tram et al. 2003, 2006; Ferree et al. 2008; Fukui et al. 2015). Evolutionary biologists were quick to note the negative impacts on host fitness (Moran et al. 2008). The range of reproductive manipulations is wide, each type corresponding to how a given selfish agent is transmitted. Like mitochondria, bacterial symbionts are easily transmitted from mothers to their offspring through the egg's large volume of cytoplasm. In contrast, sperm, which are minuscule with limited cytoplasm, are lousy transporters. Therefore, these bacteria tend to alter host reproductive processes so that host sex ratios are skewed toward females or, at minimum, that infected females are selectively favored over uninfected ones (Moran et al. 2008).

To illustrate, certain strains of the rickettsia-like alphaproteobacterium, *Wolbachia*, are believed to alter the sperm-donated hereditary material in the testes of infected male insects (Table 1) (Tram et al. 2006; Riparbelli et al. 2007). If these sperm fertilize eggs from an uninfected female, then the alteration becomes realized: the sperm's chromatin becomes transformed into a "blob" instead of nicely formed chromosomes, just before the embryo begins cell division (Tram et al. 2006; Riparbelli et al. 2007). This effect renders the sperm's chromatin incapable of normal segregation, causing mitotic abnormalities, such as chromosome bridges and chromatin fragments during the first and subsequent divisions (Lassy and Karr 1996; Callaini et al. 1997; Tram 2002). In any host species that is diploid —i.e., those needing a chromosome set from each parent— this effect leads to death. However, if a *Wolbachia*-affected sperm happens to fertilize an egg from a female that is infected with the same *Wolbachia* strain, these deleterious effects are mysteriously suppressed, resulting in normal embryonic development. Another bacterium, *Cardinium* (Bacterioidetes), which infects ~9% of arthropods, also elicits the same reproductive manipulation in the parasitic wasp, *Encarsia suzannae* (Table 1) (Hunter et al. 2003; Wedell 2008; Gebiola et al. 2017). This overall effect, termed cytoplasmic incompatibility (CI), strongly favors *Wolbachia*- and *Cardinium*-infected females over uninfected ones (Tram et al. 2006; Riparbelli et al. 2007). By giving a reproductive advantage to infected hosts, these bacteria selfishly facilitate their own spread (reviewed by (Poinsot et al. 2003; Tram et al. 2003)).

Interestingly, it is not just these CI-inducing bacteria that affect the sperm-donated chromatin for selfish gain. At least three different wasps are known to carry extra, non-essential chromosomes, or B chromosomes collectively called PSR, which cause a similar effect (for Paternal Sex Ratio) (Hunter et al. 1993; Stouthamer et al. 2001; Werren and Stouthamer 2003). Like *Wolbachia*, PSR chromosomes mysteriously prevent the sperm's chromatin from transforming into individual chromosomes, causing it to be destroyed (Reed and Werren 1995). Wasps are haplo-diploid, which means that males normally arise from unfertilized eggs having just the egg's single chromosome set, while females develop from fertilized eggs, which have two chromosome sets, one from the egg and the other from the sperm. Because of this unique reproductive system, destruction of the sperm-donated chromatin by a PSR chromosome does not lead to death but instead forces female-destined eggs to develop into males. At the population level, the host sex ratio becomes severely male-biased. One may figure that these effects must somehow benefit the spread of the PSR chromosome, and indeed, they do. Unlike maternally transmitted *Wolbachia*, a PSR chromosome is transmitted within the sperm's nucleus to new progeny (i.e., paternally) (Reed and Werren 1995). The PSR chromosome enters the egg at fertilization along with the sperm's poisoned hereditary material, but it mysteriously escapes the poisoning effect (Reed and Werren 1995). During the first cell division, the PSR chromosome is replicated normally and transforms into individualized chromatids, which successfully migrate with the egg's chromatids into daughter cells, and, eventually, to all cells of the adult animal. The upshot is the production of more than 90% PSR-carrying, -transmitting males (Reed and Werren 1995).

These two examples illustrate a general rule: if the transmission of a selfish agent is restricted to one sex, then its reproductive manipulation will bias the sex ratio toward, or give a selective advantage to, that particular sex. It is also important to point out that there are other ways that selfish agents, especially bacterial symbionts, can cause sex ratio bias. For example, certain strains of bacteria including *Spiroplasma*, *Wolbachia*, and *Arsenophonus* cause female sex ratio bias by killing host males (Table 1) (Hurst and Jiggins 2000). This may sound harsh, but the rationale is as follows: males represent a dead-end for bacterial transmission, so, directly speaking, their death is not a hindrance to bacterial spread. Instead, and more importantly, male death is believed to provide infected females better access to limited resources, allowing them to produce healthier and greater numbers of infected progeny. Thus, male killing indirectly enhances bacterial spread (Riparbelli et al. 2012). An important aspect to note is that the male-killing effect is not complete; a few males usually survive in each brood. This characteristic is essential since the death of all males

Table 1 Summary of compared selfish bacterial symbionts and B chromosomes.

Agent	Class/Type	Insect Host	Effect	Effector	Targeted Process	Developmental Time	Reference
Wolbachia pipientis	Alphaproteobacteria	Coleoptera, Diptera, Isopoda, Lepidoptera, Hymenoptera, Homoptera, and Orthoptera	Cytoplasmic incompatibility	*cifA*/*cifB*	Paternal chromatin	First mitosis after fertilization	(Werren 1997)
		Diptera, Coleoptera, and Lepidoptera	Male killing	*wmk*	Dosage compensation	Early embryogenesis	(Werren 1997)
		Lepidoptera and Hemiptera	Feminization	unknown	Sex determination	Early development	(Werren 1997)
		Hymenoptera	Parthenogenesis	unknown	NA	NA	(Werren 1997)
Cardinium hertigii	Bacteroidetes	*Encarsia pergandiella*	Cytoplasmic incompatibility	unknown	Paternal chromatin	First mitosis after fertilization	(Hunter et al. 2003)
		Encarsia hispida, *Brevipalpus phoenicis*	Feminization	unknown	Sex determination	Early development	(Giorgini et al. 2009; Weeks et al. 2001)
		Encarsia pergandiella	Parthenogenesis	unknown	NA	NA	(Zchori-Fein et al. 2001)
Spiroplasma poulsonii	Mollicutes	*Drosophila* genus	Male killing	*spaid*	Dosage compensation	Mid embryogenesis	(Martin et al. 2013; Harumoto and Lemaitre 2018)
Arsenophonus nasoniae	Gammaproteobacteria	*Nasonia vitripennis*	Male killing	unknown	Maternally inherited centrosomes	Early embryogenesis	(Ferree et al. 2008)
PSR	B chromosome	*Nasonia vitripennis*	Genome elimination	*haploidizer*	Paternal chromatin	First mitosis after fertilization	(Reed and Werren 1995)
		Encarsia pergandiella	Genome elimination	unknown	Paternal chromatin	First mitosis after fertilization	(Hunter et al. 1993)
		Trichogramma kaykai	Genome elimination	unknown	Paternal chromatin	First mitosis after fertilization	(Stouthamer et al. 2001)

would prevent any fertilization, thereby harming both host and selfish agent. Different *Wolbachia* strains can cause female-biased sex ratio distortion in still other ways, including parthenogenesis (i.e., virgin birth), in which infected females produce all-female broods of progeny in the absence of fertilization by males (Pannebakker et al. 2004; Kremer et al. 2009), and feminization, in which genetic males are converted into reproductively functional females (Kageyama et al. 2017; Miyata et al. 2017; Ma and Schwander 2017).

There have been many detailed reviews written on the life histories, genetics, and evolutionary impacts of sex ratio-distorting agents (Hurst and Werren 2001; Werren and Stouthamer 2003; Werren 2011; Perlman et al. 2015; Ågren and Clark 2018; Doremus and Hunter 2020). Our primary aim here is not to replicate these works but instead to highlight a handful of select aspects for specialists and non-specialists alike. We hope so far to have conveyed some odd and fascinating aspects of these sex ratio-distorting agents, which have enticed many biologists to devote their careers to studying them. We now turn to several outstanding questions that have perplexed researchers in this field for decades—questions that fit within the greater, overarching goal of understanding how these selfish agents usurp the developmental processes of their hosts. In doing so, we highlight findings from recent studies that are finally allowing us to make mechanistic comparisons among several of the more well-studied selfish agents.

Do different selfish agents target common developmental processes?

The answer is yes and no, depending on the particular selfish agents being compared. Consider the three different bacteria capable of inducing male killing: *Spiroplasma*, *Wolbachia*, and *Arsenophonus*. *Spiroplasma*, a spirochete belonging to the Mollicute class of bacteria, infects several different species of fruit flies (of the *Drosophila* genus). Early studies showed that *Spiroplasma*-infected *Drosophila melanogaster* male embryos undergo the early mitotic "cleavage" divisions normally. However, mitotic defects, such as chromosome bridges and oddly-misshapen nuclei begin to appear shortly after the process of cellularization (i.e., when the thousands of nuclei in the young embryo obtain plasma membrane and, in doing so, become individualized) (Martin et al. 2013; Harumoto et al. 2014). Shortly thereafter, the cells of the newly forming central nervous system and other surrounding tissues begin to look highly irregular (Martin et al. 2013; Harumoto et al. 2014), and as time goes on, these embryos undergo programmed cell death (Martin et al. 2013; Harumoto et al. 2014).

To uncover the cause of these cellular defects, researchers aspired to determine which male-specific process is disrupted by *Spiroplasma*. Several clues led to the conclusion that this bacterium targets dosage compensation, a process that adjusts the level of transcription of genes located on the male's single X chromosome to match gene expression levels in females, which have two X chromosomes. Dosage compensation in *D. melanogaster* is mediated by a conglomerate of proteins and RNA called the dosage compensation complex (DCC), which only forms in males (Gelbart et al. 2009). The DCC contains an enzymatic component that acetylates one of the main histones, H4, across the entirety of the male's X chromosome, tweaking its chromatin into a state that leads to higher gene expression (Gelbart et al. 2009). In one experiment, loss-of-function mutations in several genes that encode DCC proteins were shown to alleviate the male-killing effect (Veneti 2005). In another experiment, forced formation of the DCC in females through some genetic trickery caused female death if the females were *Spiroplasma*-infected, but not if they were uninfected (Cheng et al. 2016). And finally, microscopic imaging of infected male embryos revealed that the DCC, which normally associates with the male's single X chromosome, becomes sticky to the non-sex chromosomes (Cheng et al. 2016). This effect likely leads to global gene mis-expression, which was found to occur just before the embryonic cells begin to look abnormal (Cheng et al. 2016). The interpretation is that this gene mis-expression leads to the observed tissue abnormalities and cell death (Cheng et al. 2016).

Certain strains of *Wolbachia* also can cause male killing in a number of fly species including *Drosophila bifasciata*, *D. innubila*, *D. borealis*, and *D. recens* (Hurst et al. 2000; Jaenike 2007; Unckless and Jaenike 2012). Interestingly, the CI-causing wMel strain of *Wolbachia* appears to have the genetic machinery to elicit male killing, although it does not do so in its natural host, *D. melanogaster* (Metcalf et al. 2014; Richardson et al. 2016; Perlmutter et al. 2019). In a subsequent section we will mention a recently identified wMel phage gene that causes male killing when expressed transgenically. However, what is important to note here are the resulting cellular defects: the appearance of chromatin bridges that become hyperacetylated and show signs of DNA damage, reminiscent of what happens in *Spiroplasma*-infected males (Riparbelli et al. 2012; Perlmutter et al. 2019). These findings suggest that certain strains of *Wolbachia*, like *Spiroplasma*, induce male embryonic death by disrupting DCC activity (Veneti 2005; Harumoto et al. 2016, 2018).

Now, consider *Arsenophonus*, a member of the Gammaproteobacteria class, which kills males in the jewel wasp, *Nasonia vitripennis* (Table 1) (Ferree et al. 2008). The cellular target of *Arsenophonus* is a population of organelles

that are unique to wasps and many other insects with haplo-diploid reproduction—we will refer to these organelles as maternal centrosomes (Schatten 1994; Callaini et al. 1999). In animals with purely diploid reproduction, such as fruit flies and humans, all eggs must be fertilized. A major reason is that a part of the sperm's tail transforms into a pair of centrosomes, or microtubule organizing centers, just after fertilization (Schatten 1994; Callaini et al. 1999). These organelles build the microtubule-based spindle apparatus, which is needed to facilitate cell division. In the absence of these sperm-derived centrosomes, an egg cannot proceed into embryonic development. However, in haplo-diploid insects, eggs possess the unique ability to form hundreds of centrosomes de novo, or entirely from components in the egg's cytoplasm (Tram and Sullivan 2000; Ferree et al. 2006). These maternal centrosomes appear in all *N. vitripennis* eggs immediately after they are laid. If fertilization occurs, the egg preferentially uses the sperm-derived centrosomes to initiate cell division, while the maternal centrosomes go unused and disappear. If, instead, the egg is not fertilized, two of the maternal centrosomes are selected to facilitate cell division (Tram and Sullivan 2000; Ferree et al. 2006). Interestingly, when *Arsenophonus* is present, the maternal centrosomes are blocked from forming (Ferree et al. 2008). As a result, the egg-derived chromatin attempts to undergo mitosis, but without an organized spindle apparatus no cell division occurs, and development is arrested. To be clear, fertilized, female-destined embryos are not affected because they do not rely on maternal centrosomes. It is currently a mystery how *Arsenophonus* blocks maternal centrosome formation, but one possibility is that the bacterium interferes with specialized vesicles called accessory nuclei, which form during egg development and appear to seed maternal centrosome formation when the egg is laid (Ferree et al. 2008).

It is evident from these studies that *Spiroplasma* and *Wolbachia* target a completely different developmental process compared to *Arsenophonus*. In each case, the disrupted process is a unique feature of the male development of each host insect. However, CI-causing bacteria and the PSR chromosome target the same developmental process: as already mentioned, both agents alter the sperm's chromatin, causing it to be destroyed immediately after fertilization (Reed and Werren 1995). This fact begs a more specific question: do these agents target the same molecular aspect of the sperm-donated chromatin?

It may be somewhat premature to answer this question because there have been so few studies geared toward this fine scale. In addition, the experiments conducted on these two agents have been performed in different organisms and with different methodological tools, making comparisons difficult. For example, one study tested whether the sperm-donated chromatin undergoes several important steps between fertilization and the first cell division in fruit fly embryos that are infected with CI-causing *Wolbachia* (Landmann et al. 2009). It was observed that protamines, specialized non-histone proteins that package sperm DNA, seemed to be properly removed just after fertilization (Landmann et al. 2009). However, there was a lag in the subsequent appearance of the transitional histone, H3.3, as well as a delay in replication of the sperm-derived DNA, after protamine removal (Landmann et al. 2009). A different study examined the patterns of important chemical marks to histone proteins in jewel wasp embryos carrying the PSR chromosome. Three different chemical marks to histones, out of a total of ~20 tested, appeared abnormally spread across the sperm-donated chromatin, instead of appearing in distinct regions as they should (Aldrich et al. 2017). Protamine removal and histone H3.3 appearance were not directly addressed in this study. However, it was concluded that these events likely occur normally because they are requirements for loading of the main histones, which did appear on the sperm-donated DNA (Aldrich et al. 2017).

The results from these studies demonstrate that both CI-causing *Wolbachia* and PSR do indeed disrupt the integrity of the sperm-donated chromatin. However, in each case it could not be determined whether the particular chromatin defects observed were the result of direct disruption or were instead secondary effects from some other chromatin disruption. Nevertheless, the odds are in favor of these two agents targeting different molecular factors. Why might this be? For one reason, the chromatin blob caused by *Wolbachia* is considerably less compact than that caused by PSR, indicating some difference in chromatin structure (Reed and Werren 1995). Another reason is more speculative—that there are many different chromatin-related genes, such as *sesame/hira*, *chd1*, and *maternal haploid*—each of which when disrupted by mutation, results in a sperm-donated chromatin blob (Loppin et al. 2001; Bonnefoy et al. 2007; Konev et al. 2007). These genes, and others, play critical roles in the remodeling of the sperm's chromatin during spermatogenesis and after fertilization (Rathke et al. 2014). In effect, there is a whole repertoire of steps in this pathway, any of which could be affected by *Wolbachia* or PSR to destroy the sperm's chromatin. Much remains to be determined regarding the host factors being targeted, and these genes are excellent candidates for experimental testing.

Is the developmental time of manipulation by different selfish agents similar?

The selfish agents highlighted so far, and others that have been less extensively studied, cause reproductive manipulations that are manifested very early in development—during embryogenesis. With few exceptions, the main stages of

development are similar enough among insects that we can use the fruit fly as a good proxy for consideration here. The time it takes for a fertilized egg to reach adulthood in this insect is 12 days at room temperature (Ashburner 1989). The first period, embryogenesis, is complete by ~24 h after egg laying. Subsequently, the animal transforms into a crawling larva whose sole job during the next 5 or so days is to eat and grow (Ashburner 1989). The final 5–6 days involve pupal development, involving formation of the adult tissues and appendages. Interestingly, most reproductive manipulations occur well within the first half of development. Elimination of the sperm-donated chromatin, caused by *Wolbachia* and PSR chromosomes, happens within the first hour after fertilization (Reed and Werren 1995). The blocking of maternal centrosome formation by *Arsenophonus* in the jewel wasp (whose developmental trajectory and timing are nearly identical to those of the fruit fly) happens even earlier, during egg laying (Ferree et al. 2008). Disruption of dosage compensation by *Spiroplasma* ensues between 8 and 10 h into embryogenesis (Cheng et al. 2016). Embryonic defects caused by *Wolbachia*-induced male killing appear slightly earlier (3–4 h after egg laying) (Perlmutter et al. 2019). A couple of outlier examples are feminization, which occurs in some butterfly species through hormonal manipulation during the late pupal stage (Narita et al. 2007; Negri 2012), and late male killing (Hurst 1993), which has been documented to occur during late larval development in some mosquito species (Andreadis and Hall 1979; Andreadis 1985) and during pupation in the oriental tea tortrix (moth), *Homona magnanima* (Morimoto et al. 2001).

Why do most reproductive disruptions happen rather early in development? A large number of fundamental developmental decisions are made during embryogenesis and shortly thereafter. These include the establishment of cell fates, delineation of the main body regions, and formation of the tissues, organs, and limbs. Embedded within these landmark events are certain cellular differences that distinguish the sexes. In principle, it is possible for a sex ratio-distorting agent, such as *Spiroplasma* or *Arsenophonus* to kill males during late development or adulthood. However, the male-specific features and processes that are targeted by these particular endosymbionts, as well as those targeted by other selfish agents, appear very early. It stands to reason that the reproductive manipulations will ensue whenever the sex-specific developmental differences are first manifested during development.

Are there functional similarities among the effector genes produced by sex ratio-distorting agents?

There are two sides of the metaphorical coin when attempting to understand the effects of selfish agents. The first side, already discussed, is knowing which host cellular processes are disrupted, and in what ways. The other side is identifying how selfish agents target these processes.

An unspoken assumption has been that selfish agents produce factors—proteins or perhaps structural RNAs—that bungle the targeted reproductive processes (Akbari et al. 2013; Li et al. 2017). The past several years have been a very fruitful period for the discovery of the effector genes that produce these factors. One of the first to be identified is a *Spiroplasma*-encoded gene termed spaid (for *Spiroplasma poulsonii* androcidin), which plays a role in male killing in the fruit fly (Harumoto and Lemaitre 2018). Three different effector genes were found to be encoded by *Wolbachia* bacteriophages: *wmk*, which also is implicated in male killing, independently of *spaid* (Perlmutter et al. 2019), and *cifA* and *cifB*, which are involved in CI in *D. melanogaster* (LePage et al. 2017) and the mosquito *Culex pipiens* (Beckmann et al. 2017). Finally, an effector gene called *haploidizer* is expressed by PSR to cause paternal genome elimination in the jewel wasp (Dalla Benetta et al. 2020).

What do we know so far about these effector genes? For starters, four of them—*spaid*, *wmk*, and *cifA*/*cifB*—are capable of recapitulating the reproductive manipulation of its respective selfish agent when expressed transgenically. Thus, each gene, or set of genes in the case of *cifA*/*cifB*, is sufficient for reproductive manipulation (Beckmann et al. 2017; LePage et al. 2017; Perlmutter et al. 2019).

There is also mounting evidence for how these effector genes function. *spaid* encodes a protein with two notable regions—one containing several ankyrin repeats and another with homology to the deubiquitinase gene *otu* (*ovarian tumor*), which is found in higher eukaryotes. Both the ankyrin repeats and the OTU domain are conserved in proteins across eukaryotes (Harumoto and Lemaitre 2018). The Spaid protein was shown microscopically to co-localize with the DCC in lethal male embryos (Harumoto and Lemaitre 2018). When the ankyrin repeats were deleted, Spaid failed to co-localize with the DCC and the male's X chromosome (Harumoto and Lemaitre 2018). These findings support a model for male killing in which the Spaid protein associates with the DCC via its ankyrin repeats, causing this complex to mis-localize (Harumoto and Lemaitre 2018), in turn leading to a failure of normal chromatin remodeling on the X chromosome and inappropriate remodeling in other regions of the genome.

The function of the *cifA*/*cifB* genes in CI is a bit more complicated. Here we highlight some general characteristics of these genes. However, more extensive descriptions can be found in two recent reviews (Chen et al. 2020; Shropshire et al. 2020). The *cif*s (*cif* is short for CI factor) fall into two general co-expressed gene pairs: *cidA*/*cidB* and *cinA*/*cinB*. These nuanced names stem from the fact that *cidB* encodes a protein containing an active deubiquitylase

domain while *cinB*'s encoded protein has two active nuclease domains (Beckmann et al. 2017; LePage et al. 2017). Incidentally, the CidB protein contains two nuclease domains similarly to CinB but they are degenerate and so not catalytically active (Beckmann et al. 2017; LePage et al. 2017; Chen et al. 2019). Neither CidA nor CinA contain any domains conferring catalytic activity (Lindsey et al. 2018; Shropshire et al. 2018). Both gene pairs are expressed by CI-causing *Wolbachia* in *D. melanogaster* and *C. pipiens* (Beckmann et al. 2017; LePage et al. 2017). Interestingly, transgenic expression of either *cifA*/*cifB* pair in the fruit fly's testis causes elimination of the sperm-donated chromatin (Beckmann et al. 2017; LePage et al. 2017), leading to the idea that there may be two different ways to elicit CI (Chen et al. 2020). In contrast, expression of *cifA* alone can suppress this effect (Beckmann et al. 2017; LePage et al. 2017). These findings have led to the general idea that CifA and CifB together disrupt some aspect of the sperm's chromatin (the CI activity) while CifA somehow reverses or suppresses this disruption (the rescue activity) (Shropshire and Bordenstein 2019). Generally, while it is clear that CifA is needed in the egg's cytoplasm for rescue, it is not known whether the CI activity of CifA/CifB occurs in the testis or the egg just after fertilization (Chen et al. 2020; Shropshire et al. 2020). In addition, it is currently not understood whether CI results from the chemical modification of some sperm chromatin factor (which is rescued by either chemical reversal or secondary suppression in the egg) or instead from non-chemical interference of an important step in sperm chromatin dynamics (Shropshire et al. 2020; LePage et al. 2017; Chen et al. 2019, 2020; Shropshire and Bordenstein 2019).

Equally important will be experiments that reveal the specific host factors that interact with CifA and CifB. Although no microscopic studies have yet been performed on the Cif proteins, a yeast 2-hybrid screen and, separately, affinity column chromatography combined with mass spectrometry, were used to search for physical interactions of the Cif proteins with certain host proteins (Beckmann et al. 2019). Interestingly, although not surprisingly, two of the top interacting proteins for CifB were P32 and NAP1, which are involved in the exchange of protamines for histones after fertilization (Beckmann et al. 2019; Chen et al. 2019). Thus, it may be that CifA and CifB disrupt the protamine-to-histone transition. We note that this scenario appears to be inconsistent with a previous observation noted earlier: that protamines appear to be removed from the sperm's chromatin in *D. melanogaster* CI embryos at the microscopic level (Landmann et al. 2009). However, it may be that subtle perturbations, such as retention of trace amounts of protamines at levels undetectable by microscopy, are enough to cause subsequent chromatin remodeling abnormalities. Consistent with this idea, it was previously suggested that CifB could, through its nuclease domains, associate with transiently naked paternal DNA during the protamine-to-histone transition to disrupt this and downstream chromatin processes (Beckmann et al. 2019; Chen et al. 2019).

As of now, such functional studies have not been conducted on *wmk* or *haploidizer*. However, the predicted proteins of each of these two genes contain predicted DNA-binding regions that are commonly found in transcription factors (Perlmutter et al. 2019; Dalla Benetta et al. 2020). In the case of *haploidizer*, it is known that there is no noteworthy effect of this gene on the expression of the wasp's genes (Akbari et al. 2013), and elimination of the sperm-derived chromatin happens well before gene transcription begins in the young embryo (Reed and Werren 1995). Thus, at least for *haploidizer*, the DNA-binding domain of its encoded protein may provide it with a general ability to associate with chromatin.

In general, the genetic toolkits used by these agents for reproductive manipulation are quite simple, each expressing one or two major effector genes. These genes encode proteins that either have the potential or have been verified to associate with host proteins or DNA. In addition, there is mounting evidence that several of these bacterial proteins interact with host proteins of disrupted male-specific processes.

Concluding remarks

What originally stimulated interest in these organelle-sized selfish agents were their striking effects on host sex ratios and other aspects of host population dynamics. As a result, population geneticists and evolutionary biologists were the first to appreciate their importance (Dawkins 1977; Hurst and Werren 2001; Werren 2011). Subsequently, researchers in the areas of classical genetics, genomics, cell, and developmental biology became interested in understanding how selfish agents interact with the host cellular environment in order to replicate, induce reproductive alterations, and effectively transmit themselves through the gametes to progeny. Through recent and ongoing efforts, we are coming to know the genes that cause reproductive manipulation and how they work. We are also coming closer to solving some long-standing puzzles. For example, it has been known for decades that certain CI-causing strains of *Wolbachia* are incapable of suppressing genome elimination caused by different *Wolbachia* strains (Perrot-Minnot et al. 1996; Werren 1997; Charlat et al. 2005). The discovery of the Cif proteins as major effectors of CI may help to explain this bacteria-strain-incompatibility, such as if CifA and CifB from the same *Wolbachia* strain have co-evolved together for their function, perhaps through physical interaction.

In addition, there may be other benefits to this work, including the advancement of the long-held goal of using selfish agents like *Wolbachia* to control insect pests. For example, researchers have developed strategies for releasing large numbers of male *Aedes aegypti* mosquitoes infected with CI-causing *Wolbachia* into mosquito populations that are not *Wolbachia*-infected (Hoffmann et al. 2011; Zhang and Lui 2020). The hope is that the sterility effect resulting from the introduced males mating with uninfected females will lead to substantial reductions in mosquito populations, thus hindering insect spread (and, concomitantly, the human disease-causing viruses and other pathogens that they carry). This strategy has certain downfalls: to name a few, (1) it is energy-intensive, relying on iterative releases of adult infected male mosquitoes, (2) accidental release of infected females could result in the *Wolbachia* strain sweeping through the population, thereby undermining the sterilizing effect, and (3) efficient *Wolbachia* transmission in insect populations can be sensitive to environmental factors, such as fluctuation in temperature (Perrot-Minnot et al. 1996; Charlat et al. 2005; Ye et al. 2016). Now, with the knowledge of the CI genes in hand, researchers may be able to develop artificial gene drive systems based on the CI genes to sweep certain "cargo" genes through pest insect populations. Such cargo genes could encode factors that cause insect sterility or lethality, or they could affect insect mating behavior (Burt 2003; Alphey 2014). Moreover, certain reproductive manipulations, such as PSR's genome elimination activity, show nearly unwavering strength across varying environmental conditions (Werren and Stouthamer 2003), making them promising as more "unbreakable" systems for pest manipulation once their underlying mechanisms are better understood. Broadly, just as the last few decades have seen great progress in understanding how selfish bacteria and B chromosomes manipulate their insect hosts, the next few hold potential for equally exciting advances in this area.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict ofinterest.

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