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Host Modulation of Cytoplasmic Incompatibility in Drosophila simulans

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HOST MODULATION OF CYTOPLASMIC INCOMPATIBILITY IN *DROSOPHILA SIMULANS*

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To: Dean Michael R. Heithaus
College of Arts, Sciences and Education

This dissertation, written by AJM Zehadee Momtaz, and entitled Host Modulation of Cytoplasmic Incompatibility in *Drosophila simulans,* having been approved in respect to style and intellectual content, is referred to you for judgment.

We have read this dissertation and recommend that it be approved.

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DEDICATION

I dedicate this dissertation to my beloved parents and all the well wishers.

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ABSTRACT OF THE DISSERTATION

HOST MODULATION OF CYTOPLASMIC INCOMPATIBILITY

IN *DROSOPHILA SIMULANS.*

by

AJM Zehadee Momtaz

Florida International University, 2022

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Wolbachia are one of the most widespread bacterial endosymbionts, infecting mites, crustaceans and filarial nematodes as well as about half of all insect species. The prevalence of *Wolbachia* in nature results from an ability to manipulate the host reproduction to favor the success of infected females. The best-known reproductive modification induced by *Wolbachia* is sperm-egg cytoplasmic incompatibility (CI). In CI, the sperm of *Wolbachia*-infected males cause embryonic lethality in crosses with uninfected females, which is attributed to paternal chromatin segregation defects in early mitotic divisions. The embryos of *Wolbachia*-infected females can “rescue” CI lethality, bringing the egg hatch rate similar to uninfected crosses. The underlying mechanism for rescue of CI remains largely untested. In this study we used a chemical feeding approach to test host cellular capacity to induce rescue of CI in *Drosophila simulans*. Chemical inhibitors were fed to uninfected females, and the resulting egg hatch rate was scored from CI crosses associated with native (*w*Ri) and transinfected (*w*Mel) *Wolbachia* strains. We found that treatment with seven chemicals was able to significantly increase CI egg hatch rates associated with paternal *w*Ri *Wolbachia* infection. These chemicals reputedly affect DNA integrity, cell cycle control and protein turnover, implicating these functions in CI suppression. Three of these chemical treatments, associated with DNA

integrity and protein turnover, were also able to significantly increase CI egg hatch rates associated with paternal *w*Mel *Wolbachia* infection. These results implicate DNA integrity as a focal aspect of rescue induction/ CI suppression across *Wolbachia* strains. The framework presented here can be applied to diverse, genetically intractable CI models. Further studies will enrich our knowledge of the mechanisms underlying host reproductive manipulation by insect endosymbionts.

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CHAPTER I: INTRODUCTION

1.1 Symbiosis

Symbioses are widespread in nature and contributed to the major transitions in the evolution of life on the Earth (Oliver & Russell, 2016). The term 'symbiosis' is derived from the Greek words sym, meaning 'together', and biosis, 'living', and is generally refers to as a close and often long-term association/interaction between two or more organisms of different species (Oliver & Russell, 2016; Angelard & Bever, 2013). Almost all plants and animals, including humans, harbor microbial symbionts which can have substantial effects on the host ecology and evolution (Oliver & Russell, 2016). By convention, the larger partner in a symbiotic association is usually called the 'host' and the smaller partners are called 'symbionts' (Oliver & Russell, 2016; Moran, 2006).

Symbiosis has been a source of interest to the scientific community for a long time. In long-time symbiotic association, the symbiont can become integral to the biology of the host. According to the symbiotic hypothesis of eukaryotic cellular origin, the uptake and symbiotic association of mitochondria and chloroplasts with proto-eukaryotic cells began approximately 1.7 to 2 billion years ago (Angelard & Bever, 2013; Cavalier-Smith, 1992). The integration of a proto-mitochondrial cell into a free-living alpha-proteobacterium is assumed to have aided in production of energy and the divergence of early eukaryotic cells (Margulis, 1996; Degli Esposti, 2014 ; Angelard & Bever, 2013). Likewise, the origin of chloroplasts resulted from uptake of cyanobacteria by early eukaryotic lineages, imparting the ability to turn light into chemical energy through photosynthesis (Cavalier-Smith, 1992). Thus, the incorporation of symbionts into host cells would have increased the possibility of survival of both the host and symbiont in various environments.

1.3 Ectosymbiosis and endosymbiosis

Symbiosis can be classified dependent on the relationship of the symbiont and host, as endosymbiosis and ectosymbiosis (Das & Varma, 2009). Endosymbiosis is the symbiotic association where the symbiont lives within the tissues of the host, or in the intracellular space. Examples of endosymbiosis include nitrogen-fixing bacteria (ie. rhizobia) living in the root nodules of legumes and single-celled algae inside reef-building corals (Das & Varma, 2009). Conversely, ectosymbiosis, also known as exosymbiosis, is an association in which the symbiont lives on the exterior surface of the host, the ducts of exocrine glands, or the inner surface of the digestive tract (Das & Varma, 2009). The examples of ectosymbiotic relations include the Remona fish that form a commensal relationship with the lemon shark, or ectoparasites such as lice, living on humans and other warm-blooded animals.

1.4 The symbiotic spectrum: commensalism, mutualism and parasitism

Depending on the nature of their relationship, symbiotic association can be divided into three different categories, such as mutualism, commensalism and parasitism. In mutualism, both the host and symbiont reciprocally benefit from the symbiotic association. In commensalism, only one partner, usually the symbiont, benefits from the association without benefitting or harming the other host. In contrast, in parasitism the symbiont partitions resources from the host causing direct or indirect harm (Leung & Poulin, 2008). Sometimes it is possible for a symbiont to have one kind of symbiotic relationship with one host and a completely different type of relationship with another host. For example, the enteric bacteria *Salmonella* have a commensal association with poultry, while it is a pathogenic bacteria in humans (Pan & Yu, 2014; White et al., 1997).

1.5 Bacterial insect symbiosis

Bacterial insect symbiosis is widespread in nature and it has been estimated that about 15-20% of all insects live in a symbiotic association with bacteria (Gil. R et al., 2004). Bacterial symbionts have various effect on its insect hosts ranging from provision of essential nutrients and protection from natural enemies, to modifying host reproduction (Ferrari & Vavre, 2011). Depending on the nature of the symbiosis, the bacterial symbionts in insects are divided into two groups, obligate or primary symbiont and facultative or secondary symbionts (Ferrari & Vavre, 2011). A primary symbiont is essential for host survival and reproduction, and neither the insect nor the bacteria can survive without the other. In many cases, the primary bacterial endosymbionts can not be maintained outside the host (Ferrari & Vavre, 2011). Primary symbionts are maternally transmitted and often reside in a specialized organ-like structure inside the insects called bacteriocytes (Douglas, 2014). Typically, the genome of primary symbionts are extremely reduced, yet also have many effects on host biology including provision of vitamin and nutrients, fecundity, interaction with natural enemies, as well as with other species (Gil. R et al., 2004; Ferrari & Vavre, 2011). For example, the primary symbiont of aphid *Buchnera aphidicola* provides its host with essential amino acids that the aphid is unable to get from its primary diet of plant sap (Ferrari & Vavre, 2011; Douglas, 2014). Secondary endosymbionts are symbionts those are not essential for viability of the bacteria or insect host that can exist without one another. Although in most cases these bacteria are maternally transmitted (ie. vertically), transmission can occur horizontally (Ferrari & Vavre, 2011). The advantage of secondary symbiosis generally depends on the environment and has much broader effects ranging from mutualism to parasitism (Gil. R et al., 2004; Ferrari & Vavre, 2011). The advantages that are provided by secondary endosymbiont include increased resistance to natural

enemies, protection from other species, tolerance of heat shock and increased fecundity (Gil. R et al., 2004; Ferrari & Vavre, 2011; Douglas, 2014). For example, the secondary symbiont of pea aphids *Regiella insecticola* protects the aphids from the lethal fungus *Pandora neoaphidis*. The *Hamiltonella* secondary endosymbiont of pea aphid *A. pisum* encodes a toxin that targets eukaryotic tissues (Ferrari & Vavre, 2011). Despite rising interest in the scientific community, the basic knowledge of bacterial insect symbiosis and its impact in nature is still lacking. Thus, the primary focus of my research is to explore the interaction between insects and their bacterial symbionts. I will be focusing on the complex relationship between *Drosophila* and endosymbiotic *Wolbachia* bacteria.

1.5.1 *Wolbachia*: a brief background

Wolbachia are gram-negative bacteria which is one of the most widespread endosymbiont in nature. *Wolbachia* was first discovered in 1924 by Wolbach and Hertig in the mosquito *Culex pipiens* (Hertig & Wolbach, 1924; Lo et al., 2007; Shropshire et al., 2020). Initially, *Wolbachia* were known as Rickettsia-like bacteria, but later named *Wolbachia pipientis* by Hertig in 1936 (Hertig, 1936), belonging to the phylum α-proteobacteria, the order Rickettsiales, and the family Anaplasmataceae (Sarwar et al., 2018). *Wolbachia* are known to be present in a broad range of arthropods, mites, crustaceans and nematodes including, *Brugia malayi, Brugia timori, Wuchereria bancrofti, Onchocerciasis volvulus* (Shropshire et al., 2020; Punkosdy et al., 2003; Serbus et al., 2008). *Wolbachia* are also present in plant-parasite nematodes, such as, *Pratylenchus* and *Radopholus* (Wasala et al., 2019). Recent studies have estimated *Wolbachia* is present in almost half of all insect species (Lefoulon et al., 2020), although it has also been suggested that *Wolbachia* to be widespread among the insect species and not necessarily within the same insect species (Sazama et al., 2019).

Phylogenetic analysis showed *Wolbachia* to be highly diverse in nature, divided into 17 ‘supergroups’ (denoted A-S, excluding G and R) (Shropshire et al., 2020). Some supergroups have taxonomically wide host ranges, while some are restricted to a single taxon or even a single species (Ros, Fleming et al., 2009). The most widely identified supergroups are supergroup A and B, which can infect exclusively arthropods (Ros et al., 2009). Supergroups C, D and J are restricted to filarial nematodes (Lefoulon et al., 2020). Supergroups E and H are less widespread, and supergroup E is found only in springtails (*Collembola*) and supergroup H is found to be present in one genus of termites (*Isoptera*). Supergroup F is found in arthropods and nematodes (Ros et al., 2009). *Wolbachia* from Australian spiders have been assigned as supergroup G (Ros et al., 2009). *Wolbachia* strains from flea ecotypes (Siphonaptera) are assigned to supergroup I, and the *Wolbachia* from filarial nematode *D. gracile* (Spirurida) correspond to the supergroup J. (Ros et al., 2009). Supergroup K *Wolbachia* are present in spider mite species of the genus *Bryobia* (Ros et al., 2009). Supergroup L contains *Wolbachia* from plant parasitic nematodes (Haegeman et al., 2009; Lefoulon et al., 2020), and *Wolbachia* from the pseudoscorpion *Atemnus politus* (wApol) and *Cordylochernes scorpioides* (wCsco) were described as supergroup S (Lefoulon et al., 2020). The proposed supergroup G and R are no longer considered as separate supergroups and have been included as part of supergroup B and A, respectively (Lefoulon et al., 2020). The rapid generation of sequencing data has made it possible for the extensive expansion of the supergroup range.

1.5.2 *Wolbachia* as an example of insect-bacterial symbiosis

Wolbachia has a vast host range and servers as an excellent model for studying insect bacterial symbiosis. The symbiotic association of *Wolbachia* ranges from mutualistic to parasitic depending on the host (Werren et al., 2008). In symbiosis with its

nematode host, *Wolbachia* is an obligate endosymbiont, necessary for host reproduction. It is also suggested that *Wolbachia* provides the host nematode with ATP, FAD and revoflavin (Foster 2005; Darby et al., 2012). *Wolbachia* might also contribute to the heme biosynthesis which is crucial for the survival of the host (Wu et al., 2009). In association with the parasitic wasp *Asobara tabida, Wolbachia* is required for oogenesis for its ability to inhibit programmed cell death (Oliver & Russell, 2016). It is also been found that *Wolbachia* can provide viral protection to its arthropod host, limiting infection of some RNA viruses (Teixeira et al., 2008). Conversely, *Wolbachia* can also have a parasitic relationship with its hosts. Certain *Wolbachia* strains can cause severe tissue degeneration and premature death of *Drosophila melanogaster* (Strunov & Kiseleva, 2016). *Wolbachia* can also act as a reproductive parasite in arthropod hosts, modifying normal reproduction (Werren et al., 2008). The range of reproductive modifications caused by *Wolbachia* are: parthenogenesis, feminization of male, male killing and cytoplasmic incompatibility (CI), all of which aim to favor proliferation of the bacteria by infected females (Werren et al., 2008; Pietri et al., 2016). Among the four reproductive modifications, CI is the most abundant, and addressing associated molecular underpinnings are the goal of this thesis.

1.6 What is cytoplasmic incompatibility (CI)?

Cytoplasmic incompatibility (CI) is the most common reproductive manipulation induced by the endosymbionts. Cytoplasmic incompatibility occurs when the sperm from *Wolbachia*-infected male is incompatible with eggs from an uninfected female or a female infected with a different *Wolbachia* strain (Werren et al., 2008). In diploid organisms, CI normally results in lethality of the embryo but in haplodiploid hosts, CI results as haploid embryos and thus, male development (Werren et al., 2008). However, when a female is infected with the same *Wolbachia* strain, embryonic lethality is rescued

and the cross results in viable progeny. This scenario is termed ‘Rescue’. Complicating this scenario, CI can be unidirectional or bidirectional. In unidirectional CI, as explained in above, the male is infected with one type of *Wolbachia* and female is uninfected. In bidirectional CI, the male and female are infected with different *Wolbachia* strains (Telschow et al., 2005). Previous studies have reported CI-inducing *Wolbachia* are present in the orders Diptera, Hymenoptera, Coleoptera, Hemiptera, Orthoptera, Lepidoptera, Thysanoptera, Acari, Isopoda and Arachnids (Shropshire et al., 2020). In addition to *Wolbachia*, *Cardinium* sp. were also found to cause CI in the parasitic wasp *Encarsia pergandiella and the spider mite Eotetranychus suginamensis (Penz et al., 2012; Gotoh et al., 2007). Apart from these, unknown symbionts of coconut beetles Brontispa longissimi and parasitoid wasps Lariophagus distinguendus have been proposed to cause CI* (Shropshire et al., 2020)

1.6.1 The genetic basis of CI

Researchers have been trying to understand the genetic basis of CI for several decades, while substantial progress has been achieved in recent years. Progress in understanding the genetic basis of CI started with sequencing the genome of *w*Mel, the *Wolbachia* strain specific to *Drosphila melanogaster* in 2004 (Wu et al., 2004). The *w*Mel strain has a streamlined genome with a few mobile genetic elements, including *Wolbachia* phage WO. The prophage WO encodes a number of proteins termed a eukaryotic association module. The genome of *w*Mel also encodes multiple ankyrin proteins, which are involved in protein-protein interactions in eukaryotes (Al-Khodor et al., 2010; Jernigan & Bordenstein, 2014). Conversely, the genome of *w*BM, the *Wolbachia* associated with the nematode *Brugia malayia* that does not induce CI, does not contain the WO phage genome nor any ankyrin proteins (Foster et al., 2005). These

findings suggest a relationship between the genes of phage WO and the ankyrin proteins with the CI phenotype.

In a later study, mass spectroscopy and SDS page analysis of spermatheca extract from *Wolbachia*-infected *Culex pipiens* females identified a phage derived protein WPIP0282, which strengthen the relation between prophage WO derived genes and CI (Beckmann & Fallon, 2013). Another study compared the CI inducing *w*Mel genome with non-CI inducing *w*Au (a strain of *Wolbachia* from *D. simulans*) and identified nine genes absent in *w*Au but present in *w*Mel. These genes include several prophage WO genes, including WD0631, which is the *w*Mel homolog of *w*Pip WPIP0282 and the adjacent WD0632 gene (Sutton et al., 2014). Another sequencing study of the *w*Rec genome from *Drosophila recens* revealed a highly reduced prophage WO genome, including the *w*Rec homolog of WPIP0282 (Metcalf et al., 2014; Beckmann & Fallon, 2013). Another genome comparison from CI inducing and non-CI inducing strain identified two phage genes WD0631 and WD0632 in the eukaryotic association module, as CI candidate genes in *w*Mel (LePage et al., 2017). These two genes WD0631 and WD0632 were thus termed cytoplasmic incompatibility factors A and B (cifA and cifB) respectively (LePage et al., 2017). When referring to the *cif* genes, the specific strain that the specific *cif* gene comes from can be defined with the strain name as a subscript, such as $cif_{w\text{Mel}}$ or $cif_{w\text{Pip}}$.

Two independent studies explored the relationship between cif genes ($cif_{w\text{Mel}}$ and $cif_{w\text{Pip}}$) and their ability to induce CI in a transgenic *w*Mel expression system (LePage et al., 2017; Beckmann et al., 2017). Transgenic expression of either $\text{cifA}_{w\text{Mel}}$ or $\text{cifB}_{w\text{Mel}}$ in *D. melanogaster* males failed to induce CI, but duel expression of $cifA_{w\text{Mel}}$ and $cifB_{w\text{Mel}}$ was able to induce CI-like hatch rates, as well as CI-like cytological defects. This finding suggested that *cif* genes can induce CI when expressed together (LePage et al., 2017). A similar finding was found when $cidA_{w\text{Pip}}$ and $cidB_{w\text{Pip}}$ (*w*Pip homologs of *cif*A and *cif*B)

were expressed together in transgenic *D. melanogaster* males, but these expressions failed to induce rescue (Beckmann et al., 2017). Another similar transgenic study revealed that *cifA*$_{w\text{Mel}}$ expression in transgenic *D. melanogaster* females can rescue CI (Shropshire et al., 2018). These findings suggest a two by one model for CI and rescue, where expression of both *cifA* and *cifB* can induce CI and expression of *cifA* in ovaries can induce rescue.

The *cif* gene set can vary in copy number in the CI inducing *Wolbachia* strain and strength of CI correlates with gene copy number (Lindsey et al., 2018). Also, the *cif* gene has multiple paralogs in different *Wolbachia* strains. A recently proposed nomenclature of *cif* genes suggests using "*cif*" when discussing CI genes in general, and to use more specific "*cid*" and "*cin*", when the function of a particular protein is known or strongly predicted (Chen et al., 2019; Beckmann et al., 2019). To denote different functional categories, it is suggested the *cif* operon be called "*cid*", as CI-inducing DUB (deubiquitylating enzyme) and "*cin*" as CI-inducing nuclease, and also called "*cnd*" when these operons are predicted to have both functions (Beckmann et al., 2019). Within each *cif* operon, the first gene is labeled A (*cid*A or *cin*A), and the second gene is labeled as B (*cid*B or *cin*B) (Beckmann et al., 2019).

Structural homology-based analyses suggest that the CifA protein (Type 1) has three putative domains, a catalase-related (catalase-rel) domain, a domain of unknown function (DUF) and a sterile-like transcriptional regulator (STE) (Lindsey et al., 2018). CifA cannot contribute to CI and rescue when disrupted in the putative catalase-rel domain or in the unannotated N-terminal region of CifA$_{w\text{Mel}}$. It has also been found that when mutated in DUF domain of CifA, it loses the ability to cause CI, but maintains the ability to induce rescue (Shropshire et al., 2020). These findings suggested that the DUF domain is important for causing CI, where the N-terminal region of CifA is crucial for both

CI and rescue. Additionally, CifB (from both *w*Mel and *w*Pip) encode a putative ubiquitin-like protease (Ulp1) domain (Beckmann et al., 2017; LePage et al., 2017; Lindsey et al., 2018). When exposed to different ubiquitin chains, Ulp1 cleaves K6-, K11-, K27-, K29-, K33-, K48-, and K63-linked ubiquitin and a single mutation in the Ulp catalytic region prevents the cleavage of these ubiquitins *in vitro* (Beckmann et al., 2017). Expression of a mutant Ulp catalytic domain of CidB along with CidA in transgenic *D. melanogaster* failed to induce CI. This finding suggests that the Ulp catalytic domain is important for inducing CI (Beckmann et al., 2017; Shropshire et al., 2020). All CifB proteins have a dimer of the PD-(D/E)XK nuclease domain (CinB). CinB has DNase activity and $CinB_{wPip}$ can degrade both linearized and circular double-stranded DNA *in vitro* (Chen et al., 2019). Expression of the cinA-cinB operon in transgenic male *D. melanogaster* were able to induce the CI phenotype and expression of CinA in females were able to rescue the defects in egg hatch rate (Chen et al., 2019). It has also been shown that mutation in PDDEXK catalytic sites in $CifB_{wPip}$ prevents nuclease activity *in vitro* and CI-inducibility when expressed in *D. melanogaster*. (Chen et al., 2019). All these findings strengthen the possibility that *cif* genes are responsible for CI and rescue. Although cif genes (or their homologs) are considered to cause CI, these genes are absent in *Cardinium* sp., which also shows a CI phenotype (Mann et al., 2017). This suggests the possibility of other factors involved in an overall mechanism, particularly in *Cardinium* sp.-mediated CI.

Phylogenetic analysis of *cifA* and *cifB* revealed considerable gene divergence, dividing them into five different clades referred to as types 1-5. (Lindsey et al., 2018; Martinez et al., 2021). The *w*Mel Cif proteins belong to type1, and *w*Pip has both type 1 and type 4 Cif proteins. Only type 1 and type 4 *cif* genes have been experimentally confirmed to cause CI and rescue (Beckmann et al., 2017; Chen et al., 2019; LePage et

al., 2017; Shropshire et al., 2018; Shropshire & Bordenstein, 2019). Some unpublished data also suggest type 2 *cif* genes both induce and rescue CI (Shropshire et al., 2020). The phenotypic output of type 3 and type 5 have not yet been experimentally assessed and are unknown. However, *w*No of *D. simulans*, which can cause CI, and *w*Stri of *La. striatellus* have type3 and type5 genes respectively (Shropshire et al., 2020).

1.6.2 The cytological basis of CI

Several studies have been carried out to understand the cytological basis of CI. All these studies seek to understand the alterations occuring during spermatogenesis and embryogenesis. Although these findings provide insight to the mechanisms by which *Wolbachia* modify host reproduction, It is still unclear how these observations are related to Cif proteins or whether they are the byproduct of *Wolbachia* symbiosis in the testes. The process of spermatogenesis is a complex process in *Drosophila* sp. beginning with cells from the germline stem cell niche that replicate into spermatogonia and yield a spermatocyst with 16 spermatocytes. Each spermatocyte then undergoes two rounds of meiosis and develop into a total of 64 spermatids. The spermatids then undergo elongation, where histones are replaced with protamine and a sperm tail is formed. In the final stage, the spermatids undergo individualization, removing excess cytoplasm, becoming mature sperm and entering the seminal vesicle for storage.

Previous studies have found abnormalities both before fertilization, during sperm development, and also after the fertilization process. It has been speculated that *Wolbachia* might affect the spermatogenesis process that leads to downstream sperm defects. Previous studies have found that *Wolbachia*-infected *D. simulans* and *Ephestia* moths produce fewer sperm when compared to their uninfected counterparts (Awrahman et al., 2014). Additionally, *Wolbachia*-infected *D. simulans* and *D. melanogaster* males mate at a higher rate than uninfected males (Awrahman et al., 2014). *Wolbachia*-

infected sperm also showed abnormal morphology, including some sperm undergoing incomplete individualization with sperm fusing together and some sperm exhibiting randomly oriented axoneme– mitochondrial complexes within elongated cysts (Riparbelli et al., 2007). In *w*Ri-infected *D. simulans*, *Wolbachia* were also found to be asymmetrically distributed in tastes, with only some spermatocysts harboring *Wolbachia* (Clark et al., 2003). This finding suggests that the CI-causing factors (or Cif proteins) might act in early stage of spermatogenesis or are diffusible factors that can travel between spermatocysts (Clark et al., 2002; Clark et al., 2003; Riparbelli et al., 2007).

Apart from the abnormalities stated above, CI embryos also show abnormalities after fertilization. Lassy and Karr reported that CI is caused by a mitotic defect and aberrant segregation of male and female chromosomes, which is viewed as a key cytological outcome in CI (Lassy & Karr, 1996). In chronological order, the abnormalities in CI embryos are: abnormal maternal H3.3 histone deposition on the male pronucleus; delayed activation of the proliferating cell nuclear antigen (PCNA) and the cell cycle regulator Cdk1; and delayed nuclear envelope breakdown(found in wasp), prior to the first mitosis (Landmann et al., 2009; Tram & Sullivan, 2002). These abnormalities result in a segregation defect in male chromatin and extensive chromatin bridging between the two pronuclei and finally, results in embryonic lethality (Callaini et al., 1996; Lassy & Karr, 1996; Tram et al., 2006). It is possible that CI is caused by any of the early mitotic defects (eg. abnormal maternal histone deposition) that lead to a cascade of effects resulting in the other embryonic defects. However, it still remains as open question as to how Cif proteins interact with the host to cause CI defects. It is unknown whether Cif proteins that are transferred with sperm directly cause the CI embryo defects or the initial interaction of Cif proteins with sperm during spermatogenesis later causes the same defects.

1.6.3 Other host factors contributing to CI

Researchers have been trying to understand the correlation between *Wolbachia* infection status and host expression phenotypes (RNA/ proteins). A number of studies have been conducted to correlate *Wolbachia* infection and host expression in *D. melanogaster, D. simulans, La. striatellus, Cu. pipiens* and *A. albopictus* (Biwot et al., 2020; Brennan et al., 2008; Brennan et al., 2012; Clark et al., 2006; Huang et al., 2019; Ju et al., 2017; LePage et al., 2014; Liu et al., 2014; Pinto et al., 2013, Yuan et al., 2015; Zheng et al., 2019; Zheng et al., 2011). The most promising candidate proteins or pathways associated with CI are considered those that can be over- or under-expressed to recapitulate CI-like hatch rates as well as cytological defects. It is also important to observe whether those CI-like hatch rates can be rescued in the presence of *Wolbachia*. There have been several studies identifying host factors (RNA/ proteins) that can recapitulate CI-like abnormalities and can be rescued by *Wolbachia* infection in females. These host factors include the histone chaperone Hira in *D. melanogaster* and *D. simulans* (Zheng et al., 2011), a juvenile hormone protein (JHI-26) involved in development in *D. melanogaster* (Liu et al., 2014), two seminal fluid proteins Spn3 and Peb in *D. melanogaster* (Yuan et al., 2015), the sRNA nov-miR-12 which negatively regulates the DNA-binding protein pipsqueak (psq) in *D. melanogaster* (Zheng et al., 2019), the immunity-related gene kenny (key) in *D. melanogaster* (Biwot et al., 2020), the aminotransferase iLve in *La. striatellus* (Ju et al., 2017) and the cytosol amino-peptidase-like protein in *La. striatellus* (Huang et al., 2019). Apart from these, studies also identified host factors that show CI-like egg hatch rates and cytological defects but were unable to show rescue effects in the presence of *Wolbachia*. These host factors are often considered to be non-CI associated host factors (Clark et al., 2006). These include the overexpression of the tumor suppressor gene lethal giant larvae [l(2)gl] and

myosin II gene *zipper* in uninfected *D. simulans* males inducing a considerable reduction in egg hatch rate, accompanied with CI- associated cytological defects (Clark et al., 2006). But when l(2)gl and *zipper* over-expressing males were mated to *Wolbachia*-infected females, no rescue effect was observed (Clark et al., 2006).

Apart from the host factors described earlier, expression of CI can also be related to the level of reactive oxygen species (ROS) of the host. Previous studies have found *Wolbachia* infection in *D. melanogaster, D. simulans, A. albopictus, A. polynesiensis*, and *T. urticae* males often show higher ROS in testis than uninfected males (Brennan et al., 2008; Brennan et al., 2012; Zug & Hammerstein, 2015). It has also been suggested that an increased ROS level leads to increased DNA damage in the spermatocyte of *Wolbachia*-infected *D. simulans* (Brennan et al., 2012). Additionally, overexpression of a *D. melanogaster* gene *key* causes an increase in ROS level in transinfected male and increased DNA damage, while mimicking CI-like hatching and embryonic defects rescuable by *Wolbachia* infection in females (Biwot et al., 2020). Although it is still unclear about a direct connection between CI and ROS levels, these findings suggest a possible role of ROS in the overall CI mechanism.

1.7 Factors causing variations of CI strength

The strength of CI can be variable in different host *Wolbachia* combinations, with the embryonic death rate varying between 10-100% (Shropshire et al., 2020). There are biotic and abiotic factors that correlate with the strength of CI, including rearing temperature, male and paternal grandmother age, male mating rate, male developmental timing, rearing density and nutrient availability. *Wolbachia* density, which positively correlate with the CI strength, is a major factor which seems to drive most of the relationship (Breeuwer & Werren, 1993). Temperature is another major factor correlating the CI strength in different host species. According to the phage density

model of CI, the WO phage may respond to extreme temperatures by increasing viral replication and lysing *Wolbachia* cells, causing a decrease in *Wolbachia* density and thus lowering the CI strength. Temperatures above 27°C have been shown to negatively affect the CI strength in *A. aegypti*, *A. scutellaris, A. albopictus*, *D. melanogaster*, *D. simulans*, *T. urticae* and *Nasonia* sp. (Ross et al., 2020; Trpis et al., 1981; Wiwatanaratanabutr & Kittayapong, 2009; Hoffmann et al., 1986; Van Opijnen & Breeuwer, 1999; Bordenstein & Bordenstein, 2011). High temperature also impacts *Wolbachia* densities in *A. albopictus*, *A. aegypti*, *N. vitripennis* and *T. urticae* (Foo et al., 2019; Ross et al., 2020; Bordenstein & Bordenstein, 2011; Lu et al., 2012), even curing *Wolbachia* infection in some species (Jia et al., 2009).

In addition to high temperature, cooler temperature can also impact *Wolbachia* density and CI strength. Temperatures below 19°C can cause a decrease in CI strength in *D. simulans* and *N. vitripennis* (Bordenstein & Bordenstein, 2011; Reynolds & Hoffmann, 2002). Apart from the examples above, higher temperature can also increase *Wolbachia* replication in some *D.simulans* lines and *Leptopilina heterotoma* wasps, but cause a decrease in CI strength (Clancy & Hoffmann, 1998; Mouton et al., 2006). CI caused by *Cardinium* sp. show a different relationship between *Cardinium* density and CI strength. In *E. suzannae*, high temperature cause reduction of *Cardinium* densities and lower CI strength, while cooler temperature also cause reduced *Cardinium* densities but increased CI strength (Doremus et al., 2019).

In addition to the temperature, male age can also act as a determining factor of CI strength, correlating negatively with male age. The *w*Mel *Wolbachia* of *D. melanogaster* induces considerably strong CI in first two days after males hatch but showed weaker CI in next 3-5 days (Reynolds & Hoffmann, 2002). Similar result have been observed in *D. simulans* and *N. vitripennis*, but the CI strength can be variable

(Breeuwer & Werren, 1993; Karr et al., 1998). It has also been found that *Wolbachia* density decreases with male age in *D. melanogaster, D. simulans,* and *N. vitripennis* hosts (Binnington & Hoffmann, 1989; Clark et al., 2002; Karr et al., 1998; Reynolds & Hoffmann, 2002; Riparbelli et al., 2007; Turelli & Hoffmann, 1995;Veneti et al., 2003; Weeks et al., 2007). So, it is possible that the decrease in CI strength with male age may also be correlated with *Wolbachia* density in the host.

In addition to male age, the age of paternal grandmother also correlates with CI strength. Although *Wolbachia* density decreases with age, it has been found that older virgin females have more *Wolbachia* than non-virgins (Layton et al., 2019). When the females are aged longer before mating, the male offspring contain higher *Wolbachia* densities, thus causing stronger CI (Layton et al., 2019). It is possible that the impact of age on the density of symbionts and the strength of CI is limited to some strains of *Wolbachia*. The male age has no effect on CI induced by *Cardinium* sp. in *E. pergandiella* (Perlman et al., 2014). The mating rate of the male also correlates negatively with CI strength. It have been hypothesized that the strength of CI will correspond to the amount of time the sperm remains in contact with *Wolbachia* (Karr et al., 1998). *Wolbachia*-infected *D. simulans* males mate at a higher rate than the uninfected males and induce weaker CI in later mating (Awrahman et al., 2014). Male developmental time is also negatively correlated with the CI strength. *D. melanogaster* males carrying *w*Mel show relatively stronger CI with the first immerged males (Yamada et al., 2007). The younger brothers from the same parents and at approximately same age showed relatively weaker CI, although they take longer time to develop (Yamada et al., 2007).

Rearing density and nutrition can also have an impact on CI strength. *w*Mel bearing *D. melanogaster* shows weaker CI when reared in higher densities in

comparison to the flies reared in lower densities (Yamada et al., 2007). It is hypothesized that the higher rearing densities cause nutritional stress which can result in less *Wolbachia* (Yamada et al., 2007). Studies have found that *w*Ri-containing *D. simulans* males showed weaker CI when exposed to nutritional stress compared to the males with abundant resources (Clancy & Hoffmann, 1998; Sinkins et al., 1995). It has also been found that the rearing density doesn't affect the CI strength in *w*AlbA and *w*AlbB carrying *A. albopictus* (Dutton & Sinkins, 2004), suggesting that the effect of rearing densities on CI strength might not be a generalized trait across all *Wolbachia*-host combinations.

Although relatively little is known, host genetics certainly plays an important role in determining CI strength. Some *Wolbachia* strains show a difference in CI strength when transferred from a native host to a non-native host species. For example, *w*Mel *Wolbachia* induces comparatively weak CI in native *D. melanogaster,* whereas it induces strong CI when transinfected into a *D. simulans* or *A. aegypti* hosts (Poinsot et al., 1998; Walker et al., 2011). Similarly, *w*VitA of *N. vitripennis* wasps causes weak CI in the native host, but induces strong CI when transinfected into *N. giraulti* (Chafee et al., 2011). These observations suggest that both host and *Wolbachia* genetics play a role in CI phenotypes.

1.8 Objective of this dissertation and organization

Despite the current advancement of information about CI and rescue, the knowledge of how *Wolbachia* infection in female rescue the embryonic lethality is still limited. My overall research goal is to understand the cellular basis of rescue of CI, using *Drosophila* as a model for host *Wolbachia* interaction. I am particularly interested in understanding the involvement of cellular pathways and processes those are involved in *Wolbachia*-mediated rescue of CI.

To understand the pathways involved in overall rescue process my Chapter II describe a suitable CI-rescue model which could be further used in this study. As CI trait is highly variable depending on the host and other biotic and abiotic factors, here we established a strong CI model which can also be rescued. We have used a *Drosophila simulans* host which is infected with both native *Wolbachia w*Ri and transinfected *w*Mel strain. Both of the host-*Wolbachia* combinations showed a strong CI phenotype in our assay. Later I elucidated the embryonic lethality observed in CI, was caused by the symbiont *Wolbachia* and not because of an artifact of mating failure. I also showed the embryonic lethality in CI crosses can be rescued by modification of host pathways by chemical feeding. I observed that the HDAC inhibitor sodium butyrate (NaBu) can induce partial rescue of CI. I also observed the effect of insulin signaling pathway as well as acetic acid in inducing partial rescue of CI.

Chapter III demonstrates the design of an assay technique using 24well tissue culture plate. Using this technique, it was possible to observe the CI and rescue phenotype. Using this technique, we were able to determine how many wells were necessary to constantly distinguish CI and rescue phenotype. By using 24-well tissue culture plate it was also possible to observe the partial rescue effect of NaBu feeding as we have observed in Chapter II. Using this technique, it was also possible to determine how many wells (within the 24-well tissue culture plate) are necessary to determine any rescue effect with chemical feeding.

In Chapter IV, I explored the involvement of different host pathways and processes in overall rescue process. The pathways were selected by exploring available literature. Chemical inhibitors were used to inhibit these pathways and processes and their effect on overall rescue process were observed. A total of 24 chemical inhibitors of cellular pathways were tested for their ability to induce rescue effect in CI crosses. Both

the native and transgenic CI system were used in this study. To our knowledge, this is the first study to explore the underlying cellular mechanism involved in overall rescue mechanism. Result from Chapter IV will provide us with knowledge about the host processes/pathways involved in rescue of CI.

References

Al-Khodor, S., Price, C. T., Kalia, A., & Kwaik, Y. A. (2010). Functional diversity of ankyrin repeats in microbial proteins. *Trends in Microbiology*, *18*(3), 132–139.

Angelard, C., & Bever, J. D. (2013). *Symbionts, Genetics of*. *Brenner's Encyclopedia of Genetics: Second Edition* (Vol. 6). Elsevier Inc. https://doi.org/10.1016/B978-0-12-374984-0.01496-0

Awrahman, Z. A., Champion de Crespigny, F., & Wedell, N. (2014). The impact of W olbachia, male age and mating history on cytoplasmic incompatibility and sperm transfer in D rosophila simulans. *Journal of Evolutionary Biology*, *27*(1), 1–10.

Beckmann, J. F., Bonneau, M., Chen, H., Hochstrasser, M., Poinsot, D., Merçot, H., … Charlat, S. (2019). The toxin–antidote model of cytoplasmic incompatibility: genetics and evolutionary implications. *Trends in Genetics*, *35*(3), 175–185.

Beckmann, J. F., & Fallon, A. M. (2013). Detection of the *Wolbachia* protein WPIP0282 in mosquito spermathecae: implications for cytoplasmic incompatibility. *Insect Biochemistry and Molecular Biology*, *43*(9), 867–878.

Beckmann, J. F., Ronau, J. A., & Hochstrasser, M. (2017). A *Wolbachia* deubiquitylating enzyme induces cytoplasmic incompatibility. *Nature Microbiology*, *2*(5), 1–7.

Binnington, K. C., & Hoffmann, A. A. (1989). *Wolbachia*-like organisms and cytoplasmic incompatibility in *Drosophila* simulans. *Journal of Invertebrate Pathology*, *54*(3), 344–352.

Biwot, J. C., Zhang, H., Liu, C., Qiao, J., Yu, X., & Wang, Y. (2020). *Wolbachia*-induced expression of kenny gene in testes affects male fertility in *Drosophila* melanogaster. *Insect Science*, *27*(5), 869–882.

Bordenstein, S. R., & Bordenstein, S. R. (2011). Temperature affects the tripartite interactions between bacteriophage WO, *Wolbachia*, and cytoplasmic incompatibility. *PloS One*, *6*(12), e29106.

Breeuwer, J. A., & Werren, J. H. (1993). Cytoplasmic incompatibility and bacterial density in Nasonia vitripennis. *Genetics*, *135*(2), 565–574.

Brennan, L J, Haukedal, J. A., Earle, J. C., Keddie, B., & Harris, H. L. (2012). Disruption of redox homeostasis leads to oxidative DNA damage in spermatocytes of *Wolbachia*-infected *Drosophila* simulans. *Insect Molecular Biology*, *21*(5), 510–520.

Brennan, Lesley J, Keddie, B. A., Braig, H. R., & Harris, H. L. (2008). The endosymbiont *Wolbachia* pipientis induces the expression of host antioxidant proteins in an Aedes albopictus cell line. *PLoS One*, *3*(5), e2083.

Callaini, G., Riparbelli, M. G., Giordano, R., & Dallai, R. (1996). Mitotic defects associated with cytoplasmic incompatibility in*Drosophila* simulans. *Journal of Invertebrate Pathology*, *67*(1), 55–64.

Cavalier-Smith, T. (1992). The number of symbiotic origins of organelles. *Biosystems*, *28*(1–3), 91–106.

Chafee, M. E., Zecher, C. N., Gourley, M. L., Schmidt, V. T., Chen, J. H., Bordenstein, S. R., … Bordenstein, S. R. (2011). Decoupling of host–symbiont–phage coadaptations following transfer between insect species. *Genetics*, *187*(1), 203–215.

Chen, H., Ronau, J. A., Beckmann, J. F., & Hochstrasser, M. (2019). A *Wolbachia* nuclease and its binding partner provide a distinct mechanism for cytoplasmic incompatibility. *Proceedings of the National Academy of Sciences*, *116*(44), 22314–22321.

Clancy, D. J., & Hoffmann, A. A. (1998). Environmental effects on cytoplasmic incompatibility and bacterial load in *Wolbachia*-infected *Drosophila* simulans. *Entomologia Experimentalis et Applicata*, *86*(1), 13–24.

Clark, M. E., Heath, B. D., Anderson, C. L., & Karr, T. L. (2006). Induced paternal effects mimic cytoplasmic incompatibility in *Drosophila*. *Genetics*, *173*(2), 727–734.

Clark, M. E., Veneti, Z., Bourtzis, K., & Karr, T. L. (2002). The distribution and proliferation of the intracellular bacteria *Wolbachia* during spermatogenesis in *Drosophila*. *Mechanisms of Development*, *111*(1–2), 3–15.

Clark, M. E., Veneti, Z., Bourtzis, K., & Karr, T. L. (2003). *Wolbachia* distribution and cytoplasmic incompatibility during sperm development: the cyst as the basic cellular unit of CI expression. *Mechanisms of Development*, *120*(2), 185–198.

Das, A., & Varma, A. (2009). Symbiosis: the art of living. In *Symbiotic Fungi* (pp. 1–28). Springer.

Degli Esposti, M. (2014). Bioenergetic evolution in proteobacteria and mitochondria. *Genome Biology and Evolution*, *6*(12), 3238–3251.

Doremus, M. R., Kelly, S. E., & Hunter, M. S. (2019). Exposure to opposing temperature extremes causes comparable effects on Cardinium density but contrasting effects on Cardinium-induced cytoplasmic incompatibility. *PLoS Pathogens*, *15*(8), e1008022.

Douglas, A. E. (2014). The molecular basis of bacterial-insect symbiosis. *Journal of Molecular Biology*, *426*(23), 3830–3837. https://doi.org/10.1016/j.jmb.2014.04.005

Dutton, T. J., & Sinkins, S. P. (2004). Strain-specific quantification of *Wolbachia* density in Aedes albopictus and effects of larval rearing conditions. *Insect Molecular Biology*, *13*(3), 317–322.

Ferrari, J., & Vavre, F. (2011). Bacterial symbionts in insects or the story of communities affecting communities. *Philosophical Transactions of the Royal Society B: Biological Sciences*, *366*(1569), 1389–1400.

Foo, I. J.-H., Hoffmann, A. A., & Ross, P. A. (2019). Cross-generational effects of heat stress on fitness and *Wolbachia* density in Aedes aegypti mosquitoes. *Tropical Medicine and Infectious Disease*, *4*(1), 13.

Foster, J., Ganatra, M., Kamal, I., Ware, J., Makarova, K., Ivanova, N., … Posfai, J. (2005). The *Wolbachia* genome of Brugia malayi: endosymbiont evolution within a human pathogenic nematode. *PLoS Biology*, *3*(4), e121.

Gil, R., Latorre, A., & Moya, A. (2004). Bacterial endosymbionts of insects: insights from comparative genomics. *Environmental Microbiology*, *6*(11), 1109–1122.

Gotoh, T., Noda, H., & Ito, S. (2007). Cardinium symbionts cause cytoplasmic incompatibility in spider mites. *Heredity*, *98*(1), 13–20.

Haegeman, A., Vanholme, B., Jacob, J., Vandekerckhove, T. T. M., Claeys, M., Borgonie, G., & Gheysen, G. (2009). An endosymbiotic bacterium in a plant-parasitic nematode: member of a new *Wolbachia* supergroup. *International Journal for Parasitology*, *39*(9), 1045–1054.

Hertig, M. (1936). The rickettsia, *Wolbachia* pipientis (gen. et sp. n.) and associated inclusions of the mosquito, Culex pipiens. *Parasitology*, *28*(4), 453–486.

Hertig, M., & Wolbach, S. B. (1924). Studies on rickettsia-like micro-organisms in insects. *The Journal of Medical Research*, *44*(3), 329.

Hoffmann, A. A., Turelli, M., & Simmons, G. M. (1986). Unidirectional incompatibility between populations of *Drosophila* simulans. *Evolution*, *40*(4), 692–701.

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*, *113*, 103211.

Jernigan, K. K., & Bordenstein, S. R. (2014). Ankyrin domains across the Tree of Life. *PeerJ*, *2*, e264.

Jia, F.-X., Yang, M.-S., Yang, W.-J., & Wang, J.-J. (2009). Influence of continuous high temperature conditions on *Wolbachia* infection frequency and the fitness of Liposcelis tricolor (Psocoptera: Liposcelididae). *Environmental Entomology*, *38*(5), 1365–1372.

Ju, J.-F., Hoffmann, A. A., Zhang, Y.-K., Duan, X.-Z., Guo, Y., Gong, J.-T., … Hong, X.-Y. (2017). *Wolbachia*-induced loss of male fertility is likely related to branch chain amino acid biosynthesis and iLvE in Laodelphax striatellus. *Insect Biochemistry and Molecular Biology*, *85*, 11–20.

Karr, T. L., Yang, W., & Feder, M. E. (1998). Overcoming cytoplasmic incompatibility in *Drosophila*. *Proceedings of the Royal Society of London. Series B: Biological*

Sciences, *265*(1394), 391–395.

Landmann, F., Orsi, G. A., Loppin, B., & Sullivan, W. (2009). *Wolbachia*-mediated cytoplasmic incompatibility is associated with impaired histone deposition in the male pronucleus. *PLoS Pathogens*, *5*(3), e1000343.

Lassy, C. W., & Karr, T. L. (1996). Cytological analysis of fertilization and early embryonic development in incompatible crosses of *Drosophila* simulans. *Mechanisms of Development*, *57*(1), 47–58.

Layton, E. M., On, J., Perlmutter, J. I., Bordenstein, S. R., & Shropshire, J. D. (2019). Paternal grandmother age affects the strength of *Wolbachia*-induced cytoplasmic incompatibility in *Drosophila* melanogaster. *MBio*, *10*(6), e01879-19.

Lefoulon, E., Clark, T., Borveto, F., Perriat-Sanguinet, M., Moulia, C., Slatko, B. E., & Gavotte, L. (2020). Pseudoscorpion *Wolbachia* symbionts: diversity and evidence for a new supergroup S. *BMC Microbiology*, *20*(1), 1–15.

LePage, D. P., Jernigan, K. K., & Bordenstein, S. R. (2014). The relative importance of DNA methylation and Dnmt2-mediated epigenetic regulation on *Wolbachia* densities and cytoplasmic incompatibility. *PeerJ*, *2*, e678.

LePage, D. P., Metcalf, J. A., Bordenstein, S. R., On, J., Perlmutter, J. I., Shropshire, J. D., … Bordenstein, S. R. (2017). Prophage WO genes recapitulate and enhance *Wolbachia*-induced cytoplasmic incompatibility. *Nature*, *543*(7644), 243–247.

Leung, T. L. F., & Poulin, R. (2008). Parasitism, commensalism, and mutualism: exploring the many shades of symbioses. *Vie et Milieu*, *58*(2), 107.

Lindsey, A. R. I., Rice, D. W., Bordenstein, S. R., Brooks, A. W., Bordenstein, S. R., & Newton, I. L. G. (2018). Evolutionary genetics of cytoplasmic incompatibility genes cifA and cifB in prophage WO of *Wolbachia*. *Genome Biology and Evolution*, *10*(2), 434–451.

Liu, C., Wang, J.-L., Zheng, Y., Xiong, E.-J., Li, J.-J., Yuan, L.-L., … Wang, Y.-F. (2014). *Wolbachia*-induced paternal defect in *Drosophila* is likely by interaction with the juvenile hormone pathway. *Insect Biochemistry and Molecular Biology*, *49*, 49–58.

Lo, N., Paraskevopoulos, C., Bourtzis, K., O'Neill, S. L., Werren, J. H., Bordenstein, S. R., & Bandi, C. (2007). Taxonomic status of the intracellular bacterium *Wolbachia* pipientis. *International Journal of Systematic and Evolutionary Microbiology*, *57*(3), 654–657.

Lu, M.-H., Zhang, K.-J., & Hong, X.-Y. (2012). Tripartite associations among bacteriophage WO, *Wolbachia*, and host affected by temperature and age in Tetranychus urticae. *Experimental and Applied Acarology*, *58*(3), 207–220.

Mann, E., Stouthamer, C. M., Kelly, S. E., Dzieciol, M., Hunter, M. S., & Schmitz-Esser, S. (2017). Transcriptome sequencing reveals novel candidate genes for Cardinium hertigii-caused cytoplasmic incompatibility and host-cell interaction. *MSystems*,

2(6), e00141-17.

Margulis, L. (1996). Archaeal-eubacterial mergers in the origin of Eukarya: phylogenetic classification of life. *Proceedings of the National Academy of Sciences*, *93*(3), 1071–1076.

Martinez, J., Klasson, L., Welch, J. J., & Jiggins, F. M. (2021). Life and death of selfish genes: comparative genomics reveals the dynamic evolution of cytoplasmic incompatibility. *Molecular Biology and Evolution*, *38*(1), 2–15.

Metcalf, J. A., Jo, M., Bordenstein, S. R., Jaenike, J., & Bordenstein, S. R. (2014). Recent genome reduction of *Wolbachia* in *Drosophila* recens targets phage WO and narrows candidates for reproductive parasitism. *PeerJ*, *2*, e529.

Moran, N. A. (2006). Symbiosis. *Current Biology*, *16*(20), 866–871.

Mouton, L., Henri, H., Bouletreau, M., & Vavre, F. (2006). Effect of temperature on *Wolbachia* density and impact on cytoplasmic incompatibility. *Parasitology*, *132*(1), 49–56.

Oliver, K. M., & Russell, J. A. (2016). Symbiosis, Introduction to. *Encyclopedia of Evolutionary Biology*, *4*, 282–290. https://doi.org/10.1016/B978-0-12-800049-6.00186-4

Penz, T., Schmitz-Esser, S., Kelly, S. E., Cass, B. N., Müller, A., Woyke, T., … Horn, M. (2012). Comparative genomics suggests an independent origin of cytoplasmic incompatibility in Cardinium hertigii. *PLoS Genet*, *8*(10), e1003012.

Perlman, S. J., Dowdy, N. J., Harris, L. R., Khalid, M., Kelly, S. E., & Hunter, M. S. (2014). Factors affecting the strength of Cardinium-induced cytoplasmic incompatibility in the parasitic wasp Encarsia pergandiella (Hymenoptera: Aphelinidae). *Microbial Ecology*, *67*(3), 671–678.

Pietri, J. E., DeBruhl, H., & Sullivan, W. (2016). The rich somatic life of *Wolbachia*. *Microbiologyopen*, *5*(6), 923–936.

Pinto, S. B., Stainton, K., Harris, S., Kambris, Z., Sutton, E. R., Bonsall, M. B., … Sinkins, S. P. (2013). Transcriptional regulation of Culex pipiens mosquitoes by *Wolbachia* influences cytoplasmic incompatibility. *PLoS Pathogens*, *9*(10), e1003647.

Poinsot, D., Bourtzis, K., Markakis, G., Savakis, C., & Merçot, H. (1998). *Wolbachia* transfer from *Drosophila* melanogaster into *D. simulans*: host effect and cytoplasmic incompatibility relationships. *Genetics*, *150*(1), 227–237.

Punkosdy, G. A., Addiss, D. G., & Lammie, P. J. (2003). Characterization of antibody responses to *Wolbachia* surface protein in humans with lymphatic filariasis. *Infection and Immunity*, *71*(9), 5104–5114.

Reynolds, K. T., & Hoffmann, A. A. (2002). Male age, host effects and the weak

expression or non-expression of cytoplasmic incompatibility in *Drosophila* strains infected by maternally transmitted *Wolbachia*. *Genetics Research*, *80*(2), 79–87.

Riparbelli, M. G., Giordano, R., & Callaini, G. (2007). Effects of *Wolbachia* on sperm maturation and architecture in *Drosophila* simulans Riverside. *Mechanisms of Development*, *124*(9–10), 699–714.

Ros, V. I. D., Fleming, V. M., Feil, E. J., & Breeuwer, J. A. J. (2009). How diverse is the genus *Wolbachia*? Multiple-gene sequencing reveals a putatively new *Wolbachia* supergroup recovered from spider mites (Acari: Tetranychidae). *Applied and Environmental Microbiology*, *75*(4), 1036–1043.

Ross, P. A., Axford, J. K., Yang, Q., Staunton, K. M., Ritchie, S. A., Richardson, K. M., & Hoffmann, A. A. (2020). Heatwaves cause fluctuations in w Mel *Wolbachia* densities and frequencies in Aedes aegypti. *PLoS Neglected Tropical Diseases*, *14*(1), e0007958.

Sarwar, M. S., Jahan, N., & Shahbaz, F. (2018). Molecular Detection and Characterization of *Wolbachia* pipientis from Culex quinquefasciatus Collected from Lahore, Pakistan. *The American Journal of Tropical Medicine and Hygiene*, *98*(1), 154–161.

Sazama, E. J., Ouellette, S. P., & Wesner, J. S. (2019). Bacterial endosymbionts are common among, but not necessarily within, insect species. *Environmental Entomology*, *48*(1), 127–133.

Serbus, L. R., Casper-Lindley, C., Landmann, F., & Sullivan, W. (2008). The genetics and cell biology of *Wolbachia*-host interactions. *Annual Review of Genetics*, *42*, 683–707.

Shropshire, J. D., & Bordenstein, S. R. (2019). Two-By-One model of cytoplasmic incompatibility: Synthetic recapitulation by transgenic expression of cifA and cifB in *Drosophila*. *PLoS Genetics*, *15*(6), e1008221.

Shropshire, J. D., Leigh, B., & Bordenstein, S. R. (2020). Symbiont-mediated cytoplasmic incompatibility: What have we learned in 50 years? *ELife*, *9*, 1–36. https://doi.org/10.7554/ELIFE.61989

Shropshire, J. D., On, J., Layton, E. M., Zhou, H., & Bordenstein, S. R. (2018). One prophage WO gene rescues cytoplasmic incompatibility in *Drosophila* melanogaster. *Proceedings of the National Academy of Sciences*, *115*(19), 4987–4991.

Sinkins, S. P., Braig, H. R., & O'Neill, S. L. (1995). *Wolbachia* superinfections and the expression of cytoplasmic incompatibility. *Proceedings of the Royal Society of London. Series B: Biological Sciences*, *261*(1362), 325–330.

Strunov, A., & Kiseleva, E. (2016). *Drosophila* melanogaster brain invasion: pathogenic *Wolbachia* in central nervous system of the fly. *Insect Science*, *23*(2), 253–264.

Sutton, E. R., Harris, S. R., Parkhill, J., & Sinkins, S. P. (2014). Comparative genome analysis of *Wolbachia* strain w Au. *BMC Genomics*, *15*(1), 1–15.

Teixeira, L., Ferreira, Á., & Ashburner, M. (2008). The bacterial symbiont *Wolbachia* induces resistance to RNA viral infections in *Drosophila* melanogaster. *PLoS Biol*, *6*(12), e1000002.

Telschow, A., Yamamura, N., & Werren, J. H. (2005). Bidirectional cytoplasmic incompatibility and the stable coexistence of two *Wolbachia* strains in parapatric host populations. *Journal of Theoretical Biology*, *235*(2), 265–274.

Tram, U., Fredrick, K., Werren, J. H., & Sullivan, W. (2006). Paternal chromosome segregation during the first mitotic division determines *Wolbachia*-induced cytoplasmic incompatibility phenotype. *Journal of Cell Science*, *119*(17), 3655–3663.

Tram, U., & Sullivan, W. (2002). Role of delayed nuclear envelope breakdown and mitosis in *Wolbachia*-induced cytoplasmic incompatibility. *Science*, *296*(5570), 1124–1126.

Trpis, M., Perrone, J. B., Reissig, M., & Parker, K. L. (1981). Control of cytoplasmic incompatibility in the Aedes scutellaris complex: Incompatible crosses become compatible by treatment of larvae with heat or antibiotics. *Journal of Heredity*, *72*(5), 313–317.

Turelli, M., & Hoffmann, A. A. (1995). Cytoplasmic incompatibility in *Drosophila* simulans: dynamics and parameter estimates from natural populations. *Genetics*, *140*(4), 1319–1338.

Van Opijnen, T., & Breeuwer, J. A. J. (1999). High temperatures eliminate *Wolbachia*, a cytoplasmic incompatibility inducing endosymbiont, from the two-spotted spider mite. *Experimental & Applied Acarology*, *23*(11), 871–881.

Veneti, Z., Clark, M. E., Zabalou, S., Karr, T. L., Savakis, C., & Bourtzis, K. (2003). Cytoplasmic incompatibility and sperm cyst infection in different *Drosophila*-*Wolbachia* associations. *Genetics*, *164*(2), 545–552.

Walker, T., Johnson, P. H., Moreira, L. A., Iturbe-Ormaetxe, I., Frentiu, F. D., McMeniman, C. J., … Kriesner, P. (2011). The w Mel *Wolbachia* strain blocks dengue and invades caged Aedes aegypti populations. *Nature*, *476*(7361), 450–453.

Wasala, S. K., Brown, A., Kang, J., Howe, D. K., Peetz, A. B., Zasada, I. A., & Denver, D. R. (2019). Variable abundance and distribution of *Wolbachia* and Cardinium endosymbionts in plant-parasitic nematode field populations. *Frontiers in Microbiology*, *10*, 964.

Weeks, A. R., Turelli, M., Harcombe, W. R., Reynolds, K. T., & Hoffmann, A. A. (2007). From parasite to mutualist: rapid evolution of *Wolbachia* in natural populations of *Drosophila*. *PLoS Biology*, *5*(5), e114.

Werren, J. H., Baldo, L., & Clark, M. E. (2008). *Wolbachia*: master manipulators of invertebrate biology. *Nature Reviews Microbiology*, *6*(10), 741–751.

White, P. L., Baker, A. R., & James, W. O. (1997). Strategies to control Salmonella and Campylobacter in raw poultry products. *Revue Scientifique et Technique (International Office of Epizootics)*, *16*(2), 525–541.

Wiwatanaratanabutr, I., & Kittayapong, P. (2009). Effects of crowding and temperature on *Wolbachia* infection density among life cycle stages of Aedes albopictus. *Journal of Invertebrate Pathology*, *102*(3), 220–224.

Wu, M., Sun, L. V, Vamathevan, J., Riegler, M., Deboy, R., Brownlie, J. C., … Ahmadinejad, N. (2004). Phylogenomics of the reproductive parasite *Wolbachia* pipientis w Mel: a streamlined genome overrun by mobile genetic elements. *PLoS Biology*, *2*(3), e69.

Yamada, R., Floate, K. D., Riegler, M., & O'Neill, S. L. (2007). Male development time influences the strength of *Wolbachia*-induced cytoplasmic incompatibility expression in *Drosophila* melanogaster. *Genetics*, *177*(2), 801–808.

Yuan, L.-L., Chen, X., Zong, Q., Zhao, T., Wang, J.-L., Zheng, Y., … Yang, F. (2015). Quantitative proteomic analyses of molecular mechanisms associated with cytoplasmic incompatibility in *Drosophila* melanogaster induced by *Wolbachia*. *Journal of Proteome Research*, *14*(9), 3835–3847.

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*(4), e19512.

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*. *Journal of Insect Physiology*, *118*, 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. *Frontiers in Microbiology*, *6*, 1201.

CHAPTER II: SETTING UP A CI MODEL

2.1 Abstract

Wolbachia is one of the most widespread endosymbionts in nature that can infect both arthropod and nematode hosts. The success of endosymbiotic relationships relies on efficient endosymbiont transmission from mother to offspring. To achieve this, *Wolbachia* use a variety of host reproductive modifications, among which cytoplasmic incompatibility (CI), is considered the most successful. CI renders the uninfected female egg incompatible with infected male sperm, and causes embryonic lethality in incompatible crosses. Interestingly, *Wolbachia*-infected females can reverse this lethal effect and produce viable offspring in crosses with both uninfected and infected males, in a process termed “rescue”. Although investigations have been carried out to determine the underlying mechanism(s) of CI, there have currently been no studies investigating the mechanism(s) underlying rescue. In this chapter, we set up a chemical feeding approach to study host cellular pathways potentially contribute to the rescue process. We have used the naturally *w*Ri-infected and *w*Mel-transinfected *D. simulans* as models for both CI and rescue. We have designed a feeding assay to assess the effectiveness of chemicals and inhibitors in the context of uninfected female flies. We observed the HDAC inhibitor sodium butyrate was able to induce partial rescue in CI crosses. Thus, it is possible that pathways modified by sodium butyrate affect rescue of CI. This finding sets the stage for a more complete understanding of the cellular pathways influenced and involved in the rescue process.

2.2 Introduction

Symbiosis is the successful association between two or more species. Microbial symbiosis is ubiquitous among insect species, and most insects harbor an array of microbes in their gut, body cavity, externally and within specific cells. Among these

associations, some symbionts are parasitic, with detrimental effects on their host, some are commensalistic, with no discernible effect on the host, and others are mutualistic, providing beneficial effects to their hosts (Hosokawa & Fukatsu, 2020). The term 'symbiont' refers to the bacterial portion of the symbiotic association. In many symbiotic relationships, prokaryotic bacteria act as an 'endosymbiont', where the physical association is within the cells and tissues of the host organism (Kikuchi, 2009).

Wolbachia are maternally inherited gram-negative endosymbionts which are widespread in nature. *Wolbachia* are found in about 52% of aquatic insect species and ~60% (58%-63%) of terrestrial insects (Sazama et al., 2017). *Wolbachia* are vertically transmitted and their ability to persist and spread through a host population depends on successful transmission from mother to offspring (Landmann, 2019, (Werren et.al., 2008). To achieve this, *Wolbachia* manipulate the host reproductive system to enhance the efficacy of maternal transmission. Four widely discussed reproductive phenotypes caused by *Wolbachia* are parthenogenesis, male killing, feminization and cytoplasmic incompatibility (CI). Cytoplasmic incompatibility is the most successful reproductive modification, which causes embryonic lethality in mating between *Wolbachia*-infected males and uninfected females. However, sperm from *Wolbachia*-infected males is compatible with both *Wolbachia*-infected and uninfected embryos, resulting in viable progeny. This *Wolbachia*-associated reduction in embryonic lethality is termed 'rescue'. Thus, *Wolbachia* spread within a host population, favoring infected females, by selectively killing uninfected embryos and permitting infected embryos to live.

The mechanism underlying CI has been investigated for many years. Recently, genetic and cytological studies have shed some light on the biological basis of CI. Cytological studies indicated that CI is caused by a mitotic defect, where maternal chromatin completes segregation to the opposite pole in anaphase, whereas paternal

chromatin remains stuck at the metaphase plate (Lassy & Karr, 1996). This leads to aneuploidy and embryonic lethality. A later study by Tram and Sullivan demonstrated the the mitotic defect resulted in a timing mismatch between male and female pronuclei in the first mitotic division (Tram & Sullivan, 2002). CI embryos also show abnormal maternal histone 3.3 deposition in the male pronucleus and delayed activation of proliferating cell nuclear antigen (PCNA) (Landmann et al., 2009). All these defects lead to embryonic lethality in CI crosses (ie. matings between infected males and uninfected females). By contrast, *Wolbachia*-infected embryos showed no segregation defects and result in viable embryos in crosses with *Wolbachia*-infected females and uninfected males. Recent transgenic expression studies demonstrated that expression of two *Wolbachia* genes, cifA and cifB, are responsible for causing CI phenotypes, and expression of cifA can rescue CI (Beckmann et al, 2017; LePage et al., 2017; Shropshire et al., 2018).

Since the strength of CI can be variable depending on somatic and genetic factors, it is important to determine the strength of CI in any host model before performing any assay (Yamada et al., 2007). In different experimental models, the strength of CI is determined in various assay techniques using crosses between *Wolbachia*-infected males and uninfected females, counting the egg hatch rate or, in the case of the wasp model, the male and female ratio. A strong CI model should have a lower egg hatch rate, or in the thecase of halodiploid organisms, a higher male to female ratio. *Drosophila simulans,* which is naturally infected with *w*Ri *Wolbachia,* has been shown to induce strong CI (Hoffmann et al., 1986). *w*Mel-transinfected *Drosophila simulans* also exhibits a strong CI phenotype (Poinsot et al., 1998). This makes *D. simulans* an excellent model for studying CI and rescue.

Chemical feeding has been successfully used as a experimental tool for studying development in the *D. melanogaster* model organism (Yi et al., 2021). Chemical feedings can affect many biological processes in both wild type and mutant *Drosophila* lines whose effects include changes in lifespan, fecundity, fertility, climbing ability, insulin signaling, resistance to heat stress, cold and starvation resistance and expression of different genes (Yi et al., 2021). In general, chemical feedings affect the whole-body of *Drosophila* rather than targeting a host tissue. So, the ability to observe tissue specific effects can be limited by the chemical feeding techniques. Despite this limitation, chemical feeding that targets specific host pathways can be broadly informative in species where specific genetic modifications are not readily available.

While some advances in elucidating the underlying pathways of CI have occurred, an advancement of understanding the rescue phenotype is particularly limited. To achieve a better understanding of pathways involved in rescue, Chapter II presents a CI assay with naturally *Wolbachia* (*w*Ri) infected and transinfected (*w*Mel) *Drosophila simulans* within a high throughput setup. This assay distinguishes true embryonic lethality in CI from any lethal effect induced by changes in mating behavior. This chapter also includes a chemical feeding assay using *w*Ri- and *w*Mel-infected *Drosophila simulans,* which can be used to study the effect of biological pathway modification on CI. This assay also demonstrates the effect of different pathways on their ability to induce the rescue effect. In this chapter, we have tested the effect of sodium butyrate (NaBu), the food microbiome and insulin signaling on CI for their ability to induce any rescue effect on CI. By exploring the ability of cellular pathways to induce rescue of CI, we implicate specific involvement of these cellular pathways in *Wolbachia-mediated* rescue of CI.

2.3 Methods and materials

2.3.1 Fly stocks & maintenance

The *Wolbachia w*Ri-infected *Drosophila simulans* strain used in this study was originally described by Hoffman, Turrelli, and Simmons (Hoffmann et al., 1986). Initially, the fly strain was obtained in Watsonville, CA and it has since been maintained under lab conditions. The uninfected *D. simulans* strain were from the same genetic background and created by curing *Wolbachia* with tetracycline (He et al., 2019; Hoffmann et al., 1986). The *w*Mel trans-infected strain was created by transferring *Wolbachia* from *D. melanogaster* (Poinsot et al., 1998), and backcrossed into a cured *D. simulans* fly stock for six generations.

All the flies used in this study were maintained with standard, minimal fly food at 25°C on a 12h light/dark cycle using an Invictus *Drosophila* incubator (Genessee Scientific, USA). The recipe for fly food was derived from Bloomington *Drosophila* stock center (*http://flystocks.bio.indiana.edu/Fly_Work/media recipes/bloomfood.htm*) (Camacho et al., 2017; Christensen et al., 2016). Briefly, the food was cooked in a large batch that contain, 377 grams of yeast, 1325 grams of yellow corn meal, 96 grams of agar, 190 grams of soy flower, 1.5 liters of Karo light corn syrup, 94 ml of propionic acid and 20 liters of water. The prepared food was stored at 4°C until it was used.

Flies were raised in standard 6 oz. square bottom polyethylene bottles containing 25-30 ml of fly food. Each bottle was seeded with approximately 80-100 flies (both male and female) and incubated for 3-6 days. After the short incubation period, the flies were transferred to a new bottle. The flies used to seed new bottles were discarded after two or three rounds of egg laying. To collect virgin flies, the bottles were cleared, and each bottle were visually checked and confirmed for the absence of any remaining flies. The newly eclosed flies were collected using standard CO_2gas pads ~5-8 hours after initial

clearing. Male and female flies were immediately separated and ~45 flies were temporarily stored in vials until experimental matings were set up. Fly sorting was limited to a period of 20-25 min to avoid any damage from prolonged exposure to CO_2.

2.3.2 Microbial 16S rRNA gene sequencing

Microbial 16s rRNA gene sequencing was carried out with ovary samples from both *Wolbachia*-infected and uninfected *D. simulans,* to confirm the infection status of the flies. The 16s rRNA gene sequencing was carried out using a protocol previously described by Christensen et. al. (Christensen et al., 2019). Briefly, all the flies were reared for 3 days in normal food and ovaries dissected on standard CO_2 gas pads. Flies were dissected in three pool of 20 ovaries from each type of fly in 0.1 M Tris HCl, 0.1 M EDTA and rinsed twice with fresh buffer. To extract the total DNA, each ovary sample were homogenized in 50 µl lysis buffer. Total DNA was extracted by with DNeasy (Qiagen) Blood and Tissue Extraction Kit according to manufacturer's protocol. Extracted DNA were estimated by using fluorimetry on a Qubit 2.0 (Life Technologies). All the DNA samples were then precipitated and dried. Samples with more than 50 ng of total DNA were sent to Omega Bioservices (Norcross, GA) for Next-Gen, PCR-targeted sequencing. To determine the infection status of each sample, primers targeting the V1-V3 regions of bacterial 16S rRNA gene were used to amplify the DNA. Samples were then sequenced on an Illumina MiSeq with V3 chemistry. Results were then analyzed via Illumina's BaseSpace 16S rRNA application module, using the Illumina-curated version of May 2013 Greengenes taxonomic database in parallel with the Ribosomal Database Project for taxonomic classification of constituent microbial populations.

2.3.3 Food preparation for chemical feeding assays

For the chemical feeding assays, each food was prepared by mixing stock chemicals into standard food (described previously). For chemical feeding assays, a

100x stock solution was prepared using an appropriate solvent. To make NaBu food and acetic acid food, the appropriate amount of chemical was mixed with melted food and thoroughly mixed by stirring. To make yeast containing food, dried yeast was mixed with water to make a mixture of toothpaste consistency. Yeast mixture was then put in a microwave to kill the live yeast. One third of the yeast paste was mixed with two third melted food and thoroughly mixed by stirring. Melted foods were then transferred to a vial to let it cool and solidify. To make “control food” for each experiment, the same amount of solvent was mixed with standard food. Each vial contained approximately 5 ml of food.

2.3.4 Dose response curve preparation

To determine the appropriate dose of each chemical used in the rescue experiments, a dose response cure was prepared using 6-7 different doses of each chemical. The concentrations used in this study were selected based on the available literature and a range was prepared to include predicted effective doses. To prepare the food with different concentrations, each chemical was diluted to their desired concentration in a beaker containing 10 ml of standard food with the addition of a blue marker dye. Each chemical containing food was mixed thoroughly for 30 sec by stirring and transferred into two vials. Control food vials were prepared by mixing the same amount of solvent to standard fly food. All the vials were cooled in the fume hood for approximately 1 hour to avoid any further condensation. Among the two sets of vials, one set was used immediately, and another set was wrapped with aluminum foil and kept at 4°C for later use.

To perform the dose-response test, 6 uninfected female and 6 uninfected male flies were put into the first set of treatment and control vials and kept for 6 days. At day 6, all the flies were transferred into the corresponding second set of vials and kept for

another 6 days. Adult mortality, egg lay, egg hatch and larval development were qualitatively scored across the 12-day period. All the measurements were scored by comparing each treatment vials with its corresponding control vial. If the treatment vials seemed similar to the treatment vials, it was designated as '+', vials were designated as 'some' if the treatment vials had some negative effect compared to control. If the treatment vials had consistent negative effect on the measurements, vials were designated as '-'. The highest concentration of chemical that had no adverse effect on flies were further selected for the feeding assays.

2.3.5 Rescue assay procedures

To determine the effect of desired chemical/pathway on the on the rescue of CI, chemicals were fed to the flies and their effects were observed in CI crosses. Virgin female *D. simulans* flies were collected and divided into two groups and kept in treatment and control food for 3 days. To avoid any chemical effect on male flies, all the male flies (both *w*Ri-infected and *w*Mel-transinfected) male flies were kept in standard fly food for three days. For the rescue crosses, corresponding *Wolbachia*-infected (*w*Ri or *w*Mel) virgin flies were kept in standard fly food for three days. A total of 15-20 flies were kept in each vial to avoid overcrowding. At day 3, both male and female flies were transferred to a new standard food vial for mating for 8 hrs. Depending on the experiment, flies were kept for single pair mating or mass mating. For single pair mating only, one male and one female fly were kept in each vial and their mating was confirmed visually. For mass mating ~30-40 flies (both male and female) were kept for mating for 8 hrs. After mating, male flies were discarded and female flies were transferred back to their corresponding treatment and control vial. At day 4, each of the female flies were transferred to individual vials with the addition of blue food coloring to the food, to improve egg visibility, and flies were allowed to lay eggs for 24 hrs. At day 5, flies were

discarded and the vials were incubated at 24°C for 24 hours to allow for egg hatch. At day 6, all the unhatched and hatched eggs were counted to determine the egg hatch rate. All the experiments were carried out in 3 separate biological replicates.

2.3.6 Statistical analysis

All data collected in this study were analyzed with appropriate statistical analyses, as per a standard decision tree (Fig 2.1). Chi Square tests of goodness of fit were performed manually as per standard procedures described previously (McDonald, 2014). For all the other tests the IBM SPSS v.23 analysis package was used (Field, 2013; Christensen et al., 2019). At first, the data were analyzed for normality using the Shapiro–Wilk test and for homogeneity of variance by Levene's test (Shapiro & Wilk, 1965; Lim & Loh, 1996; Razali & Wah, 2011). If the data was normally distributed, a t-test was used to evaluate the mean difference and if the variance was homogenous, a Welch's T-test was used if data variance was uneven (McDonald, 2014; Rietveld & van Hout, 2015). If the data is not normally distributed, the Mann-Whitney U test was performed (Rietveld & van Hout, 2015; Vargha & Delaney, 1998) for data showing homogeneity of variance, and when variance was uneven, an independent T-test with bootstrapping was performed (De Cuevas & Spradling, 1998; Lim & Loh, 1996; Rietveld & van Hout, 2015; Shapiro & Wilk, 1965; Wasserman, 1994).

2.4 Results

2.4.1 Verification of *D. simulans* endosymbiont identities by 16S analysis

The *Drosophila simulans* flies used in this study were either naturally infected with *Wolbachia w*Ri or transinfected with *w*Mel *Wolbachia* strain (Hoffmann et al., 1986; Poinsot et al., 1998). These flies have previously been shown to carry nucleoids in their germline cells by DNA staining which were consistent with *Wolbachia* infection (Serbus & Sullivan, 2007; Christensen et al., 2016). These fly lines were also confirmed as PCR

positive with *Wolbachia* surface protein (*wsp*) gene and the type of infection was also confirmed by sequencing (Christensen et al., 2016). Although these detection methods were consistent with the presence of *Wolbachia*, they do not rule out the presence of other bacterial infections.

To identify the bacteria carried by the germline tissue of *Wolbachia*-infected flies, 16S rRNA microbiome analyses were carried out. Ovary tissue samples were analyzed from both *Wolbachia* uninfected and infected *D. simulans* lines. The data indicate that *Wolbachia* spp. were the predominant taxon carried by both *w*Ri and *w*Mel-infected tissues, with 94.5–98.3% of the reads representing *Wolbachia* spp (Figure 2.3, Supplementary table 2.1). Other non-*Wolbachia* bacterial species found in the *Wolbachia*-infected samples were similar to the bacterial species found in uninfected control. As this experiment was a non-sterile assay, the presence of non-*Wolbachia* bacterial species can be contributed by contaminant from the cuticle, body cavity and residual contamination of dissection equipment. Importantly, this assay indicate the absence of any other potential *Drosophila* endosymbionts, such as *Spiroplasma* spp., which are also maternally transmitted. Thus, *Wolbachia* represented the majority of the endosymbionts present in *Wolbachia*-infected ovary samples.

2.4.2 Confirmation of baseline CI and Rescue phenotypes in a lab setting

Previous studies have shown that both naturally infected *Wolbachia* *w*Ri and transinfected *w*Mel induce a strong CI phenotype in *D. simulans* (Hoffmann et al., 1986; Poinsot et al., 1998). To determine the status of CI and rescue phenotypes in our lab setting, the egg hatch rate was determined with both CI (infected male and uninfected female) and rescue (infected male and female) crosses along with the control cross (uninfected male and female). In this experiment, matings were carried out in groups and egg hatch rates were determined from individual females. In *w*Ri-infected *D. simulans,*

the egg hatch rate from control crosses (89%) was similar to the hatch rate in rescue crosses (92%) (p-value > 0.05) (Table 2.1). Similarly, egg hatch rates in *w*Mel-infected *D. simulans* were high in both control (90%) and rescue crosses (79%) (p-value > 0.05). Egg hatch rates from CI crosses were considerably low in CI crosses in both *w*Ri-infected (10%) and *w*Mel-infected (4%) *D. simulans* flies (p-value < 0.05). This data indicates strong CI and rescue phenotypes with *D. simulans* flies in a lab setting.

2.4.3 Low hatch rate in CI crosses was not caused by a failure of mating

Wolbachia infection has been found to alter the release and perception of pheromones in *Drosophila* spp., altering mating behavior. Therefore, it is possible that the low egg hatch rate in CI crosses could be caused by a failure of mating other than true embryonic lethality. To rule out the possibility that low egg hatch rates in CI crosses might be caused by a failure in mating, we carried out the CI crosses with an additional, physical confirmation of mating. In this experiment *w*Ri-infected *D. simulans* flies were used and matings were carried out using single pairs, where mating was visually confirmed. Egg hatch rates from mating-confirmed females were 90% for the control crosses and 10% for CI crosses (Table 2.2), similar to the results seen previously in a group mating vials. These data indicate that the low egg hatch rate in CI crosses were not a result of a failure of mating, but likely resulted from true embryonic lethality.

2.4.4 Modified microbiome in food doesn't affect the egg hatch rate in CI crosses

The gut microbiome of *Drosophila* in lab-reared flies can be significantly different from naturally occurring flies of the same species (Brown et al., 2021; Elgart et al., 2016). Changes in the gut microbiome in *Drosophila* have been found to effect development, behavior, life span, and disease resistance of the host (Ludington & Ja, 2020). The microbiome from wild-type *Drosophila* have been found to alter the color of standard fly food from yellow to brown. To determine whether an altered food

microbiome can affect the egg hatch rate, CI crosses were carried out with *w*Mel-transinfected *Drosophila simulans* flies grown in standard fly food and fly food with an altered food microbiome. In this experiment, matings were carried out in groups and egg hatch rates were determined for each flies. The egg hatch rates were similar for standard food (8%) and altered biome containing food (3%) (p-value >0.05) (Table 2.3). This data indicate that the modification of the food microbiome was unable to affect the egg hatch rate in *w*Mel-induced CI in *D. simulans*.

2.4.5 Insulin signaling pathways are unable to induce rescue of CI

Insulin/IGF-like signaling is ubiquitous in multicellular organisms. In *Drosophila*, insulin signaling can be involved in growth, development, metabolic homeostasis, adult lifespan, resistance to stress and fecundity (Ikeya et al., 2009). *Wolbachia* have previously found to increase insulin/IGF-like signaling in *Drosophila*. To determine whether *Wolbachia* induce rescue of CI by inducing insulin signaling, egg hatch rates were determined in CI crosses with increased insulin signaling and compared with control crosses. Feeding yeast to *Drosophila* have been reported to induce insulin signaling (Serbus et al., 2015). In this experiment, we fed yeast-enriched food to female *D. simulans* flies and egg hatch rates were compared with CI crosses where the females had been fed standard fly food. For this experiment both *w*Ri and *w*Mel-infected *D. simulans* flies were used to induce CI, where crosses were carried out in group matings. Data indicate that egg hatch rates in yeast enriched food (7%) were similar to the egg hatch rate in control food (9%) with *w*Ri-induced CI crosses (p-value >0.05) (Table 2.4). Also, egg hatch rates in yeast enriched food (10%) were similar to the egg hatch rate in control food (12%) with *w*Mel-induced CI crosses (p-value >0.05). These results indicated that *Wolbachia*-induced rescue of CI was not achieved by inducing insulin/IGF-like signaling pathways in *Drosophila simulans* flies.

2.4.6 Acetic acid exhibits variable effects on rescue of CI

Laboratory stocks of *Drosophila*, which carry a range of prokaryotic gut microbes, have been predominately colonized by *Lactobacillus* and *Acetobaceter* species. These bacteria have been implicated in the regulation of growth, immunity, nutritional regulation, mating preference and lifespan (Elgart et al., 2016). Among them, *Acetobacter* spp. are important for fecundity and oogenesis. *Acetobacter* spp. primarily colonize the gut and assist in the breakdown of sugar from food to produce acetic acid. To determine whether the gut microbiome, particularly *Acetobacter* spp., could be involved in rescue of CI by producing acetic acid, we tested the ability of acetic acid to induce rescue in *Drosophila*.

Before proceeding to the experiment, it was important to determine the amount of acetic acid which can be used effectively without harming the host. To determine the usable amount of acetic acid, we exposed 12 uninfected *D. simulans* flies to different concentrations of acetic acid, monitoring detrimental effects in a survival curve assay. We exposed flies to acetic acid concentrations ranging from 400mM to 50mM and observed any abnormalities in their lifespan, egg production, hatch rate and larval development. The highest dose that had no adverse effect based on these criteria was selected as safe to proceed to assess its effects on CI/rescue. Data from the survival curve assay suggest that acetic acid concentrations as high as 400 mM cause abnormalities in egg production, egg hatch rate and larval development when compared with control food (Table 2.5a). No discernible defects were observed with flies kept on 200 mM acetic acid concentrations or lower, indicating that 200 mM acetic acid was the highest concentration that could be used in feeding assays of *D. simulans* flies.

To determine whether *Wolbachia* induce rescue of CI by via a pathway involving gut-associated acetic acid., acetic acid was fed to *Drosophila simulans* flies and egg hatch rates were determined in CI crosses induced by both *w*Ri and *w*Mel *Wolbachia* strains. Data from the experiment showed that egg hatch rate was 5% with acetic acid fed flies which was similar (p-value > 0.05) to the hatch rate found in control crosses (3%) (Table 2.5b) in *w*Ri-induced CI crosses. Conversely, hatch rate from *w*Mel-induced CI crosses showed 16% for acetic acid-fed flies which was significantly higher (p-value < 0.05) from the hatch rate from control cross (8%). This data indicated that acetic acid has significant rescue effect on *wMel*-induced CI, though acetic acid has no effect on *w*Ri-induced CI crosses. This also suggests that the rescue of CI might be achieved by more than one single pathway and that different *Wolbachia* variants might use different pathways to induce rescue of CI.

2.4.7 Use of NaBu to induce rescue of CI

While the underlying cause of CI is still debated, CI embryos have previously been shown to exhibit a defect in histone deposition in the paternal DNA during meiotic segregation (Landmann et al., 2009). During this process, histone acetyl transferase (HAT) enzymes transfer acetyl groups to specific histones that lower the affinity of those histones to DNA and loosen the structure of chromatin. Histone de-acetyl transferases (HDACs) reverse the effect of HAT enzymes. Several, now well-documented, HDAC inhibitors can inhibit HDAC enzymes and effectively keep the chromatin structure loose. Sodium butyrate (NaBu) is a known HDAC inhibitor and has been used in many studies (Candido et al., 1978; Boffa et al., 1978; Kruh 1981). To implicate whether chromatin remodeling is involved in *Wolbachia*-mediated rescue of CI, we fed *D. simulans* flies with NaBu, to determine any their ability to induce the rescue effect in CI crosses. As before with acetic acid, it was important to know the limit of NaBu concentrations that can be

safely fed to *D. simulans* flies without adversely affecting development. To determine this, we used a survival curve assay of different concentrations of NaBu, mixed in standard fly food, ranging from 200mM to 1mM for the ability to induce negative effects on survival, egg production, egg hatch rate and larval development. Data from the survival curve assay indicate that 100 mM and 200 mM NaBu had some adverse effects of egg production, egg hatch and larval development, while 50 mM NaBu has no adverse effect on uninfected *D. simulans* flies (Table 2.6a) and thus was chosen for further feeding assays.

To understand whether NaBu can induce any rescue effect on CI, we fed 50 mM NaBu to *D. simulans* flies and tested their ability to induce rescue effect in CI crosses, with both naturally *w*Ri-infected and *w*Mel-transinfected flies. For this experiment, crosses were carried out in the group mating format. Data from the *w*Ri-induced CI crosses indicate that egg hatch rates from NaBu fed flies (16%) were significantly higher than the control (10%) (p-value <0.05) (Table 2.6b). Similarly, for *w*Mel-induced CI crosses, egg hatch rates for NaBu fed flies were significantly higher (11%) than the control (4%) (p-value <0.05). This data suggests that the HDAC inhibitor NaBu was able to induce partial rescue of CI in *D. simulans* flies.

2.4.8 Sodium Butyrate did not influence the mating behavior of *D. simulans*

In the previous experiment we found that sodium butyrate (NaBu) was able to induce a partial rescue effect on CI crosses. To rule out the possibility that NaBu induced this rescue effect by limiting or modifying the mating behavior of *D. simulans* flies, the CI assay was carried out using visual confirmation in single pair matings and, subsequently, egg hatch rates were compared. In standard fly food, the egg hatch rate in CI crosses were characteristically low (10%) and egg hatch rate in rescue crosses were high (94%) for flies with single pair mating, as found previously for group mating. The

egg hatch rate for CI crosses where females were fed NaBu containing food was 19%, which was significantly higher than the hatch rate under control food conditions (p-value < 0.05) (Table 2.6c). The data from these mating-confirmed crosses suggests that NaBu has no effect on the mating behavior of *D. simulans* flies, and NaBu is able to induce a partial rescue effect in *D. simulans* flies.

2.5 Discussion

The overall goal of this chapter was to ascertain the potential for of chemical feedings to modify the outcome of *Wolbachia*-induced CI. Our approach was first to set up a CI model in a lab-based environment, and confirm CI strength, which can vary depending on many biotic and abiotic factors. For this study, we have used *w*Ri and *w*Mel-infected *Drosophila simulans* as the CI model, since both naturally *w*Ri-infected and *w*Mel-transinfected *D. simulans* exert strong CI. We were unable to use the *Drosophila melanogaster* model organism because, as a host, it exhibits weak CI. Both *w*Ri and *w*Mel-infected *D. simulans* showed strong CI in our lab-based experimental design, with low egg hatch rates in CI crosses and higher egg hatch rate in rescue crosses. In our study, we also confirmed low egg hatch rates in CI crosses with visual confirmation of mating. This finding suggests that the low egg hatch rate observed in CI crosses was not due to a failure of mating but indicative of true embryonic lethality.

One of the limitations of using *D. simulans* is not being able to use the diverse array of genetic tools available in *D. melanogaster*, for which there exist many lines with modifications to specific cellular pathways. For this reason, a chemical feeding approach was used in this study. Although the dosing of each chemical was standardized before each feeding experiment, these whole-body feedings lack stage- and tissue-specific responses. As this may be an important feature of *Wolbachia* infections, it could account for observing a partial rescue effect with chemical feedings, where the egg hatch rates in

Wolbachia-infected rescue crosses were ~90%. Another drawback of this study was the possibility of side effects in the host due to chemical feedings. Although we tried to limit extreme effects on development by controlling dosages in survival curve assays, it is possible that chemical dosages weren't strong enough to induce the full potential in the host, particularly in specific tissues such as the ovaries.

Our feeding assay technique creates the opportunity to test microbiome effects on CI. Specific studies have not yet been undertaken to address how the host or food microbiomes affect *Wolbachia*-induced rescue. Perhaps the strongest candidate for a secreted molecule involved in the CI and rescue process is the *Cif* proteins. Our findings do not attempt to explain how the chemicals used in our feeding trials interact with these *Cif* proteins. Although it has not yet determined to what extent *Cif* proteins interact with host factors to induce CI and rescue, it might be possible that acetic acid and NaBu interact with Cifs and/or host factors, either directly or indirectly, to induce partial rescue. It is important to consider all of this when designing follow-up experiments in association with chemical and/or altered microbiome conditions.

The gut microbiome has been found to affect many essential biological processes such as development, behavior, life span, and disease resistance in *Drosophila* (Ludington & Ja, 2020). In our study, we observed no rescue effect associated with altered food microbiomes from wild flies versus our lab-grown flies. Although insulin signaling is involved in many biological processes including fecundity, no rescue effect was observed in response to diets known that reliably induce insulin signaling. Acetic acid feeding results varied between the *w*Ri and *w*Mel-induced CI models in *D. simulans*, with no change was observed for *w*Ri-induced CI despite significant rescue effects seen for *w*Mel-induced CI in *D. simulans*.

One possible explanation for this difference could be that *Wolbachia* strains use different pathways to induce CI and rescue in the host. Another possibility is that although the most reasonable concentration of acetic acid was selected via a survival curve assay, the concentration of might not be strong enough to elicit an effect on *w*Ri-induced CI. Interestingly, sodium butyrate (NaBu) was able to induce partial rescue on both *w*Ri and *w*Mel-mediated CI in *D. simulans*. Sodium butyrate is a short chain fatty acid that can influence many biological processes including altering chromatin condensation, delaying the cell cycle and inducing DNA damage repair. Thus, it might be postulated that one or more pathways influenced by sodium butyrate could be involved in the rescue process. It is also possible that NaBu triggers core pathways involved in both *w*Ri- and *w*Mel-induced rescue of CI. As a matter of formality, our study also indicates that NaBu doesn't induce the rescue effect by influencing the mating behavior of *D. simulans* flies.

To summarize, in this chapter we demonstrate that the CI and rescue phenotypes are reproducible in a lab setting. The low hatch rates in CI crosses did not result from a failure of mating but indicate true embryonic lethality. The chemical feeding methodology presented here was able to observe at least partial induction of rescue of CI. As chemical feeding can be lethal, it was a prerequisite to determine the effective dose in these feeding assays to minimize deleterious or adverse effects. Although our study recapitulated a partial rescue effect, whereas *Wolbachia* induce nearly 100% rescue, the rescue effect observed here with chemical feeding is informative, considering it enables observation of the rescue effect artificially. Presumably by affecting sensitive biological pathways, modifying such pathways in the host can help to identify the specific pathways involved in overall rescue process. The chemical feeding

technique presented here can also be used to investigate CI and rescue in other non-*Wolbachia* models such as *Cardinium* spp.

Figures and Tables

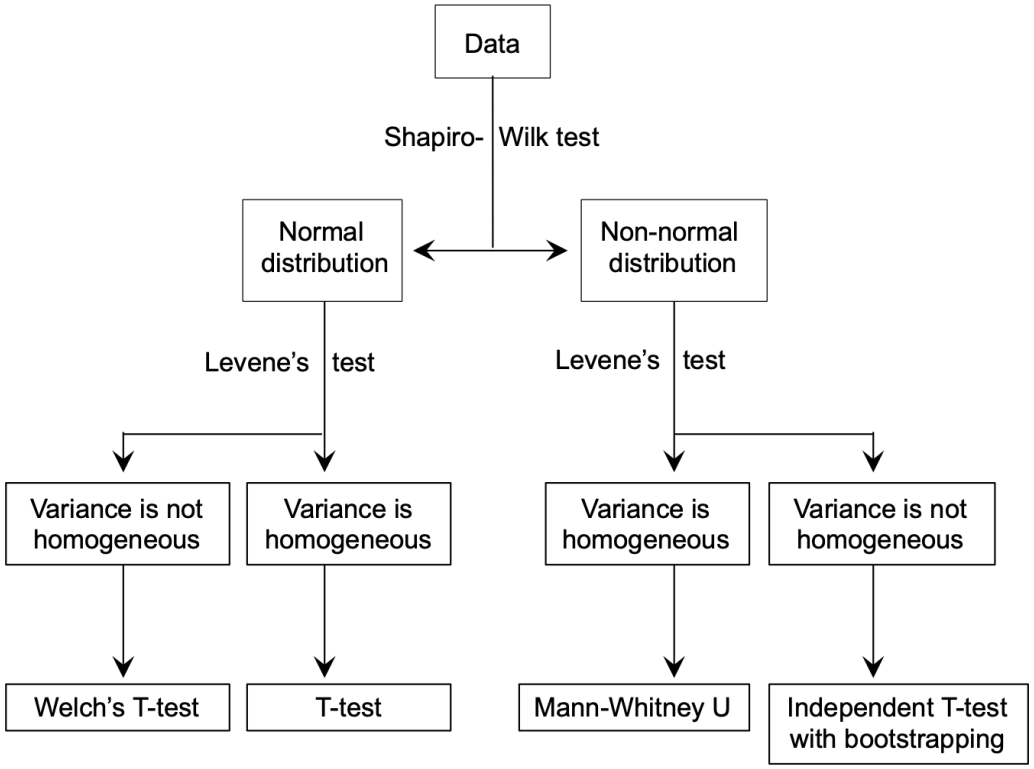

Fig. 2.1. Selection of statistical methods for pairwise data comparisons. Data normality was assessed by the Shapiro-Wilk test, and homogeneity of variance was assessed by Levene's test. Statistical analyses were subsequently performed were directed by the outcome of those tests as outlined here.

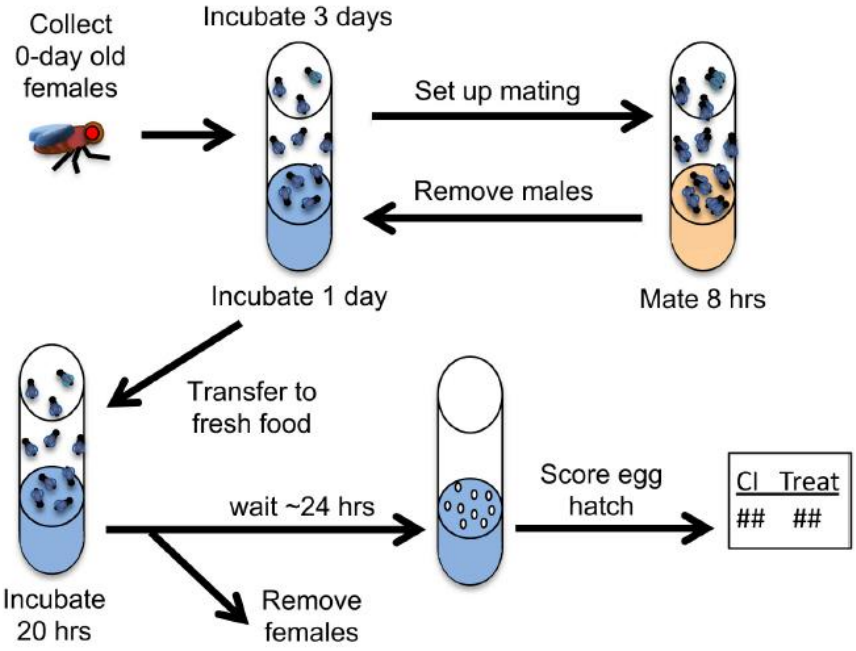

Fig. 2.2. Method used to assess CI and rescue for *D. simulans*. Blue food was used for improved visibility for eggs. See Methods for further details.

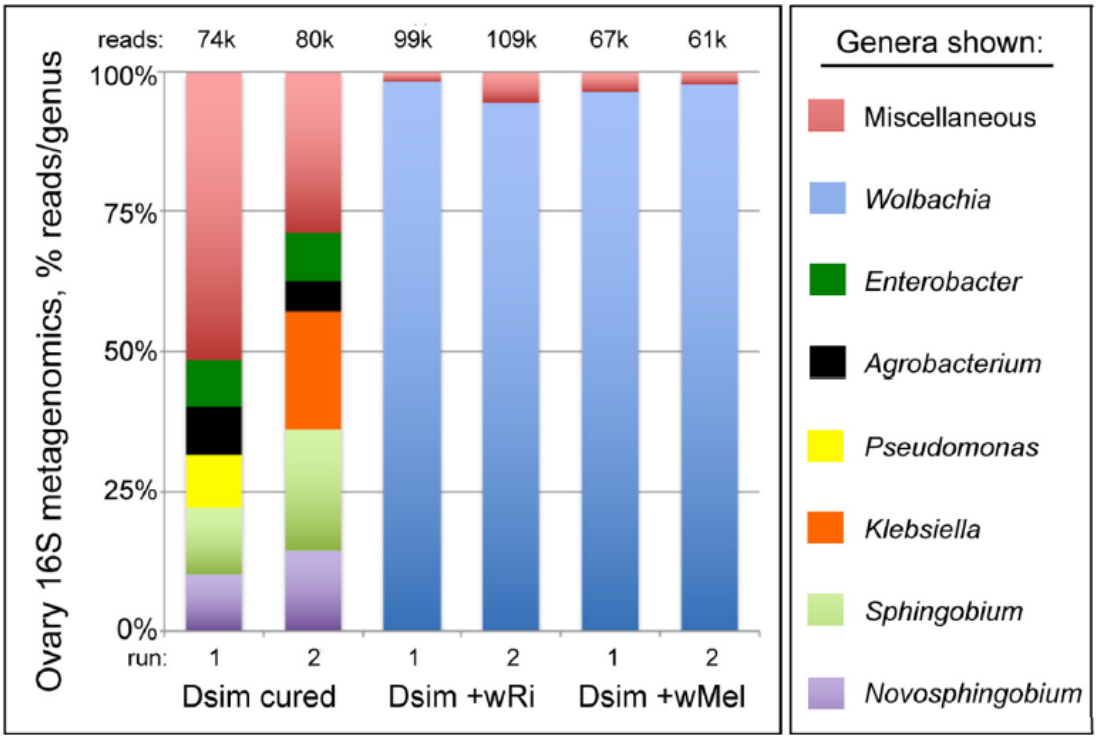

Figure. 2.3. Result from 16S rRNA microbiome profiles associated with *Drosophila simulans* ovary tissues. Both uninfected and *Wolbachia*-infected tissues are shown. Top five most abundant genera that equal or exceed 1% abundance per sample are shown. For further details, see Supplementary Table S1.

*Dsim w*Ri	♀♂	♀♂	♀♂
	Uninfected	CI	Rescue
Hatch rate	89%	10%	92
Total # eggs	606	712	575
Total # females	31	41	35

p- value < 0.05

*Dsim w*Mel	♀♂	♀♂	♀♂
	Uninfected	CI	Rescue
Hatch rate	90%	4%	79%
Total # eggs	561	546	820
Total # females	41	38	42

p- value < 0.05

Table. 2.1. Egg hatch rate in uninfected, CI and rescue crosses in *Drosophila simulans* by native *w*Ri and transinfected *w*Mel-induced CI and rescue. Egg hatch rates are consistently low in CI crosses as compared to the other types of crosses.

*Dsim w*Ri mating confirmed	♀♂ Uninfected	♀● CI
Hatch rate	90%	10%
Total # eggs	679	672
Total # females	35	43

p- value < 0.05

Table. 2.2. Egg hatch rates were still low in CI crosses compared to control in mating confirmed flies. Thus, the low hatch rate in CI crosses was not caused by a failure of mating.

*Dsim w*Mel CI outcomes	♀♂ Regular biome	♀♂ Altered biome
Hatch rate	8%	3%
Total # eggs	478	554
Total # females	26	30

p- value > 0.05

Table. 2.3. Data showing the egg hatch rate in CI crosses on normal microbiome and altered microbiome containing food. The egg hatch rate was not significantly different between food vial conditions.

*Dsim w*Ri CI outcomes	♀ ♂ Control	♀ ♂ Yeast
Hatch rate	9%	7%
Total # eggs	605	1734
Total # females	43	49

p- value > 0.05

*Dsim w*Mel CI outcomes	♀ ♂ Control	♀ ♂ Yeast
Hatch rate	12%	10%
Total # eggs	881	2566
Total # females	64	64

p- value > 0.05

Table. 2.4. Data showing the effect of nutrient-altered diets on CI crosses. Yeast feeding was previously shown to induce insulin signaling in *Drosophila*. Yeast-rich diets did not induce rescue of either *w*Ri- or *w*Mel-induced CI in *D. simulans*.

Treatment tested	Dose	Fecundity	Hatchablility	Larval development
Acetic Acid	400mM	some	some	some
	200 mM	**(+)**	**(+)**	**(+)**
	100 mM	(+)	(+)	(+)
	50 mM	(+)	(+)	(+)
	Control (water)	(+)	(+)	(+)

Table. 2.5a. Data from survival curve assay with Acetic acid. Different concentrations of acetic acid were fed to *D. simulans* flies and observed for 12 days. “some” denotes some adverse effect, “+” denotes similar to control. 400mM of acetic acid had some adverse effect on flies, 200 mM of acetic acid were used for the feeding assays.

*Dsim w*Ri CI outcomes	♀ ●	♀ ♂
	Control	Acetic Acid
Hatch rate	3%	5%
Total # eggs	465	305
Total # females	34	25

p- value > 0.05

*Dsim w*Mel CI outcomes	♀ ●	♀ ♂
	Control	Acetic Acid
Hatch rate	8%	16%
Total # eggs	822	685
Total # females	59	50

p- value < 0.05

Table. 2.5b. Effect of Acetic acid feeding on CI hatch rates. The data showed acetic acid had no significant effect on *w*Ri-induced CI. But acetic acid was able to induce significant rescue effect on *w*Mel-induced CI in *D. simulans*.

Treatment tested	Dose	Fecundity	Hatchablility	Larval development
NaBu	200 mM	some	some	some
	100 mM	(+)	some	some
	50 mM	**(+)**	**(+)**	**(+)**
	5 mM	(+)	(+)	(+)
	1 mM	(+)	(+)	(+)
	Control (water)	(+)	(+)	(+)

Table. 2.6a. Data from survival curve assay with sodium butyrate (NaBu). Different concentrations of NaBu were fed to *D. simulans* flies and observed for 12 days. “some” denotes some adverse effect, “+” denotes similar to control. 200mM and 100mM of NaBu had some adverse effect on flies, 50 mM of NaBu was used for the feeding assays.

*Dsim w*Ri CI outcomes	♀ ♂ Control	♀ ♂ NaBu
Hatch rate	10%	16%
Total # eggs	712	292
Total # females	41	21

p- value < 0.05

*Dsim w*Mel CI outcomes	♀ ♂ Control	♀ ♂ NaBu
Hatch rate	4%	11%
Total # eggs	545	544
Total # females	38	40

p- value < 0.05

Table. 2.6b. Effect of sodium butyrate (NaBu) feeding on CI. The data showed NaBu had significant rescue effect on *w*Ri- and *w*Mel-induced CI in *D. simulans*.

*Dsim w*Ri mating confirmed CI outcome	♀ ♂ CI Ctl food	♀ ♂ CI NaBu food	♀ ♂ Rescue Ctl food
Hatch rate	10%	19%	94%
Total # eggs	711	724	741
Total # females	30	32	30

p- value < 0.05

Table. 2.6c. Effect of sodium butyrate (NaBu) feeding on CI with a confirmation of mating. The data showed NaBu had significant rescue effect on *w*Ri-induced CI as observed previously. This data suggest that NaBu has no effect on the mating behavior of *D. simulans* flies

D. simulans cured				*D. simulans* + *w*Ri				*D. simulans* + *w*Mel			
Run 1 (73,944 total reads)		Run 2 (79,560 total reads*)		Run 1 (99,472 total reads)		Run 2 (109.071 total reads)		Run 1 (67,279 total reads)		Run 2 (60,883 total reads)	
Genus	%	Genus	%	Genus	%	Genus	%	Genus	%	Genus	%
Sphingobium	12.01	*Sphingobium*	21.63	*Wolbachia*	98.27	*Wolbachia*	94.50	*Wolbachia*	96.39	*Wolbachia*	97.68
Novosphingobiu	10.14	*Klebsiella*	20.93	*Desulfofrigus*	0.37	*Sphingobium*	0.86	*Desulfofrigus*	0.68	*Desulfofrigus*	0.51
Pseudomonas	9.51	*Novosphingobiun*	14.60	*Neorickettsia*	0.25	*Neorickettsia*	0.57	*Calothrix*	0.49	*Ehrlichia*	0.27
Agrobacterium	8.53	*Enterobacter*	8.66	*Bacteroides*	0.10	*Desulfofrigus*	0.49	*Ehrlichia*	0.30	*Neorickettsia*	0.21
Enterobacter	8.08	*Agrobacterium*	5.28	*Ehrlichia*	0.09	*Novosphingobium*	0.34	*Neorickettsia*	0.23	*Calothrix*	0.08
Total reads > 1%	83.29	Total reads > 1%	85.31	Total reads > 1%	98.27	Total reads > 1%	94.50	Total reads > 1%	96.39	Total reads > 1%	97.68
Total reads < 1%	16.71	Total reads < 1%	14.69	Total reads < 1%	1.73	Total reads < 1%	5.50	Total reads < 1%	3.61	Total reads < 1%	2.32

% = percentage of total bacterial reads
* = extra amplification was required in this run for detection

Table S1. Abundance of 16S metagenomic reads from *D. simulans* ovary samples.

References

Beckmann, J. F., Ronau, J. A., & Hochstrasser, M. (2017). A *Wolbachia* deubiquitylating enzyme induces cytoplasmic incompatibility. *Nature Microbiology*, *2*(5), 1–7.

Boffa, L. C., Vidali, G., Mann, R. S., & Allfrey, V. G. (1978). Suppression of histone deacetylation in vivo and in vitro by sodium butyrate. *Journal of Biological Chemistry*, *253*(10), 3364–3366.

Brown, J. J., Jandová, A., Jeffs, C. T., Higgie, M., Nováková, E., Lewis, O. T., & Hrček, J. (2021). Microbiome structure of a wild *Drosophila* community along tropical elevational gradients and comparison to laboratory lines. *BioRxiv*.

Camacho, M., Oliva, M., & Serbus, L. R. (2017). Dietary saccharides and sweet tastants have differential effects on colonization of *Drosophila* oocytes by *Wolbachia* endosymbionts. *Biology Open*, *6*(7), 1074–1083.

Candido, E. P. M., Reeves, R., & Davie, J. R. (1978). Sodium butyrate inhibits histone deacetylation in cultured cells. *Cell*, *14*(1), 105–113.

Christensen, S., Camacho, M., Sharmin, Z., Momtaz, A. J. M., Perez, L., Navarro, G., … Serbus, L. R. (2019). Quantitative methods for assessing local and bodywide contributions to *Wolbachia* titer in maternal germline cells of *Drosophila*. *BMC Microbiology*, *19*(1), 1–17.

Christensen, S., Pérez Dulzaides, R., Hedrick, V. E., Momtaz, A. J. M. Z., Nakayasu, E. S., Paul, L. N., & Serbus, L. R. (2016). *Wolbachia* endosymbionts modify *Drosophila* ovary protein levels in a context-dependent manner. *Applied and Environmental Microbiology*, *82*(17), 5354–5363.

De Cuevas, M., & Spradling, A. C. (1998). Morphogenesis of the *Drosophila* fusome and its implications for oocyte specification. *Development*, *125*(15), 2781–2789.

Elgart, M., Stern, S., Salton, O., Gnainsky, Y., Heifetz, Y., & Soen, Y. (2016). Impact of gut microbiota on the fly's germ line. *Nature Communications*, *7*(1), 1–11.

Field, A. (2013). *Discovering statistics using IBM SPSS statistics*. sage.

Hoffmann, A. A., Turelli, M., & Simmons, G. M. (1986). Unidirectional incompatibility between populations of *Drosophila* simulans. *Evolution*, *40*(4), 692–701.

Hosokawa, T., & Fukatsu, T. (2020). Relevance of microbial symbiosis to insect behavior. *Current Opinion in Insect Science*, *39*, 91–100.

Ikeya, T., Broughton, S., Alic, N., Grandison, R., & Partridge, L. (2009). The endosymbiont *Wolbachia* increases insulin/IGF-like signalling in *Drosophila*. *Proceedings of the Royal Society B: Biological Sciences*, *276*(1674), 3799–3807.

Kikuchi, Y. (2009). Endosymbiotic bacteria in insects: their diversity and culturability. *Microbes and Environments*, 908180109.

Kruh, J. (1981). Effects of sodium butyrate, a new pharmacological agent, on cells in culture. *Molecular and Cellular Biochemistry*, *42*(2), 65–82.

Landmann, F. (2019). The *Wolbachia* endosymbionts. *Microbiology Spectrum*, *7*(2), 2–7.

Landmann, F., Orsi, G. A., Loppin, B., & Sullivan, W. (2009). *Wolbachia*-mediated cytoplasmic incompatibility is associated with impaired histone deposition in the male pronucleus. *PLoS Pathogens*, *5*(3), e1000343.

Lassy, C. W., & Karr, T. L. (1996). Cytological analysis of fertilization and early embryonic development in incompatible crosses of *Drosophila* simulans. *Mechanisms of Development*, *57*(1), 47–58.

LePage, D. P., Metcalf, J. A., Bordenstein, S. R., On, J., Perlmutter, J. I., Shropshire, J. D., … Bordenstein, S. R. (2017). Prophage WO genes recapitulate and enhance *Wolbachia*-induced cytoplasmic incompatibility. *Nature*, *543*(7644), 243–247.

Lim, T.-S., & Loh, W.-Y. (1996). A comparison of tests of equality of variances. *Computational Statistics & Data Analysis*, *22*(3), 287–301.

Ludington, W. B., & Ja, W. W. (2020). *Drosophila* as a model for the gut microbiome. *PLoS Pathogens*, *16*(4), e1008398.

McDonald, R. P. (2014). *Factor analysis and related methods*. Psychology Press.

Poinsot, D., Bourtzis, K., Markakis, G., Savakis, C., & Merçot, H. (1998). *Wolbachia* transfer from *Drosophila* melanogaster into *D. simulans*: host effect and cytoplasmic incompatibility relationships. *Genetics*, *150*(1), 227–237.

Razali, N. M., & Wah, Y. B. (2011). Power comparisons of shapiro-wilk, kolmogorov-smirnov, lilliefors and anderson-darling tests. *Journal of Statistical Modeling and Analytics*, *2*(1), 21–33.

Rietveld, T., & van Hout, R. (2015). The t test and beyond: Recommendations for testing the central tendencies of two independent samples in research on speech, language and hearing pathology. *Journal of Communication Disorders*, *58*, 158–168.

Sazama, E. J., Bosch, M. J., Shouldis, C. S., Ouellette, S. P., & Wesner, J. S. (2017). Incidence of *Wolbachia* in aquatic insects. *Ecology and Evolution*, *7*(4), 1165–1169.

Serbus, L. R., & Sullivan, W. (2007). A cellular basis for *Wolbachia* recruitment to the host germline. *PLoS Pathogens*, *3*(12), e190.

Serbus, L. R., White, P. M., Silva, J. P., Rabe, A., Teixeira, L., Albertson, R., & Sullivan, W. (2015). The impact of host diet on *Wolbachia* titer in *Drosophila*. *PLoS Pathogens*, *11*(3), e1004777.

Shapiro, S. S., & Wilk, M. B. (1965). An analysis of variance test for normality (complete samples). *Biometrika*, *52*(3/4), 591–611.

Shropshire, J. D., On, J., Layton, E. M., Zhou, H., & Bordenstein, S. R. (2018). One prophage WO gene rescues cytoplasmic incompatibility in *Drosophila* melanogaster. *Proceedings of the National Academy of Sciences*, *115*(19), 4987–4991.

Tram, U., & Sullivan, W. (2002). Role of delayed nuclear envelope breakdown and mitosis in *Wolbachia*-induced cytoplasmic incompatibility. *Science*, *296*(5570), 1124–1126.

Vargha, A., & Delaney, H. D. (1998). The Kruskal-Wallis test and stochastic homogeneity. *Journal of Educational and Behavioral Statistics*, *23*(2), 170–192.

Wasserman, S. (1994). *Advances in social network analysis: Research in the social and behavioral sciences*. Sage.

Werren, J. H., Baldo, L., & Clark, M. E. (2008). *Wolbachia*: master manipulators of invertebrate biology. *Nature Reviews Microbiology*, *6*(10), 741–751.

Yamada, R., Floate, K. D., Riegler, M., & O'Neill, S. L. (2007). Male development time influences the strength of *Wolbachia*-induced cytoplasmic incompatibility expression in *Drosophila* melanogaster. *Genetics*, *177*(2), 801–808.

Yi, Y., Xu, W., Fan, Y., & Wang, H.-X. (2021). *Drosophila* as an emerging model organism for studies of food-derived antioxidants. *Food Research International*, 110307.

CHAPTER III: ESTABLISH A CHEMICAL ASSAY TECHNIQUE USING 24-WELL SCREENING PLATES

3.1 Abstract

Maternally inherited bacterial endosymbionts are extremely common in arthropods and induce a variety of phenotypes in their hosts, ranging from obligate nutritional mutualism to facultative reproductive parasitism. Bacterial endosymbionts of the genus *Wolbachia* are gram-negative endosymbiotic bacteria belonging to the class alphaproteobacteria, that infect a range of different hosts. *Wolbachia* are maternally transmitted and have the ability to modify host reproduction to support the growth of more infected females. Cytoplasmic incompatibility (CI) is the most successful reproductive modification caused by *Wolbachia,* where uninfected host females are incompatible with infected males and results nonviable progeny. However, *Wolbachia* infection in females is able to reverse or 'rescue' CI, causing viable progeny in crosses between *Wolbachia*-infected females and *Wolbachia*-infected males. The present study is aimed at understanding the underlying mechanism of rescue. In this chapter, we probe the effects of different cellular pathways for their ability to induce the rescue effect by using different chemical inhibitors. We also design a 24-well plate-based screening technique to further analyze other chemical pathways. To verify the effectiveness of the 24-well plate screening technique, sodium butyrate (NaBu) was used, which was shown to induce the rescue effect in the previous chapter. Our design of the assay technique with 24-well screening plates was able to clearly distinguish both CI and rescue phenotypes, as well as distinguish statistically relevant chemical induced rescue effects on CI

crosses. Thus, this 24-well, plate-based screening assay technique can be used to further analyze other cellular pathways.

3.2 Introduction

Bacterial/insect symbiosis is widely abundant in nature and can act as a prime example of heritable symbiosis (Bennett & Moran, 2015). Occasionally, bacteria reside inside a particular organelles of the insect called ‘bacteriocytes’ (Braendle et al., 2003). Endosymbiotic bacteria can also live in different organs inside the host (Kikuchi, 2009). These bacteria can live inside the host tissues and propagate along with reproduction of the host. The most successful endosymbionts modify host reproduction for their own benefit. Arguably one of the most successful are *Wolbachia,* gram-negative alpha-proteobacteria widely abundant in nature. *Wolbachia* are obligate, intracellular endosymbionts carried by 52%-60% of terrestrial arthropods (Sazama, et al., 2017).

Wolbachia display a wide range of symbiotic associations in arthropods, which can range from parasitism to mutualism (López-Madrigal & Duarte, 2019), though they are commonly referred to as reproductive parasites (Charlat et al., 2003). Since *Wolbachia* are maternally inherited, they spread from the mother to offspring. Thus, it is in the interest of the bacteria to favor infected females. *Wolbachia* achieve this by successful modification of host reproduction. The four reproductive modifications carried out by *Wolbachia* are feminization, parthenogenesis, male killing and cytoplasmic incompatibility (CI) all of which favor the spread of *Wolbachia*-infected females. Cytoplasmic incompatibility occurs when *Wolbachia* uninfected female is incompatible with *Wolbachia*-infected male, causing lethality of these progeny. However, *Wolbachia*-infected females can produce viable

progeny in crosses with both *Wolbachia*-infected and uninfected females, the former of which is considered “rescue”.

Researchers have been trying to understand the underlying mechanism of CI and rescue for a while. Some recent findings have given some idea on the mechanism of CI. These studies tried to address the defect in CI embryos. According to Lassy and Karr, CI is caused by a defect in the first mitotic division where one set of chromosomes stuck at metaphase plate and cause an uneven mitotic division (Lassy & Karr, 1996). A later study demonstrated that the mitotic defect is resulted by a timing mismatch between male and female pronuclei (Tram & Sullivan, 2002). According to study by Landmann, it is the paternal DNA that is defected in CI embryos, the paternal DNA showed defect in histone 3.3 deposition and delay in activation of proliferating cell nuclear antigen (PCNA) which means the male chromatin is still replicating where the maternal chromatin finished the replication (Landmann et al., 2009). All these defects accumulate to aneuploidy and cause embryonic lethality. However, embryos show no mitotic defects in rescue crosses, and the “rescued” progeny exhibit normal viability profiles. Apart from these cytological studies, some recent ransgenic expression studies have also given insight on the mechanism of CI (Beckmann et al., 2017 ; LePage et al., 2017; Shropshire et al., 2018). According to these studies, CI is caused by expression of two *Wolbachia* genes, *cif*A and *cif*B. Similarly, expression of the gene *cif*A can rescue the embryonic lethality in CI crosses (Beckmann et al., 2017 ; LePage et al., 2017; Shropshire et al., 2018).

Despite the findings about the mechanism of CI, the mechanism of how *Wolbachia* rescue the embryonic lethality in rescue crosses are still unknown. In this

study, we tried to understand the mechanism of rescue. In Chapter II, we tested some cellular pathways for their involvement in overall rescue mechanism. We designed an assay to inhibit/ influence the cellular pathways by feeding chemical and test their ability to induce a rescue effect. In our test we have found that sodium butyrate (NaBu), an HDAC inhibitor was able to induce a partial rescue effect on both *w*Ri and *w*Mel infect *Drosophila simulans* host. NaBu can be involved in a number of cellular mechanism and can modify the gene expression, modify the cell cycle and induce DNA damage repair (Kitzis et al., 1980; Coradini et.al., 2000; Smerdon et al., 1982). To determine the involvement of other cellular pathways in overall rescue process, the next step is to carry out a chemical screen, testing the contribution of candidate host pathways to the process of rescue. To do this, it is important to design an assay using a chemical screening format. The aim of this chapter to design/ replicate the assay from Chapter II using a 24-well tissue culture plate.

Overall, Chapter III expands on the discovery that chemical feeding can be used to mimic the rescue of embryonic lethality by CI, demonstrated in Chapter II. By standardizing and validating a plate-based screen of adult *Drosophila simulans* flies, this screening technique can also be used to implicate the involvement of cellular pathways in the rescue process, and for pathway determination in other studies using adult *Drosophila* models. Using *D. simulans* as a model system, we demonstrate optimization of sampling necessary to distinguish CI and rescue, as well as to observe any chemical effect on CI. The plate-based format was able to replicate the effect of NaBu in rescue of CI, demonstrating functionality of the setup. This optimized assay design will further be used in our study for screening chemicals for their ability to induce a rescue effect on CI crosses.

3.3 Methods and materials

3.3.1 Fly stocks & maintenance

The fly stocks used in this study were *Wolbachia w*Ri-infected and uninfected *Drosophila simulans*. The infected *Drosophila simulans* were originally collected in Watsonville, CA and it has been maintained under lab condition since then. The uninfected *D. simulans* line was created by curing infected line with tetracycline. Thus, all flies were from same genetic background.

All of the flies used in this study were maintained at 25°C using an Invictus *Drosophila* incubator (Genessee Scientific, USA). The flies were raised and maintained in standard minimal fly food in a 12h light/ dark cycle. The recipe of the food was derived from Bloomington *Drosophila* stock center (*http://flystocks.bio.indiana.edu/Fly_Work/media recipes/bloomfood.htm*) (Camacho et al., 2017; Christensen et al., 2016). All the flies were raised in 6oz square bottom polyethylene bottles. Each bottle contains approximately 25ml of standard fly food. To start a new bottle each bottle was seeded with approximately 80-100 flies including both male and female flies. Flies were kept there for 3-6 days and then transferred to a new bottle to seed. After 2-3 round of seeding the flies were discarded. The new progeny emerged after 12-13 days of seeding. To collect virgin flies each bottle was cleared and bottles were confirmed visually of absence of any remaining flies. The newly eclosed flies were collected after 5-8 hr of initial clearing and male and female flies were instantly separated to avoid any risk of unexpected mating. The fly collections were carried out with a standard CO_2 gas pad. After collection, approximately 45 flies (male or female) were kept in a vial temporarily until further use in an experiment.

3.3.2 Food preparation for chemical feeding assays

For the chemical feeding assay, the chemical containing food and control food were prepared in a large quantity and then distributed into the wells to keep the same concentration of chemical in all the wells. The food for chemical feeding assay were prepared by adding a 100X stock solution of each chemical into the standard fly food. 20ml of chemical containing food were prepared for each experiment. To prepare chemical containing food, fly food were melted to liquid and 20 ml of food were taken in a beaker. Appropriate amount of stock chemical was added into the food. A blue food color was also added to the food for easier visualization of fly eggs in the food. The melted food was mixed thoroughly for 30 seconds for proper mixing of chemicals. Along with the chemical containing food, a control food was also prepared at the same time by mixing same amount of solvent of the chemical to standard fly food.

3.3.3 Plate preparation for screening assay

For any experiment one pair of plate was prepared at a time. One plate was for incubation of the flies and the other plate was for egg laying. To prepare a 24-well screening plate, the wells were divided into two classes, the control food and the chemical containing food. In each 24-well plate, 8 wells contained the chemical food and 16 wells contained regular food. After preparing food (describe previously), 800ul of melted liquid food were poured into each well of 24-well plate. To avoid solidification, chemical containing food and control food were prepared separately. After preparing, the plates were kept in a fume hood for 1hr for cooling and solidification. After that one plate was used for and experiment and the other plate were wrapped with an aluminum foil and kept in 4°C for later use.

3.3.4 The CI suppression/ rescue induction test procedure in 24well plate format

To test whether modification of chemical pathways can induce suppression of CI or any rescue effect, chemical was tested in 24-well plate format too. At day 1, 10 virgin female flies were put into each well of chemical food containing wells, 10 uninfected virgin female flies were put into each of the control food containing wells as a control of CI cross. 10 virgin *Wolbachia*-infected flies were put into each well of control food for rescue cross. All the *Wolbachia*-infected male flies were kept in separate vials and kept for three days. Each female fly containing plates were covered carefully so, no fly can escape the well. The plate was incubated in 25C for 3days. At day 3 all the female flies transferred to a new plate containing control food and 10 *Wolbachia*-infected male flies were added to each well for mating for 8 hours. After mating, the male flies were discarded, and the female flies were transferred back to their original chemical food plate and their original wells. At day 4, all the female flies were transferred to the other 24well plate (prepared in day 1 and kept in 4C). Flies were kept in this plate for 24 hours for egg laying. At day 5, all the female flies were discarded, and plate was kept in incubation for 24 hr. After 24 hr, the number of hatched and unhatched egg were counted, and hatch rates were calculated at day 6.

3.3.5 Statistical Analysis

3.3.5.1 Compare the hatch rate data with control and treatment

All of the statistical tests were used in this study was carried out using The IBM SPSS v.23 analysis package (Field, 2013). All the hatch rate data collected in this study were matched with appropriate statistical analysis as per the standard

decision tree (Chapter II, Fig 2.1). All the data were analyzed for normal distribution using Shapiro-Wilk test and Levene's test was used to test for homogeneity variance (Lim & Loh, 1996; Razali & Wah, 2011; Shapiro & Wilk, 1965). For normal data showing equal variance, an Independent T-test was used. Welch's T-test were used for distribution with unequal variances (Vargha & Delaney, 1998; Wasserman, 1994). Non normal data with homogenous variances were compared using Mann-Whitney U test (Rietveld & van Hout, 2015; Vargha & Delaney, 1998). For non-normal data with unequal variances, a randomization based T-test with bootstrapping were used (De Cuevas & Spradling, 1998; Lim & Loh, 1996; Rietveld & van Hout, 2015; Shapiro & Wilk, 1965; Wasserman, 1994).

3.3.5.2 Determining the number of wells necessary to distinguish CI and rescue

To determine the suitable number of wells necessary to distinguish between CI and rescue, the egg hatch rates were randomly selected as 2 well, 4 well and 6 wells. A pairwise t-test were then performed, and the p-value were compared. Results represent 45-100 p-values per condition. For all the tests the IBM SPSS v.23 analysis package was used (Field, 2013; Christensen et al., 2019).

3.3.5.3 Determining the number of wells necessary to distinguish CI and rescue from experimental condition

To determine the number of wells necessary to distinguish the experimental condition from control CI and rescue condition a power analysis was carried out. Having collected 8 samples per subject group, power analysis was conducted to determine the smallest number of samples that would likely be needed to reveal a significant difference. To assess the power of different sample sizes, we used a program that used the bootstrap procedure programmed by Dr. Philip K. Stoddard in

MATLAB™ (Mathworks, Natick MA) that randomly sub-samples from the data to determine the sample size required to meet specified p-values. The program sub sample dataset from each condition and compare between the egg hatch rates in different condition. Sub-samples ranged from 2 to 16 data points, with 10,000 sample iterations per sample size. The significance was assessed in accordance with the normality of data being analyzed, using T-test and Mann-Whitney U test (Plonsky, 2015; Preacher & Hayes, 2004). The α-value was set at 0.01 and 0.05, two-tailed. A summary graphic for each analysis were presented by the program which indicates the proportion of significant results obtained for each sub-sample size.

3.4 Results

3.4.1 Use of plate-based screening assay to distinguish CI and rescue

To determine whether the plate base screening assay will be able to distinguish CI and rescue, here we have tested both CI and rescue in the plate-based assay format. Each of the CI and rescue crosses were tested in 8 wells of the 24-well plate (Fig 3.1). A total of three replicates were run, distinguishing between CI and rescue hatch rates with around 10% egg hatch rate for CI and 90% hatch rate for the rescue crosses (p-value <0.001) (Fig 3.2). These results suggest that it is possible to distinguish the CI and rescue phenotype using 24-well screening plate with the described method.

3.4.2 Determining how many wells are needed to distinguish CI and rescue

To determine how many wells in a 24-well plate are necessary to properly distinguish the CI and rescue conditions, we randomly sub-sampled the hatch rate data as 2-well, 4-well and 6-well subsets at a time. The data were randomly selected, compared pairwise, and their p-values analyzed. A total of three replicates were

carried out. With 2-wells, the p- value for CI verses rescue crosses ranged from <0.01 to 0.56. With 4-wells, the p-value for CI verses rescue data ranged from <0.01 to 0.16. And for 6-wells, the p-value for CI verses rescue data ranged from <0.01 to 0.03. These results suggested that a total of 6-wells was necessary to significantly distinguish between the CI and rescue crosses.

3.4.3 Determining the effect of Sodium Butyrate (NaBu) with 24-well screening plate

To determine whether the plate base screening assay will be able to distinguish the chemical feeding effect on rescue, NaBu was used as an example having previously shown a rescue effect on CI. In the 24-well screening plate, 8 wells were designated for NaBu, and 8 wells were designated for each of CI and rescue control (Fig 3.3). A total of 5 replicates were tested, using *w*Ri-infected *D. simulans* flies for the experiment. The egg hatch rate from NaBu food containing wells (20-30%) was significantly higher than from the CI control wells (11-14%) (p-value range <0.001 to 0.003) (Fig 3.4). These results suggest that the rescue effect of NaBu feeding could be recapitulated in the 24-well screening plate format, with the benefit of increasing the sample number of crosses from vials to a single plate replicates

3.4.4 Calculating the Z′ factors for CI+NaBu treatment

To further evaluate our plate-based egg hatch rate data a *Z′* factor was calculated. *Z′* factor usually used in plate-based tissue culture assay to find the ‘hit’ chemical. The *Z′* factor represents 1 – (3 times the sum of the standard deviations for each control divided by the absolute value of the difference between mean values for each control). *Z′* factors regarded as acceptable by the field range from 0–1. In our

plate-based assay the CI egg hatch rates were considered as negative control, and rescue egg hatch rate as a positive control.

According to the *Z′* factors analysis, data from the five NaBu plate replicates returned from ranging from 0.76 to 0.89 (Table 3.3, Figure 3.5). Data from CI+NaBu condition falls into the intermediate hit range which was consistently distinguishable from the CI control (Table 3.3, Figure 3.5). This observation suggested that this plate-based feeding assay can reproducibly identify the CI-suppressing/ rescue-inducing treatments.

3.4.5 How many wells were required to distinguish control from treatment conditions

To determine the quantity of wells in a screening plate required for reproducible identification of a chemical suppressor of CI/ rescue effect, the NaBu plate data were statistically analyzed. To analyze the data a MATLAB based power analysis tool was used (described previously). Data were collated and compared for every crosswise pairing of five independent plate screenings. Analysis indicated that when setting an alpha value at 0.05, data from eight or more wells per condition were sufficient to identify significant differences between CI and CI+NaBu conditions (Fig 3.6). Thus, confirming the potential to use this assay as a higher thru-put methodology, data indicate that screening with 24-well assay plates is sufficient to detect the rescue effect via chemical feeding.

3.5 Discussion

The development of an assay to determine the rescue effect of any cellular pathways could be a reasonable step toward investigating a large number of cellular pathways for their rescue capability. To achieve this, the goal of this chapter was to

design a 24-well plate-based screening method. In this chapter, we have adjusted the method used in previous chapter from vials into a 24-well plate format. After the required modification, the results were analyzed for their ability to fulfill the statistical requirements.

Although *Drosophila* fruit flies have been used in many screening studies, a plate-based assays is still not commonly reported for adult *Drosophila.* Only a few researchers have taken the approach to use screening plates to grow adult *Drosophila* (Markstein et al., 2014; Seong etal., 2020; Willoughby et al., 2013). Our approach was distinct in that it involves manual distribution of food and flies in individual wells, where the females were then transferred to another plate to lay eggs. These plates were then used to determine the egg hatch rate in different crosses. The time required to distribute the adult flies in different wells can be a limiting factor in this method. Moreover, it is not advisable to keep flies on a CO_2 gas pad for a prolong period of time. However, this can be limited with adequate practice and planning in advance. In this study, we were cautious to attempt similar experimental procedures presented in the previous chapter using vials. The rearing conditions were made as similar as possible, while using a 24-well screening plate instead of vials for the growth of flies. Initially, only the female flies were grown in the 24-well screening plate and the male were kept separately for easy handling of both flies. For our experiments, we have used *D. simulans,* as described in the previous chapter.

The male and female flies were kept separately for 3 days and then transferred into a new plate containing only the control food for mating for 8 hours. This was the only time the female flies were kept out from exposure to the chemical,

but this step was necessary to avoid any unwanted exposure of *Wolbachia*-infected male flies to the chemical. This step was critical to avoid any chemical effect to *Wolbachia*. The use of CO_2 and the handling of flies are critical factors while using a 24-well screening plate, because a prolong exposure can damage the flies, having an adverse effect on their fecundity. It was also important to not to physically damage the flies as such damage to adult *D. simulans* can affect their fecundity.

The first major question in front of us while using a 24-well screening plate was if it would be possible to distinguish the CI and rescue phenotype. Here, we were able to clearly distinguish the CI and rescue egg hatch rates where the egg hatch rate for CI were nearly 10% and the hatch rate for rescue were nearly 90%. The other major question in front of us was whether it would be possible to observe/distinguish the effect of chemical feeding on CI hatch rates. NaBu was selected as an effective chemical, since it showed to induce rescue effect in previous chapter. When NaBu was tested in this screening plate-based assay it was able to increase the egg hatch rate which was distinguishable from CI control egg hatch rate in all 5 replicates. All the data were further analyzed with Z′ factor. The Z′ factor is usually used in plate-based tissue culture system to find the efficiency of each run and the distinguish the hits from the control. Our plate-based data also support this plate-based analysis with a Z′ factors range from 0.76 to 0.89 for the five replicates. This finding further strengthens our observation that this assay technique can distinguish the chemical effect on CI with reproducibility.

The experimental methodology presented here with a thorough and systematic approach to statistical analysis. Experimental data have been traditionally accompanied by power analysis, informing the sufficiency of sample size. We

outlined a methodology for selecting appropriate statistical tests and then applied sub-sampling analyses to empirically determine appropriate sample sizes. To determine the number of wells necessary to distinguish CI and rescue, the sub-sampling was done manually, and the data was compared pairwise and the p-values analyzed. Our analysis indicates that testing only 6 wells is sufficient to distinguish hatch rates from CI and rescue. We also tried to determine the number of wells necessary to clearly observe/distinguish the effect of chemical feeding. To this end, power analysis was carried out using a MATLAB tool (described previously). An α-value of 0.05 is considered standard in many disciplines as sufficient to reject the null hypothesis and conclude that there is a statistically meaningful difference between comparison groups (Morrison & Henkel, 2006; Skipper et al., 1967). However, sub-sampling data to identify the "n" required for significance at $\alpha = 0.01$ further informs the scale of the differences observed between conditions, such as between control hatch rates vs. treatment hatch rates. Our analysis showed that a total of 8 wells were required to satisfy the α-value of 0.05. A total of 11 wells would be necessary to satisfy the α-value of 0.01. Altogether, this analysis indicates that it will be possible to distinguish any chemical treatment in 24-well tissue culture plate setting, which will be continued further in testing other chemicals/pathways for their ability to induce the rescue effect on CI.

To summarize, the chemical screening method presented here using 24-well screening plates were able to distinguish the CI and rescue, as well as the rescue effect of treatment on CI crosses. It was also clear that there were enough wells to meet statistical validation. The 24-well screening plate assay can thus further be used to test other chemicals/ pathways for their ability to induce a rescue effect on

CI. This assay technique could be translated readily to test other host *Wolbachia* CI models. This assay technique can also be used to observe the effect of other pathway modifications in *Drosophila*.

Figures and Tables

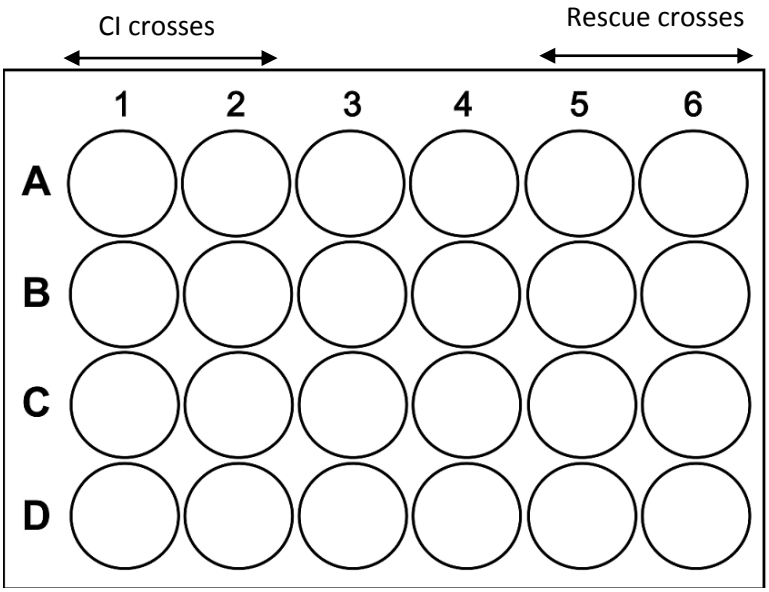

Fig. 3.1 Plate diagram for CI and rescue crosses. A total of 8 wells were used to perform each of CI and rescue crosses.

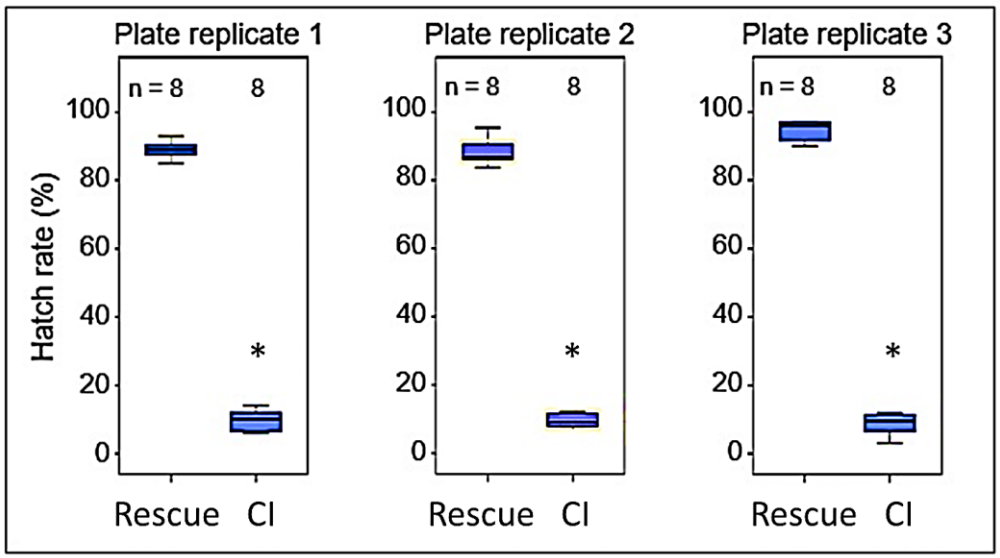

Fig. 3.2. Boxplot showing the egg hatch rate from CI and rescue crosses. The hatch rate is significantly different in all three replicates. (* denotes significant difference). “n” denotes the number of wells tested.

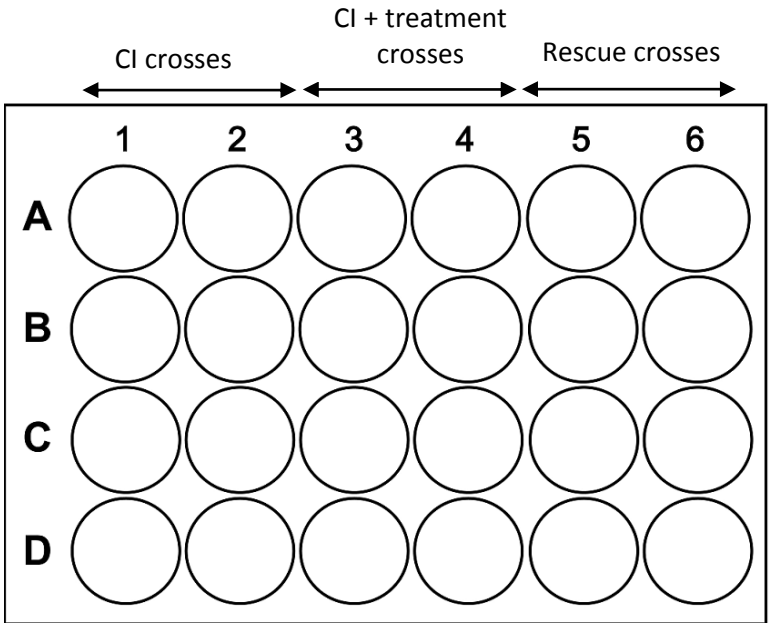

Fig. 3.3. Plate diagram for CI and rescue crosses with the treatment wells. A total of 8 wells were used to perform each of CI, rescue and CI+treatment crosses.

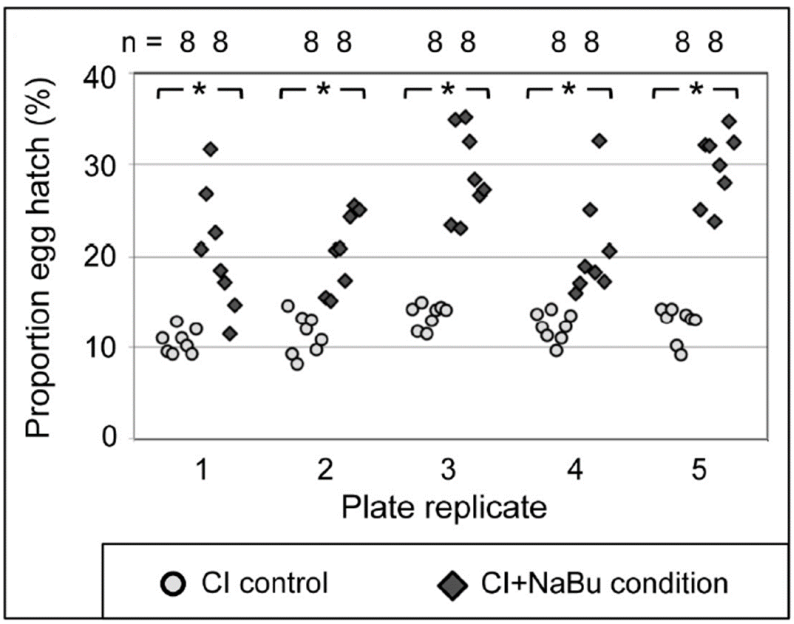

Fig. 3.4. Egg hatch rate data from assay plate showing the impact of NaBu treatment on CI. *w*Ri-infected *D. simulans* flies were used for the assay. Each symbol represents data from a single well.

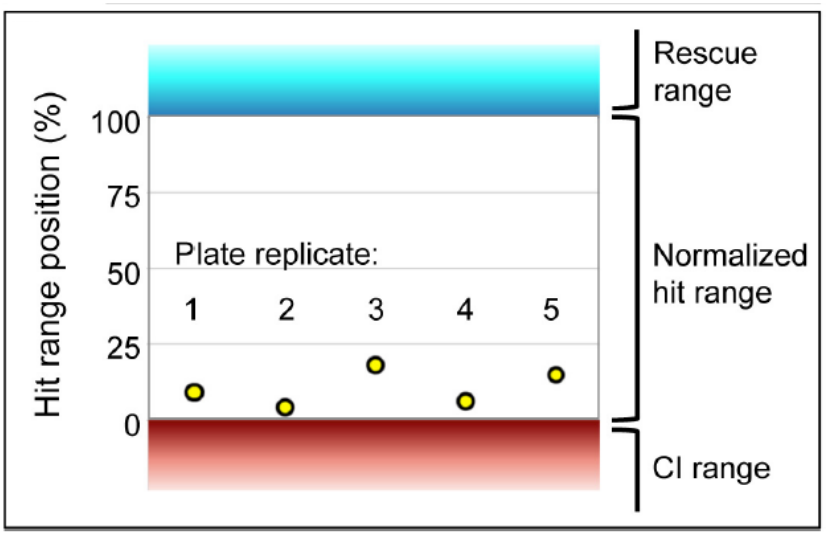

Fig. 3.5. The impact of NaBu treatment on CI egg hatch. NaBu impact on CI, in terms of conventional Z′ analysis. Range boundaries of the CI control (red) and Rescue control (cyan) are indicated. The normalized “hit range” between controls is shown in white. Yellow dots: average hatch rate for the CI+NaBu condition per screening plate, normalized to the range between CI and Rescue controls.

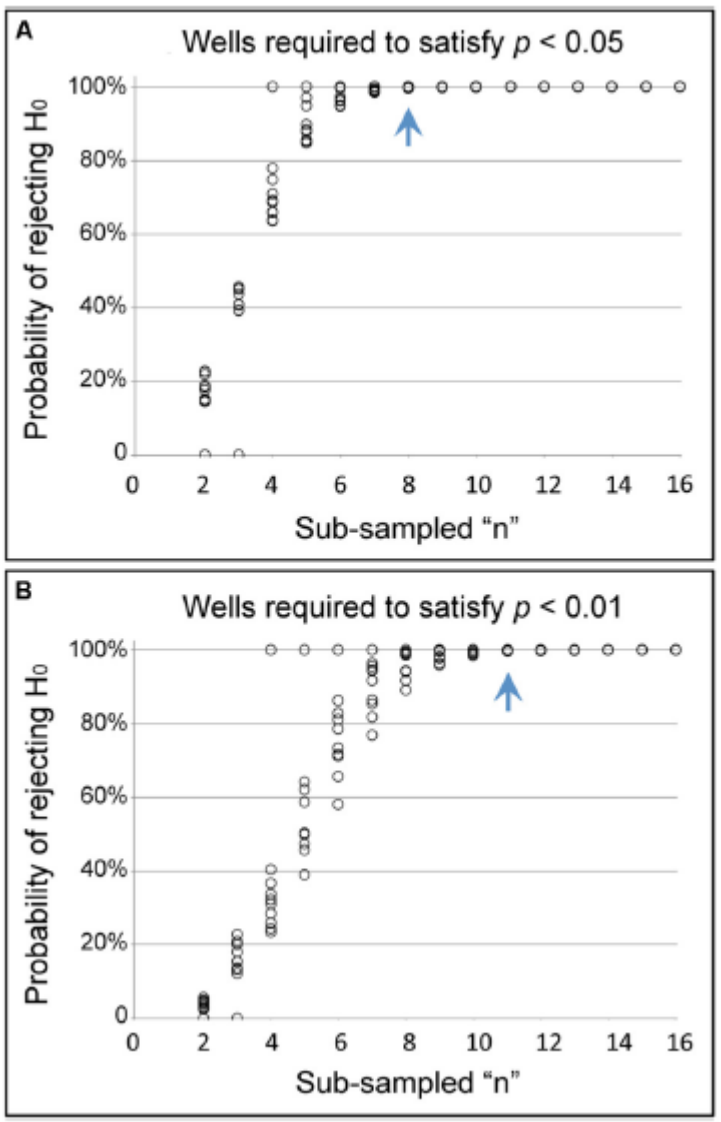

Fig. 3.6. Identifying the sample size to observe significant difference in the plate assay format. The figure shows the likelihood of seeing a significant difference in hatch rate between CI and CI+NaBu conditions, as defined by (A) the conventional α-value of 0.05, as well as (B) the more stringent a-value of 0.01. The blue arrow indicates the number of wells at which the probability of rejecting the null hypothesis has reached 99.5% or higher for all sub-sampled datasets analyzed.

Table 3.1. Impact of NaBu on *D. simulans w*Ri CI hatch rates in plate assay format.

Treatment tested	CI condition				CI + drug treatment				Rescue condition			
	Unhatched	Hatched	total	hatch rate	Unhatched	Hatched	total	hatch rate	Unhatched	Hatched	total	hatch rate
NaBu test 1	97	12	109	11.0%	103	27	130	20.8%	12	165	177	93.2%
	132	14	146	9.6%	63	23	86	26.7%	14	143	157	91.1%
	117	12	129	9.3%	80	37	117	31.6%	11	148	159	93.1%
	95	14	109	12.8%	93	27	120	22.5%	16	98	114	86.0%
	97	12	109	11.0%	106	24	130	18.5%	8	134	142	94.4%
	132	15	147	10.2%	125	26	151	17.2%	11	126	137	92.0%
	117	12	129	9.3%	131	17	148	11.5%	15	142	157	90.4%
	95	13	108	12.0%	116	20	136	14.7%	11	99	110	90.0%
NaBu test 2	76	13	89	14.6%	87	16	103	15.5%	6	87	93	93.5%
	78	8	86	9.3%	84	15	99	15.2%	5	122	127	96.1%
	78	7	85	8.2%	23	6	29	20.7%	6	104	110	94.5%
	59	9	68	13.2%	68	18	86	20.9%	13	82	95	86.3%
	51	7	58	12.1%	76	16	92	17.4%	12	145	157	92.4%
	93	14	107	13.1%	81	26	107	24.3%	7	106	113	93.8%
	111	12	123	9.8%	82	28	110	25.5%	7	116	123	94.3%
	41	5	46	10.9%	57	19	76	25.0%	12	63	75	84.0%
NaBu test 3	90	15	105	14.3%	138	42	180	23.3%	8	142	150	94.7%
	60	8	68	11.8%	73	39	112	34.8%	3	84	87	96.6%
	68	12	80	15.0%	114	34	148	23.0%	13	67	80	83.8%
	108	14	122	11.5%	83	45	128	35.2%	10	140	150	93.3%
	87	13	100	13.0%	79	38	117	32.5%	9	149	158	94.3%
	97	16	113	14.2%	81	32	113	28.3%	13	176	189	93.1%

Treatment tested	CI condition				CI + drug treatment				Rescue condition			
	Unhatched	Hatched	total	hatch rate	Unhatched	Hatched	total	hatch rate	Unhatched	Hatched	total	hatch rate
	89	15	104	14.4%	83	30	113	26.5%	10	149	159	93.7%
	103	17	120	14.2%	112	42	154	27.3%	16	149	165	90.3%
NaBu test 4	126	20	146	13.7%	84	16	100	16.0%	7	103	110	93.6%
	122	17	139	12.2%	87	18	105	17.1%	12	111	123	90.2%
	55	7	62	11.3%	64	15	79	19.0%	6	41	47	87.2%
	102	17	119	14.3%	72	24	96	25.0%	2	16	18	88.9%
	84	9	93	9.7%	89	20	109	18.3%	1	84	85	98.8%
	129	16	145	11.0%	87	42	129	32.6%	2	92	94	97.9%
	85	12	97	12.4%	43	9	52	17.3%	7	69	76	90.8%
	83	13	96	13.5%	77	20	97	20.6%	14	80	94	85.1%
NaBu test 5	126	21	147	14.3%	87	29	116	25.0%	3	119	122	97.5%
	78	12	90	13.3%	74	35	109	32.1%	0	106	106	100.0%
	102	17	119	14.3%	68	32	100	32.0%	2	123	125	98.4%
	150	17	167	10.2%	77	24	101	23.8%	0	120	120	100.0%
	167	17	184	9.2%	75	32	107	29.9%	5	126	131	96.2%
	114	18	132	13.6%	67	26	93	28.0%	2	118	120	98.3%
	92	14	106	13.2%	66	35	101	34.7%	1	102	103	99.0%
	120	18	138	13.0%	92	44	136	32.4%	0	128	128	100.0%

Table 3.2. Statistical analysis of NaBu plate assay data.

Plates analyzed	Shapiro-wilk: p-values, interpretation			Levene's test: p-values, interpretation		Outcomes: analysis of hatch rate data		
replicate #	CI control	CI+NaBu	Normal distribution?	Overall value	Variance homogeneous?	Analysis required	Resulting p-value	Sig diff?
1	0.384	0.966	both normal	0.024	no	Welch's T-test	0.003	yes
2	0.846	0.245	both normal	0.103	yes	T-test	<0.001	yes
3	0.096	0.327	both normal	0.021	no	Welch's T-test	<0.001	yes
4	0.858	0.032	one is non-normal	0.196	yes	Mann-Whitney	<0.001	yes
5	0.032	0.478	one is non-normal	0.093	yes	Mann-Whitney	<0.001	yes
1+2	0.585	0.962	both normal	0.003	no	Welch's T-test	<0.001	yes
1+3	0.201	0.867	both normal	0.001	no	Welch's T-test	<0.001	yes
1+4	0.419	0.193	both normal	0.016	no	Welch's T-test	<0.001	yes
1+5	0.074	0.410	both normal	<0.001	no	Welch's T-test	<0.001	yes
2+3	0.155	0.616	both normal	0.007	no	Welch's T-test	<0.001	yes
2+4	0.699	0.076	both normal	0.015	no	Welch's T-test	<0.001	yes
2+5	0.066	0.485	both normal	0.005	no	Welch's T-test	<0.001	yes
3+4	0.256	0.226	both normal	<0.001	no	Welch's T-test	<0.001	yes
3+5	0.019	0.202	one is non-normal	<0.001	no	Mann-Whitney	<0.001	yes
4+5	0.067	0.094	both normal	<0.001	no	Welch's T-test	<0.001	yes

Table 3.3. Z' analysis of NaBu plate assay data.

Plate replicate #	CI hatch rates (%)		CI control range (%)		Rescue hatch rates (%)		Rescue control range (%)		Z' value
	Mean	Std dev	Min	Max	Mean	Std dev	Min	Max	
1	10.66	1.31	6.73	14.59	91.27	2.61	83.44	99.09	0.85
2	11.39	2.21	4.75	18.04	91.87	4.31	78.93	100.00	0.76
3	13.53	1.31	9.61	17.45	92.47	3.93	80.67	100.00	0.80
4	12.27	1.56	7.60	16.94	91.57	4.88	76.93	100.00	0.76
5	12.65	1.89	6.98	18.32	98.69	1.37	94.59	100.00	0.89

Plate replicate #	Avg CI+NaBu hatch rate (%)	Intermediate "hit" range (%)		NaBu results: placement within normalized hit range (%)
		Min	Max	
1	20.44	14.59	83.44	8.51
2	20.56	18.04	78.93	4.13
3	28.86	17.45	80.67	18.05
4	20.75	16.94	76.93	6.35
5	29.72	18.32	94.59	14.94

References

Beckmann, J. F., Ronau, J. A., & Hochstrasser, M. (2017). A *Wolbachia* deubiquitylating enzyme induces cytoplasmic incompatibility. *Nature Microbiology*, *2*(5), 1–7.

Bennett, G. M., & Moran, N. A. (2015). Heritable symbiosis: the advantages and perils of an evolutionary rabbit hole. *Proceedings of the National Academy of Sciences*, *112*(33), 10169–10176.

Braendle, C., Miura, T., Bickel, R., Shingleton, A. W., Kambhampati, S., Stern, D. L., & Normark, B. (2003). Developmental origin and evolution of bacteriocytes in the aphid–Buchnera symbiosis. *PLoS Biology*, *1*(1), e21.

Camacho, M., Oliva, M., & Serbus, L. R. (2017). Dietary saccharides and sweet tastants have differential effects on colonization of *Drosophila* oocytes by *Wolbachia* endosymbionts. *Biology Open*, *6*(7), 1074–1083.

Charlat, S., Hurst, G. D. D., & Merçot, H. (2003). Evolutionary consequences of *Wolbachia* infections. *TRENDS in Genetics*, *19*(4), 217–223.

Christensen, S., Camacho, M., Sharmin, Z., Momtaz, A. J. M., Perez, L., Navarro, G., … Serbus, L. R. (2019). Quantitative methods for assessing local and bodywide contributions to *Wolbachia* titer in maternal germline cells of *Drosophila*. *BMC Microbiology*, *19*(1), 1–17.

Christensen, S., Pérez Dulzaides, R., Hedrick, V. E., Momtaz, A. J. M. Z., Nakayasu, E. S., Paul, L. N., & Serbus, L. R. (2016). *Wolbachia* endosymbionts modify *Drosophila* ovary protein levels in a context-dependent manner. *Applied and Environmental Microbiology*, *82*(17), 5354–5363.

Coradini, D., Pellizzaro, C., Marimpietri, D., Abolafio, G., & Daidone, M. G. (2000). Sodium butyrate modulates cell cycle-related proteins in HT29 human colonic adenocarcinoma cells. *Cell Proliferation*, *33*(3), 139–146.

De Cuevas, M., & Spradling, A. C. (1998). Morphogenesis of the *Drosophila* fusome and its implications for oocyte specification. *Development*, *125*(15), 2781–2789.

Field, A. (2013). *Discovering statistics using IBM SPSS statistics*. sage.

Kikuchi, Y. (2009). Endosymbiotic bacteria in insects: their diversity and culturability. *Microbes and Environments*, 908180109.

Kitzis, A., Tichonicky, L., Defer, N., & Kruh, J. (1980). Effect of sodium butyrate on chromatin structure. *Biochemical and Biophysical Research Communications*, *93*(3), 833–841.

Landmann, F., Orsi, G. A., Loppin, B., & Sullivan, W. (2009). *Wolbachia*-mediated cytoplasmic incompatibility is associated with impaired histone deposition in the male pronucleus. *PLoS Pathogens*, *5*(3), e1000343.

Lassy, C. W., & Karr, T. L. (1996). Cytological analysis of fertilization and early embryonic development in incompatible crosses of *Drosophila* simulans. *Mechanisms of Development*, *57*(1), 47–58.

LePage, D. P., Metcalf, J. A., Bordenstein, S. R., On, J., Perlmutter, J. I., Shropshire, J. D., … Bordenstein, S. R. (2017). Prophage WO genes recapitulate and enhance *Wolbachia*-induced cytoplasmic incompatibility. *Nature*, *543*(7644), 243–247.

Lim, T.-S., & Loh, W.-Y. (1996). A comparison of tests of equality of variances. *Computational Statistics & Data Analysis*, *22*(3), 287–301.

López-Madrigal, S., & Duarte, E. H. (2019). Titer regulation in arthropod-*Wolbachia* symbioses. *FEMS Microbiology Letters*, *366*(23), fnz232.

Markstein, M., Dettorre, S., Cho, J., Neumüller, R. A., Craig-Müller, S., & Perrimon, N. (2014). Systematic screen of chemotherapeutics in *Drosophila* stem cell tumors. *Proceedings of the National Academy of Sciences*, *111*(12), 4530–4535.

Morrison, D. E., & Henkel, R. E. (2006). *The significance test controversy: A reader*. Transaction Publishers.

Plonsky, L. (2015). *Advancing quantitative methods in second language research*. Routledge New York.

Preacher, K. J., & Hayes, A. F. (2004). SPSS and SAS procedures for estimating indirect effects in simple mediation models. *Behavior Research Methods, Instruments, & Computers*, *36*(4), 717–731.

Razali, N. M., & Wah, Y. B. (2011). Power comparisons of shapiro-wilk, kolmogorov-smirnov, lilliefors and anderson-darling tests. *Journal of Statistical Modeling and Analytics*, *2*(1), 21–33.

Rietveld, T., & van Hout, R. (2015). The t test and beyond: Recommendations for testing the central tendencies of two independent samples in research on speech, language and hearing pathology. *Journal of Communication Disorders*, *58*, 158–168.

Sazama, E. J., Bosch, M. J., Shouldis, C. S., Ouellette, S. P., & Wesner, J. S. (2017). Incidence of *Wolbachia* in aquatic insects. *Ecology and Evolution*, *7*(4), 1165–1169.

Seong, K.-H., Matsumura, T., Shimada-Niwa, Y., Niwa, R., & Kang, S. (2020). The *Drosophila* individual activity monitoring and detection system (DIAMonDS). *Elife*, *9*, e58630.

Serbus, L. R., Landmann, F., Bray, W. M., White, P. M., Ruybal, J., Lokey, R. S., … Sullivan, W. (2012). A cell-based screen reveals that the albendazole metabolite, albendazole sulfone, targets *Wolbachia*.

Shapiro, S. S., & Wilk, M. B. (1965). An analysis of variance test for normality (complete samples). *Biometrika*, *52*(3/4), 591–611.

Shropshire, J. D., On, J., Layton, E. M., Zhou, H., & Bordenstein, S. R. (2018). One prophage WO gene rescues cytoplasmic incompatibility in *Drosophila* melanogaster. *Proceedings of the National Academy of Sciences*, *115*(19), 4987–4991.

Skipper, J. K., Guenther, A. L., & Nass, G. (1967). The sacredness of. 05: A note concerning the uses of statistical levels of significance in social science. *The American Sociologist*, 16–18.

Smerdon, M. J., Watkins, J. F., & Lieberman, M. W. (1982). Effect of histone H1 removal on the distribution of ultraviolet-induced deoxyribonucleic acid repair synthesis within chromatin. *Biochemistry*, *21*(16), 3879–3885.

Tram, U., & Sullivan, W. (2002). Role of delayed nuclear envelope breakdown and mitosis in *Wolbachia*-induced cytoplasmic incompatibility. *Science*, *296*(5570), 1124–1126.

Vargha, A., & Delaney, H. D. (1998). The Kruskal-Wallis test and stochastic homogeneity. *Journal of Educational and Behavioral Statistics*, *23*(2), 170–192.

Wasserman, S. (1994). *Advances in social network analysis: Research in the social and behavioral sciences*. Sage.

Willoughby, L. F., Schlosser, T., Manning, S. A., Parisot, J. P., Street, I. P., Richardson, H. E., … Brumby, A. M. (2013). An in vivo large-scale chemical screening platform using *Drosophila* for anti-cancer drug discovery. *Disease Models & Mechanisms*, *6*(2), 521–529.

CHAPTER IV: INVOLVEMENT OF DIFFERENT CELLULAR PATHWAYS IN THE OVERALL MECHANISM OF RESCUE

4.1 Abstract

Wolbachia are gram-negative endosymbiotic bacteria carried by approximately half of all insect species. The high prevalence of *Wolbachia* in nature is a consequence of its ability to manipulate host reproduction in a variety of ways, all favoring the success of infected females. The best-known reproductive manipulation induced by *Wolbachia* is referred to as sperm-egg cytoplasmic incompatibility (CI). In CI, the sperm of *Wolbachia*-infected male cause embryonic lethality in crosses with *Wolbachia* uninfected females or in some cases, females infected with a different *Wolbachia* strain. The embryos of *Wolbachia*-infected females can "rescue" CI lethality restoring egg hatch rates to that of uninfected cross. Several studies have been carried out to understand the mechanism of CI and rescue, but the underlying mechanisms involved in *Wolbachia* induced rescue remains largely untested. In this study, we used a chemical feeding approach to test the ability to induce rescue capabilities by *Drosophila simulans* flies. We fed chemical inhibitors to uninfected females and tested their ability to increase egg hatch rates in CI crosses. We found that treatment with seven chemicals were able to significantly increase egg hatch rates in CI caused by the *w*Ri *Wolbachia* strain. These chemicals affect DNA integrity, cell cycle control and protein turnover. Three of these chemical treatments caused increase in egg hatch rates in CI induced by native *w*Ri *Wolbachia*, as well as transinfected *w*Mel *Wolbachia* infection. These three chemicals can affect DNA integrity and protein turnover. These results indicated DNA integrity is a focal aspect of rescue induction/ CI suppression for different *Wolbachia* strains. Further studies should be carried out with additional rescue-inducing chemicals and different CI models to strengthen this hypothesis.

4.2 Introduction

Symbiosis is the close interaction between two or more species (Hunter, 2006). Endosymbiosis is a specialized form of symbiosis where one organism (the endosymbiont) lives inside the cells or tissues of other organism (the host) (Archibald, 2015). Endosymbionts not only live inside their host, but also interact with host tissues for survival and reproduction (Koga et al., 2003). *Wolbachia pipientis* is one of the most widespread endosymbionts in nature, carried by half or more of all insect species (Sazama et al., 2017). *Wolbachia* are gram-negative bacteria that belong to the alpha-protobacteria, class Rickettsiales. *Wolbachia* are maternally transmitted and the success of their transmission depends on the successful loading of the endosymbiont into the egg (Breeuwer & Werren, 1990; Veneti et al., 2004; Ferree et al., 2005; Serbus et al., 2008; Fast et al., 2011). *Wolbachia* modify the host reproduction to favor the success of infected female. This is accomplished by induction of parthenogenesis, feminization of male, male killing, and sperm-egg cytoplasmic incompatibility (Serbus et al., 2008; Werren et al., 2008). Thus, *Wolbachia* can act as a reproductive parasite in many species.

Cytoplasmic incompatibility (CI) is the most widely known reproductive manipulations induced by *Wolbachia* (Hoffmann and Turelli, 1997; Werren et al., 2008). CI is characterized by embryonic lethality in crosses between *Wolbachia*-infected males and uninfected females or, in some cases, females infected with a different strain of *Wolbachia*. By contrast *Wolbachia*-infected females can produce viable embryos in crosses with both *Wolbachia*-infected and uninfected males. The ability of *Wolbachia*-infected embryos to rescue the sperm modification by *Wolbachia*-infected male is called “rescue”. It has been speculated that whatever the sperm modification done in CI embryos must be fixed in order to produce a viable embryos in rescue crosses.

The cellular basis of CI has been a point of interest for many years. Cytological experiments demonstrate some mitotic defects as consensus feature in CI embryos, where paternal chromatin becomes arrested on the metaphase plate, while the maternal chromatin segregates to opposite pole in anaphase, resulting in chromosome bridging aneuploidy and embryonic death (O'Neill & Karr, 1990; Callaini & Riparbelli, 1996; Lassy & Karr, 1996; Callaini et al., 1997; Tram & Sullivan, 2002; Landmann et al., 2009). Other studies suggest these mitotic defects are produced from a timing mismatch between male and female pronuclei at the first mitotic division (Tram & Sullivan, 2002; Landmann et al., 2009). All these defects must be resolved in order to produce a viable embryo in rescue crosses. Another study by Brennan et al, demonstrated *Wolbachia* cause oxidative damage to spermatocyte DNA, which contribute to CI lethality (Brennan et al., 2012). This finding implies that this DNA damage must be repaired in rescue crosses in order to produce viable embryos. Recent transgenic expression studies demonstrate that *Wolbachia* derived factors (Cif proteins) are contributor to CI and rescue. These studies demonstrate that the expression of two *Wolbachia* genes, cifA and cifB, are responsible for causing the CI phenotype and expression of cifA can rescue CI (Beckmann et al, 2017; LePage et al., 2017; Shropshire et al., 2018).

Despite findings about the underlying mechanism of CI, the mechanism of Rescue is largely unknown. In chapters Chapters II & III, we demonstrated that NaBu, an HDAC inhibitor, was able to induce a partial rescue effect in feeding assays with *Wolbachia* uninfected embryos. Although the rescue effect produced by NaBu was mild, it showed the possibility that cellular mechanisms effected by NaBu might be involved in the rescue process. This finding also supports the idea that some host pathways might be involved in the overall rescue process. NaBu can effect a number of cellular mechanisms and can modify gene expression, the cell cycle, and induce DNA damage

repair (Kitzis et al., 1980; Smerdon et al, 1982; Coradini et al., 2000). In this chapter, 24 chemical inhibitors were chosen, based on their ability to modify specific cellular pathways in previous studies, used in feeding assays, and observed for their ability to induce any rescue effect in CI crosses. The chemical compounds were tested on CI induced by natural *w*Ri-induced and transinfected *w*Mel-induced CI.

Overall, this chapter demonstrates the involvement of different host pathways in the overall rescue process. We investigated host pathways involving HDAC modification, DNA damage repairing mechanism and cell cycle timing. The dosing of each chemical was selected from survival curve assays using host *Wolbachia* combinations that can produce strong CI. The findings from this study will be helpful to start understanding the contribution of host mechanisms in toward the rescue process.

4.3 Methods and materials

4.3.1. Fly stocks & maintenance

*Wolbachia w*Ri-infected and uninfected *D. simulans* flies were used in this study. The *Wolbachia*-infected *D. simulans* flies were originally collected in Watsonville, CA. The collected flies have been maintained in lab conditions since then. The uninfected *D. simulans* flies were created by treating infected flies with tetracycline.

All the flies used in this study were maintained on standard minimal fly food at 25°C in a 12h light/dark cycle. The fly food recipe was derived from Bloomington *Drosophila* stock center (*http://flystocks.bio.indiana.edu/Fly_Work/media recipes/bloomfood.htm*) ((also see Chapter II). All the flies were raised in 6 oz square bottom polyethylene bottles containing approximately 25 ml of standard fly food. To start a new bottle each bottle was seeded with approximately 80-100 flies (both male and female) and kept there for 3-6 days. After that the bottle was cleared and the flies were transferred into a new bottle to seed. The new progeny flies emerged after 12-13 days.

To collect virgin flies, the bottles were cleared from any flies and each bottle was visually confirmed. The newly eclosed flies were collected after 5-8 hours using standard CO_2 gas pads. At this stage, the flies were considered virgin. The collected flies were then instantly separated into male and female populations and kept separate to avoid mating. Approximately 40-45 flies were kept in a vial until they were used in further experiments.

4.3.2 Food preparation for Dose response curves

4.3.2.1. Food preparation for single chemicals

To determine the most effective dose of any chemical used in the experiment, a dose response curve was prepared for each chemical. The procedure for preparing the dose response curve was detailed in Chapter II. Briefly, a dose response curve was prepared by using 6-7 different concentration of each chemical. The concentrations were selected using available literature. A range of different concentrations were predicted to include the effective concentration. To prepare food with different chemical concentrations, each chemical was diluted to their desired concentration and added in a beaker containing 10 ml of melted standard food with the addition of a blue marker dye. The blue marker dye helps to visualize the eggs and larvae. Each chemical-containing food was then mixed thoroughly for 30 seconds and transfered into two vials. To prepare the control food, the same amout of solvent was mixed into 10 ml of standard fly food containing blue dye and mixed thoroughly for 30 sec. The control food was then transfered into two viasl. All vials were cooled in a fume hood for ~1 hr. A total of two sets of each concentraion of vials were prepared. One set was used to begin feeding trials and the another set was wrapped with aluminum foil and kept at 4°C for later use.

4.3.2.2. Food preparation for two combined chemicals

To observe the effect of two combined chemicals, dose response curves were prepared for each combination of chemicals. The food preparation for this response

curve was similar to the procedure followed to prepare food with a single chemical. Briefly, the concentration of each chemical was selected by their survival curve assay with single chemical. A range of 6-7 different concentrations were then selected for observing their combined effect. The stock solution of each chemical was prepared at the beginning of food preparation. Then appropriate amount of each chemical was added into 10 ml of melted standard fly food mixed with blue dye. The food was mixed thoroughly for 30 sec by stirring and then transferred to two vials. Two control vials were also prepared by mixing the chemical solvent with standard fly food with blue dye thoroughly for 30 sec. Among the two set of vials, one set was used to rear flies immediately after cooling, and the other set was wrapped in aluminum foil and kept in 4°C for later use.

4.3.3. Procedure for dose response curve assay

The dose response curve experiments, for both single and multiple chemical combinations were carried out across 12 days. A total of 6 uninfected male and 6 uninfected female flies were kept in the first set of control and treatment vials and observed for 6 days, then transferred to another set of control and treatment vials for 6 days. Flies were observed and scored qualitatively for adult mortality, egg lay, egg hatch and larval development. All the measurements were scored by comparing each treatment vial with a corresponding control vial. If the treatment vials were similar to the control vial, it was designated as '+', vials are designated as 'some' if the treatment vials had some negative effect on development compared to the control. If the treatment vials had consistent negative effects on the measurement, vials were designated as '-'. The highest concentration of chemical that had no adverse effect on flies were selected as the most effective concentration and was used for feeding experiment.

4.3.4. Food preparation for chemical feeding assays

The procedure for preparing chemical containing food was explained in Chapter II, while the procedure for preparing food containing two combined chemicals was similar. Briefly, to prepare chemical containing food, a 100X solution of chemical was mixed into standard fly food. The standard fly food was melted and ~20 ml of melted fly food was taken into a beaker. Then an appropriate amount of the stock chemical was mixed with the melted food. For fly food with two combined chemicals, both chemicals were mixed with the melted fly food in their appropriate amounts. The chemicals were then mixed with the standard fly food by stirring for 30 sec. To prepare control food, the appropriate amount of solvent was added with standard fly food. A blue dye was also added for easier visualization of fly eggs in the food.

4.3.5. Plate preparation for screening assay

For CI suppression/rescue induction assays the flies were reared in a 24-well plate. The procedure of pate preparation was outlined in Chapter III. Briefly, for the assay two plates were prepared at the same time. One plate was for incubation of flies for 5 days and the other plate was for egg laying. Each 24-well plate contained wells with standard food and chemical containing food, with either one or two chemicals. In each 24-well plate, 8 wells contained the chemical food and 16 wells contained control food. To prepare a 24-well plate, 800 μl of melted liquid food were poured into each well. The control food and chemical containing food were prepared separately. Both plates were kept in the fume hood for 1 hr for cooling and solidification. After preparation, one plate was used immediately for the experiment and the other plate was kept in 4°C for later use.

4.3.6. CI suppression/rescue induction test procedure in 24-well plate format

In previous chapters, it was observed that feeding NaBu was able to induce a rescue effect on both *w*Ri- and *w*Mel-induced CI. To further investigate which cellular

pathways were involved in rescue induction, cellular pathways involved in chromatin modification, DNA damage induction and cell cycle delay were tested. A total of 24 chemical inhibitors were tested in this experiment. All the chemicals were tested using 24- well tissue culture plates. The procedure for testing a plate with one single chemical was same as the procedure for testing the effect of two combined chemicals.

The procedure was mentioned previously in Chapter III. Briefly, 10 uninfected virgin female flies were put into each of 8 wells of control food and 8 wells of chemical containing food. These flies were for CI crosses. 10 virgin *Wolbachia*-infected flies were also put into each of 8 wells of control food for rescue crosses. All the *Wolbachia*-infected male flies were kept in separate vials containing control food. All the flies (in both plate and vials) were kept at 25°C for 3 days. At day 3, all the female flies were transferred to a new plate containing control food and 10 *Wolbachia*-infected male flies were added to each well for mating for 8 hours. After mating, the male flies were discarded, and the female flies were transferred back to their original plate. At day 4, all the female flies were transferred into the second plate (prepared previously at day 1 and kept in 4°C) for egg laying. At day 5, all the flies were discarded, and the plate was incubated at 25°C for an additional 24 hr. At day 6, the number of unhatched and hatched eggs were counted, and egg hatch rates were calculated.

4.3.7. Statistical Analysis

All the egg hatch rate data collected in this study were matched with appropriate statistical analyses, as per a standard decision tree (Chapter II, Fig 2.1). All the data were analyzed for consistency with a normal distribution using the Shapiro-Wilks test, and for homogeneity of variances using Levene's test (Lim & Loh, 1996; Razali & Wah, 2011; Shapiro & Wilk, 1965). For normal data, distributions showing homogenous variances were compared by T-test. Distributions with unequal variances were

compared by Welch's T-test ((Vargha & Delaney, 1998; Wasserman, 1994). For non-normal data, distributions with homogeneous variances were compared using the Mann-Whitney U test (Rietveld & van Hout, 2015; Vargha & Delaney, 1998). For non-normal distributions with unequal variances, significance was estimated using randomization based T-tests with bootstrapping, as recommended by field literature (De Cuevas & Spradling, 1998; Lim & Loh, 1996; Rietveld & van Hout, 2015; Shapiro & Wilk, 1965; Wasserman, 1994). The IBM SPSS v.23 analysis package was used for all statistical tests performed in this study (Field, 2013).

4.4 Results

4.4.1 Testing for rescue induction/CI Suppression by short-chain fatty acids

NaBu has previously been shown to induce a rescue effect in both vial and plate-based assays. Since NaBu is a short chain fatty acid, it raises the question of whether short chain fatty acids generally exert a rescue on CI. To investigate this possibility, three other short chain fatty acids, specifically acetic acid, propionic acid and valeric acid, were tested for their ability to induce a rescue effect on *w*Ri-induced CI in *D. simulans*. The doses for each compound, as well as all others described later, were determined by survival curve assay (Table 4.1). All the chemical compounds were tested in a 24-well plate-based assay. Results from these experiments indicate that only acetic acid conferred a borderline rescue effect (p=0.047) (Table 4.2, Table 4.3 and Table 4.4). Propionic and valeric acid had no significant rescue effect on CI (Table 4.2, Table 4.3 and Table 4.4). This data indicate that rescue induction/CI suppression is not a generalized effect of short chain fatty acids.

4.4.2. Testing for rescue induction/ CI Suppression by protein acetylation modifiers

NaBu is a well known HDAC inhibitor, which suppresses HDAC 1-5 and 7-9, representing the entirety of class I and class II HDAC enzymes (Ganai et al., 2016; Tandon et al., 2016). To determine whether the maternal HDAC function can induce a rescue effect on *w*Ri-induced CI, a number of HDAC inhibitors were tested. All these tests were carried out in the plate-based assay. The HDAC inhibitors tested here include the class I and II HDAC inhibitors quisinostat (Arts et al., 2009) and trichostatin A, as well as the pan HDAC inhibitor vorinostat (SAHA) (Ganai et al., 2016; Tandon et al.,2016) and CUDC-101, which targets class I and II HDACs, as well as grth factor receptors (Lai et al., 2010). The results from these experiments showed that none of the HDAC inhibitors exerted a significant rescue effect on CI hatch rates (Table 4.2, Table 4.3 and Table 4.4). To confirm the fly stock and the assay paramenters were as observed previously, NaBu was retested, and the data confirmed that the rescue effect conferred by NaBu was still significant (p-value < 0.01) (Table 4.4). These results indicate that HDAC inhibition and associated chromatin remodeling compounds are not a generalized mechanism for rescue induction/CI suppression.

4.4.3. Testing modifiers of DNA damage for maternal rescue induction /CI suppression effects

NaBu has also been shown to promote DNA damage repair by increasing the acetylation of histone H4 (Smerdon et al., 1982; Williamson et al., 2012; Mao & Wyrick, 2016). To test whether the maternal DNA damage repair process can effect *w*Ri-induced CI, an array of inhibitors were tested. The compounds tested here include celastrol and rotenone, which can induce oxidative DNA damage and activate DNA damage repair response (Sanders & Greenamyre, 2013; Xu et al., 2013; Moreira et al., 2019) and the alkylating agent cisplatin, which generates reactive oxygen (Basu & Krishnamurthy, 2010; Podratz et al., 2011; Rezaee et al., 2013). Topoisomerase inhibitors such as

camptothecin, which prevents topoisomerase I function, and teniposide, which prevents topoisomerase II function, were also included in this study (Rowe, Wang, & Liu, 1986; Hartmann & Lipp, 2006; Nitiss, 2009). The ribosome inhibitor cycloheximide was also tested in this experiment (Yoshioka et al., 1987). The concentration of each chemical compound was adjusted through the survival curve assay (Table 4.1) and tests were carried out in 24-well plate-based feeding assay. The experimental data showed an increase in egg hatch rate in females fed with celastrol, teniposide and cycloheximide, with a *p*-value of 0.001 or less (Table 4.2, Table 4.3 and Table 4.4). These data indicate the possible role of maternal DNA damage repair processes in conferring a rescue effect on CI embryos.

4.4.4. Testing the Impact of Cell-Cycle Timing on Maternal rescue induction/ CI Suppression

In addition to promoting DNA damage repair, NaBu has been also shown to slow cell cycle timing (D'Anna et al., 1980; Lallemand et al., 1996). To test whether maternal cell cycle timing has any effect on *w*Ri-induced CI, uninfected *D. simulans* were exposed to an array of cell cycle inhibitors. The cell cycle inhibitors included the microtubule destabilizer colchicine and griseofulvin, which slow down the progression of mitosis, altering microtubule dynamics. In addition, the microtubule stabilizer taxol was tested (Singh et al., 2008; Stanton et al., 2011). As inhibitors of the anaphase promoting complex, apcin and TAME, which slow anaphase onset and exit from mitosis, were also included (Zeng et al., 2010; Sackton et al., 2014). To inhibit the progress of mitosis and the cell cycle overall, the cyclin-dependent kinase inhibitors flavopiridol and roscovitine were used (Gray et al., 1999; Cicenas et al., 2015). Proteasome inhibitors such as bortezomib and MG132 were also included (Goldberg, 2012; Rastogi & Mishra, 2012). The MAPKK(MEK) inhibitor trametinib was also included in this study, which stalls the

general re-entry into the cell cycle (Zeiser, 2014; Kurata et al., 2016). The appropriate dose of chemical inhibitors were select from survival curve assays (Table 4.1) and the cell cycle inhibitors were tested for their ability to induce a rescue effect in CI crosses. The tests were carried out in 24-well plate assay. The plate assay data revealed a significant increase in CI hatch rate when compared to control for females exposed to bortezomib, MG132 and trametinib (p-value range: 0.001–0.005) (Table 4.2 and Table 4.4). This data was consistent with the data from previous experiments, as the cell cycle delays are a consequence of DNA damage. These data suggested a possible role of embryonic cell cycle timing in induction of a rescue effect in CI embryos.

4.4.5. Re-testing rescue-inducing/ CI-suppressing compounds against transinfected *D. simulans*

If the compounds that induced a rescue effect on *w*Ri-induced CI act in a conserved mechanism, then it is expected that they can induce a rescue effect on other host strain combinations. To test whether the rescue is induced in a conserved mechanism across other CI models, the compounds that were effective were re-tested in transinfected *w*Mel-induced CI for their ability to induce the rescue effect on CI crosses. The chemical compounds tested here were NaBu, celastrol, cycloheximide, teniposide, bortezomib, MG132, trametinib and acetic acid. These chemical inhibitors were tested in the plate assay and their doses were kept the same as the dose used previously. All the parameters were kept the same as previous experiments, with the only difference being that the *w*Mel-infected male flies were used here for CI induction. The results indicate that only NaBu, celastrol and cycloheximide were able to increase CI hatch rates for *w*Mel-induced CI (*p*-value range: 0.011–0.013) (Table 4.5, Table 4.6 and Table 4.7). Teniposide, bortezomib, and MG132 treatments exhibited borderline rescue effects (*p*-value range 0.041–0.047) (Table 4.5, Table 4.6 and Table 4.7). These results suggest

that certain DNA damage and/or cell cycle timing regulators might act as general contributors of rescue induction/suppression of CI in *D. simulans*.

4.4.6. Testing combined pathway effects for induction of rescue/ suppression of CI

To test whether CI suppression/rescue induction under these treatments was due to a shared network of pathways, a dual chemical treatment was pursued. This test included the chemical hits that showed the most robust rescue effect across both native and transinfected systems. These chemicals included NaBu, celastrol and cycloheximide, as well as additional treatment combinations which exerted comparatively modest effects, including teniposide, bortezomib and MG132. The dose of each chemical in the combination was selected by survival curve assays with dual chemical feeding on uninfected *D. simulans* flies (Table 4.8). Data from survival curve assays indicated that nearly all the treatment combinations required a reduced dose/concentration of chemical inhibitors compared to their singly administered treatments (Table 4.8). The cycloheximide/bortezomib combination was the only combination where the dose of chemicals in combination was same as their single dose value. These tests were carried out in a 24-well plate-based assay with *w*Ri-infected males to induce CI. The results indicate that the cycloheximide/bortezomib combination was the only combination which was able to significantly increase the CI hatch rate when compared to the control (*p-value*= 0.006) (Table 4.9, Table 4.10 and Table 4.11). Treatment with other chemical combinations were unable to induce significant rescue effects. It is possible that the loss of rescue induction may be caused by the reduced dosage needed for these experiments. These results indicate that CI suppression/ rescue induction might be associated with the manipulation of pathways involved in protein synthesis and protein turnover.

4.5 Discussion

The aim of this chapter was to determine the involvement of different cellular pathways in *Wolbachia*-induced CI. In previous chapters, it was observed that NaBu was able to induce a rescue effect in both natural *w*Ri and transinfected *w*Mel-induced CI in *D. simulans*. NaBu can influence diverse cellular pathways such as HDAC inhibition (Ganai et al., 2016; Tandon et al., 2016), DNA damage repair (Williamson et al., 2012; Mao & Wyrick, 2016) and cell cycle timing (D'Anna et al., 1980; Lallemand et al., 1996). In this chapter, we have used an array of different chemical inhibitors to influence these mechanisms through different cellular pathways and observe their effect on overall CI egg hatch rate. One of the technical limitations inherent to the method itself was the whole-body feedings, which lack the time and tissue-specific effect afforded to *Wolbachia in vivo*. Although dosages were optimized using survival curve assays, we were unable to control the tissue/cell specific chemical dosing of the flies. This could be due to differences in ingestion, absorption, efflux, metabolism, and/or excretion rates, which may vary with each cell type and each drug. Another drawback of this feeding assay was the possibility of affecting the gut microbiome of host *D. simulans* flies. For these reasons, seeing a response in a feeding assay was informative where being unable to see an effect is not necessarily informative.

This study was carried out to test a contribution of host mechanisms such as chromatin remodeling, DNA damage repair and cell cycle timing in overall rescue of CI *in vivo*. To address this, a 24-well plate-based assay was used, previously optimized for feeding assays. A total of 24 chemical inhibitors with diverse functions were tested for their ability to induce a rescue effect on *w*Ri-induced CI. Only 7 chemical inhibitors, Celastrol, Teniposide, Cycloheximide, Bortezomib, MG132, Trametinib and NaBu were able to significantly increase the egg hatch rates in CI crosses. These chemicals can

influence DNA damage repair mechanisms, cell cycle timing and protein turnover. Acetic acid casused an increase in egg hatch rates with a borderline *p*-value of 0.047. To understand whether the involvement of these cellular pathways were universal to different host-*Wolbachia* combination, the most effective chemicals were tested on the *w*Mel-induced CI model for their ability to induce a rescue effect. Only the three chemicals NaBu, Celastrol and Cycloheximide were able to induce a significant rescue effect on *w*Mel-induced CI on *D. simulans.* These chemicals can induce DNA damage repair mechanisms and/or inhibit the cell cycle and influence protein turnover.

To understand whether these maternal cellular pathways, which might be involved in the overall rescue of CI, were part of a shared network of pathways, the chemical inhibitors were used in combination to influence multiple pathways at the same time. And total of 7 different chemical combinations were then tested for their ability to increase the egg hatch rates in CI cross. The dosing of each chemical inhibitor in the combination was selected with the survival curve assay and the chemical combinations were tested on *w*Ri-induced CI model in the 24-well plate assay format. Interestingly, only the cycloheximide/bortezomib combination was able to increase egg hatch rates significantly. The other chemical combinations were unable to induce a rescue effect on CI crosses. One possible reason behind this was the dosing of chemicals in combination were not strong enough to induce a possible rescue effect.

One question raised by these experiments is why some chemical compounds were able to affect the egg hatch rate in both *w*Ri and *w*Mel-induced CI, while some chemicals were able to affect the egg hatch rate only in *w*Ri-induced CI. A possible explanation could invovle the intrinsic difference between *w*Ri and *w*Mel-induced CI models. There might be possible involvement of certain *Wolbachia* given factors, such as Cif proteins, since Cif proteins are different between *w*Ri and *w*Mel *Wolbachia*.

Further tests with more rescue-inducing compounds, on different host/*Wolbachia* combinations, are necessary to answer this question with certainty.

To summarize, the aim of this chapter was to determine the involvement of specific cellular pathways in the *Wolbachia*-induced rescue mechanism. To test this, uninfected female *D. simulans* flies were exposed to 24 different chemical inhibitors and tested to determine whether they can increase egg hatch rates in CI crosses. The chemical inhibitors were tested on both *w*Ri- and *w*Mel-induced CI. A total of 7 different chemical compounds inducing HDAC inhibition, DNA damage repair and cell cycle delay were found to increase the egg hatch rate in *w*Ri-induced CI cross. When these 7 hit chemical compounds were tested on *w*Mel-induced CI, only 3 chemicals targeting HDAC and DNA damage repair, were able to significantly increase the egg hatch rate in CI crosses.

In general, this study found that “hit” compounds that suppressed *w*Ri-induced CI exerted overall weaker effects on *w*Mel-induced CI. To test whether the cellular pathways act in a network, the chemical inhibitors were then used in combination and tested on the native CI model. Only the cycloheximide/bortezomib combination was able to induce a significant rescue effect, which can protect DNA integrity and cause cell cycle delay. Overall, maintaining DNA integrity or cell cycle delay could be the sole mechanism involved in overall rescue of CI. Finally, the technique presented here could be used in different CI models to determine the involvement of other cellular pathways in the overall rescue mechanism.

Tables

Table 4.1. Qualitative assessment of *D. simulans* fecundity and development in response to candidate drug dose curves.

Treatment tested	Dose	Fecundity	Hatchablility	Larval development
Acetic acid	350 mM	(-)	(-)	(-)
	200 mM	(-)	(-)	(-)
	150 mM	(+)	(-)	(-)
	100 mM	**(+)**	**(+)**	**(+)**
	50 mM	(+)	(+)	(+)
	10 mM	(+)	(+)	(+)
	Control (water)	(+)	(+)	(+)
Propionic acid	500 mM	(-)	(-)	(-)
	350 mM	(-)	(-)	(-)
	150 mM	(-)	(-)	(-)
	100 mM	(-)	(-)	(-)
	50 mM	(+)	(-)	(-)
	25 mM	(+)	(-)	some
	10 mM	**(+)**	**(+)**	**(+)**
	Control (water)	(+)	(+)	(+)
Valeric acid	350 mM	(-)	(-)	(-)
	200 mM	(-)	(-)	(-)
	100 mM	(-)	(-)	(-)
	50 mM	(-)	(-)	(-)
	10 mM	(-)	(-)	(-)
	5 mM	**(+)**	**(+)**	**(+)**
	1 mM	(+)	(+)	(+)
	Control (water)	(+)	(+)	(+)
Quisinostat	10 µM	(+)	(-)	(-)
	5 µM	(+)	(-)	(-)
	2.5 µM	(+)	(+)	(-)
	1 µM	**(+)**	**(+)**	**(+)**
	500 nM	(+)	(+)	(+)
	250 nM	(+)	(+)	(+)
	100 nM	(+)	(+)	(+)
	Control (DMSO)	(+)	(+)	(+)
Trichostatin A	50 µM	(-)	(-)	(-)
	25 µM	some	some	some

Treatment tested	Dose	Fecundity	Hatchablility	Larval development
	10 µM	**(+)**	**(+)**	**(+)**
	5 µM	(+)	(+)	(+)
	2.5 µM	(+)	(+)	(+)
	1 µM	(+)	(+)	(+)
	100 nM	(+)	(+)	(+)
	Control (DMSO)	(+)	(+)	(+)
Vorinostat/SAHA	200 µM	(-)	(-)	(-)
	100 µM	(-)	(-)	(-)
	75 µM	(+)	some	(-)
	50 µM	**(+)**	**(+)**	**(+)**
	25 µM	(+)	(+)	(+)
	10 µM	(+)	(+)	(+)
	5 µM	(+)	(+)	(+)
	Control (DMSO)	(+)	(+)	(+)
CUDC-101	500 µM	(-)	(+)	(-)
	250 µM	**(+)**	**(+)**	**(+)**
	100 µM	(+)	(+)	(+)
	50 µM	(+)	(+)	(+)
	10 µM	(+)	(+)	(+)
	5 µM	(+)	(+)	(+)
	1 µM	(+)	(+)	(+)
	Control (DMSO)	(+)	(+)	(+)
Celastrol	200 µM	(-)	(-)	(-)
	100 µM	(-)	(-)	(-)
	50 µM	some	some	(+)
	20 µM	**(+)**	**(+)**	**(+)**
	10 µM	(+)	(+)	(+)
	5 µM	(+)	(+)	(+)
	1 µM	(+)	(+)	(+)
	Control (DMSO)	(+)	(+)	(+)
Rotenone	100 µM	(-)	(-)	(-)
	50 µM	(-)	(-)	(-)
	20 µM	some	some	(-)
	10 µM	**(+)**	**(+)**	**(+)**
	5 µM	(+)	(+)	(+)

Treatment tested	Dose	Fecundity	Hatchablility	Larval development
	1 µM	(+)	(+)	(+)
	Control (DMSO)	(+)	(+)	(+)
Cisplatin	350 µM	some	(-)	(-)
	200 µM	some	some	some
	100 µM	**(+)**	**(+)**	**(+)**
	60 µM	(+)	(+)	(+)
	30 µM	(+)	(+)	(+)
	15 µM	(+)	(+)	(+)
	5 µM	(+)	(+)	(+)
	1 µM	(+)	(+)	(+)
	Control (DMSO)	(+)	(+)	(+)
Camptothecin	5 mM	(-)	(-)	(-)
	1 mM	(-)	(-)	(-)
	500 µM	(-)	(-)	(-)
	300 µM	(+)	(-)	(-)
	200 µM	(+)	(-)	(-)
	100 µM	(+)	(+)	some
	50 µM	**(+)**	**(+)**	**(+)**
	20 µM	(+)	(+)	(+)
	10 µM	(+)	(+)	(+)
	Control (DMSO)	(+)	(+)	(+)
Teniposide	2 mM	(-)	(-)	(-)
	1 mM	(-)	(+)	some
	500 µM	**(+)**	**(+)**	**(+)**
	300 µM	(+)	(+)	(+)
	200 µM	(+)	(+)	(+)
	100 µM	(+)	(+)	(+)
	50 µM	(+)	(+)	(+)
	Control (DMSO)	(+)	(+)	(+)
Cycloheximide	350 µM	(-)	(-)	(-)
	250 µM	some	(-)	(-)
	100 µM	(+)	some	some
	50 µM	**(+)**	**(+)**	**(+)**
	20 µM	(+)	(+)	(+)
	10 µM	(+)	(+)	(+)

Treatment tested	Dose	Fecundity	Hatchablility	Larval development
	1 µM	(+)	(+)	(+)
	Control (DMSO)	(+)	(+)	(+)
Colchicine	50 µM	(-)	(-)	(-)
	10 µM	(-)	(-)	(-)
	5 µM	(+)	(-)	(-)
	2.5 µM	**(+)**	**(+)**	**(+)**
	1 µM	(+)	(+)	(+)
	500 nM	(+)	(+)	(+)
	250 nM	(+)	(+)	(+)
	Control (DMSO)	(+)	(+)	(+)
Griseofulvin	5 mM	(-)	(-)	(-)
	3 mM	(-)	(-)	(-)
	1 mM	some	(-)	(-)
	500 µM	some	(-)	(-)
	300 µM	**(+)**	**(+)**	**(+)**
	100 µM	(+)	(+)	(+)
	Control (DMSO)	(+)	(+)	(+)
Taxol	10 µM	(-)	(-)	(-)
	5 µM	some	some	some
	2.5 µM	some	(-)	(+)
	1 µM	**(+)**	**(+)**	**(+)**
	500 nM	(+)	(+)	(+)
	100 nM	(+)	(+)	(+)
	Control (DMSO)	(+)	(+)	(+)
Apcin	1 mM	(-)	(-)	(-)
	500 µM	(-)	(+)	some
	300 µM	**(+)**	**(+)**	**(+)**
	200 µM	(+)	(+)	(+)
	100 µM	(+)	(+)	(+)
	50 µM	(+)	(+)	(+)
	20 µM	(+)	(+)	(+)
	10 µM	(+)	(+)	(+)
	5 µM	(+)	(+)	(+)
	Control (DMSO)	(+)	(+)	(+)
TAME	50 mM	some	some	(-)

Treatment tested	Dose	Fecundity	Hatchablility	Larval development
	30 mM	(+)	(+)	some
	20 mM	**(+)**	**(+)**	**(+)**
	10 mM	(+)	(+)	(+)
	5 mM	(+)	(+)	(+)
	Control (water)	(+)	(+)	(+)
Flavopiridol	75 µM	(-)	(-)	(-)
	50 µM	(-)	(-)	(-)
	25 µM	some	some	(-)
	10 µM	**(+)**	**(+)**	**(+)**
	5 µM	(+)	(+)	(+)
	1 µM	(+)	(+)	(+)
	500 nM	(+)	(+)	(+)
	100 nM	(+)	(+)	(+)
	Control (DMSO)	(+)	(+)	(+)
Roscovitine	500 µM	(-)	(-)	(-)
	250 µM	(-)	some	(-)
	100 µM	**(+)**	**(+)**	**(+)**
	50 µM	(+)	(+)	(+)
	20 µM	(+)	(+)	(+)
	10 µM	(+)	(+)	(+)
	Control (DMSO)	(+)	(+)	(+)
Bortezomib	25µM	(-)	(-)	(-)
	10µM	(-)	(-)	(-)
	5µM	some	some	(+)
	1µM	**(+)**	**(+)**	**(+)**
	500nM	(+)	(+)	(+)
	100nM	(+)	(+)	(+)
	Control (DMSO)	(+)	(+)	(+)
MG132	200 µM	(-)	(-)	(-)
	100 µM	(+)	some	(-)
	50 µM	**(+)**	**(+)**	**(+)**
	20 µM	(+)	(+)	(+)
	10 µM	(+)	(+)	(+)
	5 µM	(+)	(+)	(+)

Treatment tested	Dose	Fecundity	Hatchablility	Larval development
	2 µM	(+)	(+)	(+)
	1 µM	(+)	(+)	(+)
	Control (DMSO)	(+)	(+)	(+)
Trametinib	5 µM	(-)	(-)	(-)
	1 µM	(-)	(-)	(-)
	500 nM	(+)	(-)	some
	250 nM	**(+)**	**(+)**	**(+)**
	100 nM	(+)	(+)	(+)
	Control (DMSO)	(+)	(+)	(+)

*Dose used is indicated in bold

Table 4.2. Impact of chemical treatments on CI egg hatch rate in *D. simulans*-*w*Ri system. Chemicals treatments were used to affect the cellular pathways and the effect of chemical treatments were observed for their ability to induce a rescue effect on *w*Ri-induced CI in *D. simulans*.

Putative Rescue effect tested by the treatment	Chemical used	Reputed cellular effect	Dose used	# wells tested (# plates)	Sig increase in hatch rate?	p-value
Diverse functions	**NaBu**	**C4 short chain fatty acid. Affects HDAC function, DNA damage repair, cell cycle**	**50 mM**	**40(5)**	**Yes**	**<0.001**
Short chain fatty acids	Acetic acid	C2 short chain fatty acid	100 mM	16(2)	Borderline	0.047
	Propionic acid	C3 short chain fatty acid	10 mM	16(2)	No	0.769
	Valeric acid	C5 short chain fatty acid	5mM	16(2)	No	0.926
Chromatin modification	Vorinostat/SAHA	HDAC inhibitor	75 uM	16(2)	No	0.913
	Trichostatin A	HDAC inhibitor	10 uM	16(2)	No	0.696
	Quisionstat	HDAC inhibitor	1 uM	16(2)	No	0.235
	CUDC-101	Inhibits HDACs, EGFR, HER2	250 uM	16(2)	No	0.610
DNA damage	**Celastrol**	**Antioxidant (also inh MEK, proteasome)**	**20 uM**	**16(2)**	**Yes**	**0.001**
	Rotenone	ROS-generating	10 uM	16(2)	No	0.149
	Cisplatin	alkylating agent, induces DNA damage	100 uM	16(2)	No	0.065
	Camptothecin	Inhibits Topol, induces DNA damage	50 uM	16(2)	No	0.059
	Teniposide	**Inhibits Topoll**	**500 uM**	**16(2)**	**Yes**	**<0.001**
	Cycloheximide	**DNA damage (also ribosome inhibitor)**	**50 uM**	**16(2)**	**Yes**	**<0.001**
Cell cycle delay	Colchicine	Suppresses microtubules	2.5 uM	16(2)	No	0.967
	Griseofulvin	Stabilizes microtubules	300 uM	16(2)	No	0.675
	Taxol	Stabilizes microtubules	1 uM	16(2)	No	0.774
	Apcin	APC/C inhibitor	300 uM	16(2)	No	0.061
	TAME	APC inhibitor	20 mM	16(2)	No	0.410

Putative Rescue effect tested by the treatment	Chemical used	Reputed cellular effect	Dose used	# wells tested (# plates)	Sig increase in hatch rate?	p-value
	Flavopiridol	CDK inhibitor	10 uM	16(2)	No	0.360
	Roscovitine	CDK inhibitor	100 uM	16(2)	No	0.175
	Bortezomib	**Proteasome inhibitor**	**1 uM**	**16(2)**	**Yes**	**0.005**
	MG132	**Proteasome inhibitor**	**50 uM**	**16(2)**	**Yes**	**0.001**
	Trametinib	**MEK inhibitor (also ribosome disruptor)**	**250 nM**	**16(2)**	**Yes**	**0.002**

Table 4.3. Impact of candidate drugs on *D. simulans w*Ri CI hatch rates in plate assay format.

Treatment tested	CI condition				CI + drug treatment				Rescue condition			
	Unhatched	Hatched	total	hatch rate	Unhatched	Hatched	total	hatch rate	Unhatched	Hatched	total	hatch rate
NaBu test 1	61	1	62	1.6%	67	3	70	4.3%	3	67	70	95.7%
	53	4	57	7.0%	78	4	82	4.9%	2	71	73	97.3%
	82	2	84	2.4%	93	9	102	8.8%	7	84	91	92.3%
	86	4	90	4.4%	94	14	108	13.0%	5	73	78	93.6%
	107	6	113	5.3%	65	7	72	9.7%	4	78	82	95.1%
	88	3	91	3.3%	76	17	93	18.3%	9	102	111	91.9%
	84	2	86	2.3%	91	13	104	12.5%	3	88	91	96.7%
	112	7	119	5.9%	78	10	88	11.4%	2	81	83	97.6%
NaBu test 2	107	3	110	2.7%	72	9	81	11.1%	8	71	79	89.9%
	92	5	97	5.2%	93	12	105	11.4%	4	79	83	95.2%
	126	8	134	6.0%	88	15	103	14.6%	13	82	95	86.3%
	115	11	126	8.7%	85	12	97	12.4%	5	96	101	95.0%
	108	9	117	7.7%	90	14	104	13.5%	6	101	107	94.4%
	78	7	85	8.2%	87	20	107	18.7%	3	91	94	96.8%
	152	13	165	7.9%	74	7	81	8.6%	7	98	105	93.3%
	121	10	131	7.6%	92	23	115	20.0%	2	106	108	98.1%
Acetic acid test 1	110	5	115	4.3%	108	10	118	8.5%	5	62	67	92.5%
	93	5	98	5.1%	123	14	137	10.2%	0	58	58	100.0%
	83	3	86	3.5%	92	13	105	12.4%	2	55	57	96.5%
	94	7	101	6.9%	87	6	93	6.5%	3	68	71	95.8%
	71	3	74	4.1%	85	4	89	4.5%	1	70	71	98.6%
	96	9	105	8.6%	76	4	80	5.0%	3	61	64	95.3%

Treatment tested	CI condition				CI + drug treatment				Rescue condition			
	Unhatched	Hatched	total	hatch rate	Unhatched	Hatched	total	hatch rate	Unhatched	Hatched	total	hatch rate
	107	2	109	1.8%	69	3	72	4.2%	3	58	61	95.1%
	111	5	116	4.3%	79	4	83	4.8%	4	62	66	93.9%
Acetic acid test 2	86	3	89	3.4%	71	6	77	7.8%	2	82	84	97.6%
	79	2	81	2.5%	56	3	59	5.1%	1	73	74	98.6%
	74	1	75	1.3%	84	2	86	2.3%	0	55	55	100.0%
	89	4	93	4.3%	53	1	54	1.9%	2	59	61	96.7%
	101	3	104	2.9%	48	0	48	0.0%	4	79	83	95.2%
	68	0	68	0.0%	57	3	60	5.0%	1	49	50	98.0%
	81	2	83	2.4%	77	5	82	6.1%	5	90	95	94.7%
	58	0	58	0.0%	61	2	63	3.2%	4	66	70	94.3%
Propionic acid test 1	115	8	123	6.5%	110	13	123	10.6%	10	120	130	92.3%
	120	10	130	7.7%	94	9	103	8.7%	3	100	103	97.1%
	80	3	83	3.6%	102	3	105	2.9%	15	126	141	89.4%
	135	6	141	4.3%	79	10	89	11.2%	10	115	125	92.0%
	118	15	133	11.3%	68	11	79	13.9%	9	125	134	93.3%
	105	7	112	6.3%	125	11	136	8.1%	1	85	86	98.8%
	78	7	85	8.2%	102	13	115	11.3%	5	100	105	95.2%
	138	15	153	9.8%	66	8	74	10.8%	11	132	143	92.3%
Propionic acid test 2	82	9	91	9.9%	106	14	120	11.7%	4	123	127	96.9%
	124	6	130	4.6%	63	3	66	4.5%	6	103	109	94.5%
	123	11	134	8.2%	103	4	107	3.7%	3	72	75	96.0%
	81	7	88	8.0%	87	0	87	0.0%	5	104	109	95.4%
	102	5	107	4.7%	53	8	61	13.1%	7	131	138	94.9%
	62	8	70	11.4%	61	1	62	1.6%	2	86	88	97.7%

Treatment tested	CI condition				CI + drug treatment				Rescue condition			
	Unhatched	Hatched	total	hatch rate	Unhatched	Hatched	total	hatch rate	Unhatched	Hatched	total	hatch rate
	87	4	91	4.4%	75	9	84	10.7%	5	91	96	94.8%
	137	13	150	8.7%	86	1	87	1.1%	4	98	102	96.1%
Valeric acid test 1	87	6	93	6.5%	83	4	87	4.6%	1	62	63	98.4%
	65	7	72	9.7%	75	8	83	9.6%	4	74	78	94.9%
	73	2	75	2.7%	81	4	85	4.7%	3	66	69	95.7%
	69	11	80	13.8%	56	3	59	5.1%	3	74	77	96.1%
	85	8	93	8.6%	54	2	56	3.6%	6	59	65	90.8%
	79	1	80	1.3%	77	7	84	8.3%	2	53	55	96.4%
	64	5	69	7.2%	69	5	74	6.8%	4	61	65	93.8%
	89	5	94	5.3%	72	7	79	8.9%	3	72	75	96.0%
Valeric acid test 2	86	6	92	6.5%	106	19	125	15.2%	4	68	72	94.4%
	69	5	74	6.8%	92	6	98	6.1%	4	81	85	95.3%
	101	2	103	1.9%	98	7	105	6.7%	4	121	125	96.8%
	35	5	40	12.5%	94	5	99	5.1%	3	97	100	97.0%
	116	10	126	7.9%	96	13	109	11.9%	2	71	73	97.3%
	96	19	115	16.5%	103	18	121	14.9%	3	97	100	97.0%
	129	11	140	7.9%	93	17	110	15.5%	3	102	105	97.1%
	95	10	105	9.5%	119	5	124	4.0%	2	58	60	96.7%
Quisinostat test 1	92	10	102	9.8%	102	7	109	6.4%	6	117	123	95.1%
	110	6	116	5.2%	96	7	103	6.8%	5	107	112	95.5%
	102	5	107	4.7%	83	5	88	5.7%	4	98	102	96.1%
	99	9	108	8.3%	125	9	134	6.7%	5	89	94	94.7%
	114	13	127	10.2%	89	6	95	6.3%	6	88	94	93.6%
	116	5	121	4.1%	86	11	97	11.3%	5	101	106	95.3%

Treatment tested	CI condition				CI + drug treatment				Rescue condition			
	Unhatched	Hatched	total	hatch rate	Unhatched	Hatched	total	hatch rate	Unhatched	Hatched	total	hatch rate
	88	7	95	7.4%	92	13	105	12.4%	3	94	97	96.9%
	92	3	95	3.2%	98	3	101	3.0%	8	102	110	92.7%
Quisinostat test 2	57	6	63	9.5%	60	7	67	10.4%	3	62	65	95.4%
	70	2	72	2.8%	92	4	96	4.2%	7	73	80	91.3%
	72	4	76	5.3%	96	8	104	7.7%	3	62	65	95.4%
	60	1	61	1.6%	82	3	85	3.5%	2	58	60	96.7%
	51	2	53	3.8%	79	7	86	8.1%	2	60	62	96.8%
	60	5	65	7.7%	96	3	99	3.0%	4	71	75	94.7%
	83	3	86	3.5%	76	6	82	7.3%	4	68	72	94.4%
	78	5	83	6.0%	98	10	108	9.3%	1	59	60	98.3%
Trichostatin test 1	95	12	107	11.2%	78	5	83	6.0%	3	94	97	96.9%
	102	4	106	3.8%	84	6	90	6.7%	4	92	96	95.8%
	85	6	91	6.6%	91	9	100	9.0%	2	90	92	97.8%
	115	7	122	5.7%	90	12	102	11.8%	2	80	82	97.6%
	110	9	119	7.6%	94	8	102	7.8%	2	81	83	97.6%
	81	12	93	12.9%	82	5	87	5.7%	4	79	83	95.2%
	89	7	96	7.3%	87	4	91	4.4%	6	83	89	93.3%
	111	5	116	4.3%	83	6	89	6.7%	4	91	95	95.8%
Trichostatin test 2	50	15	65	23.1%	88	7	95	7.4%	4	72	76	94.7%
	103	9	112	8.0%	63	10	73	13.7%	10	30	40	75.0%
	88	3	91	3.3%	26	1	27	3.7%	12	65	77	84.4%
	122	4	126	3.2%	97	5	102	4.9%	0	85	85	100.0%
	75	5	80	6.3%	63	3	66	4.5%	2	24	26	92.3%
	99	4	103	3.9%	28	4	32	12.5%	5	62	67	92.5%

Treatment tested	CI condition				CI + drug treatment				Rescue condition			
	Unhatched	Hatched	total	hatch rate	Unhatched	Hatched	total	hatch rate	Unhatched	Hatched	total	hatch rate
	79	6	85	7.1%	39	4	43	9.3%	6	38	44	86.4%
	101	10	111	9.0%	130	7	137	5.1%	2	92	94	97.9%
Vorinostat / SAHA 1	101	6	107	5.6%	190	27	217	12.4%	3	115	118	97.5%
	71	12	83	14.5%	220	20	240	8.3%	3	112	115	97.4%
	91	15	106	14.2%	160	9	169	5.3%	7	66	73	90.4%
	82	5	87	5.7%	144	18	162	11.1%	14	79	93	84.9%
	117	7	124	5.6%	186	27	213	12.7%	5	140	145	96.6%
	128	9	137	6.6%	187	43	230	18.7%	5	103	108	95.4%
	136	14	150	9.3%	207	12	219	5.5%	4	87	91	95.6%
	140	6	146	4.1%	197	10	207	4.8%	1	88	89	98.9%
Vorinostat / SAHA 2	103	15	118	12.7%	181	5	186	2.7%	0	82	82	100.0%
	121	9	130	6.9%	107	1	108	0.9%	3	143	146	97.9%
	98	4	102	3.9%	148	4	152	2.6%	6	119	125	95.2%
	95	5	100	5.0%	147	1	148	0.7%	6	72	78	92.3%
	110	8	118	6.8%	76	8	84	9.5%	16	152	168	90.5%
	161	3	164	1.8%	89	6	95	6.3%	2	157	159	98.7%
	144	10	154	6.5%	81	3	84	3.6%	7	124	131	94.7%
	138	1	139	0.7%	110	9	119	7.6%	4	155	159	97.5%
CUDC-101 test 1	61	5	66	0.076	92	3	95	0.032	10	120	130	92.3%
	42	4	46	0.087	77	2	79	0.025	3	100	103	97.1%
	70	2	72	0.028	63	2	65	0.031	15	126	141	89.4%
	49	1	50	0.020	62	5	67	0.075	10	115	125	92.0%
	59	2	61	0.033	58	12	70	0.171	9	125	134	93.3%
	34	7	41	0.171	79	7	86	0.081	1	85	86	98.8%

Treatment tested	CI condition				CI + drug treatment				Rescue condition			
	Unhatched	Hatched	total	hatch rate	Unhatched	Hatched	total	hatch rate	Unhatched	Hatched	total	hatch rate
	41	3	44	0.068	66	6	72	0.083	5	100	105	95.2%
	67	3	70	0.043	62	21	83	0.253	11	132	143	92.3%
CUDC-101 test 2	91	3	94	0.032	63	1	64	0.016	3	71	74	95.9%
	112	6	118	0.051	87	6	93	0.065	2	79	81	97.5%
	107	5	112	0.045	76	7	83	0.084	3	84	87	96.6%
	108	6	114	0.053	89	10	99	0.101	5	87	92	94.6%
	89	7	96	0.073	111	3	114	0.026	4	93	97	95.9%
	114	10	124	0.081	86	7	93	0.075	3	87	90	96.7%
	92	9	101	0.089	89	2	91	0.022	2	91	93	97.8%
	108	13	121	0.107	73	8	81	0.099	5	96	101	95.0%
Celastrol test 1	149	15	164	9.1%	100	10	110	9.1%	17	120	137	87.6%
	74	2	76	2.6%	50	11	61	18.0%	5	101	106	95.3%
	87	6	93	6.5%	95	13	108	12.0%	15	116	131	88.5%
	49	3	52	5.8%	103	9	112	8.0%	6	112	118	94.9%
	51	4	55	7.3%	120	13	133	9.8%	3	115	118	97.5%
	68	2	70	2.9%	80	12	92	13.0%	7	110	117	94.0%
	95	7	102	6.9%	107	15	122	12.3%	10	108	118	91.5%
	92	2	94	2.1%	97	16	113	14.2%	4	130	134	97.0%
Celastrol test 2	96	5	101	5.0%	72	10	82	12.2%	3	92	95	96.8%
	70	6	76	7.9%	79	8	87	9.2%	4	85	89	95.5%
	92	13	105	12.4%	93	15	108	13.9%	7	83	90	92.2%
	80	8	88	9.1%	91	7	98	7.1%	12	79	91	86.8%
	97	6	103	5.8%	123	12	135	8.9%	8	110	118	93.2%
	89	10	99	10.1%	137	7	144	4.9%	2	98	100	98.0%

Treatment tested	CI condition				CI + drug treatment				Rescue condition			
	Unhatched	Hatched	total	hatch rate	Unhatched	Hatched	total	hatch rate	Unhatched	Hatched	total	hatch rate
	95	5	100	5.0%	92	5	97	5.2%	3	101	104	97.1%
	102	6	108	5.6%	108	16	124	12.9%	5	96	101	95.0%
Rotenone test 1	127	4	131	3.1%	112	10	122	8.2%	3	98	101	97.0%
	112	18	130	13.8%	108	17	125	13.6%	2	96	98	98.0%
	125	11	136	8.1%	130	16	146	11.0%	3	102	105	97.1%
	145	2	147	1.4%	122	15	137	10.9%	2	99	101	98.0%
	128	4	132	3.0%	202	19	221	8.6%	4	108	112	96.4%
	112	2	114	1.8%	187	24	211	11.4%	1	87	88	98.9%
	134	1	135	0.7%	208	5	213	2.3%	3	114	117	97.4%
	109	3	112	2.7%	137	23	160	14.4%	2	105	107	98.1%
Rotenone test 2	106	1	107	0.9%	135	18	153	11.8%	11	110	121	90.9%
	104	16	120	13.3%	99	5	104	4.8%	5	107	112	95.5%
	135	3	138	2.2%	103	3	106	2.8%	7	103	110	93.6%
	120	6	126	4.8%	109	6	115	5.2%	10	101	111	91.0%
	119	21	140	15.0%	80	3	83	3.6%	7	93	100	93.0%
	132	15	147	10.2%	95	9	104	8.7%	4	97	101	96.0%
	118	12	130	9.2%	101	3	104	2.9%	12	118	130	90.8%
	116	9	125	7.2%	89	7	96	7.3%	4	115	119	96.6%
Cisplatin test 1	77	5	82	6.1%	42	6	48	12.5%	2	85	87	97.7%
	66	3	69	4.3%	91	9	100	9.0%	1	67	68	98.5%
	81	6	87	6.9%	72	3	75	4.0%	1	57	58	98.3%
	57	1	58	1.7%	54	3	57	5.3%	4	53	57	93.0%
	65	7	72	9.7%	74	7	81	8.6%	0	42	42	100.0%
	54	4	58	6.9%	64	8	72	11.1%	2	62	64	96.9%

Treatment tested	CI condition				CI + drug treatment				Rescue condition			
	Unhatched	Hatched	total	hatch rate	Unhatched	Hatched	total	hatch rate	Unhatched	Hatched	total	hatch rate
	69	2	71	2.8%	86	6	92	6.5%	5	46	51	90.2%
	73	2	75	2.7%	73	3	76	3.9%	3	58	61	95.1%
Cisplatin test 2	130	3	133	2.3%	136	19	155	12.3%	2	92	94	97.9%
	118	5	123	4.1%	120	4	124	3.2%	10	89	99	89.9%
	133	17	150	11.3%	145	34	179	19.0%	6	98	104	94.2%
	126	14	140	10.0%	104	23	127	18.1%	6	123	129	95.3%
	150	13	163	8.0%	150	28	178	15.7%	4	150	154	97.4%
	110	13	123	10.6%	119	18	137	13.1%	9	117	126	92.9%
	176	33	209	15.8%	142	11	153	7.2%	27	134	161	83.2%
	139	21	160	13.1%	89	23	112	20.5%	13	104	117	88.9%
Camptothecin test 1	129	3	132	2.3%	89	2	91	2.2%	2	108	110	98.2%
	138	3	141	2.1%	82	2	84	2.4%	3	105	108	97.2%
	147	4	151	2.6%	75	1	76	1.3%	4	130	134	97.0%
	116	2	118	1.7%	77	1	78	1.3%	1	124	125	99.2%
	123	2	125	1.6%	85	3	88	3.4%	0	129	129	100.0%
	140	2	142	1.4%	90	4	94	4.3%	4	114	118	96.6%
	101	5	106	4.7%	107	6	113	5.3%	1	98	99	99.0%
	97	1	98	1.0%	75	4	79	5.1%	2	96	98	98.0%
Camptothecin test 2	150	7	157	4.5%	112	21	133	15.8%	8	130	138	94.2%
	109	4	113	3.5%	118	23	141	16.3%	3	136	139	97.8%
	135	11	146	7.5%	140	16	156	10.3%	12	154	166	92.8%
	112	13	125	10.4%	165	16	181	8.8%	5	121	126	96.0%
	105	9	114	7.9%	104	17	121	14.0%	4	160	164	97.6%
	117	8	125	6.4%	121	16	137	11.7%	7	103	110	93.6%

Treatment tested	CI condition				CI + drug treatment				Rescue condition			
	Unhatched	Hatched	total	hatch rate	Unhatched	Hatched	total	hatch rate	Unhatched	Hatched	total	hatch rate
	114	5	119	4.2%	112	12	124	9.7%	7	117	124	94.4%
	102	12	114	10.5%	106	10	116	8.6%	11	110	121	90.9%
Teniposide test 1	89	9	98	9.2%	126	10	136	7.4%	3	101	104	97.1%
	102	4	106	3.8%	120	17	137	12.4%	5	105	110	95.5%
	84	6	90	6.7%	94	12	106	11.3%	3	104	107	97.2%
	99	5	104	4.8%	144	14	158	8.9%	4	82	86	95.3%
	78	9	87	10.3%	80	11	91	12.1%	6	114	120	95.0%
	108	4	112	3.6%	109	16	125	12.8%	7	93	100	93.0%
	109	6	115	5.2%	104	16	120	13.3%	3	91	94	96.8%
	146	16	162	9.9%	148	22	170	12.9%	3	106	109	97.2%
Teniposide test 2	113	10	123	8.1%	120	13	133	9.8%	4	76	80	95.0%
	126	10	136	7.4%	143	21	164	12.8%	16	129	145	89.0%
	119	14	133	10.5%	106	10	116	8.6%	8	107	115	93.0%
	94	3	97	3.1%	137	20	157	12.7%	2	90	92	97.8%
	135	12	147	8.2%	101	19	120	15.8%	3	98	101	97.0%
	89	7	96	7.3%	108	12	120	10.0%	1	93	94	98.9%
	91	5	96	5.2%	115	24	139	17.3%	1	104	105	99.0%
	118	11	129	8.5%	53	6	59	10.2%	7	82	89	92.1%
Cycloheximide test 1	71	7	78	9.0%	95	8	103	7.8%	1	81	82	98.8%
	95	5	100	5.0%	102	15	117	12.8%	5	92	97	94.8%
	105	8	113	7.1%	118	21	139	15.1%	1	101	102	99.0%
	84	4	88	4.5%	98	16	114	14.0%	3	72	75	96.0%
	120	12	132	9.1%	93	15	108	13.9%	3	80	83	96.4%
	93	6	99	6.1%	114	26	140	18.6%	1	46	47	97.9%

Treatment tested	CI condition				CI + drug treatment				Rescue condition			
	Unhatched	Hatched	total	hatch rate	Unhatched	Hatched	total	hatch rate	Unhatched	Hatched	total	hatch rate
	102	9	111	8.1%	96	20	116	17.2%	2	55	57	96.5%
	90	11	101	10.9%	92	7	99	7.1%	2	40	42	95.2%
Cycloheximide test 2	133	23	156	14.7%	92	3	95	3.2%	4	87	91	95.6%
	141	9	150	6.0%	83	13	96	13.5%	3	73	76	96.1%
	131	15	146	10.3%	96	15	111	13.5%	5	95	100	95.0%
	114	4	118	3.4%	82	23	105	21.9%	6	82	88	93.2%
	78	3	81	3.7%	77	18	95	18.9%	4	76	80	95.0%
	119	12	131	9.2%	92	28	120	23.3%	1	71	72	98.6%
	113	7	120	5.8%	127	9	136	6.6%	7	90	97	92.8%
	98	8	106	7.5%	64	11	75	14.7%	3	81	84	96.4%
Colchicine test 1	135	5	140	3.6%	120	14	134	10.4%	3	60	63	95.2%
	82	5	87	5.7%	105	5	110	4.5%	7	130	137	94.9%
	95	8	103	7.8%	125	16	141	11.3%	4	85	89	95.5%
	102	6	108	5.6%	118	4	122	3.3%	10	110	120	91.7%
	85	11	96	11.5%	120	12	132	9.1%	5	95	100	95.0%
	55	8	63	12.7%	65	8	73	11.0%	5	90	95	94.7%
	108	11	119	9.2%	120	4	124	3.2%	3	80	83	96.4%
	95	18	113	15.9%	95	4	99	4.0%	7	130	137	94.9%
Colchicine test 2	103	16	119	13.4%	110	22	132	16.7%	7	103	110	93.6%
	120	15	135	11.1%	123	11	134	8.2%	8	78	86	90.7%
	125	13	138	9.4%	103	12	115	10.4%	10	135	145	93.1%
	125	11	136	8.1%	122	19	141	13.5%	4	120	124	96.8%
	155	21	176	11.9%	140	18	158	11.4%	3	105	108	97.2%
	92	10	102	9.8%	96	19	115	16.5%	4	98	102	96.1%

Treatment tested	CI condition				CI + drug treatment				Rescue condition			
	Unhatched	Hatched	total	hatch rate	Unhatched	Hatched	total	hatch rate	Unhatched	Hatched	total	hatch rate
	83	6	89	6.7%	94	9	103	8.7%	6	130	136	95.6%
	162	13	175	7.4%	83	6	89	6.7%	3	72	75	96.0%
Griseofulvin test 1	118	9	127	7.1%	111	7	118	5.9%	7	92	99	92.9%
	86	8	94	8.5%	87	6	93	6.5%	4	76	80	95.0%
	115	8	123	6.5%	77	4	81	4.9%	5	83	88	94.3%
	145	11	156	7.1%	86	4	90	4.4%	8	101	109	92.7%
	91	4	95	4.2%	99	7	106	6.6%	6	95	101	94.1%
	125	8	133	6.0%	50	4	54	7.4%	3	74	77	96.1%
	97	11	108	10.2%	117	5	122	4.1%	9	111	120	92.5%
	68	9	77	11.7%	74	6	80	7.5%	2	94	96	97.9%
Griseofulvin test 2	165	26	191	13.6%	76	11	87	12.6%	7	94	101	93.1%
	193	18	211	8.5%	90	9	99	9.1%	8	115	123	93.5%
	168	10	178	5.6%	85	6	91	6.6%	9	89	98	90.8%
	132	4	136	2.9%	115	22	137	16.1%	5	90	95	94.7%
	143	7	150	4.7%	70	9	79	11.4%	7	110	117	94.0%
	152	21	173	12.1%	75	12	87	13.8%	9	90	99	90.9%
	165	8	173	4.6%	50	8	58	13.8%	10	95	105	90.5%
	110	21	131	16.0%	95	11	106	10.4%	11	112	123	91.1%
Taxol test 1	110	10	120	8.3%	98	12	110	10.9%	2	76	78	97.4%
	112	5	117	4.3%	95	9	104	8.7%	1	80	81	98.8%
	108	9	117	7.7%	110	13	123	10.6%	0	75	75	100.0%
	98	3	101	3.0%	97	8	105	7.6%	1	68	69	98.6%
	95	13	108	12.0%	99	16	115	13.9%	0	69	69	100.0%
	105	7	112	6.3%	115	15	130	11.5%	0	59	59	100.0%

Treatment tested	CI condition				CI + drug treatment				Rescue condition			
	Unhatched	Hatched	total	hatch rate	Unhatched	Hatched	total	hatch rate	Unhatched	Hatched	total	hatch rate
	121	11	132	8.3%	105	8	113	7.1%	2	87	89	97.8%
	95	5	100	5.0%	90	14	104	13.5%	3	57	60	95.0%
Taxol test 2	84	4	88	4.5%	85	3	88	3.4%	2	58	60	96.7%
	69	1	70	1.4%	79	1	80	1.3%	9	13	22	59.1%
	52	9	61	14.8%	88	1	89	1.1%	13	41	54	75.9%
	62	3	65	4.6%	58	2	60	3.3%	3	71	74	95.9%
	77	2	79	2.5%	59	1	60	1.7%	5	77	82	93.9%
	60	3	63	4.8%	84	0	84	0.0%	3	64	67	95.5%
	54	1	55	1.8%	81	3	84	3.6%	2	71	73	97.3%
	64	5	69	7.2%	55	2	57	3.5%	1	66	67	98.5%
Apcin test 1	63	1	64	1.6%	44	3	47	6.4%	8	88	96	91.7%
	77	1	78	1.3%	80	7	87	8.0%	4	86	90	95.6%
	56	8	64	12.5%	70	8	78	10.3%	2	88	90	97.8%
	66	4	70	5.7%	73	11	84	13.1%	2	88	90	97.8%
	68	5	73	6.8%	97	4	101	4.0%	8	88	96	91.7%
	73	5	78	6.4%	70	7	77	9.1%	6	54	60	90.0%
	68	6	74	8.1%	66	7	73	9.6%	6	82	88	93.2%
	117	4	121	3.3%	52	1	53	1.9%	8	76	84	90.5%
Apcin test 2	95	4	99	4.0%	109	15	124	12.1%	4	115	119	96.6%
	86	2	88	2.3%	89	14	103	13.6%	6	106	112	94.6%
	71	2	73	2.7%	73	12	85	14.1%	3	80	83	96.4%
	70	3	73	4.1%	88	3	91	3.3%	3	114	117	97.4%
	71	11	82	13.4%	76	4	80	5.0%	5	70	75	93.3%
	85	3	88	3.4%	78	13	91	14.3%	12	86	98	87.8%

Treatment tested	CI condition				CI + drug treatment				Rescue condition			
	Unhatched	Hatched	total	hatch rate	Unhatched	Hatched	total	hatch rate	Unhatched	Hatched	total	hatch rate
	77	12	89	13.5%	66	10	76	13.2%	3	85	88	96.6%
	75	4	79	5.1%	63	2	65	3.1%	2	111	113	98.2%
TAME test 1	151	19	170	11.2%	114	12	126	9.5%	2	112	114	98.2%
	159	20	179	11.2%	132	14	146	9.6%	3	101	104	97.1%
	116	15	131	11.5%	117	9	126	7.1%	9	95	104	91.3%
	143	18	161	11.2%	79	9	88	10.2%	4	102	106	96.2%
	130	12	142	8.5%	82	9	91	9.9%	5	101	106	95.3%
	110	9	119	7.6%	68	14	82	17.1%	6	108	114	94.7%
	144	9	153	5.9%	80	11	91	12.1%	3	129	132	97.7%
	137	13	150	8.7%	95	19	114	16.7%	8	119	127	93.7%
TAME test 2	147	25	172	14.5%	227	10	237	4.2%	1	123	124	99.2%
	235	22	257	8.6%	180	10	190	5.3%	2	97	99	98.0%
	192	20	212	9.4%	147	7	154	4.5%	2	123	125	98.4%
	183	8	191	4.2%	186	24	210	11.4%	9	93	102	91.2%
	262	9	271	3.3%	201	17	218	7.8%	1	132	133	99.2%
	157	8	165	4.8%	136	29	165	17.6%	3	164	167	98.2%
	199	28	227	12.3%	147	18	165	10.9%	1	89	90	98.9%
	212	26	238	10.9%	107	7	114	6.1%	2	105	107	98.1%
Flavopiridol test 1	86	9	95	9.5%	101	14	115	12.2%	5	94	99	94.9%
	92	10	102	9.8%	86	10	96	10.4%	4	92	96	95.8%
	103	16	119	13.4%	92	4	96	4.2%	5	90	95	94.7%
	83	4	87	4.6%	106	12	118	10.2%	4	80	84	95.2%
	88	5	93	5.4%	88	18	106	17.0%	3	81	84	96.4%
	94	13	107	12.1%	97	15	112	13.4%	3	79	82	96.3%

Treatment tested	CI condition				CI + drug treatment				Rescue condition			
	Unhatched	Hatched	total	hatch rate	Unhatched	Hatched	total	hatch rate	Unhatched	Hatched	total	hatch rate
	98	12	110	10.9%	86	11	97	11.3%	2	83	85	97.6%
	87	3	90	3.3%	99	5	104	4.8%	2	91	93	97.8%
Flavopiridol test 2	103	12	115	10.4%	66	4	70	5.7%	3	96	99	97.0%
	75	2	77	2.6%	114	7	121	5.8%	0	102	102	100.0%
	99	3	102	2.9%	30	4	34	11.8%	1	59	60	98.3%
	123	8	131	6.1%	93	4	97	4.1%	4	99	103	96.1%
	79	2	81	2.5%	102	5	107	4.7%	6	118	124	95.2%
	89	8	97	8.2%	109	3	112	2.7%	2	92	94	97.9%
	125	4	129	3.1%	102	14	116	12.1%	11	123	134	91.8%
	107	11	118	9.3%	57	2	59	3.4%	1	81	82	98.8%
Roscovitine test 1	118	11	129	8.5%	89	1	90	1.1%	7	122	129	94.6%
	113	5	118	4.2%	82	6	88	6.8%	10	92	102	90.2%
	107	9	116	7.8%	85	7	92	7.6%	13	102	115	88.7%
	100	15	115	13.0%	59	20	79	25.3%	5	98	103	95.1%
	123	9	132	6.8%	68	5	73	6.8%	16	98	114	86.0%
	120	11	131	8.4%	73	12	85	14.1%	6	115	121	95.0%
	95	6	101	5.9%	60	12	72	16.7%	10	110	120	91.7%
	115	8	123	6.5%	83	12	95	12.6%	12	99	111	89.2%
Roscovitine test 2	95	3	98	3.1%	120	8	128	6.3%	9	105	114	92.1%
	116	10	126	7.9%	134	15	149	10.1%	4	99	103	96.1%
	124	9	133	6.8%	129	10	139	7.2%	1	113	114	99.1%
	78	7	85	8.2%	96	4	100	4.0%	10	148	158	93.7%
	137	14	151	9.3%	87	3	90	3.3%	7	126	133	94.7%
	104	9	113	8.0%	125	11	136	8.1%	4	109	113	96.5%

Treatment tested	CI condition				CI + drug treatment				Rescue condition			
	Unhatched	Hatched	total	hatch rate	Unhatched	Hatched	total	hatch rate	Unhatched	Hatched	total	hatch rate
	99	6	105	5.7%	89	12	101	11.9%	0	93	93	100.0%
	95	9	104	8.7%	143	23	166	13.9%	1	82	83	98.8%
Bortezomib test 1	85	4	89	4.5%	124	2	126	1.6%	10	88	98	89.8%
	97	2	99	2.0%	138	15	153	9.8%	3	76	79	96.2%
	111	7	118	5.9%	114	8	122	6.6%	12	84	96	87.5%
	94	3	97	3.1%	98	4	102	3.9%	4	120	124	96.8%
	108	5	113	4.4%	110	8	118	6.8%	3	110	113	97.3%
	114	6	120	5.0%	89	5	94	5.3%	6	92	98	93.9%
	136	12	148	8.1%	121	19	140	13.6%	4	87	91	95.6%
	122	3	125	2.4%	112	14	126	11.1%	2	105	107	98.1%
Bortezomib test 2	103	2	105	1.9%	113	14	127	11.0%	11	29	40	72.5%
	85	15	100	15.0%	70	20	90	22.2%	2	57	59	96.6%
	85	4	89	4.5%	49	15	64	23.4%	2	28	30	93.3%
	82	3	85	3.5%	107	4	111	3.6%	15	75	90	83.3%
	71	4	75	5.3%	94	14	108	13.0%	5	83	88	94.3%
	61	6	67	9.0%	52	8	60	13.3%	5	77	82	93.9%
	115	7	122	5.7%	109	12	121	9.9%	3	82	85	96.5%
	64	4	68	5.9%	67	11	78	14.1%	7	72	79	91.1%
MG-132 test 1	63	8	71	11.3%	74	13	87	14.9%	2	76	78	97.4%
	86	2	88	2.3%	81	15	96	15.6%	6	74	80	92.5%
	64	5	69	7.2%	48	7	55	12.7%	1	76	77	98.7%
	95	7	102	6.9%	90	8	98	8.2%	5	84	89	94.4%
	91	7	98	7.1%	66	5	71	7.0%	3	59	62	95.2%
	73	5	78	6.4%	82	6	88	6.8%	1	52	53	98.1%

Treatment tested	CI condition				CI + drug treatment				Rescue condition			
	Unhatched	Hatched	total	hatch rate	Unhatched	Hatched	total	hatch rate	Unhatched	Hatched	total	hatch rate
	79	13	92	14.1%	49	8	57	14.0%	2	79	81	97.5%
	65	3	68	4.4%	73	13	86	15.1%	3	91	94	96.8%
MG-132 test 2	137	10	147	6.8%	125	15	140	10.7%	6	86	92	93.5%
	152	20	172	11.6%	85	13	98	13.3%	7	89	96	92.7%
	137	8	145	5.5%	142	20	162	12.3%	10	86	96	89.6%
	133	16	149	10.7%	101	14	115	12.2%	2	80	82	97.6%
	99	13	112	11.6%	91	18	109	16.5%	3	75	78	96.2%
	113	11	124	8.9%	106	13	119	10.9%	3	105	108	97.2%
	144	17	161	10.6%	88	16	104	15.4%	9	116	125	92.8%
	104	13	117	11.1%	90	16	106	15.1%	8	105	113	92.9%
Trametinib test 1	109	8	117	6.8%	96	7	103	6.8%	3	59	62	95.2%
	125	4	129	3.1%	121	7	128	5.5%	6	105	111	94.6%
	67	2	69	2.9%	102	21	123	17.1%	13	80	93	86.0%
	93	12	105	11.4%	72	12	84	14.3%	6	95	101	94.1%
	111	8	119	6.7%	118	17	135	12.6%	7	95	102	93.1%
	120	4	124	3.2%	61	7	68	10.3%	6	130	136	95.6%
	150	5	155	3.2%	95	10	105	9.5%	4	129	133	97.0%
	109	8	117	6.8%	83	4	87	4.6%	11	102	113	90.3%
Trametinib test 2	92	1	93	1.1%	113	9	122	7.4%	6	133	139	95.7%
	96	0	96	0.0%	86	4	90	4.4%	1	76	77	98.7%
	101	2	103	1.9%	104	6	110	5.5%	4	94	98	95.9%
	69	2	71	2.8%	103	3	106	2.8%	3	78	81	96.3%
	103	4	107	3.7%	74	17	91	18.7%	3	67	70	95.7%
	59	5	64	7.8%	129	13	142	9.2%	6	29	35	82.9%

Treatment tested	CI condition				CI + drug treatment				Rescue condition			
	Unhatched	Hatched	total	hatch rate	Unhatched	Hatched	total	hatch rate	Unhatched	Hatched	total	hatch rate
	104	3	107	2.8%	90	7	97	7.2%	8	83	91	91.2%
	83	4	87	4.6%	122	11	133	8.3%	4	77	81	95.1%

Table 4.4. Statistical analysis of chemically treated *D. simulans w*Ri CI crosses.

	Sodium Butyrate (NaBu)		Acetic Acid		Propionic Acid		Valeric Acid	
	CI	CI+Treat	CI	CI+Treat	CI	CI+Treat	CI	CI+Treat
Shapiro-Wilk p-value	0.880	0.585	0.592	0.807	0.302	0.060	0.666	0.023
normality interp.	normal	normal	normal	normal	normal	normal	normal	non-normal
test type required	parametric		parametric		parametric		non-parametric	
Levene's p-value	0.147		0.406		0.041		0.851	
HOV interp	variance is homogeneous		variance is homogeneous		unequal variance		variance is homogeneous	
test recommended	T-test		T-test		Welch's T-test		Mann-Whitney U	
Test p-value	**<0.001**		**0.047**		0.769		0.926	

	Vorinostat (SAHA)		Trichostatin A		Quisinostat		CUDC-101	
	CI	CI+Treat	CI	CI+Treat	CI	CI+Treat	CI	CI+Treat
Shapiro-Wilk p-value	0.098	0.394	0.001	0.111	0.416	0.582	0.055	0.000
normality interp.	normal	normal	non-normal	normal	normal	normal	normal	non-normal
test type required	parametric		non-parametric		parametric		non-parametric	
Levene's p-value	0.376		0.506		0.933		0.323	
HOV interp	variance is homogeneous		variance is homogeneous		variance is homogeneous		variance is homogeneous	
test recommended	T-test		Mann-Whitney U		T-test		Welch's T-test	
Test p-value	0.913		0.696		0.235		0.535	

	Celastrol		Rotenone		Cisplatin		Camptothecin	
	CI	CI+Treat	CI	CI+Treat	CI	CI+Treat	CI	CI+Treat
Shapiro-Wilk p-value	0.867	0.791	0.039	0.301	0.497	0.364	0.048	0.173
normality interp.	normal	normal	non-normal	normal	normal	normal	non-normal	normal
test type required	parametric		non-parametric		parametric		non-parametric	
Levene's p-value	0.257		0.436		0.189		0.023	
HOV interp	variance is homogeneous		variance is homogeneous		variance is homogeneous		unequal variance	
test recommended	T-test		Mann-Whitney U		T-test		Indep T-test w bootstrap	
Test p-value	**0.001**		0.149		0.065		0.059	

	Colchicine		Griseofulvin		Taxol		Apcin	
	CI	CI+Treat	CI	CI+Treat	CI	CI+Treat	CI	CI+Treat
Shapiro-Wilk p-value	0.999	0.386	0.412	0.200	0.152	0.150	0.015	0.073
normality interp.	normal	normal	normal	normal	normal	normal	non-normal	normal
test type required	parametric		parametric		parametric		non-parametric	
Levene's p-value	0.350		0.836		0.072		0.464	
HOV interp	variance is homogeneous		variance is homogeneous		variance is homogeneous		variance is homogeneous	
test recommended	T-test		T-test		T-test		Mann-Whitney U	
Test p-value	0.967		0.675		0.774		0.061	

	Flavopiridol		Roscovitine		Bortezomib		MG-132	
	CI	CI+Treat	CI	CI+Treat	CI	CI+Treat	CI	CI+Treat
Shapiro-Wilk p-value	0.136	0.107	0.146	0.289	0.006	0.241	0.4	0.141
normality interp.	normal	normal	normal	normal	non-normal	normal	normal	normal
test type required	parametric		parametric		non-parametric		parametric	
Levene's p-value	0.336		0.022		0.052		0.558	
HOV interp	variance is homogeneous		unequal variance		variance is homogeneous		variance is homogeneous	
test recommended	T-test		Welch's T-test		Mann-Whitney U		T-test	
Test p-value	0.360		0.175		**0.005**		**0.001**	

	Teniposide		Cycloheximide		TAME		Trametinib	
	CI	CI+Treat	CI	CI+Treat	CI	CI+Treat	CI	CI+Treat
Shapiro-Wilk p-value	0.489	0.519	0.492	0.48	0.447	0.142	0.153	0.141
normality interp.	normal	normal	normal	normal	normal	normal	normal	normal
test type required	parametric		parametric		parametric		parametric	
Levene's p-value	1.000		0.116		0.392		0.164	
HOV interp	variance is homogeneous		variance is homogeneous		variance is homogeneous		variance is homogeneous	
test recommended	T-test		T-test		T-test		T-test	
Test p-value	**<0.001**		**<0.001**		0.410		**0.002**	

Table 4.5. Impact of chemical treatments on CI egg hatch rate in the *D. simulans*-*w*Mel system. Chemicals that were considered as a hit previously were tested for their ability to also rescue *w*Mel-induced CI in *D. simulans.*

Putative Rescue effect tested by the treatment	Chemical Used	Reputed cellular effect	Dose used	# wells tested (# plates)	Sig increase in hatch rate?	p-value
Diverse functions	**Nabu**	**C4 short chain fatty acid. Affects HDAC function, DNA damage repair, cell cycle**	**50 mM**	**16(2)**	**Yes**	**0.011**
Short chain fatty acids	Acetic Acid	C5 short chain fatty acid	100 mM	16(2)	No	0.408
DNA damage	**Celastrol**	**Antioxidant (also inh MEK, proteasome)**	**20 uM**	**16(2)**	**Yes**	**0.013**
	Cycloheximide	**DNA damage (also ribosome inhibitor)**	**500 uM**	**16(2)**	**Yes**	**0.013**
	Teniposide	Inhibits Topoll	50 uM	16(2)	Borderline	0.041
Cell cycle delay	Bortezomib	Proteasome inhibitor	1 uM	16(2)	Borderline	0.047
	MG132	Proteasome inhibitor	50 uM	16(2)	Borderline	0.047
	Trametinib	MEK inhibition	250 nM	16(2)	No	0.096

Table 4.6. Impact of candidate drugs on *D. simulans w*Mel CI hatch rates in plate assay format.

Treatment tested	CI condition				CI + drug treatment			
	Unhatched	Hatched	total	hatch rate	Unhatched	Hatched	total	hatch rate
NaBu test 1	108	7	115	6.1%	30	2	32	6.3%
	98	4	102	3.9%	17	2	19	10.5%
	101	3	104	2.9%	82	6	88	6.8%
	89	5	94	5.3%	47	2	49	4.1%
	107	16	123	13.0%	53	14	67	20.9%
	99	1	100	1.0%	59	1	60	1.7%
	123	17	140	12.1%	11	5	16	31.3%
	92	10	102	9.8%	36	13	49	26.5%
NaBu test 2	110	5	115	4.3%	76	9	85	10.6%
	98	2	100	2.0%	81	10	91	11.0%
	74	7	81	8.6%	75	6	81	7.4%
	96	5	101	5.0%	69	16	85	18.8%
	106	3	109	2.8%	88	13	101	12.9%
	120	9	129	7.0%	65	5	70	7.1%
	83	1	84	1.2%	71	2	73	2.7%
	138	4	142	2.8%	73	9	82	11.0%
Acetic acid test 1	58	7	65	10.8%	104	12	116	10.3%
	108	11	119	9.2%	113	16	129	12.4%
	63	4	67	6.0%	102	7	109	6.4%
	117	9	126	7.1%	96	8	104	7.7%

Treatment tested	CI condition				CI + drug treatment			
	Unhatched	Hatched	total	hatch rate	Unhatched	Hatched	total	hatch rate
	53	3	56	5.4%	82	4	86	4.7%
	85	10	95	10.5%	125	15	140	10.7%
	98	12	110	10.9%	92	11	103	10.7%
	101	9	110	8.2%	109	10	119	8.4%
Acetic acid test 2	97	5	102	4.9%	84	5	89	5.6%
	93	10	103	9.7%	77	6	83	7.2%
	84	2	86	2.3%	99	9	108	8.3%
	116	11	127	8.7%	103	3	106	2.8%
	120	9	129	7.0%	68	8	76	10.5%
	59	4	63	6.3%	115	10	125	8.0%
	96	7	103	6.8%	62	7	69	10.1%
	89	8	97	8.2%	94	10	104	9.6%
Celastrol test 1	113	10	123	8.1%	78	2	80	2.5%
	98	5	103	4.9%	44	1	45	2.2%
	95	4	99	4.0%	73	5	78	6.4%
	101	4	105	3.8%	69	6	75	8.0%
	105	6	111	5.4%	103	7	110	6.4%
	76	5	81	6.2%	88	10	98	10.2%
	89	3	92	3.3%	71	4	75	5.3%
	110	8	118	6.8%	66	3	69	4.3%
Celastrol test 2	104	9	113	8.0%	123	10	133	7.5%
	151	2	153	1.3%	165	41	206	19.9%
	79	6	85	7.1%	149	23	172	13.4%
	63	0	63	0.0%	131	10	141	7.1%
	174	13	187	7.0%	117	18	135	13.3%

Treatment tested	CI condition				CI + drug treatment			
	Unhatched	Hatched	total	hatch rate	Unhatched	Hatched	total	hatch rate
	147	12	159	7.5%	114	11	125	8.8%
	108	4	112	3.6%	48	7	55	12.7%
	92	7	99	7.1%	112	20	132	15.2%
Cycloheximide test 1	77	5	82	6.1%	62	6	68	8.8%
	63	1	64	1.6%	60	2	62	3.2%
	81	2	83	2.4%	67	10	77	13.0%
	61	2	63	3.2%	77	3	80	3.8%
	58	3	61	4.9%	65	5	70	7.1%
	78	4	82	4.9%	73	2	75	2.7%
	64	5	69	7.2%	56	4	60	6.7%
	57	4	61	6.6%	63	4	67	6.0%
Cycloheximide test 2	58	5	63	7.9%	64	13	77	16.9%
	72	7	79	8.9%	69	25	94	26.6%
	78	6	84	7.1%	77	15	92	16.3%
	98	11	109	10.1%	72	12	84	14.3%
	85	10	95	10.5%	81	18	99	18.2%
	55	3	58	5.2%	84	7	91	7.7%
	41	1	42	2.4%	92	8	100	8.0%
	89	7	96	7.3%	91	14	105	13.3%
Teniposide test 1	73	4	77	5.2%	65	6	71	8.5%
	68	2	70	2.9%	53	2	55	3.6%
	77	2	79	2.5%	67	10	77	13.0%
	61	3	64	4.7%	81	3	84	3.6%
	55	0	55	0.0%	59	5	64	7.8%
	52	0	52	0.0%	74	2	76	2.6%

Treatment tested	CI condition				CI + drug treatment			
	Unhatched	Hatched	total	hatch rate	Unhatched	Hatched	total	hatch rate
	72	3	75	4.0%	80	4	84	4.8%
	64	5	69	7.2%	67	4	71	5.6%
Teniposide test 2	51	6	57	10.5%	54	5	59	8.5%
	66	4	70	5.7%	96	11	107	10.3%
	48	1	49	2.0%	67	14	81	17.3%
	91	3	94	3.2%	58	7	65	10.8%
	69	7	76	9.2%	71	9	80	11.3%
	63	9	72	12.5%	66	4	70	5.7%
	57	5	62	8.1%	52	7	59	11.9%
	78	10	88	11.4%	66	8	74	10.8%
Bortezomib test 1	95	3	98	3.1%	66	4	70	5.7%
	137	13	150	8.7%	92	6	98	6.1%
	98	4	102	3.9%	78	5	83	6.0%
	83	6	89	6.7%	104	11	115	9.6%
	112	9	121	7.4%	107	10	117	8.5%
	64	2	66	3.0%	99	8	107	7.5%
	120	11	131	8.4%	81	7	88	8.0%
	96	3	99	3.0%	51	4	55	7.3%
Bortezomib test 2	65	7	72	9.7%	72	12	84	14.3%
	103	2	105	1.9%	79	2	81	2.5%
	74	0	74	0.0%	131	7	138	5.1%
	143	18	161	11.2%	61	8	69	11.6%
	61	2	63	3.2%	46	6	52	11.5%
	182	4	186	2.2%	64	1	65	1.5%
	62	1	63	1.6%	96	2	98	2.0%

Treatment tested	CI condition				CI + drug treatment			
	Unhatched	Hatched	total	hatch rate	Unhatched	Hatched	total	hatch rate
	141	4	145	2.8%	100	11	111	9.9%
MG-132 test 1	100	1	101	1.0%	55	0	55	0.0%
	64	7	71	9.9%	102	1	103	1.0%
	85	7	92	7.6%	88	2	90	2.2%
	118	0	118	0.0%	53	3	56	5.4%
	131	11	142	7.7%	82	5	87	5.7%
	104	0	104	0.0%	79	9	88	10.2%
	80	3	83	3.6%	71	4	75	5.3%
	76	0	76	0.0%	108	12	120	10.0%
MG-132 test 2	108	11	119	9.2%	83	9	92	9.8%
	86	7	93	7.5%	61	4	65	6.2%
	92	6	98	6.1%	104	19	123	15.4%
	65	3	68	4.4%	93	10	103	9.7%
	95	5	100	5.0%	101	12	113	10.6%
	54	2	56	3.6%	76	9	85	10.6%
	98	8	106	7.5%	51	6	57	10.5%
	87	5	92	5.4%	112	16	128	12.5%
Trametinib test 1	143	4	147	2.7%	125	10	135	7.4%
	171	16	187	8.6%	84	13	97	13.4%
	114	15	129	11.6%	127	11	138	8.0%
	129	8	137	5.8%	150	12	162	7.4%
	123	17	140	12.1%	159	10	169	5.9%
	147	6	153	3.9%	138	14	152	9.2%
	121	3	124	2.4%	180	17	197	8.6%
	153	18	171	10.5%	162	23	185	12.4%

Treatment tested	CI condition				CI + drug treatment			
	Unhatched	Hatched	total	hatch rate	Unhatched	Hatched	total	hatch rate
Trametinib test 2	82	0	82	0.0%	70	4	74	5.4%
	129	13	142	9.2%	98	19	117	16.2%
	164	7	171	4.1%	116	10	126	7.9%
	138	5	143	3.5%	109	1	110	0.9%
	157	4	161	2.5%	125	12	137	8.8%
	152	6	158	3.8%	128	6	134	4.5%
	146	8	154	5.2%	105	15	120	12.5%
	103	3	106	2.8%	97	1	98	1.0%

Table 4.7. Statistical analysis of chemically treated *D. simulans w*Mel CI crosses.

	Sodium Butyrate (NaBu)		Acetic Acid		Celastrol		Cycloheximide	
	CI	CI+Treat	CI	CI+Treat	CI	CI+Treat	CI	CI+Treat
Shapiro-Wilk p-value	0.118	0.047	0.455	0.404	0.090	0.523	0.379	0.161
normality interp.	normal	non-normal	normal	normal	normal	normal	normal	normal
test type required	non-parametric		parametric		parametric		parametric	
Levene's p-value	0.057		1.000		0.051		0.032	
HOV interp	variance is homogeneous		variance is homogeneous		variance is homogeneous		unequal variance	
test recommended	Mann-Whitney U		T-test		T-test		Welch's T-test	
Test p-value	**0.011**		0.408		**0.013**		**0.013**	

	Teniposide		Bortezomib		MG-132		Trametinib	
	CI	CI+Treat	CI	CI+Treat	CI	CI+Treat	CI	CI+Treat
Shapiro-Wilk p-value	0.555	0.587	0.053	0.493	0.104	0.245	0.063	0.777
normality interp.	normal	normal	normal	normal	normal	normal	normal	normal
test type required	parametric		parametric		parametric		parametric	
Levene's p-value	0.939		0.725		0.440		0.848	
HOV interp	variance is homogeneous		variance is homogeneous		variance is homogeneous		variance is homogeneous	
test recommended	T-test		T-test		T-test		T-test	
Test p-value	**0.041**		**0.047**		**0.047**		0.096	

Table 4.8. Qualitative assessment of *D. simulans* fecundity and development in response to drug combinations.

Treatment tested	Dose	Fecundity	Hatchablility	Larval development
NaBu/Celastrol	50 mM / 20 μM	(+)	some	some
	50 mM / 10 μM	(+)	some	some
	25 mM / 20 μM	(+)	(+)	some
	25 mM / 10 μM	**(+)**	**(+)**	**(+)**
	Control (DMSO)	(+)	(+)	(+)
NaBu/Cycloheximide	50 mM / 50 μM	some	some	some
	50 mM / 25 μM	some	some	some
	25 mM / 50 μM	(+)	(+)	some
	25 mM / 25 μM	**(+)**	**(+)**	**(+)**
	Control (DMSO)	(+)	(+)	(+)
NaBu/MG132	50 mM / 50 μM	(-)	(-)	(-)
	50 mM / 25 μM	(+)	(+)	some
	25 mM / 50 μM	(-)	(-)	some
	25 mM / 25 μM	**(+)**	**(+)**	**(+)**
	Control (DMSO)	(+)	(+)	(+)
Teniposide/Celastrol	500 μM / 500 μM	(-)	(-)	(-)
	500 μM / 20 μM	(-)	(-)	(-)
	250 μM / 500 μM	**(+)**	**(+)**	**(+)**
	250 μM / 20 μM	(+)	(+)	(+)
	Control (DMSO)	(+)	(+)	(+)
Celastrol/Cycloheximide	20 μM / 50 μM	some	(-)	(-)
	20 μM / 25 μM	(+)	(+)	(+)
	10 μM / 50 μM	some	(-)	(-)
	10 μM / 25 μM	**(+)**	**(+)**	**(+)**
	Control (DMSO)	(+)	(+)	(+)
Cycloheximide/Bortezomib	**50 μM / 1 μM**	**(+)**	**(+)**	**(+)**
	50 μM / 0.5 μM	(+)	(+)	(+)
	25 μM / 1 μM	(+)	(+)	(+)
	25 μM / 0.5 μM	(+)	(+)	(+)
	Control (DMSO)	(+)	(+)	(+)
Teniposide/MG-132	500 μM / 50 μM	(+)	(+)	(-)
	500 μM / 25 μM	(+)	(+)	(-)

Treatment tested	Dose	Fecundity	Hatchablility	Larval development
	250 µM / 50 µM	(+)	(+)	(-)
	250 µM / 25 µM	**(+)**	**(+)**	**(+)**
	Control (DMSO)	(+)	(+)	(+)

*Dose used is indicated in bold

Table 4.9. Impact of chemical combinations on CI egg hatch rate in the *D. simulans*-*w*Ri system.

Putative Rescue effect tested by the treatment	**Combination Used**	**Doses used**	**# wells tested (# plates)**	**Sig increase in hatch rate?**	**p-value**
Diverse functions/ DNA damage	Nabu/Celastrol	25 mM / 10 μM	16(2)	No	0.119
Diverse functions/ DNA damage	Nabu/Cycloheximide	25 mM / 25 μM	16(2)	No	0.817
Diverse functions/ Proteasome inhibitor	Nabu/MG132	25 mM / 25 μM	16(2)	No	0.201
DNA damage	Teniposide/Celastrol	250 μM / 500 μM	16(2)	No	0.287
DNA damage	Celastrol/Cycloheximide	10 μM / 25 μM	16(2)	No	0.565
DNA damage/ Cell cycle delay	**Cycloheximide/Bortezomib**	**50 μM / 1 μM**	**16(2)**	**Yes**	**0.006**
DNA damage/ Cell cycle delay	Teniposide/MG132	250 μM / 25 μM	16(2)	No	0.264

Table 4.10. Impact of combinatorial candidate drugs on *D. simulans* wRi CI hatch rates in plate assay format

Treatment tested	CI condition				CI + drug treatment				Rescue condition			
	Unhatched	Hatched	total	hatch rate	Unhatched	Hatched	total	hatch rate	Unhatched	Hatched	total	hatch rate
NaBu/Celastrol	176	18	194	9.3%	157	24	181	13.3%	20	103	123	83.7%
(25 mM / 10 μM)	130	4	134	3.0%	149	10	159	6.3%	15	146	161	90.7%
	195	11	206	5.3%	120	13	133	9.8%	17	97	114	85.1%
Replicate 1	140	7	147	4.8%	138	12	150	8.0%	5	88	93	94.6%
	156	17	173	9.8%	128	12	140	8.6%	10	137	147	93.2%
	153	3	156	1.9%	132	12	144	8.3%	24	122	146	83.6%
	118	15	133	11.3%	101	15	116	12.9%	9	112	121	92.6%
	124	16	140	11.4%	80	6	86	7.0%	31	125	156	80.1%
NaBu/Celastrol	165	9	174	5.2%	140	20	160	12.5%	11	131	142	92.3%
(25 mM / 10 μM)	123	10	133	7.5%	133	11	144	7.6%	20	145	165	87.9%
	111	3	114	2.6%	145	13	158	8.2%	9	123	132	93.2%
Replicate 2	97	7	104	6.7%	92	11	103	10.7%	0	106	106	100.0%
	134	14	148	9.5%	112	9	121	7.4%	1	95	96	99.0%
	150	20	170	11.8%	110	7	117	6.0%	5	130	135	96.3%
	122	10	132	7.6%	86	12	98	12.2%	7	116	123	94.3%
	100	8	108	7.4%	124	10	134	7.5%	4	98	102	96.1%
NaBu/Cycloheximide	143	1	144	0.7%	126	5	131	3.8%	5	138	143	96.5%
(25 mM / 25 μM)	192	10	202	5.0%	144	23	167	13.8%	1	119	120	99.2%
	147	0	147	0.0%	137	17	154	11.0%	0	146	146	100.0%
Replicate 1	139	16	155	10.3%	108	11	119	9.2%	2	149	151	98.7%
	198	14	212	6.6%	150	6	156	3.8%	1	97	98	99.0%
	141	9	150	6.0%	131	12	143	8.4%	3	135	138	97.8%
	143	15	158	9.5%	185	13	198	6.6%	4	103	107	96.3%
	155	4	159	2.5%	140	7	147	4.8%	0	112	112	100.0%

Treatment tested	CI condition				CI + drug treatment				Rescue condition			
	Unhatched	Hatched	total	hatch rate	Unhatched	Hatched	total	hatch rate	Unhatched	Hatched	total	hatch rate
NaBu/Cycloheximide	174	18	192	9.4%	98	2	100	2.0%	3	97	100	97.0%
(25 mM / 25 µM)	178	20	198	10.1%	112	0	112	0.0%	1	103	104	99.0%
	125	12	137	8.8%	113	5	118	4.2%	0	125	125	100.0%
Replicate 2	107	7	114	6.1%	130	14	144	9.7%	6	174	180	96.7%
	156	15	171	8.8%	139	6	145	4.1%	4	92	96	95.8%
	101	3	104	2.9%	77	3	80	3.8%	0	77	77	100.0%
	170	18	188	9.6%	154	22	176	12.5%	2	119	121	98.3%
	123	16	139	11.5%	121	6	127	4.7%	5	131	136	96.3%
NaBu/MG132	186	24	210	11.4%	113	5	118	4.2%	1	131	132	99.2%
(25 mM / 25 µM)	175	17	192	8.9%	155	8	163	4.9%	0	102	102	100.0%
	130	1	131	0.8%	171	15	186	8.1%	5	156	161	96.9%
Replicate 1	76	3	79	3.8%	173	11	184	6.0%	2	110	112	98.2%
	157	2	159	1.3%	111	33	144	22.9%	1	128	129	99.2%
	197	13	210	6.2%	93	2	95	2.1%	4	130	134	97.0%
	134	3	137	2.2%	92	4	96	4.2%	8	173	181	95.6%
	226	9	235	3.8%	136	16	152	10.5%	6	118	124	95.2%
NaBu/MG132	153	11	164	6.7%	144	6	150	4.0%	0	99	99	100.0%
(25 mM / 25 µM)	115	0	115	0.0%	100	4	104	3.8%	2	114	116	98.3%
	132	1	133	0.8%	66	2	68	2.9%	5	107	112	95.5%
Replicate 2	140	5	145	3.4%	105	0	105	0.0%	0	118	118	100.0%
	129	6	135	4.4%	79	2	81	2.5%	4	121	125	96.8%
	122	1	123	0.8%	61	4	65	6.2%	0	76	76	100.0%
	117	0	117	0.0%	97	3	100	3.0%	1	91	92	98.9%
	109	7	116	6.0%	120	12	132	9.1%	1	96	97	99.0%
Teniposide/Celastrol	143	7	150	4.7%	125	9	134	6.7%	2	130	132	98.5%
(250 µM / 500 µM)	136	5	141	3.5%	117	13	130	10.0%	0	63	63	100.0%

Treatment tested	CI condition				CI + drug treatment				Rescue condition			
	Unhatched	Hatched	total	hatch rate	Unhatched	Hatched	total	hatch rate	Unhatched	Hatched	total	hatch rate
	139	1	140	0.7%	97	6	103	5.8%	0	98	98	100.0%
Replicate 1	158	5	163	3.1%	142	11	153	7.2%	4	111	115	96.5%
	129	2	131	1.5%	113	10	123	8.1%	1	61	62	98.4%
	170	6	176	3.4%	151	2	153	1.3%	1	129	130	99.2%
	239	22	261	8.4%	128	8	136	5.9%	1	85	86	98.8%
	147	6	153	3.9%	143	5	148	3.4%	0	147	147	100.0%
Teniposide/Celastrol	120	2	122	1.6%	115	2	117	1.7%	4	136	140	97.1%
(250 µM / 500 µM)	90	1	91	1.1%	44	7	51	13.7%	1	69	70	98.6%
	117	2	119	1.7%	53	6	59	10.2%	0	105	105	100.0%
Replicate 2	60	10	70	14.3%	110	4	114	3.5%	3	127	130	97.7%
	110	3	113	2.7%	64	2	66	3.0%	5	92	97	94.8%
	130	7	137	5.1%	84	2	86	2.3%	0	108	108	100.0%
	146	5	151	3.3%	70	1	71	1.4%	1	111	112	99.1%
	50	0	50	0.0%	65	0	65	0.0%	3	119	122	97.5%
Celastrol/Cycloheximide	156	11	167	6.6%	143	2	145	1.4%	19	103	122	84.4%
(10 µM / 25 µM)	176	17	193	8.8%	195	4	199	2.0%	21	120	141	85.1%
	189	18	207	8.7%	184	18	202	8.9%	18	92	110	83.6%
Replicate 1	170	13	183	7.1%	165	12	177	6.8%	23	105	128	82.0%
	171	11	182	6.0%	140	6	146	4.1%	33	89	122	73.0%
	156	14	170	8.2%	144	8	152	5.3%	34	125	159	78.6%
	160	10	170	5.9%	137	12	149	8.1%	22	100	122	82.0%
	188	9	197	4.6%	152	10	162	6.2%	36	120	156	76.9%
Celastrol/Cycloheximide	153	17	170	10.0%	135	6	141	4.3%	1	113	114	99.1%
(10 µM / 25 µM)	159	6	165	3.6%	171	15	186	8.1%	6	111	117	94.9%
	140	3	143	2.1%	134	8	142	5.6%	0	96	96	100.0%
Replicate 2	137	9	146	6.2%	129	6	135	4.4%	13	109	122	89.3%
	176	23	199	11.6%	140	12	152	7.9%	7	126	133	94.7%

Treatment tested	CI condition				CI + drug treatment				Rescue condition			
	Unhatched	Hatched	total	hatch rate	Unhatched	Hatched	total	hatch rate	Unhatched	Hatched	total	hatch rate
	205	19	224	8.5%	118	16	134	11.9%	5	101	106	95.3%
	163	21	184	11.4%	120	15	135	11.1%	10	113	123	91.9%
	148	13	161	8.1%	127	19	146	13.0%	18	130	148	87.8%
Cycloheximide/Bortezomib	122	3	125	2.4%	96	8	104	7.7%	2	98	100	98.0%
(50 µM / 1 µM)	103	0	103	0.0%	126	3	129	2.3%	0	101	101	100.0%
	121	0	121	0.0%	120	55	175	31.4%	5	117	122	95.9%
Replicate 1	72	0	72	0.0%	141	10	151	6.6%	10	149	159	93.7%
	115	1	116	0.9%	147	21	168	12.5%	1	107	108	99.1%
	109	7	116	6.0%	122	5	127	3.9%	4	153	157	97.5%
	134	3	137	2.2%	122	7	129	5.4%	7	126	133	94.7%
	127	11	138	8.0%	137	5	142	3.5%	0	105	105	100.0%
Cycloheximide/Bortezomib	172	0	172	0.0%	172	8	180	4.4%	3	137	140	97.9%
(50 µM / 1 µM)	178	0	178	0.0%	164	4	168	2.4%	1	104	105	99.0%
	169	2	171	1.2%	113	2	115	1.7%	3	156	159	98.1%
Replicate 2	151	11	162	6.8%	154	5	159	3.1%	7	193	200	96.5%
	138	4	142	2.8%	132	14	146	9.6%	0	118	118	100.0%
	124	0	124	0.0%	149	9	158	5.7%	1	128	129	99.2%
	195	22	217	10.1%	201	10	211	4.7%	6	142	148	95.9%
	199	5	204	2.5%	159	6	165	3.6%	4	121	125	96.8%
Teniposide/MG132	87	8	95	8.4%	85	15	100	15.0%	21	135	156	86.5%
(250 µM / 25 µM)	110	4	114	3.5%	105	18	123	14.6%	34	90	124	72.6%
	113	8	121	6.6%	130	12	142	8.5%	37	142	179	79.3%
Replicate 1	73	5	78	6.4%	136	9	145	6.2%	30	85	115	73.9%
	102	9	111	8.1%	124	20	144	13.9%	28	118	146	80.8%
	107	9	116	7.8%	97	3	100	3.0%	25	107	132	81.1%
	131	12	143	8.4%	88	8	96	8.3%	8	68	76	89.5%

Treatment tested	CI condition				CI + drug treatment				Rescue condition			
	Unhatched	Hatched	total	hatch rate	Unhatched	Hatched	total	hatch rate	Unhatched	Hatched	total	hatch rate
	140	18	158	11.4%	126	10	136	7.4%	24	96	120	80.0%
Teniposide/MG132	108	13	121	10.7%	100	11	111	9.9%	3	97	100	97.0%
(250 µM / 25 µM)	107	14	121	11.6%	98	11	109	10.1%	4	85	89	95.5%
	73	8	81	9.9%	120	12	132	9.1%	2	101	103	98.1%
Replicate 2	140	19	159	11.9%	77	13	90	14.4%	11	68	79	86.1%
	98	11	109	10.1%	69	5	74	6.8%	3	52	55	94.5%
	106	9	115	7.8%	75	8	83	9.6%	0	61	61	100.0%
	121	6	127	4.7%	84	13	97	13.4%	8	110	118	93.2%
	117	5	122	4.1%	89	4	93	4.3%	5	99	104	95.2%

Table 4.11. Statistical analysis of combinatorial drug treated *D. simulans w*Ri CI crosses.

	NaBu/Celastrol		NaBu/Cycloheximide		NaBu/MG132	
	CI	CI+Treat	CI	CI+Treat	CI	CI+Treat
Shapiro-Wilk p-value	0.542	0.035	0.179	0.310	0.110	0.001
normality interp.	normal	non-normal	normal	normal	normal	non-normal
test type required	non-parametric		parametric		non-parametric	
Levene's p-value	0.373		0.836		0.697	
HOV interp	variance is homogeneous		variance is homogeneous		variance is homogeneous	
test recommended	Mann-Whitney U		T-test		Mann-Whitney U	
Test p-value	0.119		0.817		0.201	

	Teniposide/Celastrol		Celastrol/Cycloheximide		Cycloheximide/Bortezomib		Teniposide/MG-132	
	CI	CI+Treat	CI	CI+Treat	CI	CI+Treat	CI	CI+Treat
Shapiro-Wilk p-value	0.002	0.373	0.993	0.857	0.002	0.000	0.256	0.385
normality interp.	non-normal	normal	normal	normal	non-normal	non-normal	normal	normal
test type required	non-parametric		parametric		non-parametric		parametric	
Levene's p-value	0.157		0.238		0.435		0.121	
HOV interp	variance is homogeneous		variance is homogeneous		variance is homogeneous		variance is homogeneous	
test recommended	Mann-Whitney U		T-test		Mann-Whitney U		T-test	
Test p-value	0.287		0.565		**0.006**		0.264	

References

Ahmad Ganai, S., Ramadoss, M., & Mahadevan, V. (2016). Histone Deacetylase (HDAC) Inhibitors-emerging roles in neuronal memory, learning, synaptic plasticity and neural regeneration. *Current Neuropharmacology*, *14*(1), 55–71.

Arts, J., King, P., Mariën, A., Floren, W., Beliën, A., Janssen, L., … Gilissen, R. (2009). JNJ-26481585, a novel "second-generation" oral histone deacetylase inhibitor, shows broad-spectrum preclinical antitumoral activity. *Clinical Cancer Research*, *15*(22), 6841–6851.

Basu, A., & Krishnamurthy, S. (2010). Cellular responses to Cisplatin-induced DNA damage. *Journal of Nucleic Acids*, *2010*.

Beckmann, J. F., Ronau, J. A., & Hochstrasser, M. (2017). A *Wolbachia* deubiquitylating enzyme induces cytoplasmic incompatibility. *Nature Microbiology*, *2*(5), 1–7.

Breeuwer, J. A. J., & Werren, J. H. (1990). Microorganisms associated with chromosome destruction and reproductive isolation between two insect species. *Nature*, *346*(6284), 558–560.

Brennan, L. J., Haukedal, J. A., Earle, J. C., Keddie, B., & Harris, H. L. (2012). Disruption of redox homeostasis leads to oxidative DNA damage in spermatocytes of *Wolbachia*-infected *Drosophila* simulans. *Insect Molecular Biology*, *21*(5), 510–520.

Callaini, G., Dallai, R., & Riparbelli, M. G. (1997). *Wolbachia*-induced delay of paternal chromatin condensation does not prevent maternal chromosomes from entering anaphase in incompatible crosses of *Drosophila* simulans. *Journal of Cell Science*, *110*(2), 271–280.

Callaini, G., Riparbelli, M. G., Giordano, R., & Dallai, R. (1996). Mitotic defects associated with cytoplasmic incompatibility in*Drosophila* simulans. *Journal of Invertebrate Pathology*, *67*(1), 55–64.

Christensen, S., Pérez Dulzaides, R., Hedrick, V. E., Momtaz, A. J. M. Z., Nakayasu, E. S., Paul, L. N., & Serbus, L. R. (2016). *Wolbachia* endosymbionts modify *Drosophila* ovary protein levels in a context-dependent manner. *Applied and Environmental Microbiology*, *82*(17), 5354–5363.

Cicenas, J., Kalyan, K., Sorokinas, A., Stankunas, E., Levy, J., Meskinyte, I., … Valius, M. (2015). Roscovitine in cancer and other diseases. *Annals of Translational Medicine*, *3*(10).

Coradini, D., Pellizzaro, C., Marimpietri, D., Abolafio, G., & Daidone, M. G. (2000). Sodium butyrate modulates cell cycle-related proteins in HT29 human colonic adenocarcinoma cells. *Cell Proliferation*, *33*(3), 139–146.

D'Anna, J. A., Tobey, R. A., & Gurley, L. R. (1980). Concentration-dependent effects of sodium butyrate in Chinese hamster cells: cell-cycle progression, inner-histone acetylation, histone H1 dephosphorylation, and induction of an H1-like protein. *Biochemistry*, *19*(12), 2656–2671.

De Cuevas, M., & Spradling, A. C. (1998). Morphogenesis of the *Drosophila* fusome and its implications for oocyte specification. *Development*, *125*(15), 2781–2789.

Fast, E. M., Toomey, M. E., Panaram, K., Desjardins, D., Kolaczyk, E. D., & Frydman, H. M. (2011). *Wolbachia* enhance *Drosophila* stem cell proliferation and target the germline stem cell niche. *Science*, *334*(6058), 990–992.

Ferree, P. M., Frydman, H. M., Li, J. M., Cao, J., Wieschaus, E., & Sullivan, W. (2005). *Wolbachia* utilizes host microtubules and Dynein for anterior localization in the *Drosophila* oocyte. *PLoS Pathogens*, *1*(2), e14.

Field, A. (2013). *Discovering statistics using IBM SPSS statistics*. sage.

Goldberg, A. L. (2012). Development of proteasome inhibitors as research tools and cancer drugs. The Rockefeller University Press.

Gray, N., Detivaud, L., Doerig, C., & Meijer, L. (1999). ATP-site directed inhibitors of cyclin-dependent kinases. *Current Medicinal Chemistry*, *6*(9), 859–876.

Hartmann, J. T., & Lipp, H.-P. (2006). Camptothecin and podophyllotoxin derivatives. *Drug Safety*, *29*(3), 209–230.

Hoffmann, A. A., and Turelli, M. (1997). Cytoplasmic incompatibility in insects, 42–80.

Hunter, P. (2006). Entente cordiale: multiple symbiosis illustrates the intricate interconnectivity of nature. *EMBO Reports*, *7*(9), 861–864.

Kitzis, A., Tichonicky, L., Defer, N., & Kruh, J. (1980). Effect of sodium butyrate on chromatin structure. *Biochemical and Biophysical Research Communications*, *93*(3), 833–841.

Koga, R., Tsuchida, T., & Fukatsu, T. (2003). Changing partners in an obligate symbiosis: a facultative endosymbiont can compensate for loss of the essential endosymbiont Buchnera in an aphid. *Proceedings of the Royal Society of London. Series B: Biological Sciences*, *270*(1533), 2543–2550.

Kurata, K., Onoda, N., Noda, S., Kashiwagi, S., Asano, Y., Hirakawa, K., & Ohira, M. (2016). Growth arrest by activated BRAF and MEK inhibition in human anaplastic thyroid cancer cells. *International Journal of Oncology*, *49*(6), 2303–2308.

Lai, C.-J., Bao, R., Tao, X. U., Wang, J., Atoyan, R., Qu, H., … Forrester, J. (2010). CUDC-101, a multitargeted inhibitor of histone deacetylase, epidermal growth factor receptor, and human epidermal growth factor receptor 2, exerts potent anticancer activity. *Cancer Research*, *70*(9), 3647–3656.

Lallemand, F., Courilleau, D., Sabbah, M., Redeuilh, G., & Mester, J. (1996). Direct inhibition of the expression of cyclin D1 gene by sodium butyrate. *Biochemical and Biophysical Research Communications*, *229*(1), 163–169.

Landmann, F., Orsi, G. A., Loppin, B., & Sullivan, W. (2009). *Wolbachia*-mediated cytoplasmic incompatibility is associated with impaired histone deposition in the male pronucleus. *PLoS Pathogens*, *5*(3), e1000343.

Lassy, C. W., & Karr, T. L. (1996). Cytological analysis of fertilization and early embryonic development in incompatible crosses of *Drosophila* simulans. *Mechanisms of Development*, *57*(1), 47–58.

LePage, D. P., Metcalf, J. A., Bordenstein, S. R., On, J., Perlmutter, J. I., Shropshire, J. D., … Bordenstein, S. R. (2017). Prophage WO genes recapitulate and enhance *Wolbachia*-induced cytoplasmic incompatibility. *Nature*, *543*(7644), 243–247.

Lim, T.-S., & Loh, W.-Y. (1996). A comparison of tests of equality of variances. *Computational Statistics & Data Analysis*, *22*(3), 287–301.

Mao, P., & Wyrick, J. J. (2016). Emerging roles for histone modifications in DNA excision repair. *FEMS Yeast Research*, *16*(7).

Moreira, H., Szyjka, A., Paliszkiewicz, K., & Barg, E. (2019). Prooxidative activity of celastrol induces apoptosis, DNA damage, and cell cycle arrest in drug-resistant human colon cancer cells. *Oxidative Medicine and Cellular Longevity*, *2019*.

Nitiss, J. L. (2009). Targeting DNA topoisomerase II in cancer chemotherapy. *Nature Reviews Cancer*, *9*(5), 338–350.

O'Neill, S. L., & Karr, T. L. (1990). Bidirectional incompatibility between conspecific populations of *Drosophila* simulans. *Nature*, *348*(6297), 178–180.

Podratz, J. L., Knight, A. M., Ta, L. E., Staff, N. P., Gass, J. M., Genelin, K., … Windebank, A. J. (2011). Cisplatin induced mitochondrial DNA damage in dorsal root ganglion neurons. *Neurobiology of Disease*, *41*(3), 661–668.

Rastogi, N., & Mishra, D. P. (2012). Therapeutic targeting of cancer cell cycle using proteasome inhibitors. *Cell Division*, *7*(1), 1–10.

Razali, N. M., & Wah, Y. B. (2011). Power comparisons of shapiro-wilk, kolmogorov-smirnov, lilliefors and anderson-darling tests. *Journal of Statistical Modeling and Analytics*, *2*(1), 21–33.

Rezaee, M., Sanche, L., & Hunting, D. J. (2013). Cisplatin enhances the formation of DNA single-and double-strand breaks by hydrated electrons and hydroxyl radicals. *Radiation Research*, *179*(3), 323–331.

Rietveld, T., & van Hout, R. (2015). The t test and beyond: Recommendations for testing the central tendencies of two independent samples in research on speech, language and hearing pathology. *Journal of Communication Disorders*, *58*, 158–168.

Rowe, T. C., Wang, J. C., & Liu, L. F. (1986). In vivo localization of DNA topoisomerase II cleavage sites on *Drosophila* heat shock chromatin. *Molecular and Cellular Biology*, *6*(4), 985–992.

Sackton, K. L., Dimova, N., Zeng, X., Tian, W., Zhang, M., Sackton, T. B., … Yu, H. (2014). Synergistic blockade of mitotic exit by two chemical inhibitors of the APC/C. *Nature*, *514*(7524), 646–649.

Sanders, L. H., & Greenamyre, J. T. (2013). Oxidative damage to macromolecules in human Parkinson disease and the rotenone model. *Free Radical Biology and Medicine*, *62*, 111–120.

Sazama, E. J., Bosch, M. J., Shouldis, C. S., Ouellette, S. P., & Wesner, J. S. (2017). Incidence of *Wolbachia* in aquatic insects. *Ecology and Evolution*, *7*(4), 1165–1169.

Serbus, L. R., Casper-Lindley, C., Landmann, F., & Sullivan, W. (2008). The genetics and cell biology of *Wolbachia*-host interactions. *Annual Review of Genetics*, *42*, 683–707.

Shapiro, S. S., & Wilk, M. B. (1965). An analysis of variance test for normality (complete samples). *Biometrika*, *52*(3/4), 591–611.

Shropshire, J. D., On, J., Layton, E. M., Zhou, H., & Bordenstein, S. R. (2018). One prophage WO gene rescues cytoplasmic incompatibility in *Drosophila* melanogaster. *Proceedings of the National Academy of Sciences*, *115*(19), 4987–4991.

Singh, P., Rathinasamy, K., Mohan, R., & Panda, D. (2008). Microtubule assembly dynamics: an attractive target for anticancer drugs. *IUBMB Life*, *60*(6), 368–375.

Smerdon, M. J., Lan, S. Y., Calza, R. E., & Reeves, R. (1982). Sodium butyrate stimulates DNA repair in UV-irradiated normal and xeroderma pigmentosum human fibroblasts. *Journal of Biological Chemistry*, *257*(22), 13441–13447.

Stanton, R. A., Gernert, K. M., Nettles, J. H., & Aneja, R. (2011). Drugs that target dynamic microtubules: a new molecular perspective. *Medicinal Research Reviews*, *31*(3), 443–481.

Tandon, N., Ramakrishnan, V., & Kumar, S. K. (2016). Clinical use and applications of histone deacetylase inhibitors in multiple myeloma. *Clinical Pharmacology: Advances and Applications*, *8*, 35.

Tram, U., & Sullivan, W. (2002). Role of delayed nuclear envelope breakdown and mitosis in *Wolbachia*-induced cytoplasmic incompatibility. *Science*, *296*(5570), 1124–1126.

Vargha, A., & Delaney, H. D. (1998). The Kruskal-Wallis test and stochastic homogeneity. *Journal of Educational and Behavioral Statistics*, *23*(2), 170–192.

Veneti, Z., Clark, M. E., Karr, T. L., Savakis, C., & Bourtzis, K. (2004). Heads or tails: host-parasite interactions in the *Drosophila-Wolbachia* system. *Applied and Environmental Microbiology*, *70*(9), 5366–5372.

Wasserman, S. (1994). *Advances in social network analysis: Research in the social and behavioral sciences*. Sage.

Werren, J. H., Baldo, L., & Clark, M. E. (2008). *Wolbachia*: master manipulators of invertebrate biology. *Nature Reviews Microbiology*, *6*(10), 741–751.

Williamson, E. A., Wray, J. W., Bansal, P., & Hromas, R. (2012). Overview for the histone codes for DNA repair. *Progress in Molecular Biology and Translational Science*, *110*, 207–227.

Xu, Z., Wu, G., Wei, X., Chen, X., Wang, Y., & Chen, L. (2013). Celastrol induced DNA damage, cell cycle arrest, and apoptosis in human rheumatoid fibroblast-like synovial cells. *The American Journal of Chinese Medicine*, *41*(03), 615–628.

Yoshioka, A., Tanaka, S., Hiraoka, O., Koyama, Y., Hirota, Y., Ayusawa, D., … Wataya, Y. (1987). Deoxyribonucleoside triphosphate imbalance. 5-Fluorodeoxyuridine-induced DNA double strand breaks in mouse FM3A cells and the mechanism of cell death. *Journal of Biological Chemistry*, *262*(17), 8235–8241.

Zeiser, R. (2014). Trametinib. *Small Molecules in Oncology*, 241–248.

Zeng, X., Sigoillot, F., Gaur, S., Choi, S., Pfaff, K. L., Oh, D.-C., … King, R. W. (2010). Pharmacologic inhibition of the anaphase-promoting complex induces a spindle checkpoint-dependent mitotic arrest in the absence of spindle damage. *Cancer Cell*, *18*(4), 382–395.

CHAPTER V: CONCLUSIONS AND FUTURE DIRECTIONS

Cytoplasmic incompatibility (CI) is one of the most widespread reproductive modification induced in insects by *Wolbachia* (Yamada et al., 2007). CI causes embryonic mortality when infected males mate with uninfected females or, in many cases, when females are infected with a different strain of *Wolbachia* (Engelstädter & Telschow, 2009). On the other hand, *Wolbachia*-infected females are capable of producing viable embryos with either uninfected or infected males, thus increasing the infected population by population replacement (Caragata & Moreira, 2017). As the mating between an infected male and infected female can effectively limit or negate embryonic mortality, this cross is termed a "rescue" cross. The mechanism or mechanisms underlying CI and rescue have become a topic of intense interest in recent years, since they influence the genetic makeup of insect populations so severely and can be used as biological and ecological tools in the fight against human-related tropical diseases (Kamtchum-Tatuene et al., 2017).

Although cytological studies have described the mechanism of CI, they have been unable to explain the underlying mechanism of rescue (Lassy & Karr, 1996, Tram & Sullivan, 2002, Landmann et al., 2009). Transgenic expression studies reported that expression of two *Wolbachia* genes, *cifA* and *cifB,* are primarily responsible for CI, while the expression of the *cif*A gene is responsible for "rescue" (Beckmann et al., 2017; LePage et al., 2017; Shropshire et al., 2018). This type of toxin/antitoxin system can be invoked as the causative agents associated with CI/rescue, yet the underlying cellular mechanism(s) of rescue are still unknown. Therefore, the overall goal of this thesis was to identify the cellular components and pathways involved in rescue of CI. Due to the

relatively high CI rates, which mimic wild-type rates, we have used both native *Wolbachia w*Ri-infected, and *Wolbachia w*Mel-transinfected *Drosophilla simulans* as a model organism to address our queries.

In chapter II we designed a CI assay to determine whether we could observe the CI and rescue phenotypes in a lab setting. The assay technique was successfully able to differentiate the CI and rescue phenotypes, with the observation that decreased egg hatch rates in CI crosses were due to true embryonic mortality and not a result of simple mating failure. We then designed a feeding assay which also able to determine the effect of chemicals/cellular pathways on CI with statistical certainty. With our assay, we initially tested chemicals such as sodium butyrate (NaBu), acetic acid, changes in the food microbiome and insulin signaling pathways. It was also important to determine the most effective concentrations of chemicals in these feeding experiments, to avoid any adverse effect of these chemicals on the flies. This was accomplished with the use of survival curve assays. We were able to identify the histone deacetylase inhibitor sodium butyrate (NaBu) as able to induce a rescue effect on both *w*Ri- and *w*Mel-induced CI crosses in *Drosophila simulans*. The increased hatch rate (aka. rescue effect) with NaBu was also demonstrated not to be an artifact of a change in mating behavior. Overall, the methodology we present can readily be used to test CI and rescue phenotypes in other non-*Drosophila* CI models such as *Cardinium* spp.

After identifying that chemical feeding can induce the rescue effect on CI, presumably through modifying cellular pathways in the host, the next step was to prepare for a chemical screening assay. This would allow numerous pathways to be evaluated for their involvement in the rescue process. Though the plate-based assay is more cumbersome to set up than individual vials, the shared headspace

across conditions has the important advantage of controlling internally for release of undetected volatiles for conditions run in parallel. In Chapter III, we designed an assay using 24-well tissue culture plates, by modifying the feeding assay designed in chapter II. This assay was able to distinguish the CI and rescue phenotypes in a 24-well tissue culture format. Statistical analysis (pairwise comparisons mentioned in Chapter II) revealed that a total of six wells were necessary to distinguish CI and rescue, in all cases. Using this assay technique, we were successfully able to confirm the rescue effect of NaBu on CI crosses observed previously. Statistical analysis of the data revealed that for a particular chemical feeding, a total of 8 wells was necessary to observe any rescue effect (detailed in Chapter III). Thus, the assay with 24-well tissue culture plates is readily available to test the involvement of any cellular pathway in any chemical screening setting.

In Chapter IV we demonstrate the involvement of specific cellular pathways in overall rescue of CI. We tried to mimic the rescue of CI, naturally facilitated by *Wolbachia* infection of the female, by artificially inhibiting certain cellular mechanisms using chemical inhibitors, determined through extensive prior studies to effect specific proteins or pathways. Any chemical was considered effective if it was able to induce a statistically measurable rescue effect on CI, ie. an increase in egg hatch rate. A total of 24 chemicals, targeting various cellular mechanisms, were tested for their ability to induce the rescue effect on CI crosses. The major cellular processes tested here include HDAC function, DNA damage repair, chromatin modification and cell-cycle timing. The primary test was carried out on the native CI model *Drosophila simulans* infected with *Wolbachia w*Ri, which cause natural infection and a strong CI. Seven chemicals, including NaBu, Celastrol, Teniposide, Cycloheximide, Bortezomib, MG132, Trametinib

were able to induce the rescue effect on *w*Ri-induced CI in *D. simulans*. These chemicals effect cellular functions such as DNA damage repair, cell cycle delay and protein turnover. Among them, the HDAC inhibitor NaBu can have influence over multiple pathways such as HDAC function, DNA damage repair, cell cycle delay and protein turnover. These findings suggested that all/any of these pathways could be involved in rescue of *w*Ri-induced CI in *D. simulans*.

To understand whether the effect of these cellular pathways on overall rescue were conserved across different CI models, the chemicals with the greatest ability to induce the rescue effect on the native *w*Ri-induced CI, were tested on a transinfected *w*Mel-induced CI model. Among the seven most effective chemicals, three chemicals, NaBu, Celastrol and Cycloheximide were able to induce the rescue effect on *w*Mel-induced CI on *D. simulans*. These chemicals can affect cellular pathways/functions such as HDAC function, DNA damage and cell cycle and protein turnover. The chemicals specifically targeting the cell cycle (Bortizomib, MG132, Trametinib) were unable to induce any rescue effect on this transinfected CI model. This finding suggests that chemical pathways such as HDAC function, DNA damage, cell cycle timing and protein turnover might be involved in rescue of *w*Mel-induced CI in *D. simulans*. This finding also suggests that only some specific pathways or mechanisms might be involved in the rescue across different CI models.

To determine whether inhibiting multiple pathways at the same time can increase the rescue capability on CI, two chemical inhibitors were combined and tested for their ability to inhibit multiple pathways, as well as testing their ability to a induce a stronger rescue of CI. A total of 7 different combinations of chemical inhibitors, targeting different pathways, were tested on the native *w*Ri-induced CI in *D. simulans*. The concentration

of each chemical in the combination was selected by survival curve assay. Interestingly, only the Cycloheximide/Bortezomib combination was able to induce a significant rescue effect on the tested CI model. Since the individual chemical concentrations used in combination were lower that used singularly, one possible explanation for this observation could be that the inhibitors were unable to produce a cellular effect strong enough to produce a rescue effect at that concentration. The other possible explanation involves possible crosstalk between different pathways and the modification of one pathway subsiding the effect of another pathway. Thus, this experimental procedure can be a framework for determining the effect of multiple cellular pathways by using chemical inhibitors, with the caveat that maximum tolerable dosages limit resolution of the assay.

Some transgenic studies with *D. melanogaster* demonstrate two *Wolbachia* genes related to CI, generally known as *cif* (CI factor) genes. *cif* is a two gene operon *cifA* and *cifB*. The first gene in the cif operon is called *cif*A, and the second gene is called *cif*B. CifB proteins have either deubiquitlyase (Beckmann et al., 2017) (Cid) or nuclease (Chen et al., 2019) (Cin) or both (Cnd) enzymatic functions. In transgenic studies the expression of CidA and CidB proteins together in male caused CI and expression of CidA induced rescue. Although a number of studies have been carried out that investigate the molecular mechanism of CidB that causes CI, it is still not clear about the molecular mechanism of CidA that causes rescue*. cidB* genes encode deubiquitylases (DUBs), which cleave ubiquitin from target proteins. CidB may have multiple direct targets. It might deubiquitylate many of the proteins found in the cell, for instance, and CI results from the accumulated defects caused by these changes (Beckmann 2019). A recent study demonstrated that CidB can interact with nuclear transporter karyopherins Kap-α2 and Moleskin as well as with the histone chaperone P32 in *Drosophila*

(Beckmann 2019). This study also identified CidA interacting proteins, where the top hits are a predicted nucleotide exchange factor Roe1, a lipid kinase Pi3K92E, and aminolevulinic acid synthase. It is still unclear whether any of these interactions is relevant to CI induction (Beckmann 2019), but it is possible that its main function being tight association with CidB. Overexpression of P32 and karyopherin-α in female *Drosophila* can suppress the CI induced by wMel-infected male (Beckmann 2019).

Our study was designed to address the contribution of host chromatin remodeling, DNA damage repair and cell cycle timing impacts on CI suppression/ rescue induction in vivo. At present, our result can't distinguish between existing models of CI and rescue, but it may open models that may better reflect the cell biology of CI. CidB is a deubiquilating enzyme that can act on many proteins in cell. Ubiquitylation of Kap-α may also be important for its ability to promote nuclear import of a key maternal protein(s) involved in protamine-histone exchange. Histone H2A and H2B are well characterized as ubiquitylated proteins. Its ubiquitylation may promote histone H3.3 loading and nucleosome formation. CI is characterized by its abnormal maternal histone deposition. So, it might be possible that CidB is involved in the abnormal maternal histone deposition process which might be inhibited by presence of CidA in eggs. In our study we have found that NaBu is a chromatin remodeler which may suppress CI may be by correcting the abnormalities in histone deposition. It can also be supported by the findings that overexpression of P32, a protamine histone chaperone in female can suppress the wMel-induced CI in *Drosophila melanogaster.* CidB has also found to be interacting with proteins involved in DNA replication, repair and packaging as well as cell division (Beckmann 2019). This finding suggested that CidB might be interact with these pathways to induce CI. It is also characterized in CI embryos a replication defect between maternal and paternal embryos.

The interaction between CidB and DNA repair proteins can also be informative. Furthermore, the CidB paralog CinB has nuclease activity and can also cause CI. In our study we found CI-suppressing / rescue-inducing compounds involved in DNA repair pathways. It might be possible that the replicating DNA might be repaired by these pathways and thus resulting the fixation of CI defect. DNA damage repair is facilitated by ubiquitination of histones and DNA repair pathway proteins, followed by their deubiquitination upon completion of the repair (Cohn and D'Andrea, 2008; Stadler and Richly, 2017). Cid B also interact with proteins involved in cell division. It is also important to note that all the 7 compounds found to induce a rescue of CI are inhibitors of the cell cycle. It might be possible that these compounds acted on pathways to synchronize the cell division between maternal and paternal counterpart, thus solving the problem of asynchrony in CI embryos. Our study doesn't readily explain the basic mechanism of rescue of CI, but it can link the pathways found in our study to the available literature of CI. Further studies involving the rescue-inducing factor CidA will be necessary to further unravel the mechanism involved in overall rescue of CI.

We were unable to find any clear association of the chemical inhibitors in the case of affecting protein turnover. While NaBu, Teniposide and Trametinib induce proteasomal activity (Giuliano et al., 1999; Yusenko et al., 2018; Lin et al., 2020), the other four compounds, Celastrol, Cycloheximide, Bortezomib and MG132, inhibit proteasomal degradation (Yang et al., 2006; Hanna et al., 2003; Berkers et al., 2005) (Figure 5.1). We were also unable to find any clear association of the chemical inhibitors to induce a cell cycle delay. All the compounds identified here as CI-suppressing/ rescue-inducing agents, act as consistent suppressors of cell cycle timing. DNA damage and cell cycle delay are intrinsically connected, and the DNA damage triggers a

checkpoint mechanism and causes cell cycle delay (Alberts et al., 2015; Chao et al., 2017) (Figure 5.1). A clearer association was evident between existing literature and CI suppression/rescue induction outcomes in this study from the perspective of DNA integrity. The four compounds that suppress *w*Ri-induced CI are known inducers of DNA damage (Long et al., 1985; Jacquemont & Taniguchi, 2007; Maertens et al., 2019) (Figure 5.1), whereas the three compounds that suppress both *w*Ri- and *w*Mel-induced CI, reportedly support DNA integrity. Celastrol can exert detrimental impacts on DNA (Han et al., 2018; Wang et al., 2020), it has also been shown to suppress radiation-induced damage (Xu et al., 2013; Moreira et al., 2019). Cycloheximide treatments prevent formation of single- and double-strand DNA breaks (Yoshioka et al., 1987). NaBu protects DNA integrity by upregulating antioxidant pathways and by facilitating DNA repair (Smerdon et al., 1982; Mao & Wyrick, 2016) (Figure 5.1). This suggests that DNA integrity is a dynamic, focal aspect of CI suppression with respect to different *Wolbachia* strains in *D. simulans.* The pathways related to DNA integrity might be further tested as a pathway involved in overall rescue of CI. This pathway can also be tested in other CI models for further insight into potential rescue mechanisms.

Overall, this project has created a template for understanding the underlying mechanism(s) of *Wolbachia*-induced rescue of CI. Using native and transinfected CI models in *Drosophila simulans*, our study demonstrated that specific cellular pathways/processes are involved in *Wolbachia*-induced rescue mechanisms that have largely remained unknown until now. The tools that we have introduced here will provide a better understanding of how these mechanisms can be tested. This work also provides a framework for how such investigation should be carried out with statistical validation. In conclusion, this work opens up the possibility to understand reproductive manipulation

mechanisms across different CI and rescue model/systems, informing us of the cellular basis of rescue of CI in greater detail. Later studies with compounds targeting DNA integrity might be greatly informative across different CI systems to resolve the mysteries of the overall rescue mechanism.

Figures

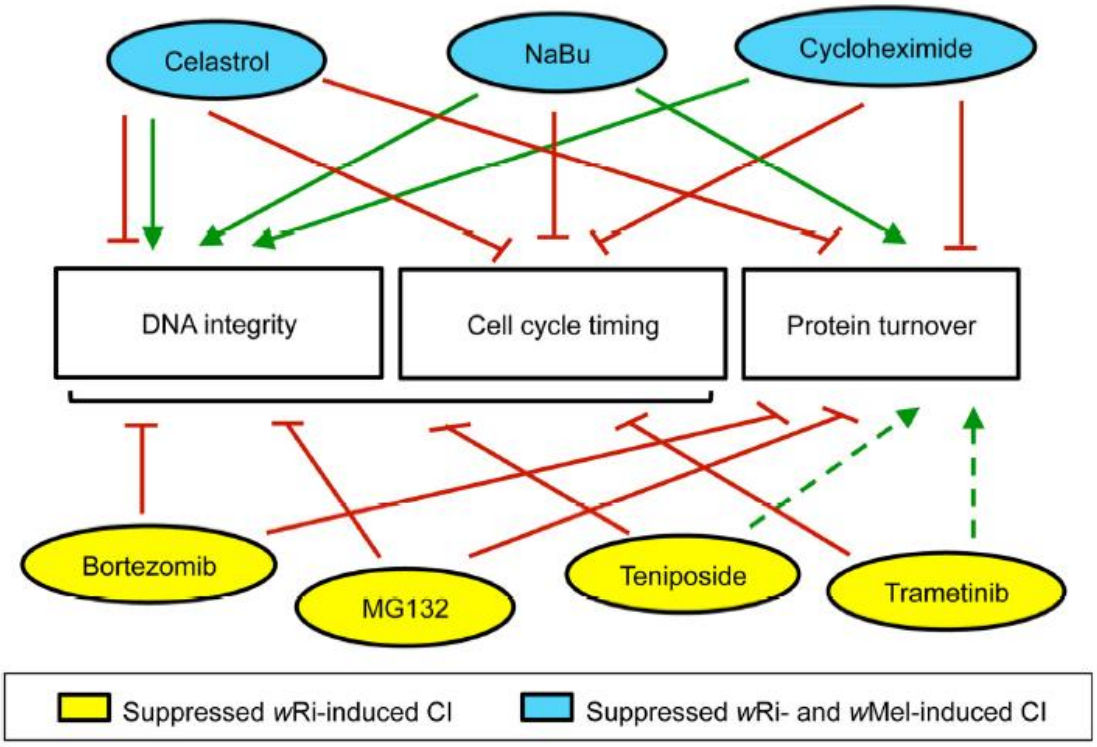

Figure.5.1. Summary of maternal impacts that significantly increased *D. simulans* CI hatch rates. Green arrows: positive impact. Red lines: negative effect. Dotted lines: interpretation based upon partial datasets. Bracket: includes multiple categories.

References

Alberts, B., Bray, D., Hopkin, K., Johnson, A. D., Lewis, J., Raff, M., … Walter, P. (2015). *Essential cell biology*. Garland Science.

Beckmann, J. F., Ronau, J. A., & Hochstrasser, M. (2017). A Wolbachia deubiquitylating enzyme induces cytoplasmic incompatibility. *Nature Microbiology*, *2*(5), 1–7.

Berkers, C. R., Verdoes, M., Lichtman, E., Fiebiger, E., Kessler, B. M., Anderson, K. C., … Galardy, P. J. (2005). Activity probe for in vivo profiling of the specificity of proteasome inhibitor bortezomib. *Nature Methods*, *2*(5), 357–362.

Caragata, E. P., & Moreira, L. A. (2017). Using an endosymbiont to control mosquito-transmitted disease. In *Arthropod Vector: Controller of Disease Transmission, Volume 1* (pp. 123–142). Elsevier.

Chao, H. X., Poovey, C. E., Privette, A. A., Grant, G. D., Chao, H. Y., Cook, J. G., & Purvis, J. E. (2017). Orchestration of DNA damage checkpoint dynamics across the human cell cycle. *Cell Systems*, *5*(5), 445–459.

Engelstädter, J., & Telschow, A. (2009). Cytoplasmic incompatibility and host population structure. *Heredity*, *103*(3), 196–207.

Giuliano, M., Lauricella, M., Calvaruso, G., Carabillo, M., Emanuele, S., Vento, R., & Tesoriere, G. (1999). The apoptotic effects and synergistic interaction of sodium butyrate and MG132 in human retinoblastoma Y79 cells. *Cancer Research*, *59*(21), 5586–5595.

Han, X., Tan, Y., Fang, Y., & Li, F. (2018). Protective effects of celastrol against γ irradiation‐induced oxidative stress in human umbilical vein endothelial cells. *Experimental and Therapeutic Medicine*, *16*(2), 685–694.

Hanna, J., Leggett, D. S., & Finley, D. (2003). Ubiquitin depletion as a key mediator of toxicity by translational inhibitors. *Molecular and Cellular Biology*, *23*(24), 9251–9261.

Jacquemont, C., & Taniguchi, T. (2007). Proteasome function is required for DNA damage response and fanconi anemia pathway activation. *Cancer Research*, *67*(15), 7395–7405.

Kamtchum-Tatuene, J., Makepeace, B. L., Benjamin, L., Baylis, M., & Solomon, T. (2017). The potential role of Wolbachia in controlling the transmission of emerging human arboviral infections. *Current Opinion in Infectious Diseases*, *30*(1), 108.

Landmann, F., Orsi, G. A., Loppin, B., & Sullivan, W. (2009). Wolbachia-mediated cytoplasmic incompatibility is associated with impaired histone deposition in the male pronucleus. *PLoS Pathogens*, *5*(3), e1000343.

Lassy, C. W., & Karr, T. L. (1996). Cytological analysis of fertilization and early embryonic development in incompatible crosses of Drosophila simulans. *Mechanisms of Development*, *57*(1), 47–58.

LePage, D. P., Metcalf, J. A., Bordenstein, S. R., On, J., Perlmutter, J. I., Shropshire, J. D., … Bordenstein, S. R. (2017). Prophage WO genes recapitulate and enhance Wolbachia-induced cytoplasmic incompatibility. *Nature*, *543*(7644), 243–247.

Lin, L., Ding, D., Xiao, X., Li, B., Cao, P., & Li, S. (2020). Trametinib potentiates TRAIL-induced apoptosis via FBW7-dependent Mcl-1 degradation in colorectal cancer cells. *Journal of Cellular and Molecular Medicine*, *24*(12), 6822–6832.

Long, B. H., Musial, S. T., & Brattain, M. G. (1985). Single-and double-strand DNA breakage and repair in human lung adenocarcinoma cells exposed to etoposide and teniposide. *Cancer Research*, *45*(7), 3106–3112.

Maertens, O., Kuzmickas, R., Manchester, H. E., Emerson, C. E., Gavin, A. G., Guild, C. J., … Hatchi, E. (2019). MAPK pathway suppression unmasks latent DNA repair defects and confers a chemical synthetic vulnerability in BRAF-, NRAS-, and NF1-mutant melanomas. *Cancer Discovery*, *9*(4), 526–545.

Mao, P., & Wyrick, J. J. (2016). Emerging roles for histone modifications in DNA excision repair. *FEMS Yeast Research*, *16*(7).

Moreira, H., Szyjka, A., Paliszkiewicz, K., & Barg, E. (2019). Prooxidative activity of celastrol induces apoptosis, DNA damage, and cell cycle arrest in drug-resistant human colon cancer cells. *Oxidative Medicine and Cellular Longevity*, *2019*.

Shropshire, J. D., On, J., Layton, E. M., Zhou, H., & Bordenstein, S. R. (2018). One prophage WO gene rescues cytoplasmic incompatibility in Drosophila melanogaster. *Proceedings of the National Academy of Sciences*, *115*(19), 4987–4991.

Smerdon, M. J., Lan, S. Y., Calza, R. E., & Reeves, R. (1982). Sodium butyrate stimulates DNA repair in UV-irradiated normal and xeroderma pigmentosum human fibroblasts. *Journal of Biological Chemistry*, *257*(22), 13441–13447.

Tram, U., & Sullivan, W. (2002). Role of delayed nuclear envelope breakdown and mitosis in Wolbachia-induced cytoplasmic incompatibility. *Science*, *296*(5570), 1124–1126.

Wang, H., Ahn, K. S., Alharbi, S. A., Shair, O. H. M., Arfuso, F., Sethi, G., … Tang, F. R. (2020). Celastrol alleviates gamma irradiation-induced damage by modulating diverse inflammatory mediators. *International Journal of Molecular Sciences*, *21*(3), 1084.

Xu, Z., Wu, G., Wei, X., Chen, X., Wang, Y., & Chen, L. (2013). Celastrol induced DNA damage, cell cycle arrest, and apoptosis in human rheumatoid fibroblast-like synovial cells. *The American Journal of Chinese Medicine*, *41*(03), 615–628.

Yamada, R., Floate, K. D., Riegler, M., & O'Neill, S. L. (2007). Male development time

influences the strength of Wolbachia-induced cytoplasmic incompatibility expression in Drosophila melanogaster. *Genetics*, *177*(2), 801–808.

Yang, H., Chen, D., Cui, Q. C., Yuan, X., & Dou, Q. P. (2006). Celastrol, a triterpene extracted from the Chinese “Thunder of God Vine,” is a potent proteasome inhibitor and suppresses human prostate cancer growth in nude mice. *Cancer Research*, *66*(9), 4758–4765.

Yoshioka, A., Tanaka, S., Hiraoka, O., Koyama, Y., Hirota, Y., Ayusawa, D., … Wataya, Y. (1987). Deoxyribonucleoside triphosphate imbalance. 5-Fluorodeoxyuridine-induced DNA double strand breaks in mouse FM3A cells and the mechanism of cell death. *Journal of Biological Chemistry*, *262*(17), 8235–8241.

Yusenko, M., Jakobs, A., & Klempnauer, K.-H. (2018). A novel cell-based screening assay for small-molecule MYB inhibitors identifies podophyllotoxins teniposide and etoposide as inhibitors of MYB activity. *Scientific Reports*, *8*(1), 1–11.

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Publications and presentations

Momtaz, A. J. M., Ahumada Sabagh, A. D., Gonzalez Amortegui, J. G., Salazar, S. A., Finessi, A., Hernandez, J., Christensen,S. & Serbus, L. R. (2020). A Role for Maternal Factors in Suppressing Cytoplasmic Incompatibility. *Frontiers in microbiology*, *11*, 2839.

Christensen, S., Camacho, M., Sharmin, Z., Momtaz, A. Z., Perez, L., Navarro, G., Triana, J., Samarah, H., Turelli, M. & Serbus, L. R. (2019). Quantitative methods for assessing local and bodywide contributions to Wolbachia titer in maternal germline cells of Drosophila. BMC microbiology, 19(1), 1-17.

Serbus, L. R., Garcia, B. R., Sharmin, Z., Momtaz, A. Z., & Christensen, S. (2017). Predictive Genomic Analyses Inform the Basis for Vitamin Metabolism and Provisioning in Bacteria-Arthropod Endosymbioses. *G3: Genes, Genomes, Genetics*, g3-117.

Christensen, S., Dulzaides, R. P., Hedrick, V. E., Momtaz, A. Z., Nakayasu, E. S., Paul, L. N., & Serbus, L. R. (2016). Wolbachia Endosymbionts Modify Drosophila Ovary Protein Levels in a Context-Dependent Manner. Applied and Environmental Microbiology, 82(17), 5354-5363.

Nahar, S., Afrad, M. H., Matthijnssens, J., Rahman, M. Z., Momtaz, Z., Yasmin, R.,& Rahman, M. (2013). Novel intergenotype human norovirus recombinant GII. 16/GII. 3 in Bangladesh. Infection, Genetics and Evolution, 20,

Momtaz AJMZ, Salazar S, Finessi A, Serbus L. Understanding the Cellular Basis for Rescue of *Wolbachia*-induced Embryonic Lethality. Bio symposium, FIU, 2018