University of Alberta

Unique Cellular Interactions Between the Obligate Intracellular Bacteria *Wolbachia pipientis* and its Insect Host

by

Lesley Jean Brennan

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Abstract

Wolbachia are maternally inherited obligate intracellular bacteria found in arthropods, where they induce feminization, male-killing, parthenogenesis, and cytoplasmic incompatibility (CI). CI is conditional male sterility, in which *Wolbachia*-infected males successfully mate with infected females, but crosses between infected males and uninfected females result in embryonic death. How sperm are modified and how the *Wolbachia*-infected egg rescues them, resulting in normal embryonic development, is unknown.

The objective of this thesis is to contribute to an understanding of the cellular biology of *Wolbachia*-host interactions, including the mechanism of CI. Protein expression in *Wolbachia*-infected and uninfected *Aedes albopictus* cells was evaluated by 2D PAGE. Proteins expressed exclusively in the presence of *Wolbachia* were identified, and included host actin depolymerizing factor and bacterial single-strand binding protein, GroES, 3,4-dihydroxy-2-butanone 4-phosphate synthase, nucleoside diphosphate kinase, and proteins involved in bacterial protein synthesis.

Three host proteins (copper zinc superoxide dismutase, glutathione peroxidase, and peroxiredoxin) and two bacterial proteins (iron superoxide dismutase and bacterioferritin) having antioxidant activity were also identified. Antioxidants neutralize reactive oxygen species (ROS) generated by aerobic respiration or an immune response and induce cellular damage. Flow cytometric

and microscopic analysis confirmed that ROS is elevated in infected cells and is associated with *Wolbachia*-containing vacuoles in the host cell cytoplasm. In *Drosophila simulans* flies, antioxidant assays showed that ROS is elevated in infected reproductive tissues, particularly the testes. To evaluate the effect of ROS accumulation, DNA damage was measured in *Ae. albopictus* cell lines by DNA dot blotting for the oxidative lesion 8-oxo-dG, which revealed an 8% increase in damage in DNA from infected cells. In *D. simulans* flies, analysis of 8-oxo-dG in DNA from whole males by mass spectrometry showed a slight increase in the lesion in infected flies, while single cell gel electrophoresis of spermatocytes revealed a 20% increase in single and double-stranded breaks as a result of *Wolbachia* infection. The conclusion from these results is that redox homeostasis is maintained in *Wolbachia*-infected insects as a whole. However in the densely infected testes *Wolbachia*-mediated ROS production exceeds antioxidant capacity resulting in oxidative DNA damage. The potential role of this damage in cytoplasmic incompatibility is discussed.

This thesis is dedicated to my parents. Thank you for your love and guidance.

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2D PAGE	2 dimensional polyacrylamide gel electrophoresis
8-oxo-dG	7,8-dihydro-8-oxo-2'-deoxyguanosine
Aa23	*Aedes albopictus* embryonic cell line, infected with *Wolbachia*
Aa23T	*Aedes albopictus* embryonic cell line , cured of *Wolbachia*
ADF	Actin depolymerising factor
AH	Androgenic hormone
Ahp	alkyl hydroperoxide reductase
AIF	Apoptosis-inducing factor
AP	Abasic site (apurinic/apyrimidinic site)
BCP	bacterial comigratory protein
BER	Base excision repair
Bfr	Bacterioferritin
carboxy-H_2DCFDA	5-(and-6)-carboxy-2′,7′-dichlorodihydrofluorescein diacetate
Cdk-1	Cyclin dependent kinase-1
CEB	Cell extraction buffer
CI	Cytoplasmic incompatibility
D. mel	*Drosophila melanogaster*
DAPI	4',6-Diamidino-2-phenylindole dihydrochloride
DIF	Dorsal-related immunity factor
Dps	DNA-binding protein from starved cells

DSR	*Drosophila simulans* Riverside, infected with *Wolbachia*
DSRT	*Drosophila simulans* Riverside, cured of *Wolbachia*
DTT	Dithiothreitol
Duox	Dual oxidase
EF-Tu	Elongation factor Tu
ERK	Extracellular signal-regulated kinase
FtnA	Ferritin-A
Fur	Ferric uptake regulator
GGR	Global genome repair
GNBP	Gram negative binding protein
GPx	Glutathione peroxidise
GSC	Germline stem cell
GSH	Reduced glutathione
HPI	Hydroperoxidase I
HPII	Hydroperoxidase II
HR	Homologous recombination
IAA	Iodoacetamide
IMD	Immune deficiency
ird	Immune response defective
ISC	Intestinal stem cell
JNK	Jun kinase

LCV	*Legionella*-containing vacuole
MLST	Multilocus sequence typing
MMR	Mismatch repair
Ndk	Nucleoside diphosphate kinase
NEB	Nuclear envelope breakdown
NER	Nucleotide excision repair
Nf-KB	Nuclear factor kappa B
NHEJ	Non-homologous end-joining
Nox	NADPH oxidase
Ns-Gpx	Nonselenium glutathione peroxidase
PBS	Phosphate-buffered saline (pH 7.2-7.6)
PCR	Polymerase chain reaction
PG	Peptidoglycan
PGRP	Peptidoglycan receptor protein
PI	Parthenogensis inducing
PLCβ	Phospholipase C-β
Prx	Peroxiredoxin
ROS	Reactive oxygen species
RRF	Ribosome releasing factor
SCGE	Single cell gel electrophoresis
SDS	Sodium dodecyl sulfate

SOD	Superoxide dismutase
SSB	Single strand binding protein
TBHP	*tert*-butyl hydroperoxide
TCR	Transcription-coupled repair
TEMPO	2,2,6,6-tetramethylpiperidine 1-oxyl
TNF	Tumour necrosis factor
TPx	Thioredoxin peroxidase
Trx	Thioredoxin
*w*Bm	*Wolbachia* strain found in *Brugia malayi*
*w*Mel	*Wolbachia* strain found in *Drosophila melanogaster*
*w*Ri	*Wolbachia* strain found in *Drosophila simulans* Riverside
wsp	*Wolbachia* surface protein

1. INTRODUCTION

1.1 Bacterial endosymbionts of insects

Symbiotic relationships between prokaryotes and eukaryotes are widespread in nature, although they often receive little attention. One of the most fascinating partnerships is that of insects and inherited symbiotic bacteria. These associations have evolved over millions of years and have contributed to the diversity and success of insects. Many insects thrive on nutritionally deficient food sources (phloem, xylem sap, blood) because symbionts supplement their dietary needs (Douglas, 2009; Gosalbes et al., 2010). Some symbionts provide their hosts with protection against pathogens, parasites, and predators (Haine, 2008). In return, bacterial symbionts gain a safe and protected environment. However, they often act as parasites, manipulating the host to their advantage (Bandi et al., 2001). As a result, symbiotic relationships between insects and bacteria have evolved to become a complex balancing act of interactions (Harris et al., 2010). An understanding of these interactions is invaluable to comprehending prokaryote-eukaryote interactions in a broader sense.

1.1.1 Primary and secondary endosymbionts

Endosymbiotic bacteria are found exclusively in the intracellular environment. Two categories of endosymbionts have been described, based on their host dependence. The primary, or P-endosymbionts are vertically

transmitted obligate mutualists, display long evolutionary histories with their host and are found within specialized organs called bacteriomes which consist of a group of host cells referred to as bacteriocytes (Moran et al., 2008). The best studied example is *Buchnera aphidicola*, the primary endosymbiont of the pea aphid *Acyrthosiphon pisum. Buchnera* are gamma-proteobacteria located in a bacteriome adjacent to the ovariole within the insect hemocoel (reviewed in Baumann et al., 1995; Brinza et al., 2009). This relationship is estimated to be over 180 million years old and both partners have lost the ability to function independently (Moran et al., 1993). The genome of *Buchnera* is significantly reduced, a consequence of an intracellular lifestyle, leaving it dependent upon the host for many nutrients, including most non-essential amino acids (Shigenobu et al., 2000). However, *Buchnera* has retained genes required to provide its host with essential amino acids that are lacking in the aphid diet of phloem sap (Douglas, 1998; Moran et al., 2003; Wilkinson and Ishikawa, 2000). Non-essential amino acids are often precursors for the biosynthesis of essential amino acids; *Buchnera* and its aphid host therefore not only complement each other metabolically, but are dependent on each other for survival. *Blochmannia floridanus* (gamma-proteobacteria), the primary endosymbiont of carpenter ants, also supplements the host with essential amino acids (Feldhaar et al., 2007), while *Wigglesworthia glossinidia* (gamma-proteobacteria), provides

vitamins and is required for female fecundity in tsetse flies (*Glossina* spp.) (Pais et al., 2008).

The secondary symbionts , or S-symbionts have more recently established associations with their host, are not restricted to vertical transmission, are found in various cell types, and are facultative from the standpoint of the host (Harris et al., 2010; Moya et al., 2008). *Wolbachia pipientis* are well-known S-symbionts, however, the variety of S-symbionts includes *Hamiltonella defensa*, *Regiella insecticola*, and *Serratia symbiotica,* all of which are gamma-proteobacteria common to aphids (Moran et al., 2005). Whiteflies harbour bacteria in the genus *Hamiltonella*, in addition to *Wolbachia* and *Rickettsia,* the gamma-proteobacteria *Arsenophonus,* and the sphingobacterium *Cardinium* (Chiel et al., 2007; Skaljac et al., 2010). Mites are also popular hosts for S-symbionts; in addition to *Wolbachia* and *Cardinium*, they often carry *Spiroplasma* (Mollicutes)(Enigl and Schausberger, 2007).

1.1.2 Genome evolution of endosymbionts

Endosymbiotic bacterial genomes exhibit a bias towards adenine and thymine base composition, erosion of the bacterial genome due to a lack of selection, and deletional bias (Mira et al., 2001; Moran et al., 2008; Rio et al., 2003). This is especially true of the P-endosymbionts, which have some of the smallest functional genomes documented (Akman et al., 2002; Gil et al., 2002;

Gil et al., 2003). S-symbionts appear to be in various stages of evolutionary transition, having genomes of intermediate size compared to free-living relatives which are littered with repetitive DNA, mobile genetic elements, and pseudogenes (Dale et al., 2006; Darby et al., 2010; Degnan et al., 2010; Degnan et al., 2009; Wu et al., 2004). As the bacterial genome evolves, the symbiont becomes increasingly reliant on its host for critical cell processes. Because one insect may harbour several endosymbionts simultaneously, isolating and studying these fastidious bacteria is a considerable challenge.

1.2 *Wolbachia pipientis*

Wolbachia are inherited S-symbionts of arthropods belonging to the alpha-proteobacteria, order *Rickettsiales*, and are closely related to the genera *Neorickettsia, Anaplasma, and Ehrlichia* (Dumler et al., 2001; Hotopp et al., 2006; O'Neill et al., 1992). They are small (0.5-1.2 µm), Gram negative, and coccoid to rod-shaped (Hertig, 1936; Popov et al., 1998). *Wolbachia* were first described in the ovaries of *Culex pipiens* (Hertig and Wolbach, 1924) and have since been detected in 20-76 % of arthropod species (Jeyaprakash and Hoy, 2000; Kikuchi and Fukatsu, 2003; Tagami and Miura, 2004; Werren and Windsor, 2000; Werren et al., 1995b). A recent meta-analysis predicts that approximately 66 % of arthropods may be *Wolbachia*-positive (Hilgenboecker et al., 2008). *Wolbachia* have been documented in all of the major insect orders (Harris and Braig, 2003;

Werren et al., 1995a), and while different strains have been identified based upon their host association, overall similarity has led to the designation of a single type species, *Wolbachia pipientis, ,* Other invertebrates, including several species of filarial nematodes (Bandi et al., 1998; Casiraghi et al., 2001a; Casiraghi et al., 2001b; Keiser et al., 2008) and isopod crustaceans (Bouchon et al., 1998; Cordaux et al., 2004; Wiwatanaratanabutr et al., 2009) also harbour *Wolbachia*.

Maternal inheritance through the egg cytoplasm (Serbus et al., 2008; Serbus and Sullivan, 2007; Tram et al., 2003) is the common mode of transmission for *Wolbachia*, but horizontal transmission is also known to occur (Baldo et al., 2008; Batista et al., 2009; Huigens et al., 2004; Huigens et al., 2000; Vavre et al., 1999). *Wolbachia* are found within the host cell cytoplasm bound by three membranes: an outer layer, which is of host origin, followed by the bacterial cell wall and plasma membrane (Louis and Nigro, 1989). *Wolbachia* are believed to replicate within these vacuoles, as multiple bacteria are often observed within a single locale (O'Neill et al., 1997). *Wolbachia* are consistently found in reproductive tissue and have also been identified in the hemolymph and somatic tissues including muscle, fat body, midgut, brain, salivary glands, and Malpighian tubules (Cheng et al., 2000; Clark et al., 2005; Dobson et al., 1999).

1.2.1 The *Wolbachia* genome

Wolbachia genomes from several insect hosts have been sequenced and annotated, including *w*Mel from *Drosophila melanogaster,* 1.27 Mb (Wu et al., 2004), *w*Pip from *Culex pipiens,* 1.48 Mb (Klasson et al., 2008) and *w*Ri from *Drosophila simulans*, 1.45 Mb (Klasson et al., 2009b). *E. coli,* a free-living proteobacteria, has a genome size of approximately 4.6 Mb (Blattner et al., 1997). Like most obligate bacterial symbionts, the *Wolbachia* genomes are significantly reduced as a result of deletional bias. For example, *w*Mel has lost genes involved in cell envelope biogenesis and cell wall synthesis; numerous other genes are defective and in various stages of degradation (Wu et al., 2004).

The genomes of *w*Mel, *w*Ri, and *w*Pip are similar in G + C content (34.3-35.2%), and contain unusually large amounts of repetitive DNA and DNA corresponding to mobile genetic elements, including several prophages (Klasson et al., 2008; Klasson et al., 2009b; Wu et al., 2004). A unique feature of the *Wolbachia* genomes is the presence of numerous genes coding for ankyrin repeat domains, which are tandem repeats commonly found in eukaryotic proteins, but less frequently in prokaryotes. Ankyrin repeats mediate protein-protein interactions and are involved in many cellular processes, including cell cycle regulation, ionic transport, cytoskeleton interactions, signal transduction, development, and differentiation (reviewed in Hryniewicz-Jankowska et al., 2002; Li et al., 2006a; Sedgwick and Smerdon, 1999). Ankyrin-repeat containing

proteins are believed to mediate *Wolbachia*-host interactions and play a role in the manipulation of host reproductive biology (Iturbe-Ormaetxe et al., 2005; Walker et al., 2007).

It is interesting to note that lateral gene transfer from *Wolbachia* to its host is not uncommon. It has been documented within both insect and nematode hosts (Hotopp et al., 2007). The transfer of short insertions up to nearly the entire genome has been reported (Hotopp et al., 2007; Klasson et al., 2009a; Kondo et al., 2002). Such transfer may play an important role in the evolution of the host (Klasson et al., 2009a).

1.2.2 Reproductive manipulations

Wolbachia have the remarkable ability to alter host reproduction to give infected females a selective advantage. Since *Wolbachia* are maternally inherited, this enhances the spread and persistence of infection. *Wolbachia* are therefore referred to as reproductive parasites. Reproductive manipulations include feminization, male-killing, parthenogenesis, and cytoplasmic incompatibility (reviewed by Duron et al., 2008; Engelstadter and Hurst, 2009).

1.2.2.1 Feminization

The development of chromosomal males as functional phenotypic females is known as feminization. *Wolbachia*-induced feminization is common in

isopods (Bouchon et al., 1998; Rousset et al., 1992). *Wolbachia* are believed to inhibit differentiation of the androgenic gland, which produces the male androgenic hormone (AH), and is often hypertrophied in infected intersexes (Nasr et al., 2010; Rigaud et al., 1997; Rigaud et al., 1999). In some isopods, *Wolbachia* may also interfere with AH receptors, preventing masculinization even in the presence of AH (Rigaud et al., 1997; Rigaud et al., 1999). Feminizing *Wolbachia* have been identified in only two insects: the leafhopper *Zyginidia pullula* (Hemiptera) (Negri et al., 2006) and the moth *Eurema hecabe* (Lepidoptera) (Hiroki et al., 2002). While the exact mechanism of feminization is unknown, recent work in *Z. pullula* suggests that epigenetic modification is involved. In this case, W*olbachia* disrupts normal male genomic imprinting by altering cytosine methylation patterns, presumably modifying the expression of genes involved in sex determination and development (Negri et al., 2009). Narita and colleagues (2007) found that in the *E. hecabe*, for *Wolbachia* to successfully feminize genetic males, it must act continuously on its host throughout larval development. Sex determination in *Z. pullula* and *E. hecabe* differs significantly: the former by XO/XX, in which the absence of a second sex chromosome determines a male and the latter by ZZ/ZW, in which the female is heterogametic (Sanchez, 2008), and this may influence the mechanism of feminization. However, both studies found that the intensity of feminization is correlated with *Wolbachia* density (Narita et al., 2007; Negri et al., 2009).

1.2.2.2 Male-Killing

Male-killing *Wolbachia* have been identified in arachnids (Zeh et al., 2005) and three insect orders: Coleoptera (Fialho and Stevens, 2000; Hurst et al., 1999), Lepidoptera (Dyson et al., 2002; Hurst et al., 1999; Jiggins et al., 2000; Kageyama and Traut, 2004; Mitsuhashi et al., 2004), and Diptera (Dyer and Jaenike, 2004; Hurst et al., 2000; Sheeley and McAllister, 2009). The result of *Wolbachia* in these arthropods is a sex ratio that is either exclusively female or highly female-biased. *Wolbachia*-induced male-killing occurs during embryonic stages; cannibalism of these eggs by sister siblings upon emergence provides a valuable source of nourishment to infected females (Hurst and Majerus, 1993). The mechanism of male-killing is unknown; however, recent research has provided unique insight. In the butterfly *Hypolimnas bolina,* inhibition of *Wolbachia* proliferation in females by bacteriostatic antibiotics delayed male-killing until larval stages, and treatment of infected male larva with bacteriocidal antibiotics permitted full rescue of males (Charlat et al., 2007). This suggests that *Wolbachia's* effect is not limited to embryonic stages of development. In moths in the genus *Ostrinia,* males infected with *Wolbachia* die during larval development, but females require *Wolbachia* for survival (Kageyama and Traut, 2004; Sakamoto et al., 2007). The *Wolbachia* present are capable of feminizing the host, and complete feminization is believed to be the basis of male mortality (Kageyama and Traut, 2004; Sakamoto et al., 2007).

1.2.2.3 Parthenogenesis

Wolbachia are capable of inducing thelytokous parthenogenesis in infected females, a phenomenon commonly found in insects which employ haplodiploid sex determination, wherein males develop from unfertilized haploid eggs, and females develop from fertilized diploid eggs. Parthenogenesis-inducing (PI) *Wolbachia* have been documented in Hymenoptera (Arakaki et al., 2000; Gottlieb et al., 2002; Plantard et al., 1998; Reumer et al., 2010) and Thysanoptera (Arakaki et al., 2001) and other arthropods such as mites (Acari) (Weeks and Breeuwer, 2001). Female hosts infected with PI *Wolbachia* produce eggs that develop into females, whether they are fertilized or not. Restoration of diploidy in unfertilized eggs by gamete duplication has been described in several *Wolbachia*-infected insects by two different mechanisms. In the first, common to parasitic wasps in the genera *Trichogramma* and *Leptopilina*, the two sets of chromosomes fail to separate in the first mitotic anaphase, resulting in a nucleus with two sets of identical chromosomes (Pannebakker et al., 2004; Stouthamer and Kazmer, 1994). In the second, described in the wasp *Muscidifurax uniraptor,* gamete duplication occurs by fusion of haploid nuclei following the first mitotic division (Gottlieb et al., 2002). Both types of gamete duplication result in completely homozygous female offspring. In mites of the genus *Bryobia*, instead of gamete duplication, *Wolbachia* induce apomictic parthenogenesis, in which

meiosis does not occur and the genotype of the offspring is heterozygous and identical to the mother (Weeks and Breeuwer, 2001).

Many insects, including the parasitic wasps *Leptopilina clavipes*, *Telenomus nawai*, and *Asobara japonica*, and the thrips *Franklinothrips vespiformis* have evolved with PI *Wolbachia* to fixation, resulting in populations which reproduce exclusively by thelytokous parthenogenesis and females that have lost the ability to reproduce sexually (Arakaki et al., 2001; Jeong and Stouthamer, 2004; Kremer et al., 2009a; Pannebakker et al., 2005). The progression of *Wolbachia* from a facultative reproductive parasite to obligate reproductive mutualist is apparent in such cases.

1.2.3 Cytoplasmic incompatibility (CI)

Wolbachia induced cytoplasmic incompatibility (CI) was first identified in 1971 (Yen and Barr) and is conditional male sterility. Crosses between *Wolbachia*-infected males and uninfected females result in nonviable embryos, while all other crosses are successful (Sinkins, 2004) (Figure 1.1). CI is the most common *Wolbachia*-induced reproductive phenotype, and has been noted in all the major insect orders (Harris and Braig, 2003). The intensity of CI is dependent on numerous factors including environment, host age and genetic background, and strain and density of *Wolbachia* (Bordenstein and Werren, 2007; Clancy and Hoffmann, 1998; Turelli and Hoffmann, 1995). Hatch rates may be reduced by as

little as 15%, as seen in some populations of *D. melanogaster* (Hoffmann et al., 1994) to nearly 100%, as in *Culex pipiens* (Duron et al., 2007; Rasgon and Scott, 2003). When more than one *Wolbachia* strain are present in a population, both parents must harbour the same or compatible strains for eggs to be viable; this is called bidirectional CI (Bordenstein and Werren, 2007; O'Neill and Karr, 1990). *Wolbachia* are maternally inherited, and by inhibiting the success of matings which fail to yield infected individuals, CI causes an increase in the frequency of infection within a population with each generation.

While the mechanism of CI is unknown, two important details are widely agreed upon: (1) *Wolbachia* somehow "modify" the sperm within an infected male and, (2) the *Wolbachia*-infected egg "rescues" the modification, resulting in normal embryonic development (Werren, 1997) .

1.2.3.1 Spermatogenesis: CI modification

Spermatogenesis in *Drosophila* has been reviewed in detail by Fuller (1993)(Figure 1.2). It begins with the asymmetric division of a germline stem cell (GSC) at the apex of the testes yielding a new stem cell and a primary spermatogonium. The spermatogonium is encapsulated by two somatic cyst cells, and then undergoes four mitotic divisions to form a cyst of 16 primary spermatocytes connected by cytoplasmic bridges called ring canals. Primary spermatocytes undergo growth and gene expression during this time, increasing

in volume 25 fold. As the cyst migrates distally within the testis, each primary spermatocyte divides meiotically to form 32 secondary spermatocytes and then 64 haploid spermatids, which elongate and differentiate to produce mature spermatozoa. Individualization then occurs, whereby an actin-based complex remodels the sperm membrane and strips away cytoplasm and organelles, forming a waste bag at the distal tip of the sperm bundle (Fabrizio et al., 1998; Noguchi and Miller, 2003). Towards the end of spermatogenesis, the sperm chromatin undergoes extensive remodelling. Histones, core structural components of chromatin, are replaced with basic proteins called protamines, which compact the sperm DNA and protects it from mutagens (Jayaramaiah Raja and Renkawitz-Pohl, 2005; Rathke et al., 2010).

In *D. simulans*, which exhibits strong CI (Weeks et al., 2007), *Wolbachia* are present in spermatocytes and spermatids but are removed with the waste bag during individualization, indicating that sperm are modified prior to this point in development (Clark et al., 2002a; Riparbelli et al., 2007; Snook et al., 2000). In *D. melanogaster*, which exhibits low CI, *Wolbachia* are scarce in spermatocytes and occur in variable numbers in spermatids prior to removal during spermiogenesis, suggesting that *Wolbachia's* presence within the cyst itself is a major factor in sperm modification (Clark et al., 2003). However, in the parasitic wasp *Nasonia vitripennis* (Hymenoptera) and the beetle *Chelymorpha alternans* (Coleoptera) *Wolbachia* presence within developing sperm cells is not

compulsory for induction of CI, suggesting that modification is not the result of ongoing contact between *Wolbachia* and sperm (Clark et al., 2008). Interestingly, in *Drosophila*, the strength of CI decreases with age, as does *Wolbachia* density in the testes (Clancy and Hoffmann, 1998; Clark et al., 2002a; Reynolds and Hoffmann, 2002; Turelli and Hoffmann, 1995). However, the modification imposed on developing sperm remains unknown.

1.2.3.2 Fertilization and development: Maternal rescue

Insect fertilization has been studied in detail and differs significantly from mammalian fertilization (reviewed in Foe et al., 1993; Loppin and Karr, 2005). In insects, the gamete plasma membranes do not fuse. Following entry of the sperm into the egg, the sperm plasma membrane and nuclear envelope are removed and replaced with a nuclear envelope derived from maternal components. During this time, the sperm chromatin decondenses as protamines are replaced with maternally-supplied histones (Bonnefoy et al., 2007; Loppin et al., 2005). As the male and female pronuclei migrate toward each other, the DNA replicates. Upon apposition, the chromosomes condense and nuclear envelope breakdown (NEB) occurs. The male and female pronuclei undergo the first mitotic division independently, using a shared spindle (gonomeric division). Genome fusion occurs after the completion of telophase to form two diploid nuclei.

In *Drosophila* and *Nasonia*, CI is associated with aberrant condensation of the paternal chromosomes and failure to separate at the first mitotic division, resulting in extensive chromosome bridging at anaphase and embryo death (Figure 1.3) (Callaini et al., 1997; Lassy and Karr, 1996; Reed and Werren, 1995; Serbus et al., 2008; Tram et al., 2006). In *Nasonia*, the breakdown of the male pronuclear envelope is delayed, causing it to lag behind the female pronucleus in mitotic entry (Tram and Sullivan, 2002). Nuclear envelope breakdown and mitotic entry is induced by cyclin-dependant kinase-1 (Cdk1) activation, suggesting that *Wolbachia* interfere with the activity of cell cycle regulators (Tram and Sullivan, 2002). Recent work in *Drosophila* proposes that chromatin defects during interphase, including delayed histone deposition and incomplete DNA replication, activate cell cycle checkpoints and are responsible for Cdk1 inhibition (Landmann et al., 2009).

In a rescue cross, in which the oocyte is infected with *Wolbachia*, modified sperm is restored and development proceeds normally (Lassy and Karr, 1996; Tram and Sullivan, 2002). However, this process is highly specific. While multiple *Wolbachia* may infect a common host, strains of the same compatibility type must be present in both the male and female for rescue to occur (Werren et al., 2008). Compatibility is often restored if an identical strain occurs in the female (Bordenstein and Werren, 2007; Braig et al., 1994a; Charlat et al., 2001; O'Neill and Karr, 1990; Zabalou et al., 2008). Occasionally, one strain of

Wolbachia can partially or completely rescue sperm modified by a different strain, but this is dependent upon the genotype of *Wolbachia* and the host (Duron et al., 2010; Zabalou et al., 2008).

Several models have been proposed for CI (reviewed by Serbus et al., 2008). However, the most plausible is the "lock-and-key" model, whereby the male chromatin is modified in such a way that only the proper strain of *Wolbachia* can identify and reverse the alteration, permitting normal pronuclear formation and embryonic development (Poinsot et al., 2003; Werren, 1997).

1.3 The insect immune response

The presence of bacteria generally induces strong responses in metazoans. Insects lack adaptive immunity and therefore rely exclusively on innate immunity for survival, which is an effective defense against foreign invaders including viruses, bacteria, fungi, and some metazoan parasites. Of key importance to intracellular bacteria is the generation of antimicrobial peptides, phagocytosis, and the formation of reactive oxygen species (ROS). It is unclear if bacteria such as *Wolbachia*, which reside in host-derived cytoplasmic vacuoles, elicit recognition by the host immune response; this question is central to this research.

1.3.1 Antimicrobial peptides

Antimicrobial peptides (AMPs) are small, immune-inducible effectors produced by the fat body and secreted into the hemolymph (Bulet et al., 1999). Seven classes of AMPs have been described in *Drosophila*, based upon their activity (Lemaitre and Hoffmann, 2007; Lemaitre et al., 1997). Diptericin, attacin, cecropin and drosocin are active against Gram negative bacteria, defensin is active against Gram positive bacteria, and drosomycin and metchnikowin have antifungal properties (Imler and Bulet, 2005). Gram positive bacteria and fungi activate the Toll signalling pathway and Gram negative bacteria activate the Imd signalling pathway through host peptidoglycan receptor proteins (PGRP)(Choe et al., 2002; Michel et al., 2001). Activation of Toll and Imd initiates an intracellular proteolytic cascade culminating in the cleavage of transcription factors DIF/Dorsal and Relish, which translocate to the nucleus and bind to nuclear factor-kB (NF-kB) response elements, stimulating the expression of antimicrobial peptides (Ferrandon et al., 2007). Induction of Toll and Imd pathways were previously believed to be mutually exclusive processes. However, recent work demonstrates that the pathways work synergistically with cross-regulation mediated by NF-kB related transcription factors and contributing to an enhanced broad-spectrum host response (Tanji et al., 2007).

PGRPs including Toll receptors PGRP-SA, PGRP-SD, and Imd receptor PGRP-LE are secreted (Ferrandon et al., 2007). Others, such as Toll receptor

PGRP-LC are embedded in the cell membrane, and act extracellularly (Ferrandon et al., 2007). An intracellular form of PGRP-LE recognizing monomeric PGN of gram negative bacteria was recently identified in the cytoplasm of *Drosophila* cells (Kaneko et al., 2006).

Wolbachia induce no change in the expression of transcripts encoding antimicrobial peptides in *D. simulans* and *Aedes albopictus,* both of which are naturally infected (Bourtzis et al., 2000). In an artificial infection generated *in vitro* using *Drosophila* S2 cells, increased expression of AMP genes and several genes in the Toll and Imd pathways, including Relish and Dorsal were reported (Xi et al., 2008). Down regulation of *ird5*, a key component of the Imd pathway, was also noted (Xi et al., 2008). *Wolbachia* were cleared from the cells over time, confirming an immune reaction and suggesting that while *Wolbachia* can stimulate a host response, bacterial-host interactions differ between inherited and artificial infections. *Wolbachia*'s location within a host-derived vacuole may provide camouflage from host recognition in natural infections (Siozios et al., 2008). This evasion may be the consequence of another defense mechanism: phagocytosis.

1.3.2 Phagocytosis

Phagocytosis of invading bacteria in insects is typically accomplished by a class of hemocytes called plasmatocytes (Lavine and Strand, 2002). Bacteria are

recognized by phagocyte membrane receptors (eg. Eater, Nimrod, dSR-CI, and Dscam) which promote their uptake (Kocks et al., 2005; Kurucz et al., 2007; Nehme et al., 2011; Ramet et al., 2001; Watson et al., 2005). Following internalization, the phagosome undergoes maturation, fusing with endosomes and lysosomes and creating an acidic and hydrolytic environment which destroy and degrade the ingested particle (Stuart and Ezekowitz, 2008; Vieira et al., 2001). Formation of the NADPH oxidase complex is also stimulated, which assembles on the phagosomal membrane and pumps toxic reactive oxygen species (ROS) into the vacuole in what is known as an oxidative burst (Babior et al., 1973; Bergin et al., 2005; Rada and Leto, 2008; Renwick et al., 2007).

Many bacterial pathogens of mammals avoid destruction following phagocytosis. Bacteria such as *Listeria monocytogenes* lyse the phagosome and escape into the cytoplasm (Beauregard et al., 1997; Goldfine et al., 2007; Tilney and Portnoy, 1989). *Mycobacterium tuberculosis*, *Legionella pneumophila,* and *Anaplasma phagocytophilum* survive within the phagosome by inhibiting maturation (Fortier et al., 2007; Fratti et al., 2003; Huang et al., 2010a; Pethe et al., 2004). In *Salmonella typhimurium,* acidification of the phagosome promotes expression of virulence genes, including those involved in acid tolerance (Alpuche Aranda et al., 1992; Bearson et al., 1998; Prost and Miller, 2008). Many bacteria, such as *M. tuberculosis* and *Burkholderia cenocepacia* express antioxidants which neutralize ROS in the phagosome (Keith and Valvano, 2007;

Ng et al., 2004); others, including *Coxiella burnetii*, *A. phagocytophilum* and *Ehrlichia chaffeensis* resist the oxidative burst by inhibiting the proper assembly of the NADPH oxidase complex on the phagosomal membrane (Ijdo and Mueller, 2004; Lin and Rikihisa, 2007; Siemsen et al., 2009). *C. burnetii* also has an impressive complement of genes involved in maintaining genome integrity under exposure to ROS, including the SOS response, an inducible DNA repair network (Mertens et al., 2008).

In ticks as well as humans. *A. phagocytophilum* expresses proteins which localize to the phagosomal membrane, presumably contributing to the generation of the modified vacuole which acts as a symbiosome (Huang et al., 2010b; Huang et al., 2010c). In *Drosophila* whole flies and S2 cells, *L. monocytogenes* escapes the phagosome into the cytosol as it does in mammalian cells, and has proven to be a good model for bacterial pathogenesis (Cheng and Portnoy, 2003; Mansfield et al., 2003). *L. pneumophila*, *Mycobacterium fortuitum,* and *Chlamydia trachomatis* all survive and replicate within the *Drosophila* phagosome (Dorer et al., 2006; Elwell and Engel, 2005; Philips et al., 2005).

While it is has been hypothesized that the vacuole in which *Wolbachia* reside is a modified phagosome (Siozios et al., 2008), the composition of the host membrane which surrounds *Wolbachia* and its contents is unknown. However, experimental work and genome analysis provide clues as to how *Wolbachia* may

interact with its host and avoid destruction. *Wolbachia* have what appears to be a complete and functional Type IV Secretion System (T4SS) (Rances et al., 2008; Wu et al., 2004), which plays an integral role in phagosome remodelling and intracellular survival in *Legionella pneumophila, B. cenocepacia* and *Brucella abortus* (Comerci et al., 2001; Molmeret et al., 2004; Sajjan et al., 2008). *Wolbachia* also have an extensive repertoire of genes containing ankyrin repeats (Iturbe-Ormaetxe et al., 2005; Klasson et al., 2008; Klasson et al., 2009b; Wu et al., 2004). Ankyrin proteins are known bacterial T4SS effectors (Pan et al., 2008). In *L. pneumophila,* secreted ankyrin B (AnkB) promotes phagosome decoration with polyubiquitinated proteins, contributing to biogenesis of the unique *Legionella*-containing vacuole (LCV) which supports intracellular replication in protozoans and human macrophages (Al-Khodor et al., 2008; Price et al., 2009; Price et al., 2010).

1.3.3 *Wolbachia* effects on host immunity

Recent work shows that the presence of *Wolbachia* affects the host response to other invaders, including parasitoids and viruses. Infected *D. simulans* show increased susceptibility to the parasitoid wasp *Leptopilina heterotoma,* and the eggs of uninfected parasitoids better survive the *Drosophila* immune response than infected eggs (Fytrou et al., 2006). *Wolbachia* infection enhances resistance to RNA viruses in *D. melanogaster* (Hedges et al., 2008;

Teixeira et al., 2008) and *Culex quinquefasciatus* (Glaser and Meola, 2010). In artificially infected *Anopheles gambiae* and *Aedes aegypti*, *Wolbachia* upregulate the expression of immune genes and inhibit infection by RNA viruses (Bian et al., 2010; Moreira et al., 2009) and *Plasmodium gallinaceum* and *Plasmodium berghei* (Kambris et al., 2010; Moreira et al., 2009).

1.4 Reactive oxygen species, antioxidants, and cellular damage

Reactive oxygen species (ROS) are an important component of the insect and mammalian innate immune response. They are also central to aerobic metabolism and intracellular signalling. High levels of ROS create a state of oxidative stress and induce cellular damage; therefore the balance between their generation and neutralization by antioxidants is crucial in every aerobic organism.

1.4.1 Reactive oxygen species

1.4.1.1 Chemistry and formation

Reactive oxygen species (ROS), including superoxide ($O_2^{\cdot}$-), hydrogen peroxide (H_2O_2), and hydroxyl radicals ($OH^{\cdot}$) are oxygen intermediates which have one or more unpaired electrons (reviewed in Fridovich, 1999). They are common by-products of respiration. Electrons leak from the mitochondrial electron transport chain and react with molecular oxygen to form superoxide,

which is quickly reduced to hydrogen peroxide either spontaneously or via the antioxidant enzyme superoxide dismutase [1] (Boveris and Cadenas, 1975; Dionisi et al., 1975; Loschen et al., 1974; McCord and Fridovich, 1969; Murphy, 2009; St-Pierre et al., 2002). Hydrogen peroxide may be fully reduced to water by antioxidants such as catalase [2] or peroxidases [3] (reviewed in Bayir, 2005; Chae et al., 1999; Cohen and Hochstein, 1963; de Haan et al., 1998; Keilin and Hartree, 1938; Mills, 1960; Netto et al., 1996) or it can react with free iron via the Fenton reaction to generate the most damaging form of ROS, hydroxyl radicals [4] (Graf et al., 1984; Imlay et al., 1988; Lloyd et al., 1997). Unreduced superoxide contributes to the formation of hydroxyl radicals by inactivating enzymes containing iron-sulfur clusters, releasing iron into the intracellular environment (Flint et al., 1993; Keyer and Imlay, 1996) (Figure 1.4).

$$2O_2^{\cdot -} + 2H^+ \rightarrow 2H_2O_2 + O_2 \qquad [1]$$

$$2H_2O_2 \rightarrow 2H_2O + O_2 \qquad [2]$$

$$H_2O_2 \rightarrow H_2O \qquad [3]$$

$$Fe(II) + H_2O_2 \rightarrow OH^{\cdot} + OH^- + Fe(III) \qquad [4]$$

Adapted from (Bayir, 2005)

In mammals and in insects, the generation of ROS is among the first lines of defense against invading microbes (Ha et al., 2005b; Hoffmann, 2003). In

vertebrates, ROS is generated by a family of NADPH oxidases. Seven exist in mammals: Nox1, Nox2, Nox3, Nox4, Nox5 which generate superoxide, and Duox1 and Duox2, which produce hydrogen peroxide (reviewed in Rada and Leto, 2008). Nox2, the phagocytic NADPH oxidase, plays a central role in immunity and is well characterized. Nox2 is a protein complex consisting of several subunits: cytosolic proteins $p40^{phox}$, $p47^{phox}$, $p67^{phox}$, the small GTP-binding protein rac, and phagosomal membrane proteins $p22^{phox}$ and $gp91^{phox}$ (which complex to form cytochrome b_{558}) (El-Benna et al., 2005; Nauseef, 2008). While the complex is normally inactive in resting cells, upon stimulation $p22^{phox}$, $p40^{phox}$, $p47^{phox}$, and $p67^{phox}$ are phosphorylated, causing the migration of cytosolic proteins to the membrane of the phagosome, where they complex with cytochrome b_{558} (El-Benna et al., 2008; Groemping et al., 2003; Nauseef, 2004; Taura et al., 2009). NADPH oxidase transfers electrons from cytosolic NADPH to the intraphagosomal space where they combine with molecular oxygen to generate superoxide anions from which additional ROS are formed in what is known as the oxidative burst (El-Benna et al., 2005; Hampton et al., 1998; Roos et al., 2003).

The relatives of Nox2 are characterized mainly by the mammalian cell types in which they are found and while non-phagocytic in nature, they share many structural and functional attributes with Nox2, including C-terminal NADPH and FAD binding sites, six transmembrane domains, two membrane-embedded

heme groups, and the ability to generate ROS (Rada and Leto, 2008). Nox1, 3, and 4 are most structurally similar to Nox2. In humans, Nox1 is expressed predominantly in the colon, but has been identified in other tissues, including the prostrate, uterus, and vascular smooth muscle cells (Banfi et al., 2000; Geiszt et al., 2003a; Hilenski et al., 2004). Nox4 was first identified in the kidneys, where it is abundant (Geiszt et al., 2000; Shiose et al., 2001). It has since been found in a variety of tissues and cell types, including vascular smooth muscle, placenta, fetal tissues, endothelial cells, brain, and thyroid (Ago et al., 2004; Cheng et al., 2001; Hilenski et al., 2004; Li et al., 2009; Vallet et al., 2005; Weyemi et al., 2010; Xu et al., 2008). Nox3 is found mainly in the inner ear and some fetal tissues (Banfi et al., 2004; Cheng et al., 2001). Work in mice suggests that Nox3 it is important in balance and the perception of gravity (Paffenholz et al., 2004). Nox5 is expressed primarily in the testes, spleen, and lymph nodes, although it has also been identified in the uterus and some fetal tissues (Banfi et al., 2001; Cheng et al., 2001). Nox5 has an additional cytoplasmic region containing four N-terminal EF-hand Ca+ binding domains and is calcium-activated (Banfi et al., 2001). No immune role for Nox5 has been identified. Duox1 and 2 (dual oxidase) have an additional transmembrane domain, and an N-terminal region containing an extracellular peroxidase-like domain and two cytosolic EF-hand Ca+ binding domains (De Deken et al., 2000; Dupuy et al., 1999). Duox enzymes are commonly expressed in the thyroid and mucosal epithelia where they produce

hydrogen peroxide in a calcium- dependant manner (Ameziane-El-Hassani et al., 2005; Bae et al., 2010; Forteza et al., 2005; Geiszt et al., 2003b).

In addition to their role in immunity, ROS are also cell signalling molecules and their generation is critical to many cellular processes (Hoidal, 2001). Extensive research shows that ROS signalling mediates cell proliferation and differentiation (Arnold et al., 2001; Li et al., 2006b; Sauer et al., 2001; Tsukagoshi et al., 2010) adhesion and migration (Pan et al., 2010; Sangrar et al., 2007) and apoptosis (Aikawa et al., 2010; Cerella et al., 2009).

1.4.1.2 ROS generation and insect immunity

In insects, superoxide generative reactions mimic the oxidative burst seen in vertebrates (Whitten and Ratcliffe, 1999). In *Galleria mellonella* hemocytes, a phagocytic NADPH oxidase with protein homologs of $gp91^{phox}$, $p47^{phox}$, $p67^{phox}$, and rac were identified by immunofluorescence and immunoblotting (Bergin et al., 2005; Renwick et al., 2007). This NADPH oxidase assembles on the phagosomal membrane and generates superoxide in response to pathogens (Bergin et al., 2005; Renwick et al., 2007). Insects including *D. melanogaster, Apis mellifera*, and *Anopheles gambiae* lack Nox2, but contain Nox5 and Duox (Kawahara et al., 2007). Both of these are characterized by a calcium-binding EF-hand domain, are ubiquitous in eukaryotes, and appeared early in the evolutionary history of Nox enzymes (Kawahara et al., 2007). In *Drosophila* a

duox (dDuox) with a vital role in gut immunity has been identified in the mucosal epithelia where it releases ROS into the lumen (Ha et al., 2009a; Ha et al., 2005a). Expression and activation of dDuox are coordinated to generate the correct intensity of ROS depending upon the presence of invasive or indigenous microbes (Bae et al., 2010; Ha et al., 2009b). Activity of dDuox is Ca^{2+} - dependent, and gut microbes mobilize intracellular Ca^{2+} from ER stores in a peptidoglycan (PG)-independent manner by activating the Gαq-phospholipase C-β pathway (PLCβ) (Ha et al., 2009a). Basal ROS generation via this pathway is critical for host survival in the presence of commensal microbes and activation of dDuox is enhanced under an increasing microbial burden (Ha et al., 2009a). Expression of dDuox, on the other hand, is positively and negatively regulated depending upon the composition of microbes in the gut (Ha et al., 2009b). Pathogenic microbes activate ATF2 through p38 kinase in both a PG-dependent and PG-independent manner, resulting in elevated dDuox expression and ROS production (Ha et al., 2009b). However, under normal conditions, p38 activity is suppressed by PLCβ, thus limiting dDuox expression and preventing excessive amounts of ROS (Ha et al., 2009b). The complex interaction between dDuox activity and expression permits the level of ROS to be tailored to the composition of gut microbes in *Drosophila*. Recently, it was found that the dDuox-mediated oxidative burst promotes intestinal stem cell (ISC) activation, presumably by inducing cellular damage, and this epithelial regeneration contributes to gut

homeostasis during bacterial infection (Buchon et al., 2009a; Buchon et al., 2009b).

Drosophila also has a Nox5 ortholog (dNox5), which regulates ovarian muscle contraction, thus playing an essential role in female fertility (Ritsick et al., 2007). Recently dNox5 was identified in the *Drosophila* seminal receptacle (Prokupek et al., 2010).

Numerous studies emphasize the importance of ROS generation in insect immunity. Early work suggested a role for ROS in *Drosophila* encapsulation of eggs of the parasite *Leptopilina boulardi* (Nappi and Vass, 1998; Nappi et al., 1995) and in the response of *Rhodnius prolixus* to the parasite *Trypanosoma rangeli* in the hemolymph (Whitten et al., 2001). An ROS-mediated response to bacteria and fungi in the hemolymph was noted in the cockroach *Blaberus discoidalis* (Whitten and Ratcliffe, 1999) and *G. mellonella* (Bergin et al., 2005; Renwick et al., 2007). *Bombyx mori* expresses an NADPH-oxidase-like protein (BmNox) in the gut which provides viral resistance (Selot et al., 2010). In *Anopheles gambiae* high levels of ROS are important immune effectors generated in response to *Plasmodium* and bacteria (Herrera-Ortiz et al., 2011; Kumar et al., 2003; Molina-Cruz et al., 2008).

1.4.2 Antioxidants

ROS can also induce significant cellular damage. Thus, redox homeostasis in aerobic organisms is maintained by balancing ROS formation with antioxidants, which include a complement of enzymatic and non-enzymatic defences (Figure 1.4).

Superoxide dismutase (SOD) catalyzes the breakdown of superoxide anions to hydrogen peroxide and molecular oxygen. Mammals and insects express two copper-zinc SODs: one in the cytoplasm (SOD1) and one which is secreted (SOD3), and one manganese SOD (SOD2) expressed predominantly in mitochondria (Corona and Robinson, 2006; Parker et al., 2004; Zelko et al., 2002).

The product of SOD activity, hydrogen peroxide is also a damaging form of ROS and may be broken down via several methods. Catalase directly converts H_2O_2 into water and dioxygen and has been characterized in *Drosophila* (Orr et al., 1996; Park et al., 2004). Peroxiredoxins (Prx) are a ubiquitous family of antioxidants which reduce peroxides in a thiol-dependent manner. Based on the number of cysteine residues present, two subgroups have been identified, the 1-Cys and 2-Cys Prxs (McGonigle et al., 1998). All Prxs utilize a redox-active cysteine (peroxidatic cysteine) which is oxidized to form a cysteine sulfenic acid (Cys-SOH) in the process of reducing peroxide substrates (Wood et al., 2003). Cys-SOH must then be regenerated by reduction via a resolving cysteine. The 2-

Cys Prxs are divided into two subgroups based on the pathway they use. The typical 2-Cys Prxs form an obligate dimer, in which the resolving cysteine from one subunit forms a stable disulfide bond with the peroxidatic cysteine of the other (Hall et al., 2009). The atypical 2-Cys Prxs are monomeric and fold upon themselves, forming an intramolecular disulfide bond and reducing their own peroxidatic cysteine (Wood et al., 2003). In both cases, the disulfide bond is reduced, often by using thioredoxin (Trx) as an electron donor (Chae et al., 1994; Park et al., 2000; Radyuk et al., 2001; Seo et al., 2000). The 1-Cys Prxs, in contrast, contain only a peroxidatic cysteine, which must be resolved by a thiol-containing electron donor. Proposed sources include cyclophilin, lipoic acid, vitamin C and glutathione (Lee et al., 2001; Manevich et al., 2004; Monteiro et al., 2007; Peshenko and Shichi, 2001).

Six mammalian peroxiredoxins have been identified; 1 through 4 are typical 2-Cys Prxs, 5 is an atypical 2-Cys Prx, and 6 is a 1-Cys Prx (reviewed in Shuvaeva et al., 2009; Wood et al., 2003). Six peroxiredoxins have also been identified in *D. melanogaster,* 4 of the 2-Cys variety, and 2 of the 1-Cys variety, all of which exhibit antioxidant capabilities (Michalak et al., 2008; Radyuk et al., 2009; Radyuk et al., 2001).

Glutathione peroxidase (GPx) catalyzes the reduction of hydrogen peroxide and organic hydroperoxides. The first GPx discovered (cytosolic GPx-1) relied on reduced glutathione (GSH) as an electron donor (Cohen and Hochstein,

1963; Mills, 1957, 1959), and it was presumed that this was always the case. However, substrates such as thioredoxin and glutaredoxin are also effective electron donors, depending upon the GPx examined (Bjornstedt et al., 1994). Seven functional mammalian GPxs with known subcellular localizations have been identified to date (Margis et al., 2008). A putative GPx-8 with unknown function was recently identified (Toppo et al., 2008). Most mammalian GPxs (GPx-1, 2, 3, 4, and sometimes 6, depending on the species) are selenoproteins utilizing a seleno-cysteine at the catalytic site (Flohe et al., 1973; Kryukov et al., 2003; Toppo et al., 2008). The others contain a cysteine in place of the seleno-cysteine, and maintain antioxidant function (Chabory et al., 2009; Utomo et al., 2004).

The *Drosophila* genome contains two GPx homologs (Adams et al., 2000). Both are cysteine-containing enzymes; one, referred to as GPx-like (CG15116) has not yet been biochemically characterized, and the other, called *D. melanogaster* GPx, (*Dm*GPx) (CG12013), utilizes reduced thioredoxin (Trx) as an electron donor and has been described as a GPx homolog with TPx (thioredoxin peroxidase) activity (Maiorino et al., 2007; Missirlis et al., 2003b). Interestingly, in response to paraquat (*N*,*N*'-dimethyl-4,4'-bipyridinium dichloride) stress, expression of *Dm*GPx is upregulated, while the GPx-like protein is repressed, suggesting diversity in their functional roles (Girardot et al., 2004).

Antioxidant activity is not limited to eukaryotes. Bacteria are often aerobic and the target of ROS and as a result they have developed advanced defense networks. *E. coli,* like *Wolbachia*, are Gram negative proteobacteria known to express numerous antioxidant proteins, including three superoxide dismutases – an Mn SOD (Keele et al., 1970), an Fe SOD (Carlioz et al., 1988; Sakamoto and Touati, 1984; Yost and Fridovich, 1973) and a CuZn SOD (Benov and Fridovich, 1994). Mn SOD and Fe SOD, termed SodA and SodB respectively, are cytoplasmic and their expression is controlled by the global regulatory system Fur (ferric uptake regulator) (Niederhoffer et al., 1990). CuZn SOD (SodC) is secreted into the periplasm (Imlay and Imlay, 1996) where it likely protects the bacteria from the phagocyte respiratory burst, making it integral to pathogenesis (Battistoni, 2003; Gort et al., 1999). SodC is expressed primarily during the stationary phase (D'Orazio et al., 2008; Gort et al., 1999).

Hydrogen peroxide and other hydroperoxides are broken down by several mechanisms. *E. coli* expresses two catalases, referred to as hydroperoxidase I (HPI) and hydroperoxidase II (HPII). HPI is a bifunctional enzyme with peroxidase activity in addition to catalase activity, encoded by KatG (Loewen et al., 1985b; Triggs-Raine et al., 1988), while HPII is a monofunctional catalase encoded for by KatE (Loewen et al., 1985b; von Ossowski et al., 1991). HPI is expressed during logarithmic growth and in response to H_2O_2, while HPII is

expressed primarily during stationary phase and is unresponsive to H_2O_2 (Loewen et al., 1985a; Schellhorn, 1995).

Recent work has characterized an oxidative-stress inducible glutathione peroxidase in *E. coli* which preferentially uses thioredoxin as a reducing agent (Arenas et al., 2010). *E. coli* also expresses several cytoplasmic peroxiredoxins, including alkyl hydroperoxide reductase (AhpC), thiol peroxidase (p20), and bacterial comigratory protein (BCP) (Link et al., 1997; Tao, 2008). AhpC is a typical 2-Cys peroxiredoxin specifically reduced by the flavoprotein AhpF (Jonsson et al., 2007; Seaver and Imlay, 2001). Thiol peroxidase p20 is an atypical 2-Cys peroxiredoxin, which functions as a lipid hydroperoxide peroxidase and is important during anaerobic growth (Cha et al., 1995; Cha et al., 2004). BCP is a 1-Cys peroxiredoxin (Jeong et al., 2000) which, like p20, is reduced by thioredoxin (Choi et al., 2003; Clarke et al., 2009).

The fully annotated genome of the *Wolbachia* endosymbiont of *D. melanogaster* (Wu et al., 2004), has genes which code for three of the aforementioned nine antioxidants proteins – Fe SOD, BCP, and AhpC. As previously described, *Wolbachia* has a significantly reduced genome, the result of an ongoing adaptation to an intracellular lifestyle (Fenn and Blaxter, 2006; Wu et al., 2004). Among genes that have been lost over time are several coding for bacterial antioxidants. How *Wolbachia* copes with intracellular oxidative stress in

spite of this deficiency is a focus of my work and provides insight into obligate host-symbiont interactions.

1.4.3. Oxidative stress and cellular damage

High concentrations of ROS create a state of oxidative stress, resulting in damage to lipids, proteins, and nucleic acids. An unbalanced production of ROS has been implicated in human ageing and disease, including atherosclerosis, neurodegenerative and ophthalmologic diseases, and cancer (Dalle-Donne et al., 2006).

DNA damage by ROS includes single and double-stranded breaks, base and deoxyribose modifications, and DNA cross-linking (reviewed in Valko et al., 2006). The principal source of DNA damage is the highly reactive hydroxyl radical ($^{\cdot}OH$) , which adds to double bonds of DNA bases and abstracts hydrogen atoms from the C-H bonds of 2'deoxyribose and the methyl group of thymine (reviewed in Dizdaroglu et al., 2002). Purine and pyrimidine radicals are produced as a result of these interactions, and they react further through a variety of redox pathways. For example, H-atom abstraction from the methyl group of the purine base thymine produces the allyl radical, which reacts with oxygen to give 5-hydroxymethyluracil and 5-formyluracil (Cooke et al., 2003). Hydroxyl radical addition to pyrimidine bases leads to C5-OH adduct radicals which may be oxidized, followed by the addition of OH^- to yield cytosine glycol.

Upon deamination, cytosine glycol generates uracil glycol, 5-hydroxycytosine, and 5-hydroxyuracil (Cooke et al., 2003). More than 20 oxidatively modified bases, called DNA lesions, have been identified and are used as indicators of oxidative stress. The lesion 7,8-dihydro-8-oxo-2'-deoxyguanosine (8-oxo-dG) is generated by oxidation of deoxyguanosine (creating a C8-OH adduct radical), followed by loss of an electron and a proton (Slupphaug et al., 2003). 8-oxo-dG is the most common oxidative lesion and is a reliable biomarker of oxidatively damaged DNA (Valavanidis et al., 2009).

Both prokaryotes and eukaryotes have an extensive capacity for repairing damaged DNA and preventing abnormalities resulting from its misincorporation into the genome. Homologous recombination (HR) and non-homologous end-joining (NHEJ) are involved in the repair of double-stranded breaks, while transcription-coupled repair (TCR), global genome repair (GGR), mismatch repair (MMR), and nucleotide excision repair (NER) are important pathways for repairing DNA lesions (Slupphaug et al., 2003). Oxidative DNA lesions are most often restored by base excision repair (BER), during which a DNA glycosylase which specifically recognizes a designated lesion removes it from the DNA strand creating an abasic (AP) site (D'Errico et al., 2008). An AP endonuclease then cleaves the DNA 5'of the AP site, followed by cleavage by an end processing enzyme at the 3' end of the AP site (Zharkov, 2008). Some glycosylases also act as an AP lyase while they remove the damaged base (bifunctional glycosylases),

cleaving the DNA 3' of the AP site prior to AP endonuclease activity (Zharkov, 2008). In both cases, a single nucleotide gap is created which is then filled by DNA polymerase and DNA ligase (Seeberg et al., 1995). In mammals and *Drosophila*, the bifunctional DNA glycosylase OGG1 typically identifies and excises 8-oxo-dG, promoting the BER pathway (Bruner et al., 2000; Dherin et al., 2000). An unrelated bifunctional glycosylase called Fpg functions analogously in bacteria such as *E. coli* (Koval et al., 2010).

Many studies have demonstrated an association between ROS-induced sperm DNA damage and a reduction in human male fertility (Ishikawa et al., 2007; Kodama et al., 1997; Lopes et al., 1998; Saleh et al., 2003). Sperm that are severely damaged have a decreased capacity for fertilization; those with less damage are capable of fertilization but can pass on the damaged paternal DNA to the embryo (Aitken et al., 1998). While a small amount of ROS is generated by the sperm cells themselves, the majority is generated by infiltrating leukocytes recruited to the semen (Henkel et al., 2005; Whittington and Ford, 1999) in response to bacterial infection (Sanocka-Maciejewska et al., 2005; Urata et al., 2001; Wang et al., 1997).

1.5 Thesis overview/objectives

While this is a relatively new scientific field, interest in *Wolbachia* has gained momentum over the past several years. It is an excellent model for

prokaryote-eukaryote interactions. Furthermore, given *Wolbachia's* ability to infect and drive through arthropod populations, its potential to be used as a method of insect biological control has been recognized (Bourtzis, 2008). In order to meet this goal, the biology of *Wolbachia* must be thoroughly studied. The purpose of this work is to contribute to our understanding of the intricacies of this unique symbiotic relationship at the cellular level.

This study began with a proteomics screen of *Wolbachia*-infected and uninfected *Aedes albopictus* cells to identify factors expressed exclusively in the presence of *Wolbachia*. Many proteins contributing to the maintenance of symbiosis were identified. These include host actin depolymerizing factor (ADF), bacterial single-strand binding protein, GroES, 3,4-dihydroxy-2-butanone 4-phosphate synthase, nucleoside diphosphate kinase, and proteins involved in bacterial protein synthesis. Most importantly, it was revealed that increased antioxidant expression, both host and bacterial, is a major factor of this symbiosis.

Antioxidants are expressed to counter to reactive oxygen species (ROS), which can induce significant damage to cellular components. When antioxidant expression matches or exceeds ROS formation, redox homeostasis is achieved and oxidative damage is inhibited. However, when ROS levels surpass antioxidant capacity, a state of oxidative stress results and cellular damage is inevitable.

ROS formation was evaluated in *Wolbachia*-infected and uninfected *Ae. albopictus* cells to determine if antioxidant expression was indeed upregulated in response to changes in ROS levels. *Wolbachia* induce cytoplasmic incompatibility, and this reproductive phenotype is dependent upon an unknown activity of *Wolbachia* in the testes (sperm modification) and the ovaries (paternal chromatin rescue). ROS formation and antioxidant expression were therefore measured in the reproductive tissues of *D. simulans* flies as well.

To determine if redox homeostasis is disrupted in the presence of a *Wolbachia* infection, DNA damage was measured in *Wolbachia*-infected and uninfected Ae*. Albopictus* cells, as well as in whole male *D. simulans* flies and spermatocytes.

This study suggests that ROS acts as a critical mediator of *Wolbachia*-host interactions. *Wolbachia* infection is associated with an increase in ROS formation which is counterbalanced by overexpression of host and bacterial antioxidants. In whole organisms, it appears that redox homeostasis is maintained despite infection with *Wolbachia*. However, in densely infected tissues, such as the testes, it appears that redox homeostasis is lost, and oxidative DNA damage occurs. This damage may play a role in the modification of sperm chromatin which leads to cytoplasmic incompatibility. The details of these findings and their contribution to understanding the cellular basis of host-symbiont interactions will be discussed.

Figure 1.1 Cytoplasmic incompatibility in *Wolbachia*-infected insects. When *Wolbachia* are absent in both parents (white boxes), offspring are viable and uninfected. When *Wolbachia* are present (shaded boxes), if the female is infected, regardless of male infection status, all offspring survive and are *Wolbachia*-infected. When *Wolbachia* are absent in the female, but present in the male, the embryos die following fertilization. As *Wolbachia* are maternally inherited, this ensures that the frequency of infected individuals in each generation is maximized. Adapted from Clark et al., 2002b.

	♀ (W-) 	♀ (W+) 
♂ (W-) 	♀ ♂ ♀ ♂ 	♀ ♂ ♀ ♂ 
♂ (W+) 	X Embryonic death	♀ ♂ ♀ ♂ 

Figure 1.2 Spermatogenesis in *Drosophila*. At the apex of the testes, a germline stem cell (GSC) divides asymmetrically to give a new stem cell and a primary spermatogonium. The spermatogonium undergoes four mitotic divisions to form a cyst of 16 primary spermatocytes connected by cytoplasmic bridges called ring canals. Primary spermatocytes undergo growth and gene expression then divide meiotically to form 32 secondary spermatocytes and then 64 haploid spermatids. Spermatids elongate and differentiate to form mature spermatozoa. During individualization, an actin-based complex remodels the sperm membrane and strips away cytoplasm and organelles, forming a waste bag at the distal tip of the sperm bundle. Adapted from Fuller, 1993.

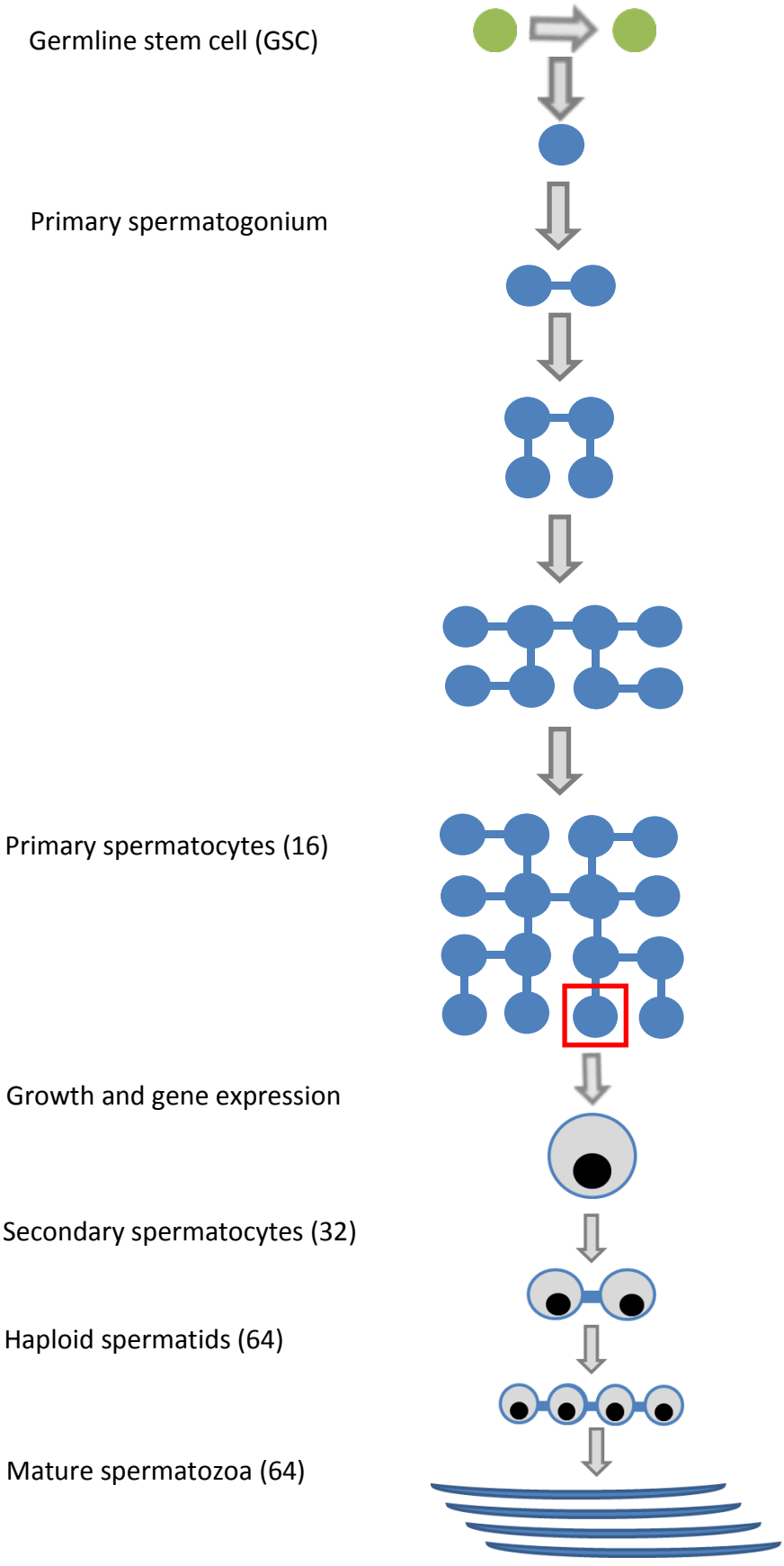

Germline stem cell (GSC)
Primary spermatogonium
Primary spermatocytes (16)
Growth and gene expression
Secondary spermatocytes (32)
Haploid spermatids (64)
Mature spermatozoa (64)

Figure 1.3 The cellular basis of cytoplasmic incompatibility. Following fertilization in a normal cross (A), DNA replication occurs as the male (blue) and female (pink) pronuclei migrate toward each other. Upon apposition, the chromosomes condense and nuclear envelope breakdown (NEB) occurs. The male and female pronuclei undergo the first mitotic division independently, using a shared spindle (gonomeric division). Genome fusion occurs after the completion of telophase to form two diploid nuclei. In an incompatible cross (B), the *Wolbachia*-modified paternal DNA is introduced into a *Wolbachia*-free environment (the uninfected egg). Nuclear envelope breakdown and chromosome condensation are delayed in the male pronucleus, resulting in extensive chromosome bridging at anaphase and embryo death. In a rescue cross (C), in which the oocyte is infected with *Wolbachia* (green dots), the male pronucleus is restored and development proceeds normally. Adapted from Werren et al., 2008.

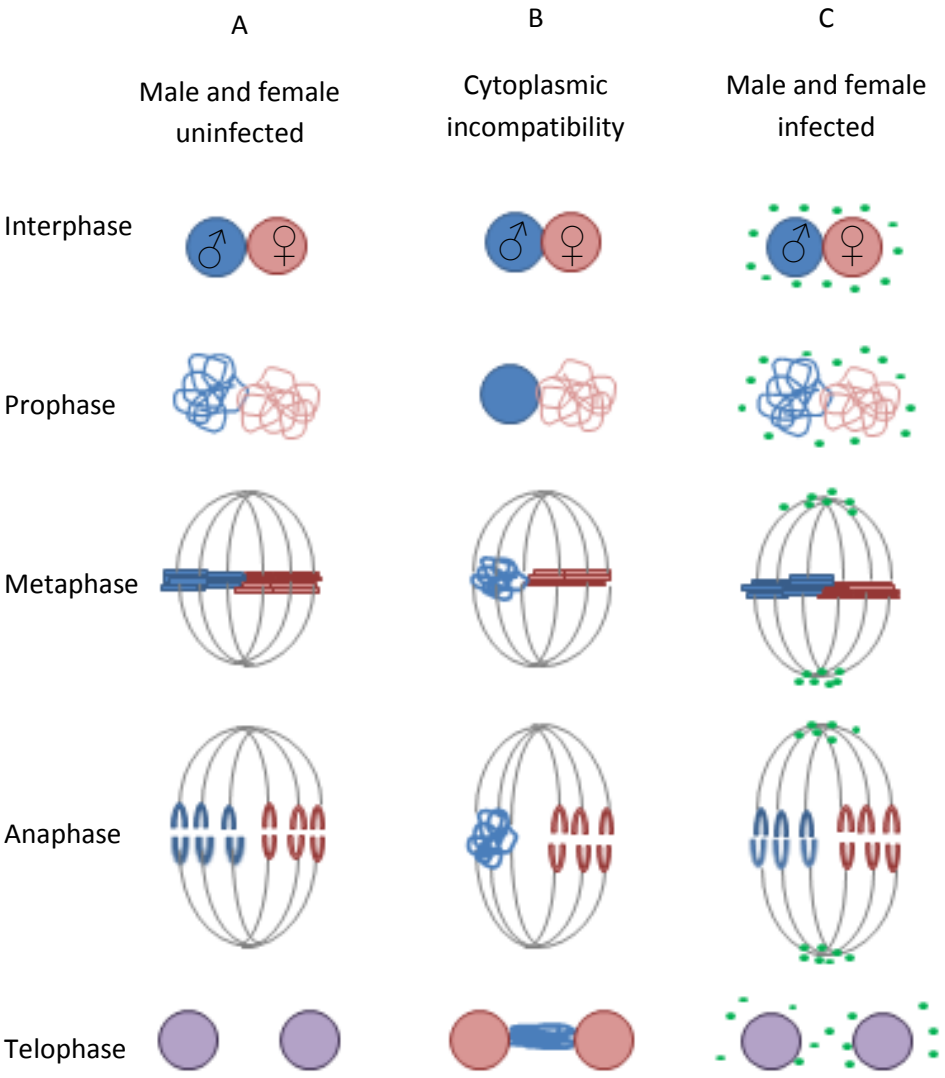
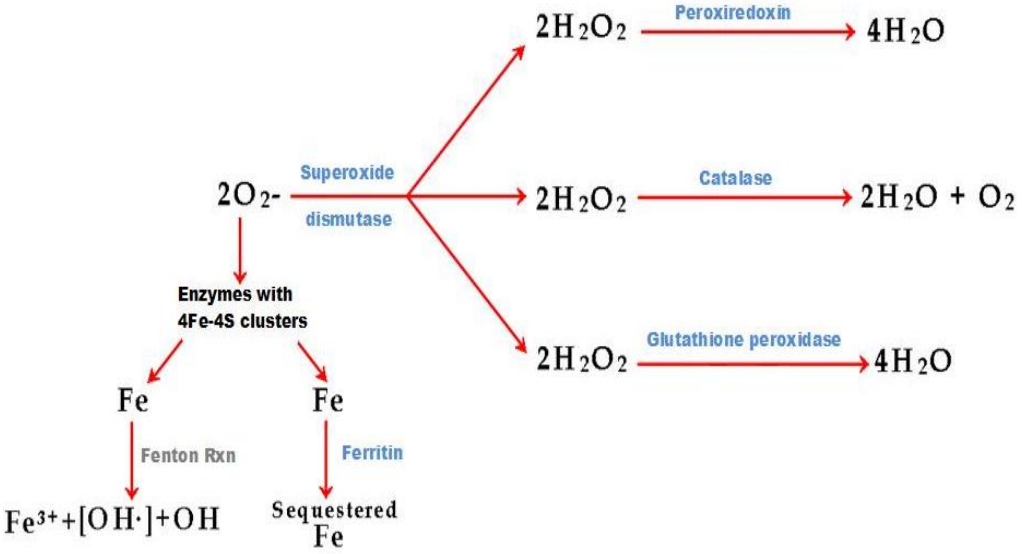

A
Male and female uninfected
B
Cytoplasmic incompatibility
C
Male and female infected
Interphase
Prophase
Metaphase
Anaphase
Telophase

Figure 1.4 The formation of reactive oxygen species (ROS) and neutralization by antioxidants. Electrons react with molecular oxygen to form superoxide, which is reduced to hydrogen peroxide either spontaneously or via the antioxidant enzyme superoxide dismutase (SOD). Hydrogen peroxide may be fully reduced to water by antioxidants such as catalase or peroxidases (such as glutathione peroxidase, GPx and peroxiredoxin, Prx) or it can react with free iron via the Fenton reaction to generate hydroxyl radicals. Unreduced superoxide contributes to the formation of hydroxyl radicals by inactivating enzymes containing iron-sulfur clusters, releasing iron into the intracellular environment. The Fenton reaction can be inhibited by binding of free iron through ferritins.


$2O_2$-
Superoxide dismutase
$2H_2O_2$
Peroxiredoxin
$4H_2O$
$2H_2O_2$
Catalase
$2H_2O + O_2$
$2H_2O_2$
Glutathione peroxidase
$4H_2O$
Enzymes with 4Fe-4S clusters
Fe
Fe
Fenton Rxn
Ferritin
$Fe^{3+}+[OH\cdot]+OH$
Sequestered Fe

2. MATERIALS AND METHODS

2.1 Model organisms

2.1.1 *Aedes albopictus* (Aa23) cell lines

Wild populations of *Aedes albopictus* mosquitoes are superinfected with two CI-inducing *Wolbachia* strains, designated *w*AlbA and *w*AlbB (Sinkins et al., 1995; Werren et al., 1995b). The Aa23 cell line was established from infected *Ae. albopictus* eggs in 1997, and while the *w*AlbA strain was lost during cultivation, the *w*AlbB infection has been stably maintained since that time. The infection can be cured by treatment with antibiotics (Fenollar et al., 2003; O'Neill et al., 1997). The Aa23 cell line used in this study was kindly supplied by Dr. Stephen Dobson at the University of Kentucky in 2005 and cultured according to O'Neill et al., (1997). Cells were cultured in 25 cm^2 plastic tissue culture flasks (Corning) containing 5 ml of SMM growth medium at approximately 27^{o}C. SMM consists of equal volumes of Mitsuhashi-Maramorosch (MM) (Handmade, Appendix I) and Schneider's insect medium (Sigma, Appendix 1) supplemented with 15- 20% heat-inactivated fetal bovine serum (Sigma). Every 4-5 days, the flasks were shaken vigorously to detach the cells from the flask surface. Approximately 90% of the medium containing cells was poured off and replaced with fresh medium. New flasks were generated as needed by placing 1ml of the existing cell/medium mixture in a new flask and adding 4 mls of fresh media. Infected cultures were maintained free of antibiotics.

An uninfected cell line (Aa23T) was generated from the original cell line by adding 10 μg/ml rifampicin to the culture medium for 7 passages (Hermans et al., 2001). Cells undergoing treatment with antibiotics were removed from the culture flask as described, transferred to a 15 ml conical tube, and centrifuged at 2,000 rpm for 5 minutes to form a soft pellet. The pellet was resuspended in 5 mls of fresh medium. One ml of this suspension was used to generate new flasks, in addition to 4mls of growth medium and 15.5 μl of a 3.2 mg/ml rifampicin (Sigma) stock solution prepared in 70% ethanol. New flasks were used for each passage to eliminate the possibility of residual *Wolbachia* in the medium. Once cleared of infection, cells were maintained without antibiotics. Infected and uninfected cell lines were observed using a digital camera attached to an inverted microscope.

2.1.2 *Drosophila simulans* Riverside (DSR) fly stocks

The *Drosophila simulans* Riverside (DSR) line originated from Riverside, California, and is naturally infected with the CI-inducing *Wolbachia* strain wRi (Hoffmann and Turelli, 1988; Hoffmann et al., 1990; Hoffmann et al., 1986; Turelli and Hoffmann, 1995; Zhou et al., 1998). The lines used in this study were a gift from Dr. H. Braig at the University of Wales at Bangor. Stocks were maintained in plastic culture bottles at room temperature (22-25^{o}C) on a standard diet containing agar (0.9%), cornmeal (8.4%), dextrose (6.8%), yeast

extract (2.7%) and methyl-4-hydroxybenzoate (0.25%, dissolved in 95% ethanol). *Wolbachia*-free lines (DSRT) were generated by adding tetracycline (0.025% w/v) (Sigma) to the diet for two generations (Giordano et al., 1995). Adult flies were placed on fresh diet every 4-6 weeks.

2.1.3 *Wolbachia* diagnostics

2.1.3.1 DNA extractions

Genomic DNA was isolated from approximately 10^6 cells or 10 flies using the Sigma GenElute Mammalian Genomic DNA MiniPrep kit and stored in TE buffer (10mM Tris-HCl, 0.5mM EDTA, pH 9.0, Sigma) at -20 ^{o}C until use. DNA concentration and quality were measured using the Nanodrop ND-1000 spectrophotometer. Nucleic acid purity is determined by the ratio of absorbance at 260 nm compared to 280 nm (~ 1.8) and 260nm compared to 230 nm (1.8-2.2). Samples with ratios significantly outside of these ranges (± 0.4) were not used for analysis. A minimum concentration of 20 ng/ul was required for experimental work.

2.1.3.2 Polymerase chain reaction

Polymerase chain reaction (PCR) was used to confirm the presence or absence of *Wolbachia* in fly stocks and cell lines using the *Wolbachia* surface protein *(wsp)* primers 81F (5' TGG TCC AAT AAG TGA TGA AGA AAC) and 691R (5'

AAA AAT TAA ACG CTA CTC CA)(Sigma) which generate a 591 bp product (Zhou et al., 1998). The universal 28S ribosomal DNA primers D3A (5'GAC CCG TCT TGA AAC ACG GA) and D3B (5'TCGGAA GGA ACC AGC TAC TA-3) (Sigma) which generate a 400bp product, were used to confirm DNA template quality (Singh et al., 2004). A PCR master mix composed of 2.5 µl 10 X buffer containing 15 mM $MgCl_2$ (Qiagen), 0.5 µl dNTPs (0.5 mM each) (Amersham Biosciences), 0.25 ul (1.25 µnits) Taq polymerase (Qiagen), 0.5 µl each forward and reverse primers (0.4 mM), 1.5 µl template DNA, and 19.25 µl sterile H_2O, per each 25 µl reaction was used. Prepared samples were denatured at 95 ^{o}C for 5 min, cycled 30 times at 95 ^{o}C (30s), 55 ^{o}C (1 min), and 72 ^{o}C (1 min), followed by a 5 minute extension at 72 ^{o}C. A 15 µl sample of each was electrophoresed on a 0.8 % agarose gel, stained with ethidium bromide (0.4 µg/ml, Sigma), and visualized on a UV light box. Photographs were taken using a digital camera.

2.1.3.3 *Wolbachia* visualization by immunofluorescence

Aa23 and Aa23T cells were cultured to approximately 50% confluency in flasks containing SMM media with 15-20% FBS. Cells were diluted 20 fold in SMM media and pipetted (2 mls) onto sterile glass coverslips in a six-well plate (Corning). The plate was wrapped with Parafilm^{R} (Pechiney Plastic Packaging Company) and incubated at room temperature overnight (16 hrs). The culture media was aspirated and the coverslips were washed twice with PBS. Cells were

fixed by adding 3.7% paraformaldehyde (Electron Microscopy Sciences) in PBS and incubating at room temperature for 10 minutes. The fixative was aspirated and the cells were washed twice with PBS. To permeabilize the cells, PBS-T (PBS + 0.2% Triton X-100, Sigma) was added and the coverslips were incubated for 5 minutes at room temperature. A primary antibody SMAUG (generated in guinea pig) was diluted 1:750 in PBS-T + 3% BSA (Sigma).

SMAUG is a RNA-binding protein which participates in the transition from maternal to zygotic gene expression in development (Dahanukar et al., 1999). The antibody was provided by Dr. Craig Smibert at the University of Toronto and gifted to our lab. Evidence suggests that it does not bind to the appropriate antigen, and in fact recognizes an unknown component of *Wolbachia* (Dr. Andrew Simmonds, personal communication). Coverslips were incubated in primary antibody for 1.5 hours at room temperature, then washed three times with PBS-T. They were incubated in secondary antibody (Cy2-labeled donkey anti-guinea pig, Jackson ImmunoResearch) diluted 1:200 in PBS-T for 20 mins at room temperature in the dark. Following three final washes with PBS-T, a drop of mounting media containing 4',6-Diamidino-2-phenylindole dihydrochloride (DAPI,1.5 µg/ml) (VECTASHIELD^{R}, Vector Laboratories) was placed on the coverslips, and they were lowered on to a glass slide. The coverslips were sealed with clear nail polish and imaged on a Zeiss Axiomat 40

fluorescent microscope with a Canon PowerShot camera. Images were processed using Photoshop.

2.2 Proteome analysis of *Wolbachia*-infected Aa23 cells

2.2.1 Protein purification from Aa23 and Aa23T cell lines

Cell-free protein extracts were prepared as described by Adrain et al. (2004). For each cell line, 5 X 10^8 cells were packed into a 2 ml Dounce homogenizer with an equal volume of ice-cold cell extraction buffer (CEB: 20mM HEPES-KOH, pH 7.5, 1.5 mM $MgCl_2$, 1mM EDTA, 1mM EGTA, 1mM DTT, 250mM sucrose, 10mM KCl, 100 µM phenylmethylsulfonyl fluoride (Sigma), 10 µg/ml leupeptin (Sigma), 2µg/ml aprotinin (Sigma)). Following a 20 minute incubation on ice, the cells were lysed by homogenization with 20-30 strokes of a B-type pestle. The homogenate was centrifuged for 15 minutes at 14,000 rpm. The pellet was resuspended in ice-cold CEB with 0.6% IGEPAL CA-630 (Sigma), in order to free membrane proteins in addition to cytoplasmic proteins and the extraction procedure was repeated. The supernatant was collected, and a Bradford Assay (Bio-Rad) was used to determine the protein concentration (µg/µl). Equivalent amounts of protein (750 µg) were precipitated in acetone overnight, then pelleted by centrifugation at 14,000 rpm for 10 min. The supernatant was removed and the pellets were dried, then resuspended in 200

µl of sterile H_20 and cleared of potential contaminants using the 2-D Clean-Up Kit (Bio-Rad).

2.2.2 2 Dimensional polyacrylamide gel electrophoresis

Aa23 and Aa23T protein pellets were suspended in 322 µl of Amersham DeStreak Rehydration Solution containing 0.5 % Amersham IPG buffer (pH 3-10 NL) and 10 mM dithriothreitol (DTT) (Sigma), then loaded onto 17 cm (pH 5-8) Bio-Rad ReadyStrip IPG strips and permitted to rehydrate passively overnight (16 hours) at room temperature. Isoelectric focusing was performed on an Amersham IPGphor isoelectric focusing unit overnight according to the following settings: 500V (1hr), 1000V (1hr), 8000V (4hr), 500V (12 hr), 8000V (1hr). Following isoelectric focusing, strips were sequentially equilibrated for 15 minutes in each of two equilibration buffers (50mM Tris-HCl, pH 8.8, 6M urea, 30% glycerol, 2% SDS, 0.002% bromophenol blue), one containing 1% DTT, the other containing 2.5% iodoacetamide (IAA) (Sigma) to prepare the strips for gel electrophoresis and minimize point streaking and artifacts (Amersham Biosciences). The strips were run on 12 % homogeneous SDS-PAGE gels with a 4 % stacking gel at 280 V for approximately 4 hours. Gels were stained with Deep Purple fluorescent stain (Amersham) and imaged on a Fugifilm FLA-500 scanner at 473nm. Coomassie blue (Sigma) was used secondarily in order to visualize protein spots. Spots present in the *Wolbachia*-infected gel but not in the

uninfected gel were excised manually and placed in a sterile 1.5 ml centrifuge tube containing 20 µl of nuclease-free H_20 and stored at 4^oC. Three replicates were run for each sample.

2.2.3 MS/MS analysis and protein identification using Mascot

Gel slices were sent on dry ice to the Southern Alberta Mass Spectrometry (SAMS) Centre for Proteomics at the University of Calgary. The samples were trypsin-digested and peptide sequences were determined by LC/MS/MS. Matching sequences were identified by the Mascot search engine using standard scoring (www.matrixscience.com). Where more than five significant hits were generated, an ions score cut-off of 20 was implemented, which eliminates low-scoring, random peptide matches and allows homologous proteins to generally collapse into a single hit. From the significant hits generated by Mascot (those indicating identity or extensive homology) protein matches from suitable organisms were identified. Since a protein database does not exist for *Aedes albopictus*, those matches from its closest relative for which a primary sequence database does exist, *Aedes aegypti,* were considered most probable. *Wolbachia* protein matches from the endosymbiont which infects *Drosophila melanogaster* (a primary sequence database also exists for this isolate), were deemed most likely when evaluating hits within the proteobacteria. Protein matches were further evaluated on the basis of

isoelectric point and molecular mass. The protein matches which best represented the location of the corresponding spot on the two dimensional gel were positively identified.

2.3 Evaluation of reactive oxygen species (ROS) formation in Aa23 cells

2.3.1 ROS labelling

ROS production in Aa23 and Aa23T cells was compared using the Image-iT LIVE Green Reactive Oxygen Species Detection Kit from Molecular Probes. This assay uses the fluorogenic marker 5-(and-6)-carboxy-2′,7′-dichlorodihydro-fluorescein diacetate (carboxy-H_2DCFDA), which permeates live cells and is deacetylated by nonspecific esterases intracellularly. In the presence of ROS, this reduced compound is oxidized, and fluoresces bright green. An inducer of ROS, *tert*-butyl hydroperoxide (TBHP) was used to generate a positive control. Cells were evaluated by flow cytometry and microscopy.

2.3.2 Flow cytometric analysis

Prior to labeling, Aa23 and Aa23T cells were harvested from cell culture medium by agitating each flask, poured into a 15 ml conical tube and gently pelleted by centrifugation (2,000 rpm for 5 min). Cells were washed twice in PBS containing 1.26 mM $CaCl_2$, 0.81 mM $MgSO_4$ and 5 mM EDTA (Buffer A) by gentle resuspension of the pellet followed by centrifugation (2,000 rpm for 5 min). They

were resuspended in Buffer A containing 25 µM carboxy-H_2DCFDA and incubated for 30 min at 27 ^{o}C in the dark. The cells were washed twice, then resuspended in 1 ml of Buffer A. Positive controls were generated by incubating cells in 150 µM *tert*-butyl hydroperoxide (TBHP) in cell culture medium for 90 min followed by labeling. Fluorescence of the cells was measured using the FACScan flow cytometer (BD Biosciences). A total of 10,000 events were acquired and analyzed using CellQuest software.

2.3.3 Microscopic analysis

Aa23 and Aa23T cells were harvested and washed in Buffer A as described above. Following the second wash, cells were resuspended in Buffer A and permitted to adhere to glass slides for 1 hour. Buffer A was replaced with 1 ml of Buffer A containing 25 µM carboxy-H_2DCFDA and the slides were incubated for 30 min at 27 ^{o}C in the dark. Cells were counterstained for the last 5 min of the incubation period by adding one 1µl of Hoechst 333342 (1mM, Molecular Probes) to the buffer to generate a final concentration of 1 µM. The buffer was removed by blotting with a Kimwipe (Kimberly-Clark), and the slides were washed three times with fresh buffer A. The slides were mounted in 1 ml of warm buffer A and immediately imaged on a Zeiss Axiomat 40 fluorescent microscope with a Canon PowerShot camera. Images were processed using Photoshop.

2.4 Analysis of superoxide dismutase (SOD) levels in DSR and DSRT reproductive tissue

2.4.1 Ovary and testis dissections and protein preparation

Testes and ovaries from a dozen four-day old flies were dissected and homogenized using a mechanical hand held homogenizer in 80 µl of Buffer B (10 mM potassium phosphate, 1mM EDTA, pH 7.4). The homogenate was centrifuged at 14,000 rpm for 15 min. The supernatant was collected and a Bradford Assay (BioRad) was used to determine the protein concentration (µg/µl). Protein aliquots (0.025 µg/µl) were prepared in Buffer B. Three biological replicates each of DSR and DSRT were prepared.

2.4.2 SOD assay

Total SOD levels were measured using a microplate SOD assay (Dojindo Laboratories). This method utilizes the tetrazolium salt WST-1 (2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt), which, upon being reduced by superoxide anions forms a water-soluble formazan dye (WST-1 formazan). Superoxide is generated by the interaction of xanthine (a purine base containing two oxygen molecules) and xanthine oxidase, which reduces xanthine to uric acid and hydrogen peroxide, producing superoxide anions as a by-product. If SOD is present, it degrades available superoxide, preventing it from forming WST-1 formazan. Based on the amount

of WST-1 formazan formed, the percentage of superoxide anion formation that is inhibited by available SOD can be determined by measuring the absorbance (at 450 nm) of the sample and comparing it to a standard curve. SOD standards were prepared from bovine liver SOD (Sigma). One unit of SOD is defined as that amount of protein which inhibits superoxide anion formation by 50%, therefore standards ranging from 0.001 units/ml to 200 units/ml were prepared by serial dilution in Buffer A.

Three biological replicates of each sample were evaluated, with the average of two technical replicates representing the determined value within each experiment. The SOD assay was run on 96-well plates (Corning) following the protocol outlined by Dojindo. Twenty µl of sample solution or standard were added to each test well, and to a well designated blank 2. Twenty µl of ddH_20 were added to two additional wells designated blank 1 and blank 3. To all wells, 200µl of WST working solution was added. Dilution buffer (20µl) was added to blank 2 and 3 while 20µl of enzyme working solution was added to the sample and blank 1. In blank 1, superoxide was generated with no inhibiter present to stop the conversion of WST-1 to WST-1 formazan. Blank 2 and blank 3 were void of enzyme, thus preventing the formation of superoxide in the presence of sample or water, respectively. The samples were mixed and incubated at 37^oC for 30 minutes. Absorbance was read on a Bio-Rad Benchmark microplate reader

at 450 nm and SOD activity (inhibition rate %) was calculated using the following formula:

$$\text{SOD Activity (\% inhibition rate)} = \left(\frac{[(\text{A blank 1} - \text{A blank 3}) - (\text{A sample} - \text{A blank 2})]}{(\text{A blank 1} - \text{A blank 3})} \right) \text{X } 100$$

By determining the difference between blank 1 and blank 3, and the sample and blank 2, colorimetric change not induced by xanthine oxidase is omitted. The amount of WST-1 formazan generated in the sample well is subtracted from the maximum amount of WST-1 formazan, and is indicative of the amount of superoxide present in this system. SOD activity is determined by dividing this value by the maximum amount of WST-1 formazan possible and converting it into a percentage. Based on SOD activity (% inhibition rate) the units/mg of SOD present in each sample was calculated through comparison with SOD standards ($y=mx + b$) and statistical significance was determined using a t-test ($p = 0.05$).

2.5 DNA damage in *Wolbachia*-infected cells and insects

2.5.1 Analysis of 8-oxo-dG in Aa23 and Aa23T cell lines

2.5.1.1 DNA extraction

Genomic DNA from Aa23 and Aa23T cells was isolated as described above using the Sigma GenElute Mammalian Genomic DNA MiniPrep kit, except DNA was eluted in sterile water instead of TE. Three biological replicates were prepared and the DNA concentration was measured using the Nanodrop ND-1000. DNA was lyophilized and resuspended in TBS (150mM NaCl, 20 mM Tris-HCl, pH 7.5) to a concentration of 250 ng/µl (first replicate) or 200 ng/µl (second and third replicates), depending upon the amount of DNA in the sample. Serial dilutions were prepared in TBS. From the 250 ng/µl stock, dilutions of 125 ng/µl, 50 ng/µl, 5 ng/µl, 0.5 ng/µl were prepared. From the 200 ng/µl stock, dilutions of 100 ng/µl, 50 ng/µl, 5 ng/µl, and 0.5 ng/µl were prepared in TBS.

2.5.1.2 DNA dot blot

Nitrocellulose membranes (Bio-Rad 0.45 µm Trans-Blot Transfer Membrane) were soaked in TBS for 5 minutes then permitted to air dry for 2 minutes. DNA samples (2 µl volumes) were pipetted onto the membrane. DNA was fixed to the membrane by UV cross-linking at (120mJ/cm^2) using the Spectrolinker ^{TM} UV Crosslinker (Spectronics Corp). The membranes were blocked in TBS containing 1 % BSA (Sigma) for 2 hours at room temperature,

then incubated at 4^{o}C with primary (anti-DNA/RNA damage) antibody (monocolonal antibody, recognizes 8-oxo-dG, made in mouse, StressMarq Biosciences) diluted 1:3000 in TBS with 1 % BSA overnight (16 hours). Membranes were washed 3 times for 5 minutes in TBS containing 0.05% Tween-20 (Sigma) then incubated in alkaline phosphatase-labeled anti-mouse IgG (KPL) diluted 1:5000 in TBS/BSA for hour at room temperature. The membrane was washed 3 times for 5 minutes in TBS containing 0.05% Tween-20. Two different methods of detection were used from this point on.

For the first replicate, a colorometric method was used. The membrane was developed for 15 minutes in 5-bromo-4-chloro-3-indolylphosphate/nitroblue tetrazolium (BCIP/NBT, KPL), which is dephosphorylated by alkaline phosphatase, generating an insoluble purple precipitate. Following development, the membrane was washed in sterile ddH_20, and imaged on a light box. A digital image was captured and processed using Photoshop.

For the second and third replicates, a more sensitive detection method based on fluorescence was employed. The membranes were incubated in ECF^{TM} substrate (GE Healthcare) for 5 minutes at room temperature then immediately scanned on a Fugifilm FLA-500 scanner using a 570 nm filter. In the presence of alkaline phosphatase the ECF substrate is converted into a product which fluoresces at 540–560 nm. Images were processed using Photoshop.

All images were adjusted for brightness and contrast and analyzed using ImageJ software (Rasband, 1997-2011) (available at http://rsbweb.nih.gov/ij/). Integrated density, which is a product of the area and mean gray value, was measured for all Aa23 and Aa23T dots within each replicate. The ratio of Aa23 compared to Aa23T was calculated for each pair and the mean difference between Aa23 and Aa23T was compared using a one-sample t-test ($p = 0.05$).

2.5.2 Quantification of 8-oxo-dG in male DSR and DSRT flies

2.5.2.1 DNA extractions

Male DSR and DSRT flies 0-5 days old were collected and stored at -80^{o}c until use. DNA was extracted in batches of fifty flies as follows. Flies were homogenized in 500 µl of lysis buffer (0.1 M Tris-HCl (pH 9.0), 0.1 M EDTA, 1% SDS) containing 20 mM 2,2,6,6-Tetramethylpiperidine 1-oxyl (TEMPO, Sigma) using 50 passes with a type A pestle in a Dounce homogenizer (Kontes Glass Co.). TEMPO is a free radical scavenger, and inhibits the formation of oxidative artifacts. Forty µl of proteinase K (Qiagen) was added followed by incubation at 56^{o}C overnight (16 hrs). Twenty µl of RNAse mix (8 µl of RNAse T1 (Fermentas) and 30 µl of RNase A (Sigma) in 614 µl of dd$H_2$0) was added and the samples were incubated at 37^{o}C for 30 min, then incubated at 70^{o}C for 30 minutes. Seventy µl of potassium acetate (8M) was added, and the samples were shaken and placed on ice for 30 minutes. They were then centrifuged for 15 minutes at

13,000 rpm and the supernatant was transferred to a new tube. One volume of phenol : chloroform : isoamyl alcohol (25:24:1, Sigma) containing 20 mM TEMPO was added, the tubes were inverted by hand for 3 minutes, and centrifuged for 5 minutes at 13,000 rpm. This step was repeated once with phenol : chloroform : isoamyl alcohol and once with chloroform (Sigma) alone. After each centrifugation, the supernatant was transferred to a new tube. Isopropanol (300 µl) was added following the last extraction and the samples were placed on ice for 30 minutes to precipitate the DNA. DNA was pelleted by centrifuging for 5 minutes at 10,000 rpm. One ml of 70% ethanol was added to each pellet followed by centrifugation at 13,000 rpm for 5 minutes. The pellets were resuspended in 100 µl of TE buffer (10 mM Tris-HCl, 5 mM EDTA, pH 7.3) containing 2 mM TEMPO. DNA concentration and purity were measured using the Nanodrop ND-1000 and three biological replicates containing 50 µg of DNA at a final concentration of 0.2 µg/µl were prepared and stored at -80^{o}C until analysis.

2.5.2.2 MS/MS analysis

DSR and DSRT DNA samples from whole male flies were sent on dry ice to the Center for Environmental Health and Susceptibility, The University of North Carolina at Chapel Hill for mass spectrometry. The frequency of 8-oxo-dG was measured by ultra-high pressure liquid chromatography–heat assisted

electrospray ionization–tandem mass spectrometry according to Boysen et al. (2010). For each biological replicate of DSR and DSRT, the number of 8-oxo-dG bases per 10^6 dG bases was determined. These values were compared using a t-test ($p = 0.05$).

2.5.3 Single cell gel electrophoresis (SCGE) of *Drosophila* spermatocytes

Spermatocytes were evaluated for DNA strand breaks by single cell gel electrophoresis (SCGE, also known as the comet assay) using the Oxiselect™ Comet Assay Kit by Cell BioLabs. The testes from 4 DSR and DSRT flies < 1 day old were dissected and placed in a drop of PBS on a glass slide. Testes were disrupted with fine dissecting pins and the dissociated cells were collected by pipette and transferred to a 1.5ml centrifuge tube. PBS containing 2mM EDTA was added to bring the volume to 200 µl. OxiSelect™ low melting point agarose was placed in a water bath at 90-95°C until it liquefied, then transferred into a 37°C water bath for 20 minutes to cool. The testes cell suspension was diluted 1/10 in 37°C agarose and mixed by gentle pipetting. Seventy-five µl of sample was pipetted onto OxiSelect™ glass slides with labeled wells. The slides were chilled at 4°C in the dark to allow the agarose to solidify, then immersed in a basin containing chilled lysis buffer (2.5 M NaCl, 100 mM EDTA, 1X OxiSelect™ lysis Solution) at 4°C in the dark for 45 minutes. Lysis buffer was replaced with chilled alkaline solution (300 mM NaOH, 1 mM EDTA) and incubated at 4°C for 30

minutes in the dark. The slides were transferred to a horizontal gel electrophoresis apparatus and it was filled with fresh alkaline solution. Voltage was applied at 1 volt/cm and 300 mA for 20 minutes. Slides were removed from the tank and placed in a basin containing chilled ddH_20. Water was aspirated after 2 minutes and the wash was repeated twice. A 70% ethanol solution was then added for 5 minutes, aspirated, and the slide was permitted to air dry at 4^oC overnight. DNA was stained with Vista Green and visualized on a Zeiss Axiomat 40 fluorescent microscope. Images were captured using a Canon PowerShot camera and processed using Photoshop. Spermatocytes were identified based on the increase in nuclear volume (~25 fold) at this stage of development compared to other stages (Cenci et al., 1994; Fuller, 1993). In this assay, single and double stranded breaks permit the migration of damaged DNA away from the nucleus, creating “comets”, with distinct head and tail regions. Alkaline conditions unwind the DNA and contribute to the formation of strand breaks at sensitive AP sites (Moller, 2006) . The comet analysis software CometScore ™ (www.autocomet.com) created by Tritek was used to quantify and compare fluorescence in comet heads and tails. Three biological replicates of 50 cells each were evaluated blind for each of DSR and DSRT. The mean percentage of DNA forming the comet tail in each spermatocyte for DSR and DSRT was recorded and compared using a t-test ($p = 0.05$). Additionally, the proportion of spermatocytes falling into defined damage categories, based on a scale of 0 to 5 was

determined. Cells with 0-20% of their DNA in the tail were scored as a “0”, those with 21-40 % were scored as a ”1” , 41-60 % were scored as a “2”, 61-80 % were scored as a “3”, and 81-100% were scored as a “4” (Figure 3.5.1 A,B).

3. RESULTS

3.1 *Wolbachia* in *Aedes albopictus* cell lines and *Drosophila simulans* flies

3.1.1 Antibiotic treatment and PCR analysis of Aa23 and Aa23T cells

The infection status of the Aa23 cell lines was confirmed by PCR using the *wsp* primers. Genomic DNA from stably infected cells generated a band at approximately 590 bp (Figure 3.1.1 A, top). DNA template quality was confirmed by the presence of a band at 400 bp with universal 28S ribosomal DNA primers D3A and D3B (Figure 3.1.1 A, bottom). Rifampicin treatment (10 μg/ml) resulted in the gradual dissipation of the *Wolbachia* signal over the course of 7 passages. No morphological differences were observed between infected and uninfected cells (Figure 3.1.1 B, I and II).

3.1.2 PCR analysis of DSR and DSRT flies

Infected (DSR) and uninfected (DSRT) *Drosophila simulans* Riverside flies were tested on a regular basis to confirm infection status. DNA from infected flies consistently showed a strong band with wsp primers at 590 bp, which was absent in DNA from DSRT flies. DNA from all flies generated a 400 bp band with 28S DNA primers (Figure 3.1.2).

3.1.3 Immunofluorescence of *Wolbachia* in Aa23 cells

Aa23 and Aa23T cells were stained with 4',6-Diamidino-2-phenylindole dihydrochloride (DAPI) and labelled with anti-SMAUG antibody which is believed to recognize *Wolbachia* (Figure 3.1.3). The nuclei of infected (I) and uninfected (II) mosquito cells were clearly visible upon staining with DAPI. In infected cells, *Wolbachia* DNA was identifiable when stained with DAPI and this signal overlapped with fluorescence generated by Cy2-labeled anti-SMAUG antibody (green).

3.2 Proteome analysis of *Wolbachia*-infected Aa23 cells [1]

3.2.1 2 dimensional protein fingerprint of Aa23 and Aa23T cell lines

2 dimensional PAGE of *Wolbachia*-infected Aa23 cells and uninfected Aa23T cells generated a consistent protein map across three biological replicates. More than 20 proteins expressed in Aa23 cells, and absent in Aa23T cells were revealed. In addition, several hundred host proteins were present regardless of the status of *Wolbachia* infection (Figure 3.2.1). Nine spots (ID #1-9) which appeared exclusively on the 2D gel from *Wolbachia*-infected Aa23 were manually excised for identification by LC/MS/MS.

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[1] A version of this chapter has been published. Brennan et al., 2008. PLoS ONE 3: doi: 10.1371/journal.pone.0002083.

3.2.2 Protein identification by MS/MS

Excised proteins were identified using the Mascot search engine which predicts the matching protein based on the amino acid sequence of isolated peptides. Five of the nine spots selected (Protein ID 2,3,6,7 and 8) correspond to host proteins which are overexpressed in response to a *Wolbachia* infection (Table 3.2.1). Two protein matches were identified at position 6. The peptides sequenced by LC/MS/MS showed amino acid sequence coverage of 12 to 56 % in matched proteins (Table 3.2.2). Eight of the 9 spots corresponded to a *Wolbachia* protein (Table 3.2.1). Amino acid sequence coverage ranged from 10 to 79 % (Table 3.2.3) Spots 1,4,5 and 9 were exclusively *Wolbachia*-generated, with spot 4 generating two bacterial matches. The remaining spots (2,3,6, and 7) matched both host and *Wolbachia* proteins.

3.2.2.1 Host (*Aedes albopictus*) proteins

The spot at position 8 was identified as a mosquito protein within the peroxiredoxin family. The remaining four spots resulted in matches to both host (*Ae. albopictus*), and endosymbiont (*Wolbachia*) proteins. Spot 6 generated two significant host matches, actin depolymerizing factor and CuZn superoxide dismutase (SOD). CuZn SOD was also identified at spots 3 and 7. The protein corresponding to spot 2 was identified as glutathione peroxidase.

Peroxiredoxin (gi|55233150), glutathione peroxidase (gi|108871565), and CuZn SOD (gi|94468490) are highly conserved antioxidant proteins which function in the neutralization of reactive oxygen species (ROS). CuZn SOD is responsible for the conversion of superoxide (O_2-) to oxygen and hydrogen peroxide. The mosquito CuZn SOD in this study is homologous to *D. melanogaster* Sod1 (CG11793), which is a cytoplasmic protein. Peroxiredoxins (Prx) are a group of thiol-dependant antioxidant enzymes which break down peroxides (Wood et al., 2003). The peroxiredoxin expressed in *Wolbachia*-infected *Ae. albopictus* cells is homologous to mammalian and *Drosophila* Prx5 (CG7217); both are atypical 2-Cys peroxiredoxins (Michalak et al., 2008; Seo et al., 2000). The mosquito glutathione peroxidase identified is homologous to the characterized GPx of *D. melanogaster* (CG12013). GPx uses reduced thioredoxin (Trx) as an electron donor to reduce hydrogen peroxide and organic hydroperoxides (Maiorino et al., 2007; Missirlis et al., 2003b).

Actin depolymerizing factor (gi|94469346) belongs to a family of proteins ubiquitous in eukaryotes which are essential to actin regulation (Moon and Drubin, 1995). ADF contributes to the turnover of actin by depolymerizing actin filaments, allowing them to be rapidly recycled (Carlier et al., 1997).

3.2.2.2 Endosymbiont (*Wolbachia*) proteins

Two highly conserved proteins involved in bacterial redox homeostasis were identified: Fe superoxide dismutase(SOD) (gi|4250581) and bacterioferritin (Bfr) (gi|42521044). Fe SOD is a bacterial antioxidant enzyme which degrades superoxide, a damaging reactive oxygen species (Fridovich, 1995). It contains iron instead of copper and zinc at the catalytic center (Wang et al., 2006). Ferritins include a broad superfamily of iron-storage proteins common to both aerobic and anaerobic organisms which play a key role in iron metabolism (Carrondo, 2003). Ferritins generally lack haem groups, however some bacterial forms contain a haem b, and are referred to as bacterioferritins (Ford et al., 1984; Stiefel and Watt, 1979).

Four of the identified proteins are conserved factors in bacterial protein synthesis and folding, including ribosomal protein L7/L12 (gi|42519941), translation elongation factor EF-Tu (gi|42520532), ribosome releasing factor (gi|42520392) , and GroES (gi|21742794). Ribosome releasing (recycling) factor (RRF) is a bacterial protein responsible for the disassembly of ribosomes from mRNA following termination of protein biosynthesis (Caskey et al., 1989). It also plays a role in preventing translational errors during peptide chain elongation (Janosi et al., 1996).

Elongation factor Tu (EF-Tu) is a prokaryotic elongation factor belonging to the guanosine triphosphatase superfamily of proteins (Bourne et al., 1991).

During polypeptide chain elongation, EF-Tu binds GTP, promoting the binding of an aminoacyl tRNA to the acceptor site of the ribosome (Gordon, 1968).

Ribosomal protein L7/L12 has been studied extensively in bacteria, where it forms a well-defined domain within the 50S ribosomal subunit and it interacts with elongation factors during protein synthesis (Gudkov, 1997). It is also involved in release factor function and in the control of translational accuracy during proofreading steps (Kirsebom and Isaksson, 1985; Tate et al., 1990).

GroES is a protein cofactor which complexes with the chaperonin GroEL to assist in the proper folding of newly synthesized proteins and the refolding of misfolded proteins (Sigler et al., 1998; Xu and Sigler, 1998).

Nucleoside diphosphate kinase (Ndk)(gi|42520980) is a ubiquitous enzyme which catalyzes the transfer of a phosphoryl group from a nucleoside triphosphate to a nucleoside disphosphate, and is therefore responsible for balancing the cellular concentrations of these molecules (Levit et al., 2002).

3,4-dihydroxy-2-butanone 4-phosphate synthase (gi|42520502) is an enzyme responsible for the generation of the riboflavin precursor L-3,4-dihydroxy-2-butanone 4-phosphatase (Volk and Bacher, 1991). It is part of a conserved pathway for riboflavin synthesis in bacteria. Riboflavin is essential to cell metabolism, as it is a precursor to flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN).

A *Wolbachia* single-strand binding protein (SSB) (gi|42520614) was identified. SSBs are essential proteins in all organisms, where they bind with high affinity to single stranded DNA and are important factors in DNA replication, recombination and repair (Lohman and Ferrari, 1994).

3.3 Evaluation of reactive oxygen species (ROS) formation in Aa23 cells [2]

Seven proteins identified by 2D PAGE have an antioxidant function, suggesting an increase in ROS within this system. ROS production was measured within *Wolbachia* infected and uninfected Aa23 cells by flow cytometry and microscopy, using the fluorogenic ROS indicator carboxy-H_2DCFDA.

3.3.1 Flow cytometric analysis

Approximately 1.54 % of uninfected Aa23T cells exhibited ROS formation when labeled with carboxy-H_2DCFDA and examined by flow cytometry (Figure 3.3.1A). This number rose to 5.47 % following induction of ROS with TBHP (Figure 3.3.1B). In contrast, 9.90 % of *Wolbachia*-infected cells stained with carboxy-H_2DCFDA fluoresced, demonstrating a 6.4 fold increase in ROS formation (Figure 3.3.1C) over uninfected cells.

[2] A version of this chapter has been published. Brennan et al., 2008. PLoS ONE 3: doi: 10.1371/journal.pone.0002083.

3.3.2 Microscopic analysis

When labelled with carboxy-H_2DCFDA and evaluated by microscopy, *Wolbachia*-infected *A. albopictus* cells exhibit greater ROS formation than uninfected cells. Fluorescence is associated with DAPI-labeled *Wolbachia* in the host cell cytoplasm (Figure 3.3.2).

3.4 Analysis of superoxide dismutase (SOD) levels in DSR and DSRT reproductive tissue

To investigate the expression of antioxidant expression and ROS formation *in vivo*, the level of superoxide dismutase (units/mg) was evaluated in protein extracts from *Wolbachia*-infected and uninfected *Drosophila simulans* ovaries and testes.

3.4.1 SOD levels in DSR/DSRT ovaries and testes

SOD levels were 394.9 units/mg of protein extract for DSR testes, 273.9 units/mg for DSRT testes, 94.3 units/mg for DSR ovaries and 83.4 units/mg for DSRT ovaries. Therefore, SOD activity is 1.44 X greater in the testes of DSR compared to DSRT flies and 1.13 X greater in the ovaries of DSR compared to DSRT flies ($p < 0.05$) (Figure 3.4.1). SOD activity is consistently greater in the testes compared to the ovaries (4.3 X greater in infected extracts, 3.3 X greater

in uninfected extracts, $p < 0.05$). SOD activity in uninfected controls reflects, at least in part, exposure to ROS during dissection and protein extraction. The increase in SOD activity in infected tissues, particularly in the testes, over uninfected tissues, is due to the presence of *Wolbachia.* These results are consistent with *in vitro* data in mosquito cells (Figure 3.3.2) and confirm that ROS formation and antioxidant expression are elevated in the reproductive tissue *in vivo*.

3.5 DNA damage in *Wolbachia*-infected cells and insects

ROS can induce damage to cell lipids, proteins and nucleic acids. The identification of excess ROS in *Wolbachia*-infected *Aedes albopictus* cells and *Drosophila simulans* flies led to an evaluation of ROS-mediated DNA damage in these systems.

3.5.1 Analysis of 8-oxo-dG in Aa23 and Aa23T cell lines

The intensities of the Aa23 DNA dots were visibly greater at each concentration than the Aa23T DNA dots across three replicate blots when stained with anti-8-oxo-dG antibody and detected by colorimetric or fluorescent methods (Figure 3.5.1A). Within each replicate, the ratio of integrated density for Aa23 dots compared to Aa23T dots was determined using ImageJ. On average, Aa23 dot blots showed a 7.93% increase in integrated density

compared to Aa23T dot blots ($p < 0.05$) (Figure 3.5.1). This represents a significant increase in 8-oxo-dG lesion formation in DNA from *Wolbachia*-infected cells.

3.5.2 Quantification of 8-oxo-dG in male DSR and DSRT flies by MS/MS analysis

The number of 8-oxo-dG bases per 10^6 dG bases ranged from 10.17 to 13.34 for male DSR flies, with an average of 11.93. For DSRT males, the number ranged from 10.61 to 11.63, with an average of 11.20. On average, DSR flies exhibited an increase of 0.73 8-oxo-dG bases per 10^6 dG bases (Figure 3.5.2 A, B, C). This represents a small, statistically insignificant difference ($p > 0.05$).

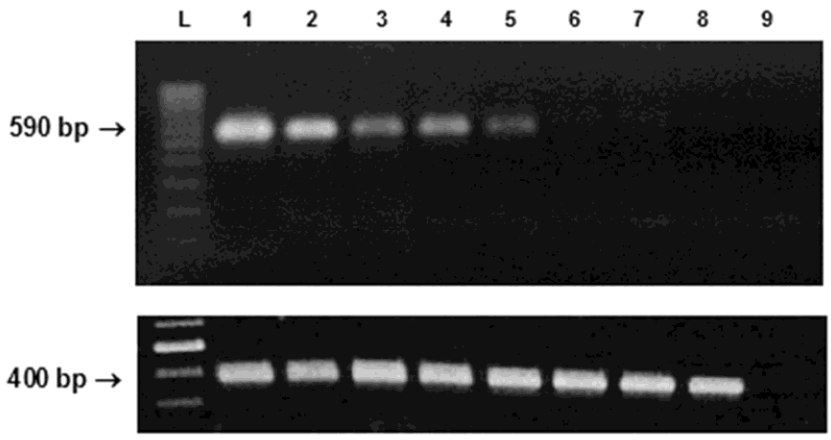
3.5.2 Single cell gel electrophoresis (SCGE) of *Drosophila* spermatocytes

Wolbachia often modifies the sperm of infected *Drosophila* in order to induce CI (Werren, 1997). Because *Wolbachia* are associated with damaging ROS, developing DSR and DSRT spermatocytes were evaluated for DNA damage using single cell gel electrophoresis (SCGE), or the comet assay. The percentage of DNA in the tail region of each comet was used for analysis. Spermatocytes were first categorized according to the scoring system shown in Figure 3.5.3 A and B. While nearly 75% of DSR spermatocytes exhibited a score of 2 or more (significant DNA damage), the majority of DSRT spermatocytes (58%) fell in the lowest damage

categories (0-1, no damage to moderate damage) (3.5.2 B). On average, the tails of DSR spermatocytes contained 55.6 % of the total DNA while in DSRT tails contained 35.4 %, representing a 20% increase in single and double stranded DNA breaks ($p < 0.05$) (Figure 3.5.2 C). This data correlates with increased oxidative stress in DSR testes, as determined by the SOD assay (Figure 3.4.1)

Figure 3.1.1 PCR analysis of *Aedes albopictus* cells naturally infected with *Wolbachia* (Aa23) and cleared of infection using antibiotics (Aa23T). (A) Amplification of *Wolbachi*a surface protein (wsp primers, top) and ribosomal DNA (28S primers, bottom) using genomic DNA from untreated Aa23 cells, and cells treated with 10 ug/ml rifampicin for seven passages. Lane L: molecular ladder. Lane 1: stably infected Aa23 cells. Lanes 2 through 8: cells treated with rifampicin for 1, 2, 3, 4, 5, 6, and 7 passages. Lane 9: negative control. (B) Brightfield image (10 X objective) of Aa23 cells and Aa23T cells cultured in SMM media containing 10-15% FBS. Bar, 100 µm.

A



B

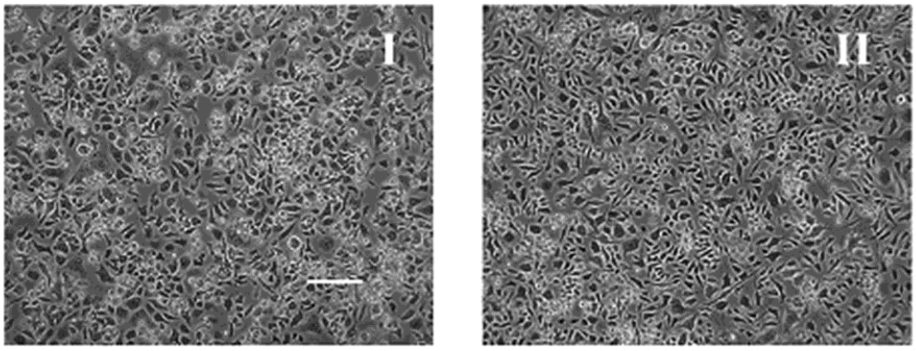


Figure 3.1.2 PCR analysis of *Drosophila simulans* Riverside flies infected with *Wolbachia* (DSR) and cleared of infection using antibiotics (DSRT). DSR flies are naturally infected with *Wolbachia,* while DSRT flies have been cleared of infection by adding tetracycline (0.025%) to the diet for two generations. PCR analysis shows amplification of genomic DNA from two DSR samples and two DSRT samples tested with ribosomal (28s) primers (lanes 1-4) for DNA template quality and *Wolbachia* surface protein (*wsp)* primers for the presence of *Wolbachia* (lanes 7-10). Lane L: molecular ladder. Lanes 1 and 3: DNA from DSR flies tested with 28S primers. Lanes 2 and 4: DNA from DSRT flies testes with 28S primers. Lane 5: negative control. Lane 6: blank. Lane 7 and 9: DNA from DSR flies tested with *wsp* primers. Lane 8 and 10: DNA from DSRT flies tested with *wsp* primers. Lane 11: negative control.

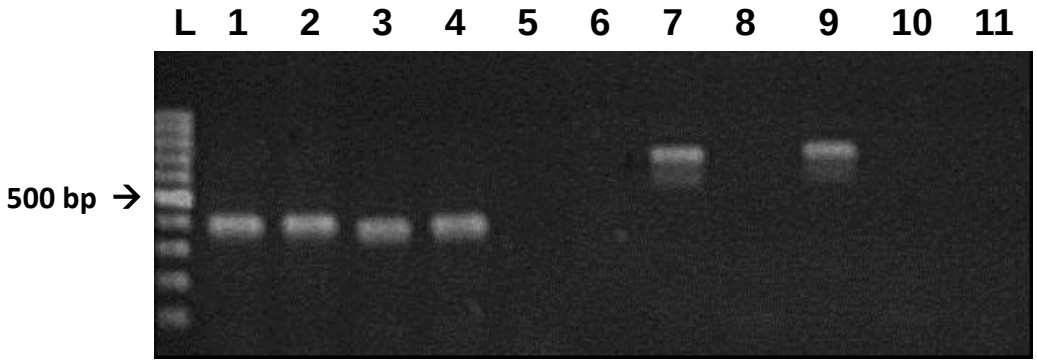

L 1 2 3 4 5 6 7 8 9 10 11
500 bp →

Figure 3.1.3 Fluorescent microscopy of *Wolbachia* -infected (I, top) and uninfected (II, bottom) *Aedes albopictus* cells. DNA is stained with DAPI (1.5µg/ml) (blue) in IA and IIA; *Wolbachia* are labelled with anti-SMAUG antibody tagged with Cy2-labeled secondary antibody (green) in IB and IIB. Overlay is shown in IC and IIC. Cells were imaged on a Zeiss Axiomat 40 fluorescent microscope under the 40X objective. Bar, 10µm.

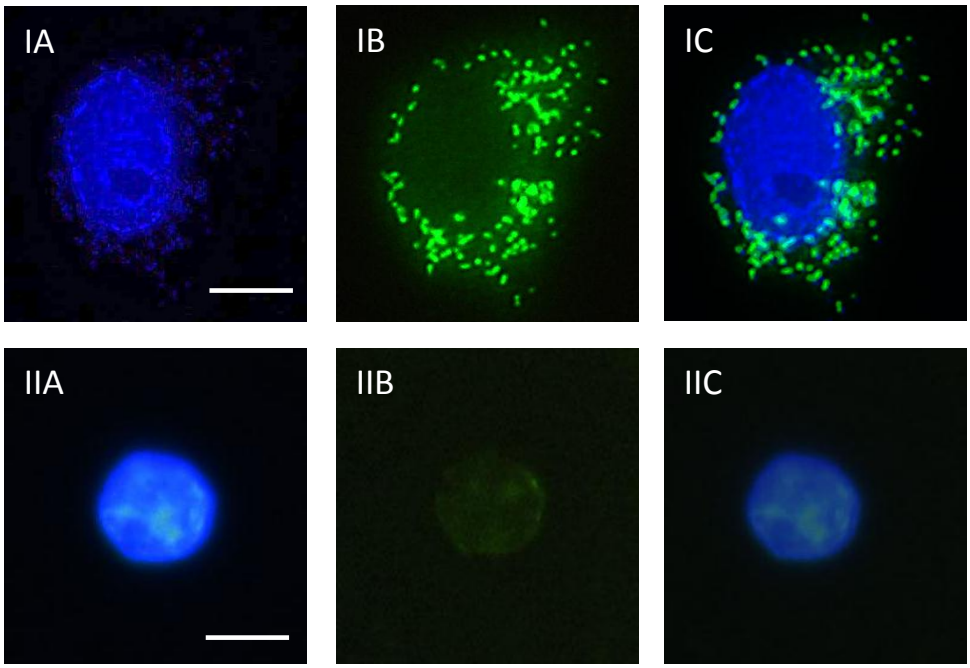
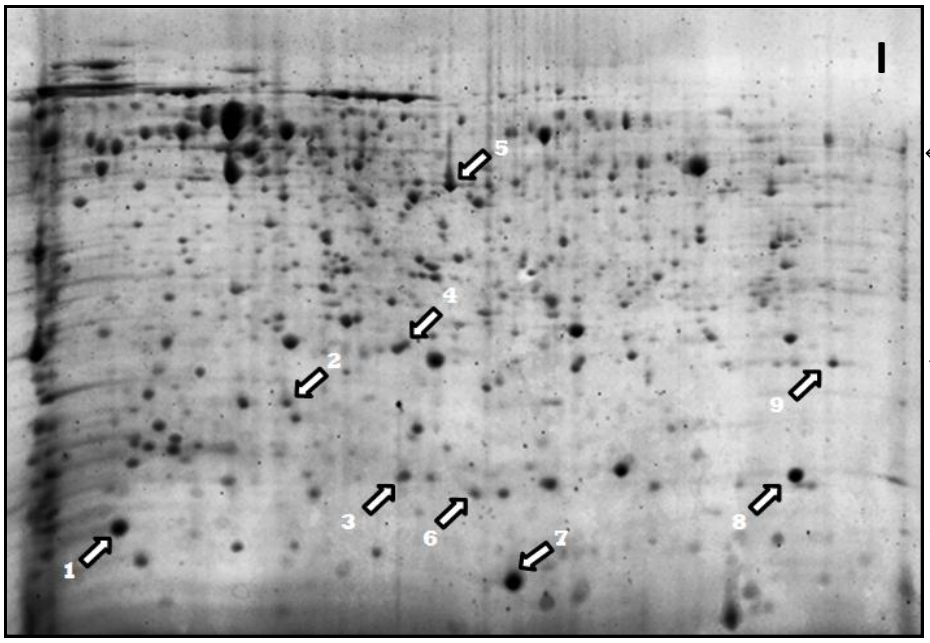

IA
IB
IC
IIA
IIB
IIC

Figure 3.2.1 Protein map of soluble extract from *Wolbachia* -infected and uninfected *Aedes albopictus* cells by 2 dimensional polyacrylamide gel electrophoresis (2D PAGE). Approximately 750 ug of protein extract from Aa23 cells (I) and Aa23T cells (II) were analyzed. Gels were stained with Deep Purple fluorescent stain (Amersham) and imaged on a Fugifilm FLA-500 scanner at 473nm. Spots 1-9 correspond to proteins visualized only in the extract containing the endosymbiont *Wolbachia*. These spots were excised and their identities were determined by mass spectrometry (shown in Table 3.2.1)


I
MW
(Da)
← 46, 877
← 22,019
← 12,185
1
2
3
4
5
6
7
8
9

5 **pI** **8**

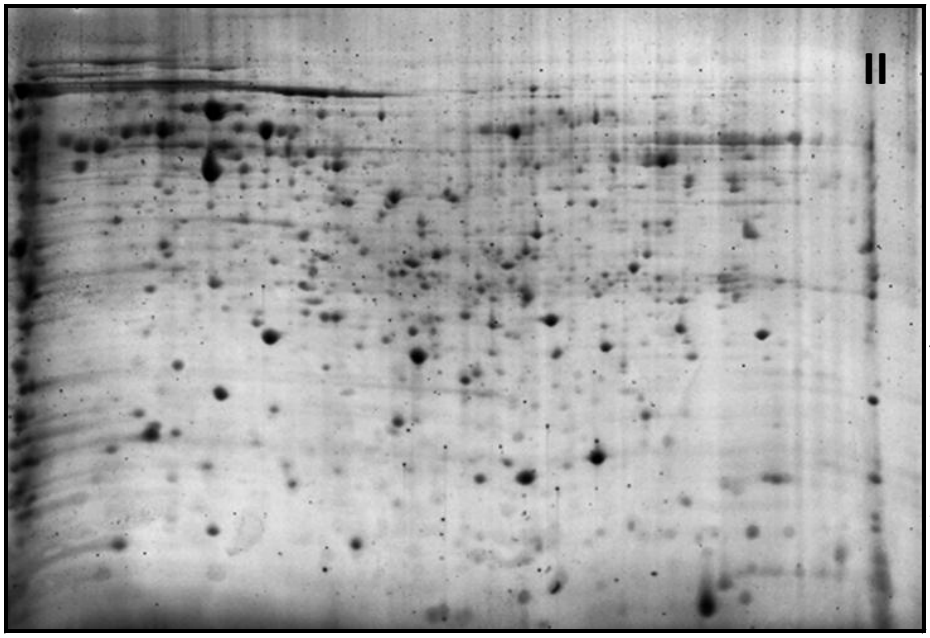

II
MW
(Da)
← 46, 877
← 22,019
← 12,185

Figure 3.3.1 Analysis of ROS formation in *Wolbachia* -infected and uninfected Aa23 cells by flow cytometry using the fluorescent ROS marker carboxy-H_2DCFDA. Histograms representative of three replicates are shown. The negative control (shaded) consists of unlabeled cells. Carboxy-H_2DCFDA positive cells are represented by the black lines. (A) Uninfected Aa23T cells, (B) uninfected Aa23 cells induced to produce ROS using TBHP and (C) infected Aa23 cells. *Wolbachia*-infected *A. albopictus* cells exhibit a 6-fold increase in fluorescence compared to uninfected cells.

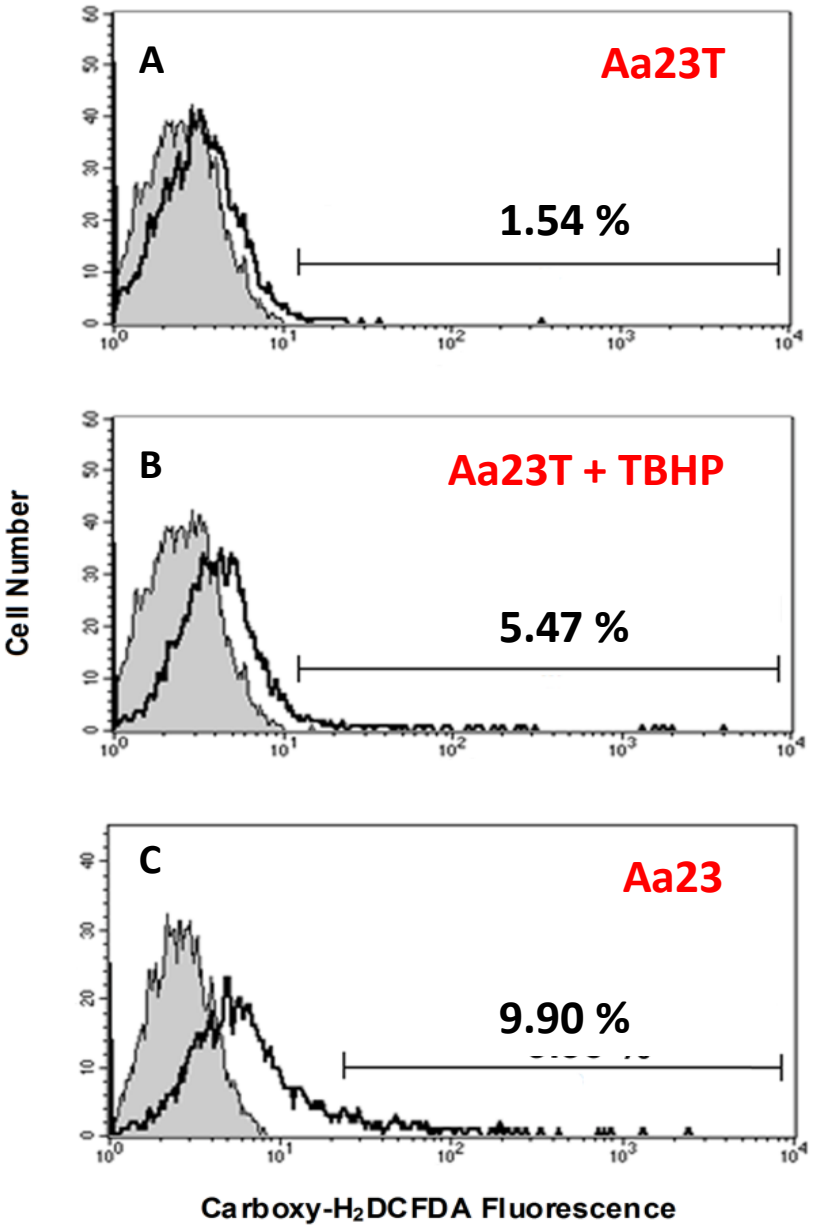

A
Aa23T
1.54 %
B
Aa23T + TBHP
5.47 %
C
Aa23
9.90 %
Cell Number
Carboxy-H2DCFDA Fluorescence

Figure 3.3.2 Microscopic analysis of ROS formation in *Wolbachia*-infected (I) and uninfected (II) *Aedes albopictus* cells labeled with the fluorescent ROS marker carboxy-H2DCFDA. Hoechst stain was used to label DNA (left panel). Carboxy-H_2DCFDA was used to label ROS (right panel). ROS formation is greater in Aa23 cells compared to Aa23T cells, and is associated with *Wolbachia* in the host cell cytoplasm. Cells were imaged on a Zeiss Axiomat 40 fluorescent microscope under the 40X objective. Bar, 10µm.Bar.

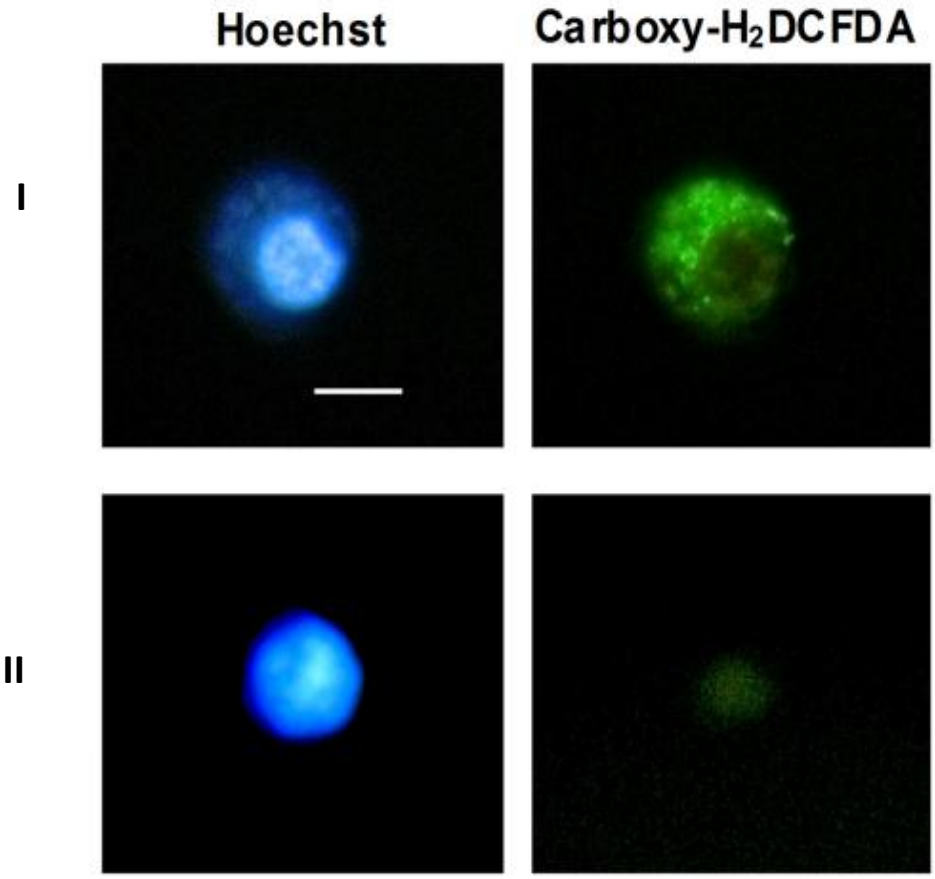

Hoechst
Carboxy-H2DCFDA
I
II

Figure 3.4.1 Assay for superoxide dismutase (SOD) actitvity in protein extracts generated from *Wolbachia* infected (DSR) and uninfected (DSRT) reproductive tissue of *Drosophila simulans* flies. The level of SOD (units/mg) is significantly greater in the both the testes (1.45 X) and ovaries (1.13 X) of DSR flies compared to DSRT ($p = 0.05$).

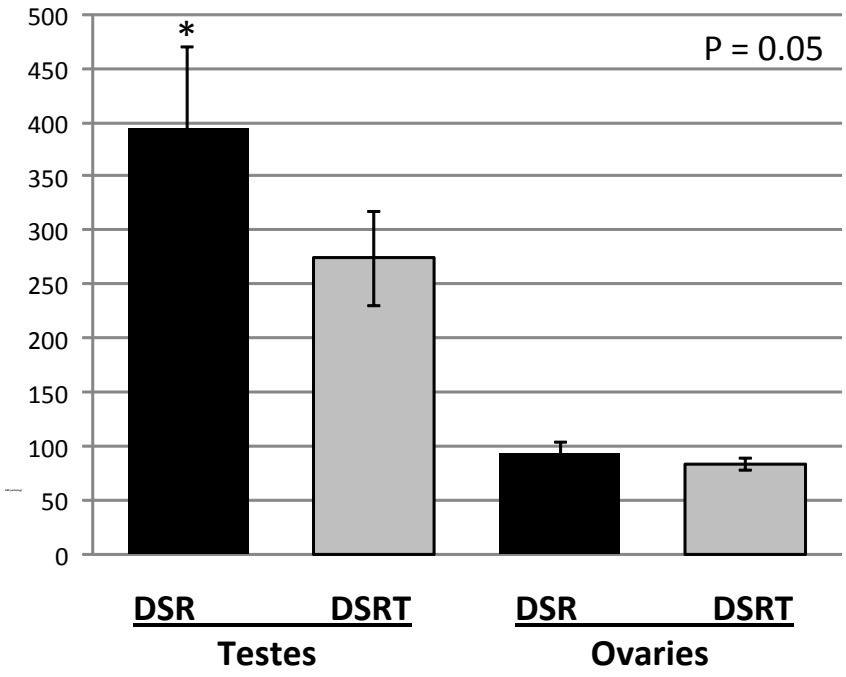
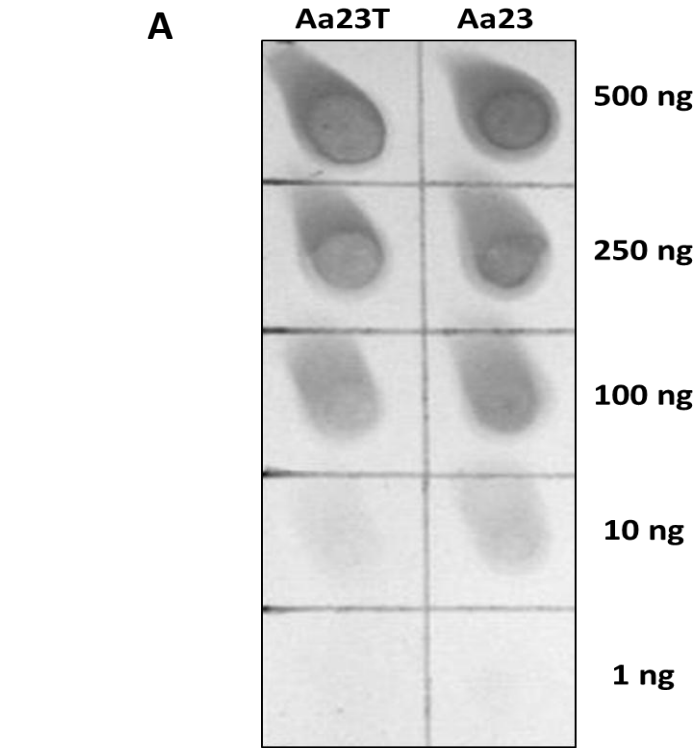

500
450
400
350
300
250
200
150
100
50
0
*
P = 0.05
DSR
DSRT
DSR
DSRT
Testes
Ovaries

Figure 3.5.1 DNA dot blot analysis of 8-oxo-dG in *Wolbachia* infected and uninfected *Aedes albopictus* cells. Serial dilutions of genomic Aa23 and Aa23T DNA were applied to a nitrocellulose membrane and probed with anti-8-oxo-dG antibody. Detection was by a colorimetric method (BCIP/NBT, KPL) or fluorescence method (ECF™,GE Healthcare). (A) A representative blot (colorimetric method). (B) The mean increase in integrated density of Aa23 dots compared to Aa23T dots from three biological replicates using ImageJ software. DNA from *Wolbachia*-infected cells shows a mean increase of 7.93 % in density compared to uninfected cells ($p = 0.05$).


A
Aa23T
Aa23
500 ng
250 ng
100 ng
10 ng
1 ng

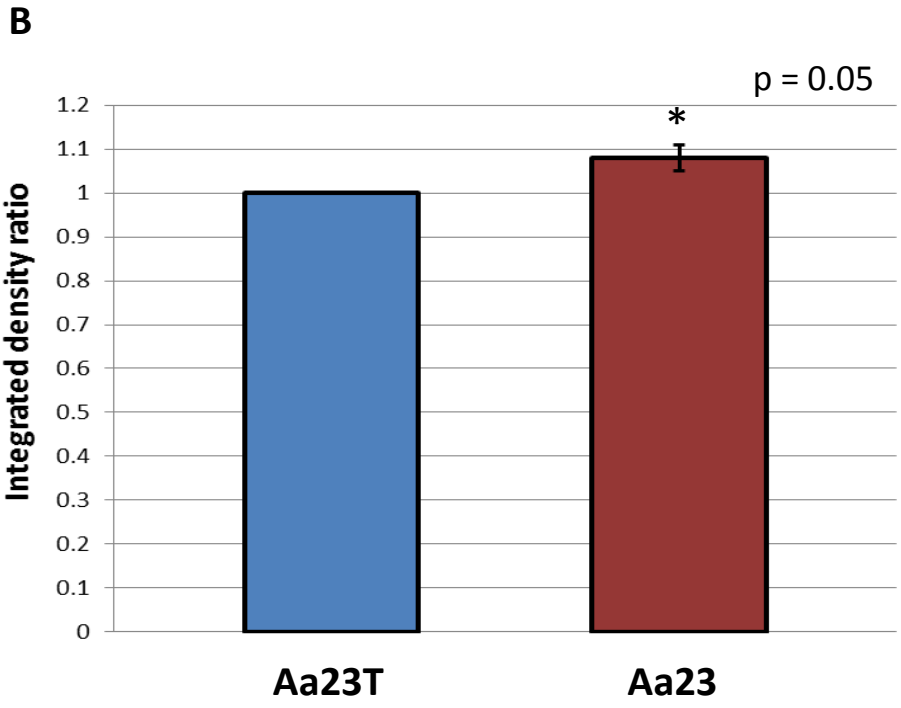
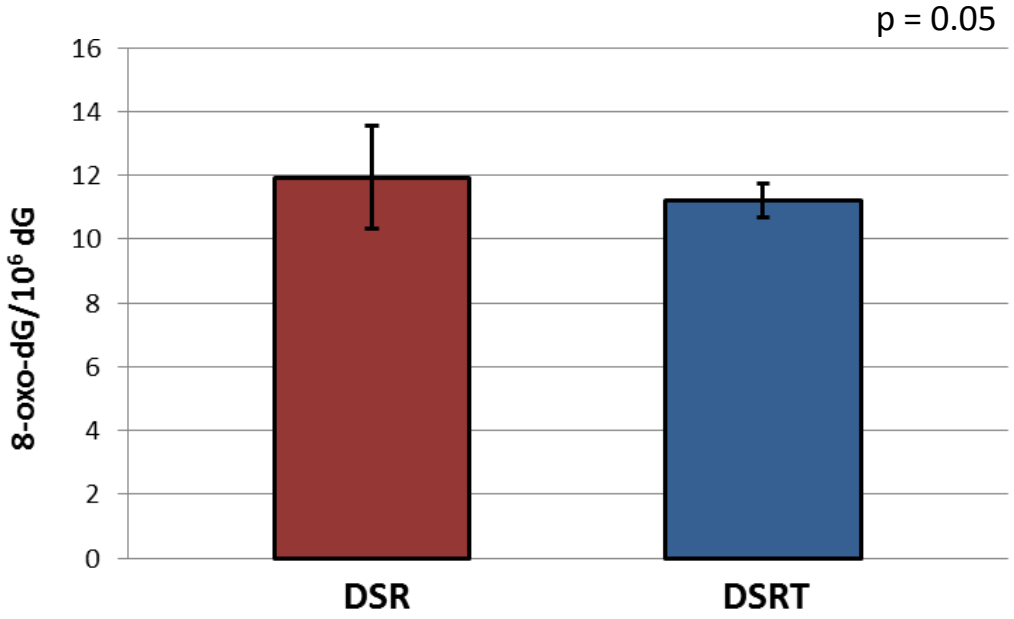

B
p = 0.05
*
Integrated density ratio
1.2
1.1
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Aa23T
Aa23

Figure 3.5.2 Quantification of 8-oxo-dG in *Wolbachia*-infected (DSR) and uninfected (DSRT) genomic DNA from male *Drosophila simulans* flies by ultra-high pressure liquid chromatography–heat assisted electrospray ionization–tandem mass spectrometry. (A) Raw data and mean number of 8-oxodG lesions per dG bases for three biological replicates of DSR and DSRT DNA. (B) There is an increase of 0.73 8-oxo-dG per 10^6 dG bases in the DNA of DSR flies compared to DSRT flies, which is not statistically significant ($p = 0.05$). (C) Representative chromatograms for DSR and DSRT DNA samples.

A

Sample	dG injected (nmol/ 275uL)	dG amount in sample (nmol)	8-oxo-dG amount (fmol) in sample	8-oxo-dG / 10^6 dG	Mean 8-oxo-dG / 10^6 dG ± SD
DSR A	9.76	10.65	108.3	**10.17**	
DSR B	8.69	9.48	116.5	**12.29**	**11.93 ± 1.61**
DSR C	10.39	11.33	151.2	**13.34**	
DSRT A	11.88	12.96	150.6	**11.62**	
DSRT B	9.80	10.70	113.5	**10.61**	**11.20 ± 0.53**
DSRT C	10.84	11.82	134.4	**11.37**	

B



C

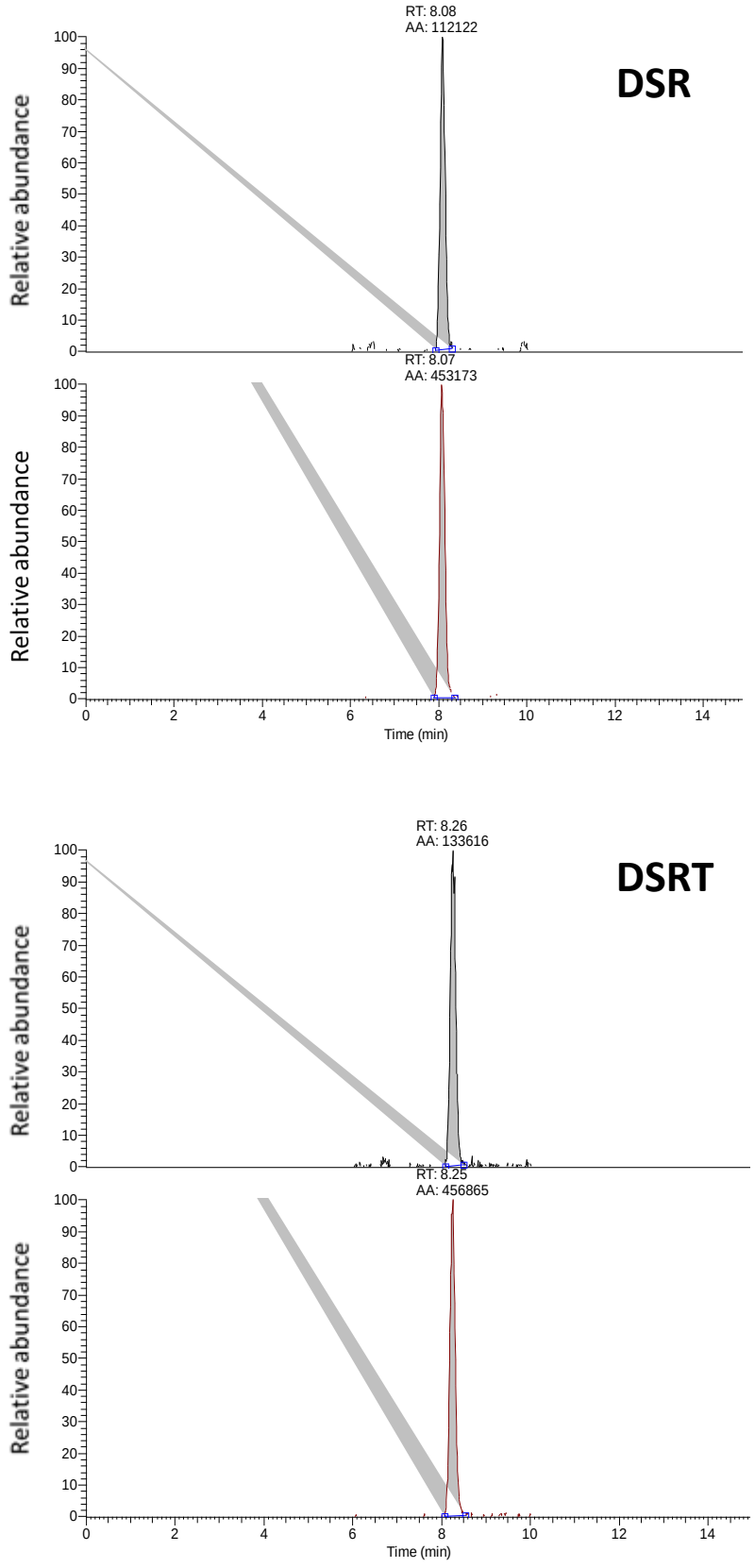
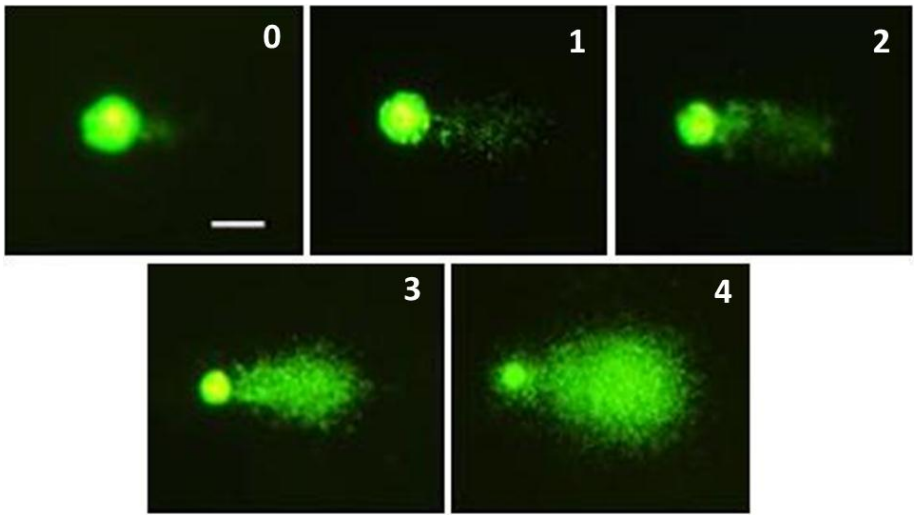

RT: 8.08
AA: 112122
DSR
Relative abundance
RT: 8.07
AA: 453173
Relative abundance
Time (min)
RT: 8.26
AA: 133616
DSRT
Relative abundance
RT: 8.25
AA: 456865
Relative abundance
Time (min)

Figure 3.5.3 Analysis of DNA damage in *Wolbachia*-infected (DSR) and uninfected (DSRT) *Drosophila simulans* spermatocytes by single cell gel electrophoresis. (A) The five categories of DNA damage, from 0 (no damage) to 4 (highly damaged) based on the percentage of DNA that has migrated to form the tail of the comet. Cells were imaged on a Zeiss Axiomat 40 fluorescent microscope under the 20X objective. Bar, 10µm. (B) The comet scoring scheme, showing the proportion of *Wolbachia*-infected and uninfected spermatocytes which fall into each category. (C) Comparison of the percentage of DNA making up the comet tails in DSR (55.6%) and DSRT (35.4%) spermatocytes. A digital image of each spermatocyte was analyzed blind using the CometScore ™ software.

A



B

Score	% DNA in Comet Tail	% of Spermatocytes	
		DSR	DSRT
0	0-20	19.39	47.27
1	21-40	7.27	10.30
2	41-60	10.30	10.91
3	61-80	51.52	26.06
4	81-100	12.73	5.45

C

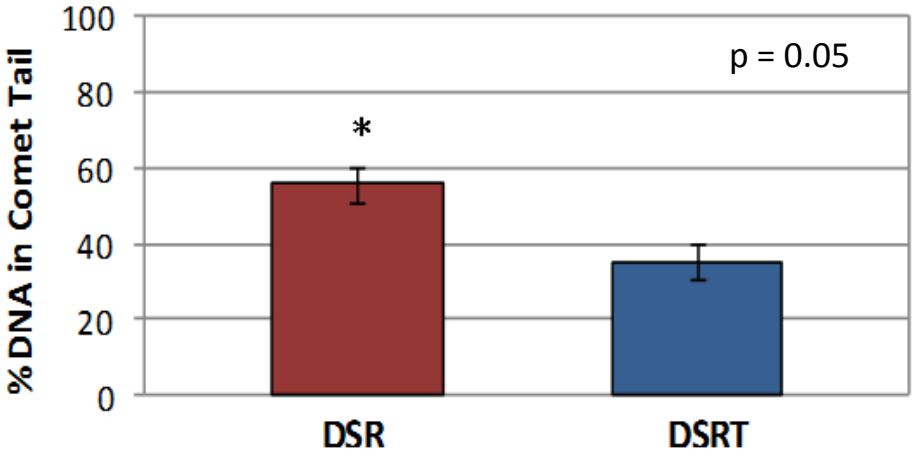


Table 3.2.1 Identification of proteins unique to *Wolbachia*- infected *Aedes albopictus* cells by LC/MS/MS. Protein matches to host (*Ae. albopictus*) and endosymbiont (*Wolbachia)* are reported, along with corresponding mowse score, isoelectric point, molecular mass, accession number, and matched peptide fragments (Mascot). Proteins denoted with a * have matches within both the host and endosymbiont databases

2D PAGE - Host Protein Matches (*Aedes albopictus*)								
Protein ID	**Match**	**Mowse Score**	**PI**	**Mass (Da)**	**% Coverage**	**Protein Name**	**Accession #**	**Matched Peptides**
2*	*Aedes aegypti*	99	6.13	19,150	14	Glutathione peroxidase	gi|108871565	K.GNYAELTELSQK.Y R.VNVNGDDAAPLYK.Y
3*	*Aedes aegypti*	59	5.77	15,616	13	Cu2+/Zn2+ superoxide dismutase	gi|94468490	R.TVVVHADPDDLGLGGHELSK.S
6*	*Aedes aegypti*	157	5.77	15,616	32	Cu2+/Zn2+ superoxide dismutase	gi|94468490	K.AVCVLSGDVK.G K.VDISDSQISLSGPLSILGR.T R.TVVVHADPDDLGLGGHELSK.S
	Aedes aegypti	186	6.74	17,323	38	Actin depolymerizing factor	gi|94469346	R.NAEYDQFLEDIQK.G R.YGLFDFEYMHQCQGTSESSK.K K.LFLMSWCPDTAK.V K.LFLMSWCPDTAK.V + Oxidation (M) K.YIQATDLSEASR.E
7*	*Aedes aegypti*	65	5.77	15, 616	12	Cu2+/Zn2+ superoxide dismutase	gi|94468490	K.VDISDSQISLSGPLSILGR.T
8	*Aedes aegypti*	355	6.71	16,862	56	Peroxiredoxin-like protein	gi|55233150	K.VNMADLCAGK.K + Oxidation (M) R.YSMVLEDGVIK.S R.YSMVLEDGVIK.S + Oxidation (M) K.IPSIDLFEDSPANK.V K.QLELGADLPPLGGLR.S K.VVLFAVPGAFTPGCSK.T K.SLNVEPDGTGLSCSLADK.I K.EGDKIPSIDLFEDSPANK.V

2D PAGE - Endosymbiont Protein Matches (*Wolbachia pipientis*)								
Protein ID	Match	Mowse Score	PI	Mass (Da)	% Coverage	Protein Name	Accession #	Matched Peptides
1	*Wolbachia (D.mel)*	136	5.06	13,924	25	Ribosomal protein L7/L12	gi|42519941	K.ILSLNLLEASELVK.V K.ELVESLPK.D K.LIEAGATKVELE.-
2*	*Wolbachia (D. mel)*	112	5.17	18,110	20	Single-strand binding protein	gi|42520614	K.VILVGNLGKDPEIR.T K.VYVEGSLR.T K.DELLDDEIPF.-
3*	*Wolbachia (D. mel)*	109	5.74	18,573	16	Bacterioferritin	gi|42521044	K.NELNEELEHANK.L K.GVPNFQDTNEISK.H
4	*Wolbachia (D. mel)*	111	5.98	24,008	22	3,4-dihydroxy-2-butanone 4-phosphate synthase, putative	gi|42520502	R.YGITTGVSAHDR.T R.YGTGIVFLAMTK.L R.ENEGDLVVLAEK.V R.YGTGIVFLAMTK.L + Oxidation (M) R.LPQLLKFAEQHK.I
	Wolbachia (D. mel)	54	5.98	24,048	10	Fe superoxide dismutase	gi|42520581	M.SFTLPELPYDK.T K.IQDDIGGFDK.F

2D PAGE - Endosymbiont Protein Matches (*Wolbachia pipientis*) - continued								
Protein ID	Match	Mowse Score	PI	Mass (Da)	% Coverage	Protein Name	Accession #	Matched Peptides
5	*Wolbachia (D.mel)*	666	5.84	42,741	44	Translation elongation factor Tu	gi|42520532	M.TAIVEAFGKPHVNVGTIGHVDHGK.T K.TTLTAAITK.H K.HYGNFVAYDQIDK.A K.HYGNFVAYDQIDKAPEER.K R.GITIATAHVEYQTEK.R R.HYAHVDCPGHADYVK.N K.NMIVGAAQMDAAILVVSGVDGPMPQTR.E K.NMIVGAAQMDAAILVVSGVDGPMPQTR.E+Oxidation (M) K.QVGVGYIVVYINK.A K.ADVADADMIDLVEMEVR.E K.ADVADADMIDLVEMEVR.E + Oxidation (M) K.SIDKLMEK.L K.TICTGVEMFK.K K.TICTGVEMFK.K Oxidation (M) K.LLDKGSAGLNVGILLR.G K.GSAGLNVGILLR.G
7*	*Wolbachia (D.mel)*	314	6.49	8457	79	GroES	gi|21742794	K.TGDKVFYR.Q K.ESDLLAVIK.- K.GEVIAIGGGSR.N K.QGGIVLPSSAEK.K K.KPTKGEVIAIGGGSR.N R.QWAGTEVEHDNEK.Y K.YVVMKESDLLAVIK.- K.YVVMKESDLLAVIK.- + Oxidation (M) R.QWAGTEVEHDNEKYVVMK.E
6*	*Wolbachia (D. mel)*	84	5.43	15,596	22	Nucleoside diphosphate kinase	gi|42520980	K.KQAELFYEIHK.D R.QIMGATDPK.Q R.GDFADDISENR.V R.QIMGATDPK.Q + Oxidation (M)
9	*Wolbachia (D.mel)*	80	6.99	20,847	10	Ribosome releasing factor	gi|42520392	K.NAILNANLNLNPVVEGSTIR.I

Table 3.2.2 Peptide alignment of host proteins (*Aedes albopictus*) identified by LC/MS/MS. *Ae. albopictus* protein sequences are shown in black with matched peptides in red. Protein name, location on the 2D map and sequence coverage (%) are listed.

Host proteins (*Aedes albopictus*)			
Spot #	**Protein name**	**% coverage**	**Sequence with peptide matches**
2	Glutathione peroxidase	14	MATESTSDYKSASSVYDFTVKDGQGEDISLEKYRGKVLLV VNIASKCGLTKGNYAELTELSQKYADKDFKILSFPCNQFGS QMPEKDGEEMVCHLRDAKADVGDVFARVNVNGDDAA PLYKYLKHKQGGSLGNFIKWNFTKFLVDKNGVPVARYSPT TNPLDIVKDIDKLL
3	Cu2+/Zn2+ superoxide dismutase	13	MPAKAVCVLSGDVKGTIFFQQNGDSDPVKVTGEVTGLKP GNHGFHIHEFGDNTNGCTSAGPHFNPHGKEHGGPDAAE RHAGDLGNVVADGSGVAKVDISDSQISLSGPLSILGRTVV VHADPDDLGLGGHELSKSTGNAGARLACGVIGICKA
6	Cu2+/Zn2+ superoxide dismutase	32	MPAKAVCVLSGDVKGTIFFQQNGDSDPVKVTGEVTGLKP GNHGFHIHEFGDNTNGCTSAGPHFNPHGKEHGGPDAAE RHAGDLGNVVADGSGVAKVDISDSQISLSGPLSILGRTVV VHADPDDLGLGGHELSKSTGNAGARLACGVIGICKA
	Actin depolymerizing factor	38	MASGVTVSDVCKTTYEEIKKDKKHRYVIFYIRDEKQIDVEVI GDRNAEYDQFLEDIQKGGPGECRYGLFDFEYMHQCQGT SESSKKQKLFLMSWCPDTAKVKKKMLYSSSFDALKKSLVG VQKYIQATDLSEASREAVEEKLRATDRQ
7	Cu2+/Zn2+ superoxide dismutase	12	MPAKAVCVLSGDVKGTIFFQQNGDSDPVKVTGEVTGLKP GNHGFHIHEFGDNTNGCTSAGPHFNPHGKEHGGPDAAE RHAGDLGNVVADGSGVAKVDISDSQISLSGPLSILGRTVV VHADPDDLGLGGHELSKSTGNAGARLACGVIGICKA
8	Peroxiredoxin-like protein	56	MVQIKEGDKIPSIDLFEDSPANKVNMADLCAGKKVVLFAV PGAFTPGCSKTHLPGYVDRADAIKSSGVQEIVCVSVNDPF VMSAWGKQHNTGGKVRMLADPAAIFTKQLELGADLPPL GGLRSKRYSMVLEDGVIKSLNVEPDGTGLSCSLADKIKV

Table 3.2.3 Peptide alignment of endosymbiont proteins (*Wolbachia pipientis*) identified by LC/MS/MS. *Wolbachia* protein sequences are shown in black with matched peptides in red. Protein name, location on the 2D map and sequence coverage (%) are listed.

Endosymbiont proteins (*Wolbachia pipientis*)			
Spot #	**Protein name**	**% coverage**	**Sequence with peptide matches**
1	Ribosomal protein L7/L12	25	MSNVTSDLVDKILSLNLLEASELVKVLEEKIGLPAGSFLGGAVG AGAPIGDNAAAPAAQEKAEYKVVIKEIDASKKIGVIKAVREVNS TLGLKEAKELVESLPKDLTANVPKDEAEKIKQKLIEAGATKVELE
2	Single-strand binding protein	20	MSGGTINKVILVGNLGKDPEIRTTQNGKEMASFSIATSESWTD KLSGMRSEKTEWHNIVIFSEGLVKIVKDFARKGSKVYVEGSLRT RKWTDQNGGERYTTEVVLYNFNSALTLLDSRNSAPNSDYKPS EYKQGETEQKDKHESFDNDIKDELLDDEIPF
3	Bacterioferritin	16	MNEEIVKHLNKLLTNELTSVRQYLLHFAILKNNGINRFAEKVKN ELNEELEHANKLAERILLFKGVPNFQDTNEISKHDGKFTKDTIRK ILEANLKLEGKGIKDIKETISIAEKEKDFVSVMLLEEMLKNEEEHF HWIEKQIDLIELMGVENYLRTQI
4	3,4-dihydroxy-2-butanone 4-phosphate synthase	22	MVQATYASMSLPGISSVEDVLEDARSGKLFILVDDESRENEGD LVVLAEKVKPEHMAFMVRYGTGIVFLAMTKLHMSKLNLEFM RKSNVDEKLTPHTAFTTSIDARYGITTGVSAHDRTHTILTAIDEK STKDDIITPGHVFPIIANEGGVLARNGHTEASVEIAKLVGLNHA AVGCELVNDDCSMMRLPQLLKFAEQHKIKLTTIDKLISYVKKLN
	Fe superoxide dismutase	10	MSFTLPELPYDKTALEPYISAKTLDFHYDKHHKGYLNKLNELVE NTDYQHVKIEELITKVHGNSGNLPIFNNAAQVWNHTFYWNS MKKNGGGKPKDGSLLAKKIQDDIGGFDKFYEEFSSHGVSQFG SGWVWLVLEKGRLGKLKITKTPNADLPIIYGQVPLLTMDVWE HAYYLDCQNRRIDYIKVFLDHLINWDFAEENLEEYMR
5	Translation elongation factor Tu	44	MTAIVEAFGKPHVNVGTIGHVDHGKTTLTAAITKHYGNFVAY DQIDKAPEERKRGITIATAHVEYQTEKRHYAHVDCPGHADYVK NMIVGAAQMDAAILVVSGVDGPMPQTREHILLAKQVGVPYI VVYINKADVADADMIDLVEMEVRELLSKYGFPGDEVPVVVGS ALKALEDDSSEYGKKSIDKLMEKLDEYVAVPPRPVDLPFLLPIED VFSISGRGTVVTGRIEKGEIKTGEEIEIIGLKATQKTICTGVEMFK KLLDKGSAGLNVGILLRGTKREEVERGQVLAKPGTITPHRKFKA EVYILKKEEGGRHTPFFANYQPQFYLRTTDVTGSIKLLDGKEMV MPGDNVSVEVELQVPIAMDKGLRFAIREGGRTVGSGVVSEILE
6	Nucleoside diphosphate kinase	22	MAIERTLSILKPDAVKNNITGNINSYIEQSGLKITAQKMMLLTK KQAELFYEIHKDRPFFGELVEFMTSGSVVVQVLVGENAVSKYR QIMGATDPKQADKGTIRGDFADDISENRVHGSDSLENARKEIA FFFAECELV
7	GroES	79	TEEKQGGIVLPSSAEKKPTKGEVIAIGGGSRNSSGERIALTVKTG DKVFYRQWAGTEVEHDNEKYVVMKESDLLAVIK
8	Ribosome releasing factor	10	MLNEIKAKTKERMLKTIQSFHDDIKGVRTGRASASLLDGIVVNI YGGHQKLNQVAGVSVIDNKTLSIKVWDISVVGEVKNAILNANL NLNPVVEGSTIRIALPDLTQETREKLVKLLHQFAENARIAIRNIR RDIMEETEKMKENKEISEDDFHGAKKEIQNITDDNIKKIDGELSI KEKDILNH

4. DISCUSSION

4.1 Symbiotic equilibrium: The balance between ROS formation and antioxidant expression in *Wolbachia*-infected insects

4.1.1 Antioxidant expression

A proteome analysis of *Ae. albopictus* cells naturally infected with *Wolbachia* (Aa23) and cured of infection using antibiotics (Aa23T) revealed five proteins, three host and two bacterial, having antioxidant capabilities (Brennan et al., 2008). Antioxidants are ubiquitous proteins which play a protective role by neutralizing damaging reactive oxygen species (ROS). Work presented here suggests that antioxidant expression contributes to the maintenance of *Wolbachia*-host symbioses.

Host antioxidants identified in this study include copper zinc superoxide dismutase (CuZn SOD), peroxiredoxin (Prx), and glutathione peroxidase (GPx). Host CuZn SOD was detected in three samples located within close proximity in gel I (Figure 3.2.1). These are likely isoforms of the same protein generated by posttranslational modification (Arai et al., 1987; Csar et al., 2001; Furukawa and O'halloran, 2006). This CuZn SOD is located in the cytoplasm and represents one of three families of SOD enzymes present in insects (Parker et al., 2004). Insects deficient in cytoplasmic CuZn SOD suffer from a reduction in lifespan and fertility, and an increase in spontaneous DNA damage (Phillips et al., 1989; Woodruff et al., 2004). In contrast, overexpression of CuZn SOD protects

neurons from damage induced upon exposure to oxidative stress in *Drosophila* (Botella et al., 2008), and in combination with catalase overexpression contributes to an extension in lifespan (Orr and Sohal, 1994).

A thiol-dependant glutathione peroxidase (GPx) with homology to *D. melanogaster* GPx (*Dm*GPx) is also upregulated in *Wolbachia* infected Aa23 cells. *Drosophila* GPx is cytoplasmic and provides protection against oxidative stress (Missirlis et al., 2003a). It contains a cysteine at its catalytic center rather than the more common seleno-cysteine and is therefore categorized as a nonselenium GPx (NS-GPx). NS-GPxs have been identified in a wide range of organisms, and many are phospholipid-hydroperoxide GPxs (PHGPxs) (Herbette et al., 2007). PHGPxs can reduce peroxidized phospholipids in addition to peroxides, preserving membrane integrity under oxidative stress (Thomas et al., 1990). *Dm*GPX too, appears to have an affinity for peroxidized phospholipids (Maiorino et al., 2007). In adult *D. melanogaster*, GPx expression is greatest in the testes (Li et al., 2003), and in humans, elevated expression of GPx in spermatozoa protects sperm lipids, and is required for fertility (Diaconu et al., 2006; Foresta et al., 2002). An analysis of expression in early development in *Drosophila* found that GPx is maternally expressed, and transcripts are found throughout the egg and early embryo (Missirlis et al., 2003a).

GPxs also play an important role in signal transduction. In plants, regulation of ROS by GPx controls cell differentiation (Faltin et al., 2010). In mice,

GPx4 acts as a redox sensor, inhibiting the mitochondrial apoptosis-inducing factor (AIF)-mediated cell death pathway, which relies on translocation of AIF from the mitochondria to the nucleus to induce apoptosis (Modjtahedi et al., 2006; Seiler et al., 2008).

A peroxiredoxin-like protein with homology to *Drosophila* Prx5 (dPrx5) was identified in *Wolbachia*-infected *Ae. albopictus* cells. *Drosophila* Prx5 is found in the cytosol, nucleus, and mitochondrion where it provides protection from oxidative stress and apoptosis, significantly extending lifespan (Radyuk et al., 2009). Peroxiredoxins are important signalling molecules and mammalian studies have detailed the mechanisms by which they modulate innate immunity. For example, Prx1 can inhibit NF-kB and JNK signalling (Hansen et al., 2007; Kim et al., 2006) while Prx4 can activate these pathways (Haridas et al., 1998). Prx2 regulates the cytokine TNF-α, by inhibiting its effect on the stress-inducible pathways JNK and p38, but enhancing its effect on ERK, which is involved in cell survival and proliferation (Kang et al., 2004). Recent work by Radyuk et al. (2010) shows that dPrx5 regulates JNK signalling. Overexpression of dPrx5 in *Drosophila* leads to decreased resistance to bacterial infection compared to controls, while *dprx5* mutants showed increased resistance to bacterial infection (Radyuk et al., 2010). Mutants also displayed induction of Imd-pathway associated antimicrobial peptides (AMPs), and the ability to rapidly clear *E. coli* infection, suggesting that Prx5 negatively controls the host response to bacteria which typically activate

the Imd pathway (i.e. Gram negative bacteria). *Wolbachia* are Gram negative proteobacteria and do not induce AMP expression (Bourtzis et al., 2000). This study shows that *Wolbachia* stimulates the induction of host Prx5, which may inhibit the host immune response and enhance intracellular survival.

Expression of host-derived catalase in response to *Wolbachia* infection was not detected in this study. Catalase is a ubiquitous antioxidant which neutralizes H_2O_2 (Bayir, 2005; Orr et al., 1996). The catalase protein of *Ae. aegypti*, the closest relative of *Ae. albpoictus* for which an annotated genome exists, has a molecular weight of 56899 Da and an isoelectric point of 7.71. It is possible that it did not migrate into the gel during 2D PAGE. Alternatively, it may have been equally expressed in both Aa23 and Aa23T cells, or may have been present in both gels at different intensities. As spots were selected based only on presence or absence, it would have been excluded from analysis. Differential gel electrophoresis (DIGE), in which several protein samples are run together with different fluorescent stains could be used to quantify changes in abundance of this protein.

Two *Wolbachia* proteins having antioxidant function were also identified by proteome analysis: bacterial iron superoxide dismutase (Fe SOD), and bacterioferritin (Bfr). Like host SOD, Fe SOD is an antioxidant enzyme which degrades superoxide (Fridovich, 1995). It is one of three superoxide dismutases identified in bacteria, along with an Mn SOD and CuZn SOD (Benov and

Fridovich, 1994; Carlioz et al., 1988; Keele et al., 1970; Sakamoto and Touati, 1984; Yost and Fridovich, 1973). Fe SOD is cytoplasmic, contains iron at its catalytic center, and is an important component of the bacterial response to ROS (Cabiscol et al., 2000; Storz and Imlayt, 1999). In some bacterial pathogens, Fe SOD contributes to intracellular survival and pathogenesis (Franzon et al., 1990; Khelef et al., 1996). The proteobacteria have been well studied in this respect. Fe SOD mutants of the human pathogen *Francisella tularensis* show increased susceptibility to oxidative stress and reduced virulence (Bakshi et al., 2006). In *Edwardsiella tarda,* a pathogen of fish, Fe SOD provides protection from oxidative damage and suppresses the host innate immune response by inhibiting macrophage activation (Cheng et al., 2010). *Helicobacter pylori*, like *Wolbachia*, lacks all bacterial SODs except Fe SOD (Spiegelhalder et al., 1993), and its loss results in increased sensitivity to ROS and DNA mutation, and a reduced ability to colonize hosts (Seyler et al., 2001).

Wolbachia bacterioferritin (Bfr) is an intracellular iron storage protein. Iron is a transition metal which participates in many critical biological processes, and is vital to all living organisms. In bacteria, iron participates in respiration, oxygen transport, gene regulation, and DNA synthesis and is therefore essential for bacterial survival and pathogenesis (Andrews et al., 2003). Host sequestration of iron as a means of restricting bacterial metabolism is a common eukaryotic response to pathogens (Ganz, 2009; Skaar, 2010). Successful pathogens

overcome iron starvation, often competing for iron by expressing their own iron scavenging proteins. Both intracellular and extracellular pathogens secrete siderophores, which bind iron and return it to the bacteria for uptake (Ratledge and Dover, 2000). Some bacterial pathogens, such as *Neisseria, Pasteurella, and Campylobacter* have outer membrane receptor proteins which directly acquire iron from host storage proteins (Gray-Owen and Schyvers, 1996; Miller et al., 2008). Once inside the cell, ferritins and ferritin-like molecules store and regulate iron for cell processes (Carrondo, 2003; Smith, 2004).

Recent research emphasizes the mutualistic effects of *Wolbachia* on manipulation of host iron. Brownlie et al. (2009) found that *D. melanogaster* flies infected with *Wolbachia* (wMel) and raised on iron restricted or overloaded diets show an increase in fecundity compared to flies raised on a conventional diet. They also noted that wild flies often have low iron levels and propose that *Wolbachia* acts as a nutritional mutualist by provisioning iron (Brownlie et al., 2009). In *Wolbachia*-infected *D. simulans* and *Ae. aegypti cells,* iron overload stimulates expression of Bfr, but has only a minor effect on host ferritin, indicating a storage role for *Wolbachia* in iron metabolism (Kremer et al., 2009b).

Kremer et al. (2009b) identified a reduction in host ferritin expression in the *Wolbachia*-infected parasitic wasp *Asobara tabida* compared to uninfected individuals. In *A. tabida*, *Wolbachia* are mutualists required for oogenesis

(Dedeine et al., 2001). In the absence of infection, excessive apoptosis of nurse cells of mid-stage egg chambers occurs, rendering females sterile (Pannebakker et al., 2007). ROS is known to activate apoptotic pathways (Bubici et al., 2006; Nakamura and Sakamoto, 2001, 2008), and free iron contributes to ROS formation by reacting with H_20_2 via the Fenton reaction. The authors suggest that (i) *Wolbachia* may repress host ferritin expression in order to make iron available for bacterial processes or (ii) it may be scavenging iron for itself via Bfr, thus limiting the need for host sequestration (Kremer et al., 2009b). In either case, it appears that *A. tabida* has evolved to rely on *Wolbachia* to regulate iron and prevent Fenton-mediated apoptosis. Even in the presence of *Wolbachia*, iron overload in *A. tabida* leads to an increase in apoptosis in the ovaries reflective of the phenotype observed in aposymbiotic individuals (Kremer et al., 2009b). Furthermore, *Wolbachia* Bfr expression remains constant under normal and excess iron conditions (Kremer et al., 2009b). The authors hypothesize that *A. tabida* is highly susceptible to iron toxicity, and they compensate poorly in the absence of *Wolbachia* or in the presence of high iron levels. Studies such as these emphasize the importance of iron in bacterial-host interactions and the ability of *Wolbachia* to manipulate iron sequestration and metabolism.

In *E. coli* and many other bacteria, expression of Fe SOD and Bfr is positively regulated by the ferric uptake regulator (fur) protein (Andrews et al., 1989; Dubrac and Touati, 2000, 2002). Activation by fur is iron-dependent

although the mechanism by which it operates is unclear. In *E.coli* it appears to be indirectly mediated by a small RNA called RyhB (Masse and Gottesman, 2002). Expression of fur is activated by OxyR and SoxR, which are global regulators of the bacterial response to oxidative stress (Zheng et al., 1999). *Wolbachia* lacks fur, OxyR and SoxR homologs (Wu et al., 2004). The loss of these genes is likely the result of genome erosion resulting from adaptation to an obligate intracellular lifestyle. How *Wolbachia* senses oxidative stress and activates the appropriate responses is unknown and provides an interesting avenue for future research.

Free iron reacts with H_2O_2 through the Fenton reaction, producing hydroxyl radicals ($OH^{\cdot}$), which are highly reactive and damaging to cell lipids, nucleic acids, and proteins (Winterbourn, 1995). Iron-mediated oxidative stress and damage is associated with numerous human diseases, underlining the importance of proper sequestration and metabolism (Ghio et al., 2008; Honda et al., 2004; McDonald et al., 2011; Oakley et al., 2007). Iron is critical to the maintenance of redox homeostasis in bacteria (Andrews et al., 2003; Cabiscol et al., 2000). Three intracellular ferritin-like molecules have been characterized in *E. coli:* bacterial ferritin (ferritin-A*,* FtnA), dodecameric ferritin (DNA-binding protein from starved cells, Dps), and bacterioferritin (Bfr) (Smith, 2004). All of these proteins contribute to iron homeostasis and protection from oxidative stress (Bellapadrona et al., 2010; Bitoun et al., 2008; Bou-Abdallah et al., 2002).

Wolbachia lacks homologs of FtnA and Dps, therefore the burden of intracellular iron metabolism and protection from ROS falls solely on Bfr. In the intracellular pathogen *Brucella abortus*, Bfr is the main iron-storage protein, accounting for 70% of the intracellular iron (Almirón and Ugalde, 2010). Bfr mutants show slower growth and increased sensitivity to ROS compared to wild-type cells (Almirón and Ugalde, 2010). Bfr mutants of *Neisseria gonorrhoeae* showed comparable effects (Chen and Morse, 1999). The storage and regulation of iron in *Wolbachia* appears to be important for a number of reasons, including a protective role against ROS and oxidative damage.

4.1.2 ROS formation

The primary function of antioxidants is to neutralize harmful reactive oxygen species (ROS). The identification of numerous proteins having antioxidant capacity (CuZn SOD, Prx, GPx, Fe SOD Bfr, Table 3.2.1) in *Wolbachia*-infected mosquito cells suggested that excess ROS is generated in this system compared to uninfected cells. *Wolbachia*-infected *Ae. albopictus* cells generate more than 6 times the ROS of uninfected cells (Figure 3.3.1), and this ROS is associated with *Wolbachia*-containing vacuoles in the host cell cytoplasm (Figure 3.3.2). Low levels of ROS in control cells represents by-products of mitochondrial respiration. Increase ROS in Aa23 cells is due to *Wolbachia* infection. There are two potential sources of excess ROS in this system, which may occur simultaneously.

First, excess ROS may be a by-product of normal bacterial metabolism. *Wolbachia* are aerobic (Wu et al., 2004), and as occurs with all respiring organisms, electrons leak from the electron transport chain and react with molecular oxygen to form superoxide, which is broken down to other forms of ROS (Esterhazy et al., 2008; Murphy, 2009). In prokaryotes, this accounts for a significant amount of ROS. In *E. coli*, the majority of H_2O_2 (up to 87%) is generated by the electron transport chain (González-Flecha and Demple, 1995).

ROS may also be generated as part of a host-mediated immune response to *Wolbachia*. The nature of the host-derived vacuole which contains *Wolbachia* remains unknown. However, if derived from a phagosome, it is possible that NADPH oxidase complex formation is triggered, causing the injection of superoxide into the vacuole via an oxidative burst (Rada and Leto, 2008). This suggests that *Wolbachia* stimulates a part of the innate immune response. NADPH oxidase activity (in the form of dDuox) has been well characterized in the *Drosophila* gut, where ROS is released into the lumen in precise amounts depending on the complement of microbes present (Buchon et al., 2009a; Buchon et al., 2009b; Ha et al., 2009a; Ha et al., 2009b; Ha et al., 2005a). Immune stimulated NADPH oxidase activity in other *Drosophila* cell types has not been studied. However this activity has been observed in hemocytes of other insects including the moth *Galleria mellonella*, the cockroach *Blaberus discoidalis,* and the bug *Rhodnius prolixus* upon challenge with bacteria or protozoans (Bergin et

al., 2005; Renwick et al., 2007; Whitten et al., 2001; Whitten and Ratcliffe, 1999). More research is needed to characterize the host-derived membrane which surrounds *Wolbachia* and to determine if immune stimulated NADPH oxidase complex assembly occurs.

4.1.3 Evolution of symbiosis and redox homeostasis

Some bacteria can manipulate the host antioxidant system to benefit their survival. This includes the intentional disruption of host cell redox status to induce pathogenesis. One example is *Rickettsia rickettsii,* an α-proteobacteria closely related to *Wolbachia* (Weinert et al., 2009). It is vectored by ticks and is an intracellular pathogen in humans, causing Rocky Mountain spotted fever (Dantas-Torres, 2007). *R. rickettsii* infects endothelial cells, replicating in the cytoplasm and inflicting host cell damage via ROS-mediated lipid peroxidation of membranes (Silverman and Santucci, 1988). In infected cells, antioxidant expression is modified in a manner that is consistent with the generation of intracellular peroxides. SOD activity is upregulated (Santucci et al., 1992), leading to generation of intracellular H_2O_2. Glutathione peroxidase and catalase activity are suppressed, and intracellular thiol levels are depleted, diminishing the cell's ability to neutralize H_2O_2 (Devamanoharan et al., 1994; Silverman and Santucci, 1990). Treatment with the antioxidant α-lipoic acid rescues thiol levels and GPx activity, reducing intracellular peroxide levels and subsequent lipid damage

(Eremeeva and Silverman, 1998a). These results demonstrate that *R. rickettsii* alters redox homeostasis resulting in induced peroxide-mediated membrane damage. Intracellular bacteria are thus capable of manipulating the host antioxidant system. Unlike pathogenesis, host antioxidant expression in *Wolbachia* symbioses may be induced to return the cell to redox homeostasis or may be adaptive, thus ensuring bacterial survival.

The evolution from free-living bacteria to obligate intracellular symbiont is inevitably accompanied by genome reduction, as bacterial genes with redundant functions found in the host become unnecessary (Wernegreen, 2002). The *Wolbachia* genome has lost about two thirds of the major antioxidant genes found in free living relatives such as *E. coli*. In stimulating host antioxidant production, *Wolbachia* has likely reduced the need for a bacterial response to ROS, resulting in a streamlined subset of bacterial antioxidants. This may contribute to *Wolbachia*'s dependence on its host, and its inability to survive extracellularly.

In any organism, if ROS formation exceeds antioxidant activity, redox homeostasis is lost and cellular damage is inevitable. In *Wolbachia*-infected *Ae. albopictus* cells, where a substantial amount of ROS is generated as a result of infection (Figure 3.3.1 and 3.3.2), an increase in the oxidative lesion 7,8-dihydro-8-oxo-2'-deoxyguanosine (8-oxo-dG) has been identified by measuring the intensity of antibody staining in a DNA dot blot experiment (Figure 3.5.1). In spite

of the DNA damage present, *Wolbachia*-infected Aa23 cells show no morphological changes indicative of stress (Figure 3.1.1B), and doubling time is unaffected by infection (Fallon, 2008). Overall, this data shows that despite the generation of host and bacterial antioxidants, DNA damage is associated with *Wolbachia* infection. In healthy cells, DNA repair processes may be sufficient to restore ROS-induced lesions and allow the persistence of *Wolbachia*.

The effect of *Wolbachia* on host fitness varies considerably depending on host genetic background (Dean, 2006). However, aside from reproductive alterations, insects naturally infected with *Wolbachia* typically fail to show negative fitness effects. Fecundity is unaffected by infection in *Nasonia vitripennis* (Bordenstein and Werren, 2000) and enhanced in *D. melanogaster* and *Ae. albopictus,* along with lifespan (Dobson et al., 2002; Fry et al., 2004). This would suggest that in spite of any negative effects induced by *Wolbachia*, insect redox homeostasis is maintained.

Symbiotic associations between insect populations and *Wolbachia* can rapidly expand. In the late 1980's and early 1990's the spread of *Wolbachia* through native *D. simulans* populations in California as a result of cytoplasmic incompatibility (CI) was documented, occurring at a rate of 100km per year (Turelli and Hoffman, 1991). Weeks et al. (2007) found that infected flies collected at the beginning of this study showed at 15-20% reduction in fecundity, but only 20 years later showed a 10% increase in fecundity compared to

uninfected flies. Symbiont modelling studies predict that selective pressure resulting from maternal transmission and symbiont spread via CI is likely to result in a reduction of negative effects and movement towards mutualism (Prout, 1994; Turelli, 1994). The results of the present study suggest that mitigation of ROS by host and bacterial antioxidant expression is an adaptation which permits the persistence of *Wolbachia* infection. While redox homeostasis in *Wolbachia*-infected insects may be disrupted in early stages of infection, as the relationship evolves, this detriment is overcome.

Some insects do not develop a stable and heritable interaction with *Wolbachia*. For example, *Wolbachia* have never been found in wild caught mosquitoes from the genus *Anopheles,* (Rasgon and Scott, 2004; Ricci et al., 2002) and artificially induced somatic transinfections of laboratory females are transient (Jin et al., 2009; Kambris et al., 2010). However, an *A. gambiae* somatic cell line (Sua5B) was stably transinfected with *Wolbachia* from *D. simulans* (*w*Ri) and *Ae. Albopictus* (*w*AlbB) (Rasgon et al., 2006). A transcriptome analysis of infected Sua5B cells revealed that gene regulation differs according to the *Wolbachia* strain present (Hughes et al., 2011). Among immunity related transcripts both *w*Ri and *w*AlbB induced AMP expression, while *w*Ri supressed receptor transcripts including Gram negative binding protein (GNBP) (Hughes et al., 2011). Furthermore, the antioxidant peroxiredoxin was decreased under both infections, and superoxide dismutase was decreased in *w*Ri- infected cells

(Hughes et al., 2011). In a similar experiment, *D. melanogaster* S2 cells artificially infected with *w*Ri showed increased expression of AMP genes and several genes in the Toll and Imd pathways, and down regulation of *ird5*, part of the Imd pathway (Xi et al., 2008). In this case, the infection was lost over the course of 18 passages (Xi et al., 2008). These experiments demonstrate that *Wolbachia* strain and host genetic background affect the response of the insect to *Wolbachia* when the infection is introduced. In contrast, the current study evaluates the interaction between a *Wolbachia* strain and its natural host.

4.2 Oxidative stress in *Wolbachia*-dense tissues: Role for oxidative damage in *Wolbachia*-induced reproductive phenotypes

4.2.1 ROS and antioxidants in *D. simulans* testes and ovaries

ROS generation colocalizes to *Wolbachia* infection (Figure 3.3.2), and since the reproductive tissues of *Wolbachia* are consistently infected, the redox status of *D. simulans* testes and ovaries was investigated. Superoxide generation and SOD activity were slightly elevated (13 %) in *Wolbachia*-ovaries, but greatly elevated (44 %) in *Wolbachia*-infected testes (Figure 3.4.1), confirming an effect of *Wolbachia* on ROS formation.

Under aposymbiotic conditions, DSR testes exhibit 3.3 X the SOD activities of DSR ovaries. Spermatogenesis involves rapid cell division accompanied by significant oxygen consumption by mitochondria within the

testes (Aitken and Roman, 2009). ROS are by-products of respiration, and various mammalian studies have found that developing sperm are highly susceptible to oxidative damage (Aitken and Baker, 2006; Aitken et al., 1989; Koppers et al., 2008). As a result, robust antioxidant systems have developed in the testes of mammals (Aitken and Roman, 2009; Kaur et al., 2006; Peltola et al., 1992; Zini and Schlegel, 1996), with the importance of SOD being well documented (Alvarez et al., 1987; Mruk et al., 2002). Likewise, insects have evolved protection for developing sperm. In *D. melanogaster* GPx is highly expressed in the testes (Li et al., 2003). The expression of several antioxidants, including catalase and SOD is greater in both the somatic and reproductive tissue of *Apis mellifera* males compared to females (Collins et al., 2004). Additionally, both testis and ovary specific thioredoxins have been characterized (Svensson et al., 2003; Svensson et al., 2007). The environment in which spermatogenesis occurs is prone to oxidative stress, thus it is not surprising that antioxidant expression is elevated in the testes under aposymbiotic conditions in comparison to ovaries.

However, infected DSR testes exhibit a greater increase (4.2 X) in the amount of SOD activity compared to DSR ovaries. An evaluation of *Wolbachia* copy number by quantitative PCR shows that DSR testes contain approximately 35 *Wolbachia* per host cell, while the ovaries contain approximately 9 *Wolbachia* per host cell (Biliske, unpublished). This suggests that *Wolbachia*-associated ROS

generation is density dependent and raises new questions regarding redox homeostasis in tissues heavily infected with *Wolbachia*.

4.2.2 Sperm DNA damage and infertility

The significant increase in ROS in *Wolbachia*-infected testes and the fact that cytoplasmic incompatibility is induced by a modification to the sperm during spermatogenesis suggested the possibility of ROS-mediated cellular damage in male *D. simulans* flies. Oxidative DNA damage was evaluated in whole male flies *in vivo* by measuring the formation of 8-oxo-dG by mass spectrometry. A slight increase in 8-oxo-dG was identified (0.73 bases per 106 d^G bases) in DSR males compared to DSRT males. This difference is not significant, supporting the fact that *Wolbachia*-associated ROS is not harmful to the host as a whole. However, *Wolbachia* density is greatest in the reproductive tissues, particularly the testes, compared to somatic tissues (Cheng et al., 2000; Clark et al., 2005; Dobson et al., 1999). In DSR, the density of *Wolbachia* in the testes is about 35 per host cell, while in whole males, it is about 7 per host cell (Biliske, unpublished). Clark et al. (2002b) observed approximately 50 *Wolbachia* per host cell in the primary spermatocytes of DSR flies. DNA damage may be a localized effect in the densely infected testes. Therefore single cell gel electrophoresis was used to evaluate DNA damage in the spermatocytes of *Wolbachia*-infected and uninfected *D. simulans* flies. DSRT spermatocytes exhibited 35.4% DNA in the comet tail, while

DSR spermatocytes showed 55.6%. This experiment revealed a 20% increase in single and double-stranded breaks in *Wolbachia*-infected nuclei compared to uninfected nuclei. This work shows that redox homeostasis is disrupted in tissues heavily infected with *Wolbachia,* such as the testes, and results in DNA damage from excess ROS.

In humans, the comet assay is used to measure sperm quality. Hughes et al. (1998; 1996; 1997) found that baseline values of human sperm are variable, and can reach 20-25% tail DNA compared to a baseline of 5% tail DNA which is characteristic of lymphocytes. This difference and is attributed to the susceptibility of the haploid genome and tight DNA packaging common to sperm (Hughes et al., 1996).

Wolbachia manipulates the reproductive behaviour of their hosts – inducing a range of phenotypes including male-killing, feminization, parthenogenesis and cytoplasmic incompatibility (CI). In CI, the sperm of *Wolbachia*-infected males is modified so that following fertilization of an uninfected egg, chromosome condensation is disrupted and the embryo dies. When the modified sperm encounters an infected egg, the modification is rescued and development proceeds normally (Werren et al., 2008). The nature of this modification remains unknown. However, research in humans suggests that DNA damage may be a factor.

In humans, numerous studies have demonstrated an association between oxidative DNA damage and a reduction in male fertility (Ishikawa et al., 2007; Kodama et al., 1997; Lopes et al., 1998; Saleh et al., 2003; Wang et al., 2003). Sperm that are severely damaged have a decreased capacity for fertilization; those with less damage are capable of fertilization but can pass on the damaged paternal DNA to the embryo (Aitken et al., 1998). Excess ROS is often generated by infiltrating leukocytes recruited to the semen in response to bacterial infection (Henkel et al., 2005; Sanocka-Maciejewska et al., 2005; Urata et al., 2001; Wang et al., 1997; Whittington and Ford, 1999). In addition to DNA damage, ROS also induces lipid oxidation of the sperm membrane (Tremellen, 2008). The plasma membrane of human sperm is high in polyunsaturated fatty acids, making them susceptible to lipid peroxidation (Aitken et al., 1989). Such damage leads to reduced sperm motility, inhibition of the acrosome reaction, and impaired sperm-oocyte fusion (Aitken et al., 1993; Williams and Ford, 2005). In CI, *Wolbachia*-modified sperm successfully fertilize eggs, suggesting that DNA damage is a likely effect of oxidative stress in *Wolbachia*-infected sperm.

4.2.3 Support for ROS mediated cytoplasmic incompatibility

Cytoplasmic incompatibility is the most common reproductive phenotype induced by *Wolbachia*, having been identified in all the major insect orders

(reviewed in Harris and Braig, 2003). The intensity of CI varies considerably (Duron et al., 2007; Hoffmann et al., 1994; Rasgon and Scott, 2003) and is dependent on a number of factors, including the density of *Wolbachia* in males (Bourtzis et al., 1996; Breeuwer and Werren, 1993; Clancy and Hoffmann, 1998). This effect appears to be correlated with age, as *Wolbachia* numbers and hatch rate of CI crosses decreases in older males (Hoffmann et al., 1990; Reynolds and Hoffmann, 2002; Weeks et al., 2007).

Much evidence points to the importance of *Wolbachia* in the developing sperm cyst in cytoplasmic incompatibility. In *D. simulans*, CI is very strong, often exceeding 90% incompatibility (James and Ballard, 2000; Turelli and Hoffmann, 1995) and *Wolbachia* are abundant within developing spermatocytes (Bressac and Rousset, 1993; Clark et al., 2002a; Clark et al., 2003). Microscopic analysis shows that *Wolbachia* replicate in spermatocytes following spermatogonial mitosis, and again during spermiogenesis (Clark et al., 2002a). In *D. melanogaster*, where CI is weaker, fewer cysts are infected and *Wolbachia* are often found between spermatocytes and in somatic cyst cells (Clark et al., 2002a). Furthermore, Clark and Karr (2002) found that while bacterial load in the testes of two different *D. simulans* strains exhibiting differing CI levels (44.4% vs 75.3%) were comparable, the strength of CI positively correlated with the number of infected cysts. Similar patterns were observed in several other *Drosophila* species (Veneti et al., 2003). Together, these results were the

foundation for the WISSH hypothesis, (*Wolbachia* Infected Spermatocyte/Spermatid Hypothesis), which proposed that *Wolbachia* presence in developing spermatocytes and spermatids is a requirement for sperm modification and CI induction (Clark et al., 2003).

In the parasitic wasp *Nasonia vitripennis*, *Wolbachia* are found in somatic cyst cells and the testis sheath and are present in the sheath cells alone in the beetle *Chelymorpha alternans* (Clark et al., 2008), demonstrating that *Wolbachia* does not have to reside within developing sperm to induce CI, and more importantly, the factor that modifies sperm can diffuse across cellular membranes. Hydrogen peroxide diffuses across mitochondrial and plasma membranes through aquaporins (Bienert et al., 2007; Bienert et al., 2006). Signalling studies have found that superoxide can move across plasma and endosomal membranes through the chloride channel-3 (ClC-3) (Hawkins et al., 2007). Superoxide generated within *Wolbachia*-containing vacuoles could enter the cytoplasm of the host cell (and potentially neighbouring cells) before or after being degraded spontaneously or by antioxidants into hydrogen peroxide. The presence of SOD in the cytoplasm of *Wolbachia* and insect cells supports this theory. Superoxide is unstable compared to hydrogen peroxide, so dismutation takes place relatively quickly, (McCord and Fridovich, 1969) leading to an accumulation of hydrogen peroxide in the host cell cytoplasm. Unreduced superoxide can inactivate enzymes containing iron-sulfur clusters, releasing iron

into the intracellular environment (Flint et al., 1993; Keyer and Imlay, 1996). Free iron and hydrogen peroxide react through the Fenton reaction to form hydroxyl radicals, which may then attack DNA (Dizdaroglu et al., 2002). Because hydroxyl radicals are so reactive, they will interact with the nearest substrate upon formation (Pryor, 1986). Thus, the Fenton reaction must occur within close proximity of DNA in order to induce base damage (Cadet et al., 1999; Marnett, 2000). During meiosis in *Drosophila*, the nuclear membrane becomes fenestrated and the chromosomes condense (Church and Lin, 1985; Kremer et al., 1986), leaving DNA particularly vulnerable to oxidative attack. The conditions for ROS-mediated DNA damage are thus optimal in *Wolbachia*-infected spermatocytes. The capacity for repair of DNA damage in *Drosophila* testes is currently unknown. Barreau et al. (2008) found that in *D. melanogaster*, mRNA transcription is halted in late primary spermatocytes, then is reactivated prior to chromatin remodelling in spermiogenesis. If oxidative DNA damage occurs primarily during meiotic stages, the opportunity for expression and activity of repair proteins during spermiogenesis would be limited, thus increasing the likelihood that DNA damage would be carried into mature sperm. The complement of DNA repair proteins in various stages of *Wolbachia*-infected and uninfected *Drosophila* spermatogenesis should be evaluated, as this may influence the intensity of DNA damage, and in turn, the strength of CI.

Post-fertilization events in incompatible crosses have been analyzed in detail. Insects are unique in that the maternal and paternal chromosomes do not fuse immediately following fertilization, but go through the first mitotic division individually, sharing the same spindle (Loppin and Karr, 2005). In a CI cross, the paternal chromosomes fail to properly condense prior to first mitosis, resulting in extensive chromosome bridging at anaphase, and later, embryonic death (Lassy and Karr, 1996; Reed and Werren, 1995; Serbus et al., 2008; Tram et al., 2006). DNA replication, condensation, and mitosis proceed normally for maternal chromatin, regardless of *Wolbachia* status (Lassy and Karr, 1996). Entry into anaphase is controlled individually for each set of chromosomes, which is why the female chromatin can proceed with segregation without the male chromatin (Callaini et al., 1997). Tram et al. (2006) proposed that in CI crosses, aberrant paternal chromosome condensation activates the mitotic spindle assembly checkpoint, which prevents progression into anaphase until all chromosomes are properly attached to the spindles by microtubules (Pinsky and Biggins, 2005). In human cells, DNA damage is known to activate the spindle assembly checkpoint activation and delay mitosis (Mikhailov et al., 2002; Nitta et al., 2004).

Tram and Sullivan (2002) identified earlier CI-associated mitotic defects, during the transition from prophase to metaphase. In CI crosses, nuclear envelope breakdown (NEB) and cyclin-dependent kinase 1 activation (Cdk1) are

delayed in the male pronucleus (Tram and Sullivan, 2002). NEB is triggered by active Cdk1, which regulates entry into mitosis, and it is suggested that the *Wolbachia* modified sperm either directly inhibits activation of Cdk1 or triggers a cell cycle checkpoint which inhibits it (Tram and Sullivan, 2002).

Landmann et al. (2009) analysed male pronuclear formation and DNA replication in incompatible embryos to better elucidate Cdk1 inactivation. *Drosophila* chromatin is organized into nucleosomes by histones, which are replaced by small and highly basic proteins called protamines during spermatogenesis, generating highly compacted sperm nuclei (Awe and Renkawitz-Pohl, 2010; Jayaramaiah Raja and Renkawitz-Pohl, 2005; Rathke et al., 2007; Rathke et al., 2010). Following fertilization, but prior to DNA synthesis, protamines are replaced with maternal histones permitting decondensation (Loppin et al., 2005). Four core histones are incorporated into each nucleosome: H3 and H4, which form a tetramer, and H2A and H2B, which form two dimers (Akey and Luger, 2003). In *Drosophila*, H4 and histone variant 3.3 are deposited in a replication-independent manner early on and H3.3 is gradually replaced following the first mitotic division with the canonical H3 (Bonnefoy et al., 2007; Tagami et al., 2004). Landmann et al. (2009) found that protamine removal in CI embryos is normal, but deposition of maternal histone H3.3 and H4 is delayed. They then examined the timing of DNA replication by staining for *Drosophila* Proliferating Cell Nuclear Antigen (dPCNA), a DNA clamp which binds DNA so that

it may be processed by polymerase (McNally et al., 2010). dPCNA is therefore characteristic of S-phase nuclei (Easwaran et al., 2007; Yamaguchi et al., 1991) While synthesis is normally completed by the time the male and female pronuclei appose following fertilization, dPCNA was detected in the *Wolbachia* modified male pronucleus into metaphase, suggesting a delay or block in DNA replication (Landmann et al., 2009). In humans, PCNA is also a reliable marker of DNA damage, as it is involved in numerous repair processes (Moldovan et al., 2007), including correction of the oxidative lesion 7,8-dihydro-8-oxo-2'-deoxyguanosine (Burkovics et al., 2009; Maga et al., 2008; van Loon and Hubscher, 2009). Landmann et al. (2009) conclude that defects in DNA replication and nucleosome assembly in the male pronucleus activate S-phase checkpoints and inhibit Cdk1 activation, leading to incompletely segregated chromosomes at anaphase. Interestingly, DNA damage is known to activate cell cycle checkpoints during both replication and mitosis in eukaryotes (Dotiwala et al., 2010; Mikhailov et al., 2002; Nyberg et al., 2002; Paulovich and Hartwell, 1995). Work presented here suggests that the replication defects observed in the male pronucleus may be the result of *Wolbachia*-mediated oxidative DNA damage during spermatogenesis, upstream of fertilization and pronuclear formation.

It is important to note that Landmann et al. (2009) crossed *Wolbachia* -infected and uninfected *D. simulans* males with uninfected *D. melanogaster*

females in their experiments. Hybrids demonstrate strong incompatibility, yet permit the utilization of transgenic markers developed in *D. melanogaster* embryos (Ferree and Sullivan, 2006). Female offspring of control crosses using uninfected males survive to adulthood but are sterile, and male offspring die during late larval or early pupal stages (Sawamura, 2000). Developmental abnormalities observed in interspecies CI crosses should therefore be analyzed with caution.

Sperm chromatin modification is part of the CI story. When sperm from a *Wolbachia*-infected male enters a *Wolbachia*-infected egg, pronuclear formation and embryonic development proceed normally. Results in male *Drosophila* show that ROS associated with *Wolbachia* in the testes contributes to DNA damage in spermatocyte nuclei. The rescue mechanism in a *Wolbachia*-infected egg therefore likely depends on activating DNA repair. The *Drosophila* genome contains a comprehensive array of DNA repair pathways (Sekelsky et al., 2000), and repair is known to occur in *Drosophila* early embryos (Hagmann et al., 1998). The zygote is transcriptionally silent until stage 14 of embryogenesis, until which time maternal RNAs deposited into the egg drive development (Foe et al., 1993). The presence of *Wolbachia* in the oocyte may initiate transcription and translation of appropriate repair proteins prior to sperm entry. As events following fertilization proceed rapidly in insects, and female chromatin remodelling progresses regardless of male chromatin status, this ‘priming’ of the

oocyte cytoplasm would be critical to restoring the paternal chromatin in time to replicate and segregate in synchrony with the female pronucleus.

Bidirectional CI occurs when the modification imparted by one *Wolbachia* strain in a population cannot be rescued by a differing strain (Bordenstein and Werren, 2007; Zabalou et al., 2008). More research is needed to characterize this phenomenon, which presumes that different *Wolbachia* strains induce different modification-rescue systems (Werren, 1997). Some bacteria manipulate the host antioxidant system to induce specific types of oxidative damage (Devamanoharan et al., 1994; Eremeeva and Silverman, 1998b; Santucci et al., 1992). Perhaps different strains of *Wolbachia* affect host redox homeostasis in a unique way, causing variations in sperm defects which are only recognized by the same or a similar strain. It also is possible that oxidative DNA damage is one of multiple factors underlying the mechanism of CI. A detailed analysis of sperm chromatin damage and oocyte repair pathways in different insects exhibiting CI will help elucidate the mechanism of this unique phenotype.

4.3 The proteome of *Wolbachia*-infected *Aedes albopictus* cells: Insight into symbiosis

Several proteins identified in this study (Table 3.2.1) have functions independent of antioxidant pathways and provide valuable information about the ongoing interactions between *Wolbachia* and its host. These include host

(*Ae. albopictus)* actin depolymerizing factor (ADF) and bacterial single stranded binding protein (SSB), ribosomal protein L7/L12, translation elongation factor EF-Tu, ribosome releasing factor (RRF), GroES, 3,4-dihydroxy-2-butanone 4-phosphate synthase, and nucleoside diphosphate kinase (Ndk).

4.3.1 Host actin depolymerizing factor: Bacterial manipulation of host machinery

Numerous critical cell processes, including cell locomotion and cell division, rely on the organization of actin within the cell, including its constant assembly and disassembly (Bamburg et al., 1999). Following escape from the phagosome, the human pathogen *Listeria monocytogenes* uses host actin filaments to move through the cytoplasm and into neighbouring cells (Theriot et al., 1992; Tilney and Portnoy, 1989). To accomplish this, *L. monocytogenes* manipulates host actin depolymerizing factor (ADF), which contributes to actin turnover by disassembling filaments (Bierne et al., 2001; Pollard and Borisy, 2003). Similar observations have been made of bacterial pathogens in the genus *Shigella* (Egile et al., 1999; Loisel et al., 1999) and *Rickettsia* (Gouin et al., 1999; Serio et al., 2010; Teysseire et al., 1992; Van Kirk et al., 2000). Although *Wolbachia* are predominantly vertically transferred, horizontal transmission is known to occur (Baldo et al., 2008; Batista et al., 2009; Huigens et al., 2004; Huigens et al., 2000; Vavre et al., 1999). When injected into a new and

permissive female host, *Wolbachia* cross several tissue layers in order to infect the germline (Frydman et al., 2006). It is plausible that such movement is associated with reorganization of host actin. There is evidence that *Wolbachia* interacts with the host cytoskeleton. The subcellular localization of *Wolbachia* during oogenesis in *Drosophila* is dependent on host microtubules, and contributes to efficient maternal transmission (Ferree et al., 2005; Serbus and Sullivan, 2007). Additionally, *Wolbachia* associates with astral microtubules of *Drosophila* embryos, ensuring proper distribution among dividing cells (Callaini et al., 1994; Kose and Karr, 1995). *Wolbachia* localization during development and movement between cells thus relies on its ability to associate with the host cytoskeleton.

Significant actin reorganization is associated with the process of phagocytosis and phagosome maturation (May and Machesky, 2001). While the composition of the vacuole which contains *Wolbachia* is unknown, it has been proposed that it is a modified phagosome (Siozios et al., 2008). ADF may therefore play a role in maintaining the intracellular niche of *Wolbachia*.

4.3.2 Bacterial replication and protein synthesis/folding: The maintenance of an intracellular lifestyle

Several bacterial proteins with functions pertaining to DNA replication and protein synthesis and folding were identified in in this study in *Wolbachia*-

infected *Ae. albopictus* cells, including bacterial single-strand binding protein (SSB), Ribosomal protein L7/L12, translation elongation factor EF-Tu, ribosome releasing factor (RRF), and the chaperone GroES.

Single-strand binding proteins (SSBs) bind ssDNA, and complex with proteins involved in DNA replication, recombination, and repair, anchoring them to the DNA (Glassberg et al., 1979; Lohman and Ferrari, 1994; Lu and Keck, 2008; Meyer et al., 1979). In *E. coli*, SSB binds DNA polymerase III, primase, and proteins involved in DNA repair by homologous recombination (HR) through the RecF pathway, including the exonuclease RecJ, the helicase RecG, and RecO, which assists RecA in homologous DNA pairing (Butland et al., 2005; Handa et al., 2009; Umezu et al., 1993; Yuzhakov et al., 1999). The RecF pathway is one of two recombinational DNA repair pathways described *E. coli*. The RecBCD pathway repairs dsDNA breaks while the RecF pathway typically repairs ssDNA breaks but can also act on dsDNA breaks (Spies and Kowalczykowski, 2005). In addition to RecJ, RecG, and RecO, numerous proteins are involved in the RecF pathway including RecA, RecR, RecF, RecQ, RecN, RuvA, RuvB, RuvC, and UvrD. (Handa et al., 2009). Of these proteins, HelD RecA, RecF, RecJ, RecO, RecR, and RuvC, are required, while RecN, RecQ, RuvA, RuvB, UvrD, and HelD help (Kuzminov, 1999). The *Wolbachia* genome appears to contain homologs of all of these proteins except RecN and HelD (Wu et al., 2004), suggesting that the RecF pathway may be a viable repair mechanism in *Wolbachia*.

Since host DNA damage is induced by oxidative stress associated with a *Wolbachia* infection, and since *Wolbachia* are located at the foci of ROS generation, the bacterial genome is also vulnerable to damage. SSBs may mediate DNA repair and contribute to the preservation of bacterial genome integrity in a damaging environment. If they are present in the *Wolbachia*-infected egg, they may also be involved in the repair of damaged sperm DNA and play a key role in rescue of CI. The *Wolbachia* RecF DNA repair pathway, which is dependent upon SSB, is a good candidate for repair of bacterial and host DNA, and should be further investigated. Additionally, SSBs likely play an important role in DNA replication of *Wolbachia,* which is required for vertical transmission and may influence CI induction (Clark et al., 2002a; McGraw et al., 2002; Ruang-areerate et al., 2004).

Ribosomal protein L7/L12, translation elongation factor EF-Tu, and ribosome releasing factor are involved in bacterial protein synthesis, and were a identified in the protein screen of *Ae. albopictus* extracts. *Wolbachia* are actively transcribing and translating proteins intracellularly, thus these factors are expected to be present.

The GroEL/GroES complex is a bacterial chaperone involved in the proper folding of proteins (Hartl, 1996; Masters et al., 2009). GroEL is cylinder-shaped, formed by two stacked heptameric rings (Braig et al., 1994b). GroES is a heptameric ring which forms a dome-shaped lid on GroEL (Hunt et al., 1996).

Hydrophobic residues within the GroEL cavity promote protein binding; the attachment of GroES causes a conformational change which buries these residues, creating a hydrophilic cavity which releases the protein for folding (Xu et al., 1997). The hydrolysis of GroEL-bound ATP triggers the release of GroES and the folded polypeptide (Weissman et al., 1995). GroEL is essential for bacterial survival and growth under both normal and stress conditions (Fayet et al., 1989; Horwich et al., 1993). It has a large repertoire of candidate polypeptides: a proteome study in *E. coli* found that GroEL interacts with approximately 250 proteins under standard conditions (Kerner et al., 2005). Other intracellular endosymbionts show unusually high expression rates of GroEL, including *Buchnera*, a common primary endosymbiont of aphids (Aksoy, 1995; Baumann et al., 1996; Charles et al., 1997; Haines et al., 2002). Due to maternal transmission, small population sizes, and a lack of recombination, endosymbionts are prone to genetic drift and accumulation of slightly deleterious mutations (Funk et al., 2001; Mira and Moran, 2002). It has been postulated that overexpression of GroEL is a mechanism of compensating for mutations over time by salvaging proteins with incorrect conformations (Fares et al., 2002a; Fares et al., 2002b; Moran, 1996). Positive selection on GroEL allows improved interaction with endosymbiont proteins, further encouraging endosymbiosis (Fares et al., 2002a; Fares et al., 2004). GroEL is an important part of the bacterial response to oxidative stress, as oxidized proteins can be

recovered through chaperone activity (Cabiscol et al., 2000; Ericsson et al., 1994; Farr and Kogoma, 1991; Susin et al., 2006; Walkup and Kogoma, 1989). Interestingly, GroEL itself is stable under oxidizing conditions, and retains its function as a chaperone (Melkani et al., 2004; Melkani et al., 2008). Constitutive overexpression of GroEL/S may permit *Wolbachia* survival under constant exposure to ROS within a cytoplasmic vacuole and is an important adaptation of *Wolbachia* to an obligate intracellular lifestyle.

4.3.3 Bacterial 3,4-dihydroxy-2-butanone 4-phosphate synthase: Evidence of a riboflavin supplement pathway

The enzyme 3,4-dihydroxy-2-butanone 4-phosophate synthase catalyzes the conversion of ribulose 5-phosphate to 3,4-dihydroxy-2-butanone 4-phosphate, intermediates in the riboflavin (vitamin B2) pathway (Richter et al., 1992; Volk and Bacher, 1990). While plants and many microorganisms use this pathway, all animals including insects are incapable of riboflavin synthesis and must assimilate it from their environment (Bacher et al., 2000; Long et al., 2010; Magee et al., 1994; Miller and Silhacek, 1995; Roje, 2007). Riboflavin is an important component of the insect diet (Tatum, 1939, 1941). It is involved in numerous cellular processes including aerobic metabolism, ROS generation, antioxidant activity, and apoptosis (Arnér and Holmgren, 2000; Massey, 2000; Nordberg and Arnér, 2001; Susin et al., 1999). *Buchnera* synthesizes riboflavin for

its aphid host (Nakabachi and Ishikawa, 1999) and *Wolbachia* endosymbionts of the bedbug *Cimex lectularius* appear to provide B vitamins (Hosokawa et al., 2010). In the latter case, *Wolbachia* reside at high densities in a bacteriome adjacent to the gonads compared to other tissues, and are vertically transmitted through the egg (Hosokawa et al., 2010). In some cases, *Wolbachia* are the only symbiont found in the bacteriome and their removal causes delayed growth and sterility, emphasizing the importance of nutrient provisioning in this relationship (Hosokawa et al., 2010). Nutrient supplementation by endosymbionts is a common phenomenon (Akman et al., 2002; Feldhaar et al., 2007; Nogge, 1976; Sabree et al., 2009; Wu et al., 2006). It allows insects to exploit diets and environments that may otherwise be unfavourable, thus conferring a selective advantage and influencing their evolution and diversity (Douglas, 2009; Gosalbes et al., 2010).

4.3.4 Bacterial nucleoside diphosphate kinase (Ndk): Potential for a unique bacterial – host interaction

Nucleoside diphosphate kinase (Ndk) is a ubiquitous enzyme which regulates the intracellular concentrations of nucleoside diphosphates and triphosphates and is therefore critical to numerous cellular processes. In bacteria, Ndks are involved in DNA/RNA synthesis, polysaccharide and protein synthesis, cell division, and signal transduction, and are therefore important to

virulence and pathogenesis (Chakrabarty, 1998; Mukhopadhyay et al., 1997; Sundin et al., 1996). In *Pseudomonas aeruginosa*, Ndk is found as a 16 kDa cytoplasmic form, and a truncated (12 kDa) membrane-associated form (Shankar et al., 1996). The molecular mass of *Wolbachia* Ndk is nearly 16 kDa and it shows strong sequence homology to the 16 kDa form of *P. aeruginosa*, indicating that it is cytoplasmic.

Ndks from many organisms including the bacteria *Mycobacterium tuberculosis, M. bovis, Vibrio cholera, Burkholderia cepacia, P. aeruginosa* and the parasitic nematode *Trichinella spiralis* are secreted (Chopra et al., 2003; Gounaris et al., 2001; Kamath et al., 2000; Melnikov et al., 2000; Punj et al., 2000; Zaborina et al., 1999). *M. tuberculosis* Ndk localizes to the nucleus of mammalian cells (human HeLa and monkey kidney COS-1), where it causes DNA damage through single-stranded nicks, contributing to host cell death (Kumar et al., 2005; Saini et al., 2004). Cleavage requires metal ions and molecular oxygen, and superoxide is generated in the process (Saini et al., 2004). This work suggests that DNA damage by *M. tuberculosis* is mediated by ROS formation. Elevated levels of ROS and DNA damage occur in *Wolbachia*-infected spermatocytes. *Wolbachia* Ndk may be a factor in this activity, therefore it is important to investigate the localization and function of this enzyme.

In bacteria, various proteins complex with Ndk as a means of manipulating NTP-synthesis (Shankar et al., 1997a). GTP-binding proteins (G-

proteins) such as *P. aeruginosa* Ras-Like Protein (Pra), and EF-Tu which rely on GTP hydrolysis for activity bind Ndk and increase synthesis of GTP (Chopade et al., 1997; Mukhopadhyay et al., 1997). *P. aeruginosa* pyruvate kinase also interacts with Ndk to generate GTP's, presumably for use by G-proteins (Sundin et al., 1996). Interestingly, *P. aeruginosa* Ras-Like Protein (Pra) can interact with eukaryotic Ndks, and *M. tuberculosis* Ndk can interact with mammalian G-proteins to promote GTP synthesis (Shankar et al., 1997b). GTP is an important signalling molecule. By binding with G-proteins it activates a plethora of cellular processes (Neves et al., 2002; Oldham and Hamm, 2008). That the activity of bacterial and host Ndks are not restricted to substrates within their own proteome provides a novel mechanism for cellular interactions in symbiosis. Bacteria may directly alter host cell signalling in manner which benefits their survival. More work is needed to characterize *Wolbachia* Ndk and its interactions with host proteins and nucleic acids.

4.4 Conclusions and future directions

The symbiotic relationship between *Wolbachia* and its insect host provides a valuable model for investigating prokaryote-eukaryote interactions. The goal of this thesis has been to gain an understanding of the exchange that occurs between symbiont and host at the cellular level, as these interactions

promote the evolution of a stable and heritable relationship and determine the effect on the host.

A proteome analysis of *Wolbachia*-infected *Ae. albopictus* cells revealed the presence of numerous proteins which contribute to this unique symbiosis. Bacterial proteins identified are involved in nutrient supplementation, cellular metabolism, protein folding, and DNA replication. Host actin depolymerising factor (ADF), which may play a critical role in *Wolbachia* localization and mobility was also identified.

The most interesting result of the proteome analysis was the presence of numerous genes of both host and bacterial origin which code for antioxidants, proteins which neutralize damaging reactive oxygen species (ROS). Further analysis revealed that *Wolbachia* symbiosis is also associated with an increase in ROS, which co-localizes with *Wolbachia* in the host cell cytoplasm. ROS may be generated as a by-product of *Wolbachia* metabolism or it may be part of a host-mediated immune response; this is an important avenue for future research. Currently, *Wolbachia* are not believed to stimulate the immune response, based on a lack of AMP induction (Bourtzis et al., 2000; reviewed in Siozios et al., 2008). However, insects harbour the components of the NADPH oxidase complex, and are capable of generating an oxidative burst in response to bacteria (Bergin et al., 2005; Ha et al., 2009a; Ha et al., 2005a; Renwick et al., 2007). It remains to be seen if the *Wolbachia*-containing vacuole acts as a scaffold for phagocyte NADPH

oxidase activity, which would suggest a role for immune regulated ROS formation. Regardless of the source of ROS, expression of host and bacterial antioxidants maintains intracellular redox homeostasis, and preserves the symbiotic relationship. This represents a novel adaptation by both the host and *Wolbachia* to symbiosis.

ROS and antioxidant expression in the reproductive tissues of *Wolbachia*-infected *Drosophila simulans* Riverside flies are also upregulated, agreeing with *in vitro* experiments. The response was strongest in the testes, where *Wolbachia* numbers are at their highest. This led to the hypothesis that while insects as a whole do not suffer from disrupted redox homeostasis when infected with *Wolbachia*, perhaps tissues which are heavily infected exhibit signs of oxidative damage. Significant DNA damage was measured in the spermatocytes of *Wolbachia*-infected *Drosophila*. This result has unique implications in the aetiology of cytoplasmic incompatibility, which is induced by a *Wolbachia*-mediated modification to the sperm of infected males and causes failed development following fertilization of an uninfected egg. A *Wolbachia*-infected egg can rescue this modification; therefore future studies should be directed at characterizing the DNA repair capacity of the egg cytoplasm.

Wolbachia are fascinating endosymbionts which hold tremendous promise in understanding the intricacies of prokaryote-eukaryote interactions.

This research lends insight into the cellular basis of this symbiosis, and the interactions which permit its continuation.

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APPENDIX I: CULTURE MEDIUM RECIPES

Schneider's Insect Medium (Sigma, #S1046)

Recipe according to (Schneider and Blumenthal, 1978)

COMPONENT	g/L
INORGANIC SALTS	
Calcium Chloride (anhydrous)	0.6
Magnesium Sulfate	1.807221
Potassium Chloride	1.6
Potassium Phosphate Monobasic	0.45
Sodium Bicarbonate	0.4
Sodium Chloride	2.1
Sodium Phosphate Dibasic	0.7
AMINO ACIDS	
β-Alanine	0.5
L-Arginine	0.6
L-Aspartic Acid	0.4
L-Cystine•HCl	0.026732
L-Cysteine	0.06
L-Glutamic Acid	0.8
L-Glutamine	1.8
Glycine	0.25
L-Histidine	0.4
L-Isoleucine	0.15
L-Leucine	0.15
L-Lysine	1.65
L-Methionine	0.15
L-Proline	1.7
L-Serine	0.25
L-Threonine	0.35
L-Tryptophan	0.1
L-Tyrosine•2Na•2H2O	0.720199
L-Valine	0.3

OTHER	
Fumaric Acid	0.06
D(+)-Glucose	2.0
α-Ketoglutaric Acid	0.35
L(-)Malic Acid	0.6
Succinic Acid	0.06
D(+)-Trehalose	2.0
Yeast Extract	2.0
pH at RT	6.5 ± 0.3

Mitsuhashi-Maramorosch Insect Medium (Handmade)
Recipe according to (Mitsuhashi and Maramorosch, 1964)

COMPONENT	g/L
INORGANIC SALTS	
Calcium Chloride (anhydrous)	0.151
Magnesium Chloride•6H2O	0.1
Potassium Chloride	0.2
Sodium Chloride	7.0
Sodium Phosphate Monobasic	0.174
Sodium bicarbonate	0.12
OTHER	
D(+)-Glucose	4.0
Lactalbumin Hydrolysate	6.5
Yeast Extract	5.0
pH at RT	6.5 ± 0.3