Wolbachia in bedbugs *Cimex lectularius*

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To Dora and Elizabeth Lewis

who inspired in me a passion to learn and explore

and helped set me on the path to where I am today.

Abstract

The global rise in numbers of bedbug infestations has been facilitated by increased global travel and climate change, and further compounded by widespread pesticide resistance. The resurgence of this blood-sucking pest is costing the hospitality industry millions of pounds each year and non-chemical control strategies are urgently needed. The recent discovery that bedbugs rely on symbiotic *Wolbachia* bacteria for the B vitamins necessary for normal egg production, growth and development, has highlighted a possible biocontrol strategy through reducing *Wolbachia* infection loads. The aim of my thesis is to better understand the nature of the *Wolbachia*-bedbug symbiosis and the mechanisms by which *Wolbachia* achieve effects in their host in order to determine whether such biocontrol strategies are likely to succeed.

I develop protocols to manipulate *Wolbachia* infection loads using heat or antibiotic treatment to test hypotheses relating to (i) the fitness effects of *Wolbachia* infection for male hosts; (ii) the fitness effects of *Wolbachia* infection for female hosts and (iii) the underlying mechanisms through which *Wolbachia* achieve their effects. My research has revealed that *Wolbachia* increase the fitness of both male and female hosts via a range of mechanisms.

The male's *Wolbachia* increase the male host's fitness through increasing the egg laying rate of the female he has mated with. I found no evidence that *Wolbachia* are transferred in the male's ejaculate. *Wolbachia* may affect egg laying rate through raising the quality of the male's ejaculate, such as via direct nutritional provisioning or by enabling the male to invest in a higher quality ejaculate. Another symbiont in male bedbugs may also influence ejaculate production.

Wolbachia increase the female host's fitness by increasing her egg hatch and development success (proportion of eggs that hatched or reached adulthood), increasing offspring body size (which will increase her indirect fitness by increasing the fitness of her offspring) and reducing the fitness costs (wounding and infection) associated with traumatic insemination. This latter effect was associated with an enhanced melanisation response.

Despite the widespread fitness benefits that *Wolbachia* provide to both male and female hosts, biocontrol methods involving a reduction in *Wolbachia* loads in natural bedbug populations are unlikely to be effective. (i) It is not possible to reduce the *Wolbachia* load (population size) of *all* individuals in a bedbug infestation due to the logistical difficulties in administering antibiotic treatment. Heat treatment to reduce *Wolbachia* loads is logistically feasible but does not present an effective alternative to antibiotics, as the reduction in bedbug fitness is too subtle (even though significant) and short-lived. (ii) A reduction in *Wolbachia* load does not kill the bedbugs outright, so infestations will remain after biocontrol has taken place. (iii) The ecology of bedbugs precludes the release of sterile males – a biocontrol strategy used for other pest insects –as a biocontrol strategy for this system.

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Abbreviations

αb	Antibiotics
AMP	Antimicrobial peptide
approx	approximately
B	B vitamins
BDMA	Dimethylbenzylamine
BLS	*BEV*-like symbiont
c	circa
$CaCl_2$	Calcium chloride
CI	Confidence interval
C.I.	Cytoplasmic incompatibility
cm	Centimetres
^oC	Degrees Celsius
C_T	Cycle threshold
DCV	*Drosophila* C virus
ddH_2O	Double-distilled water
DDSA	Dodecenyl succinic anhydride
dH_2O	Distilled water
DNA	Deoxyribonucleic acid
e.g.	For example
EDTA	Ethylenediaminetetra-acetic acid
FISH	Fluorescence in situ hybridisation
g	G force (relative centrifugal force)
HCl	Hydrochloric acid
H_2O	Water
i.e.	That is
in	Inches
LLA	Lysozyme-like activity
M	Mole
mcp	Multiple comparisons
mg	Milligram

mL	Millilitre
mm	millimetre
mRNA	Messenger RNA
Na-Cac	Cacodylic acid
NaCl	Sodium chloride
ng	Nanogram
nm	Nanometre
PBS	Phosphate buffered saline
PCR	Polymerase Chain Reaction
***p*mol**	Picomole
PO	Phenoloxidase
proPO	Prophenoloxidase
qPCR	Quantitative polymerase chain reaction
rcf	Relative centrifugal force
rDNA	Ribosomal DNA
RH	Relative humidity
RNA	Ribonucleic acid
rRNA	Ribosomal RNA
RT-PCR	Reverse transcriptase polymerase chain reaction
SD	Standard deviation
SDS	Sodium dodecyl sulphate
SE	Standard error
SIT	Sterile insect technique
SMP	Symbiont-mediated protection
TEM	Transmission electron microscopy
TI	Traumatic insemination
Tris	Tris(hydroxymethyl) aminomethane
µg	Microgram
µL	Microlitre
µM	Micromole
vits	Vitamins

Wol	*Wolbachia*
wsp	*Wolbachia* surface protein
vits	Vitamins

Terms and Definitions

Bacteriome – a specialised insect organ comprising specialised cells (bacteriocytes) that contain endosymbiotic bacteria. (Synonymous with the term “mycetome”).

Bacteriocyte – a specialised cell that contains endosymbiotic bacteria.

Bedbug –refers to the single species *Cimex lectularius* L. unless specified otherwise

Cohort − a group of bedbugs of the same age and same developmental stage, that hatched from eggs at the same time.

Cryptic female choice – the action of a multiply mated female to bias paternity to favour a subset of the males with whom she has mated.

Eclosion – the emergence of an insect from its egg or pupa case; or the progression of an insect between successive immature stages.

Endosymbiont –a symbiont that dwells within the body of its symbiotic partner (host).

Facultative symbiont – Opportunistic symbiont that may establish a relationship with a host if the opportunity presents itself but is not physiologically dependent on doing so.

Fitness – the lifetime reproductive success (number of offspring during an individual’s lifespan). Throughout this thesis the number of eggs laid within a fixed time period is used as a surrogate measure of fitness.

Germ cell – a gamete (egg or sperm cell) or one of its precursor cells.

Germ line – a series of germ cells each descended or developed from earlier cells in the series, regarded as continuing through successive generations of an organism.

Gonochorism – describes the unisex state whereby an individual is just one of at least two distinct sexes.

Gram-negative bacteria – a class of bacteria that do not hold Gram's stain on account of the high lipopolysaccharide content of their cell walls.

Gram-positive bacteria – a class of bacteria that hold Gram's stain.

Haemocoel – the haemolymph-containing component of an insect's coelom.

Haemocytes – an invertebrate's circulatory cells; analogous to blood cells in humans.

Haemolymph – the liquid component of an invertebrate's circulatory system; analogous to blood in humans.

Hemimetabolous – the classification of insect development whereby incomplete metamorphosis occurs.

Horizontal transmission – refers to the mode of symbiont transmission whereby a host acquires the symbiont either from the external environment or from another member of the same species.

Host – the larger partner of a symbiosis.

Inclusive fitness – a measure of an individual's genetic fitness that captures direct fitness (genetic success via offspring) and indirect fitness (mainly shared genetic success via relatives)

Instar – immature life stage of an arthropod between two successive moults.

Kin selection – the selection that generates traits in an individual which enhance genetic benefits gained via kins' fitness.

Load (*sensu Wolbachia* load) – population size of *Wolbachia* within a host or specified tissue, determined by qPCR analysis as the relative abundance of *Wolbachia wsp* genes to host elongation factor 1-alpha ef1α genes.

Mass of blood meal – the mass of blood taken in by an individual during a feed, calculated as the difference in the individual's body mass before and immediately after feeding.

Melt temperature − the temperature at which 50% of DNA is denatured.

Obligate symbiont – symbiont that is physiologically dependent on the relationship with its host.

Paramere – male bedbug's intromittent organ.

Prevalence – the proportion of infected individuals in a species.

Primary symbiont – obligate associate that occupies specialised host cells.

Proboscis – the tube-like, modified mouthparts of Hemipterans used to pierce through tissues and suck out liquid.

Pronotum – the first dorsal thoracic segment of an insect.

***Ad libitum* fed body mass** – the body mass of an individual immediately after feeding

Reproductive parasite – any vertically transmitted endosymbiont that manipulates host reproduction to favour female hosts.

Resistance – (immunological) a host's ability to actively or passively reduce or eliminate their parasite load via behavioural, anatomical and physiological adaptations.

Secondary (symbiont) – facultative associate that may occupy various host tissues and shows a variable distribution among hosts.

Seminal reservoir – one of a pair of male organs that stores the seminal fluid component of the ejaculate. (Other authors may use the term mesadenial reservoir).

Seminal vesicles – one of a pair of male organs that stores the sperm component of the ejaculate. (Other authors may use the term sperm vesicle).

Sexual conflict – the conflict that occurs within (intragenomic) or between (intergenomic) the genomes of the sexes of a species as a result of the expression of different trait optima in each sex.

Spermalege – an organ found only in bedbug females that has evolved to mitigate the costs of traumatic insemination. (Referred to in early studies (e.g. Arkwright, 1921, Hertig and Wolbach, 1924) as the "organ of Berlese").

Supergroups (*Wolbachia*) – officially recognised subdivisions of the *Wolbachia* genus into ten lineages (groups) that are taxonomically more closely related.

Symbiont – the smaller partner of a symbiosis.

Symbiont-mediated protection − protection conferred on the host by its symbionts, against predators or pathogens through mechanisms that increase the host's resistance, tolerance or wound healing processes.

Symbiosis –a neutral term describing the lasting partnership between two or more organisms of different species that live in close association with one another, as was first defined by the plant physiologist De Bary in 1879 (Hurst and Darby, 2009). (Other authors may use this term to refer specifically beneficial partnerships (mutualisms)).

Tolerance - (immunological) a host strategy to minimise the costs of a parasite insult by not directing resistance mechanisms at all, or fully, at the parasite.

Transovarial transmission – the maternal (vertical) transfer of symbionts to the next generation inside the egg.

Vertical transmission – refers to the mode of symbiont transmission whereby symbionts are passed on to the next generation maternally, inside the egg.

***Wolbachia* load** – see Load

Chapter 1

Introduction

"So, naturalists observe, a flea
Hath smaller fleas that on him prey;
And these have smaller fleas to bite 'em,
And so proceed Ad infinitum*"*

Swift (1667-1745)

Bedbugs have undergone a recent resurgence in North America and Worldwide (Boase, 2004, 2007, Doggett et al., 2004) possibly due to increased travel, climate change and insecticide resistance (Reinhardt and Siva-Jothy, 2007). These blood sucking pest insects are an economic concern, costing the hospitality industry millions of pounds each year in costly control efforts (Boase, 2007). New control strategies are desperately needed (Reinhardt and Siva-Jothy, 2007). One approach not yet explored involves manipulating the microorganisms that naturally occur inside the bedbug's body, one of which is *Wolbachia spp.* (Hypša and Aksoy, 1997). *Wolbachia* have been investigated as potential biocontrol agents for a number of pest and vector species including filarial nematodes (e.g. Slatko et al., 2010), mosquitoes (e.g. Kambris et al., 2009, Wang et al., 2009, Bian et al., 2013), and fruit flies (e.g. Zabalou et al., 2004), but there have been few studies in bedbugs. This is probably because the increase in bedbug infestations is a relatively recent and rapid phenomenon. The first step in implementing any such biological control strategy, is understanding the relationship between the host and its symbiont(s). The work I present here is a fundamental part of that process. As *Wolbachia* are ubiquitous bacteria infecting many pest insects, life-history and ecological immunity studies of *Wolbachia* effects on bedbugs have relevance not only to bedbug control, but also for the control of other pest insects and any diseases they vector.

First I begin by reviewing what we already know of *Wolbachia*'s role in bedbugs. Then to help in the design of my experiments, I investigate the pros and cons of heat and antibiotic

treatments (chapter 3). These techniques are widely used to manipulate *Wolbachia* loads (population size) in a range of hosts (e.g. van Opijen and Breeuwer, 1999, Wiwatanaratanabutr and Kittayapong, 2006, Charlat et al., 2007a, Pike and Kingcombe 2009). I then use these techniques to experimentally investigate the nature and underlying mechanisms of the relationship that *Wolbachia* have with their host, in male (chapter 4) and female (chapters 5-7) bedbugs.

1.1 Bedbugs

Bedbugs are small (5 mm long), reddish-brown, dorso-ventrally flattened insects belonging to the true bug order Hemiptera (Heteroptera: Cimicidae). They comprise around 90 species (Usinger, 1966, Boase, 2007), all of which are obligate haematophages, feeding solely on the blood of vertebrates. Bedbugs are nocturnal, and live clustered together hidden in crevices close to where their host sleeps: they venture out of their refugia at night in order to feed. Two bedbug species, the common, or temperate, bedbug *Cimex lectularius* L. and the tropical bedbug *Cimex hemipterus* Fabricius are most adapted to living with humans, although a range of domestic animals including chickens, rats, bats, rabbits and horses are also suitable hosts in the laboratory (De Meillon and Golberg, 1947, Usinger, 1966). The geographic distribution of *C. lectularius* is worldwide, whereas that of *C. hemipterus* is more limited to the tropics. The empirical work in this thesis will concentrate on *C. lectularius*.

Bedbugs are not known to transmit disease (Reinhardt and Siva-Jothy, 2007), but their bites are itchy, inflammation-prone, susceptible to infection and often numerous, causing the bitten person considerable distress (Potter, 2009, Reinhardt et al., 2009a,). Bedbugs disperse short distances by walking or longer distances by hitchhiking on their human host's belongings to start new infestations (Reinhardt and Siva-Jothy, 2007). Places that have a lot of people coming and going such as hostels and hotels (Boase, 2007) are particularly problematic as a source from which bedbugs can spread. Without effective control measures, a bedbug population can reach very high densities, for example one report estimates an infestation numbering between 50,000 – 100,000 individuals in one small flat (Naylor, 2012b). Widespread insecticide resistance makes infestations difficult and costly to eradicate (e.g. total annual costs to treat for a single large hotel with an

established infestation have been known to exceed €1M per year (Boase, 2007), consequently bedbugs are a pest species of considerable economic importance. An active area of bedbug research is finding new control strategies.

1.2 Insights from the bedbug laboratory model system

Bedbugs have traditionally been used as a model system in evolutionary biology to study cryptic female choice (Eberhard, 1996) and sexual conflict (Stutt and Siva-Jothy, 2001). More recently, prompted by reports that bedbug infestations are on the rise, particularly in the UK, USA and Australia (Boase, 2004, 2007, Doggett et al., 2004); there has been a shift towards ecological (e.g. How and Lee, 2010a, 2010b), behavioural (e.g. Siljander et al., 2008, Weeks et al., 2010) and genetic studies (e.g. Moriyama et al., 2012) that may help design new control methods. In other studies, for example with mosquitoes, tsetse flies and aphids, manipulation of the insect's microbiota is being explored as a strategy to control these pests (McMeniman et al., 2009, Moreira et al., 2009, Augustinos et al., 2011) or the diseases they transmit (Bian et al., 2010, Pan et al., 2012, Bian et al., 2013, Cook and McGraw, 2010, Frentiu et al., 2010, Walker et al., 2011). These approaches remain to be explored in the bedbug.

1.3 Life cycle

Bedbugs are hemimetabolous, with the nymphal phase divided into 5 instars prior to adult eclosion (Fig. 1.1). In order for development to progress, bedbugs need to take a complete blood meal between each instar and adult eclosion (Reinhardt and Siva-Jothy, 2007). Under *ad libitum* feeding conditions at 30-31 ^{o}C, generation times can be as short as 22 days (from egg to adult) (Takano-Lee et al., 2003), and the adults are sexually mature as soon as they have taken their first blood meal. Egg production in fed females begins after mating and continues until either blood supplies become limited (*ca*. 7-10 days since feeding) or sperm supplies are depleted (*ca.* 35-50 days since mating) (Reinhardt and Siva-Jothy, 2007). Longevity and egg laying rate are partly dependent on environmental temperature conditions (Usinger, 1966). In laboratory conditions (26°C 70% RH) with weekly feeds and mating, females can lay up to 5 eggs d^{-1} (Potter, 2009), over a reproductive lifespan of *ca*. 4-5 months (Reinhardt et al., 2009b).

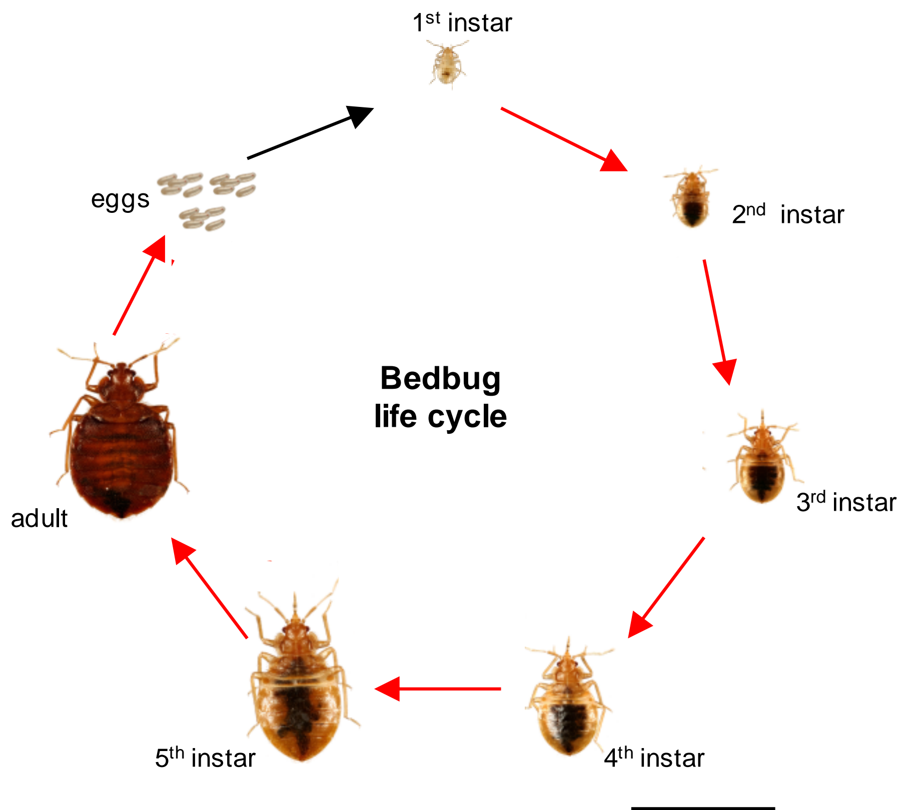


Figure 1.1 The life cycle of the bedbug *Cimex* lectularius, from egg, through five nymph stages known as instars to adult. Each red arrow represents one blood meal, a prerequisite before the nymph can eclose to the next stage. Bar, 5 mm. Individual images ©cimexstore.co.uk (Naylor, 2013)

1.4 Reproduction

Bedbugs have an unusual mode of reproduction involving extragenital traumatic insemination (TI). Mating rate is controlled by the males (Stutt and Siva-Jothy, 2001) who direct their mating attempts towards recently fed females. After feeding females have reduced mobility because of their swollen blood-filled bodies: consequently they put up less resistance to mating (Reinhardt et al., 2009c). During copulation the male pierces the female's abdominal wall with his intromittent organ (a sclerotized lanceolate paramere) injecting sperm and seminal fluid into her haemocoel (Reinhardt and Siva-Jothy, 2007). The female's 'normal' genital tract is not used for mating, but serves as a passage for eggs during oviposition (Siva-Jothy, 2006). The 'traumatic' nature of TI, which causes wounding and carries immune costs via infection (Reinhardt et al., 2003), as well as the

high male mating rates, imposes a considerable cost on females which have responded by physical and physiological adaptation(s) (Stutt and Siva-Jothy, 2001).

At the site where the male inserts his intromittent organ into the female, a spermalege has evolved, a sac-like organ containing high densities of haemocytes (blood cells associated with immune function and wound healing), manifest externally as a ventral groove on the 5th abdominal sternite (Usinger, 1966, Siva-Jothy, 2006) (Fig. 1.2). This is also the site where sperm are deposited (and sometimes phagocytosed by host haemocytes); Eberhard (1996) hypothesised that the spermalege played a role in cryptic female choice. There is however no evidence to suggest that this is so, and sperm phagocytosis is likely to function in removing aged and defunct sperm (Siva-Jothy, 2006). Experimental evidence suggests the primary function of the spermalege is in immune defence, reducing the costs of wounding (Morrow and Arnqvist, 2003) and the opportunistic infection (Reinhardt et al., 2003) associated with TI.

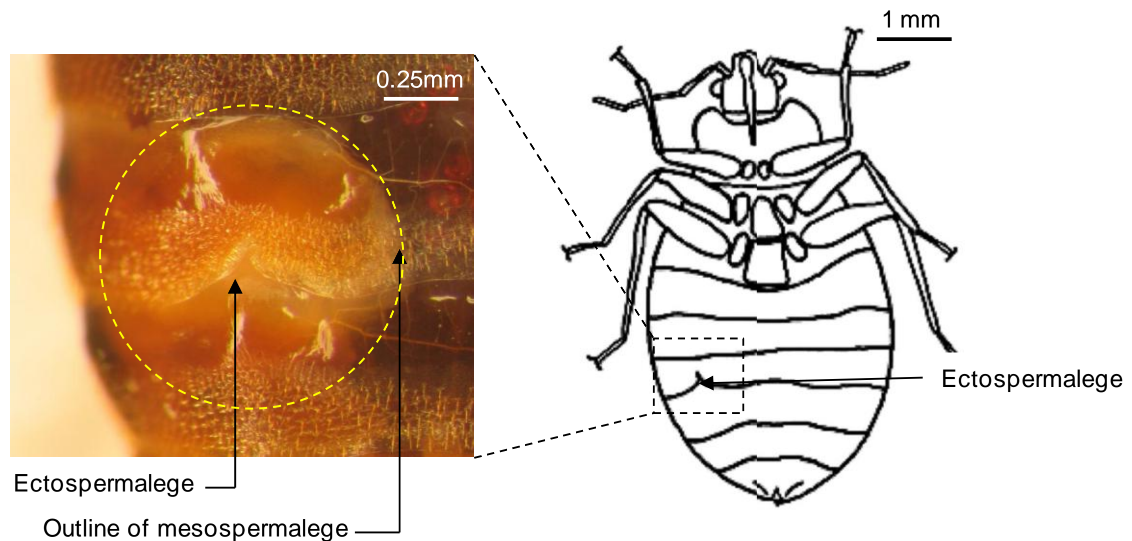


Figure 1.2 Ventral view of adult female bedbug to show the location of the spermalege, the site of TI. The spermalege consists of an outer ectospermalege (an external cuticular groove) and an inner mesospermalege (outlined in dotted yellow line, visible beneath the cuticle of newly eclosed females), into which sperm are injected. Figure based on that in Siva-Jothy (2006), with image ©cimexstore.co.uk (Naylor, 2013) and line drawing modified from (Reinhardt et al., 2003).

1.5 Immunity

It is useful to contextualise bedbug immune function by considering the generic aspects of insect immunity first. There are a number of resources that offer in-depth reviews of insect immunity (e.g. Hoffmann et al., 1996, Rolff and Siva-Jothy, 2003, Siva-Jothy et al., 2005, Rolff and Reynolds, 2009). I summarise the main points here. Insect immune systems consist of highly conserved, non-specific innate immune mechanisms; they lack the adaptive immunity of vertebrates. Innate immunity is conventionally compartmentalised into cellular mechanisms (those involving cells) and humoral mechanisms (those involving soluble immune effectors), although the two are interlinked (Siva-Jothy et al., 2005). Depending on how the cellular and humoral mechanisms are employed, these are further categorised as constitutive (a baseline or background level of immunity) or induced (up-regulated in response to pathogen infection).

1.5.1 Cellular immunity

Cellular immunity involves haemocytes (immune cells) that remove or isolate pathogens from the organism by phagocytosis, nodulation or encapsulation (Siva-Jothy et al., 2005). In the latter two processes, the haemocytes aggregate around the pathogen forming an impenetrable inert cell layer, ultimately externalising the pathogen *in situ*. In bedbugs, haemocytes occur at highest densities within the spermalege (Siva-Jothy, 2006) suggesting that this is where cellular immune mechanisms are very active. Different morphotypes can be distinguished with electron microscopy (Klein and Kallenborn, 2000, Steigner, 2001). Similar observations have been reported for the closely related *C. hemipterus*, where 5 haemocyte morphotypes are distinguishable (Sonawane and More, 1993); whether these morphotypes differ functionally is unknown.

1.5.2 Humoral immunity

Humoral immunity consists mainly of enzyme cascades and soluble proteins which play important roles in recognising, signalling and attacking non-self, and co-ordinating cellular responses. One important enzyme is phenoloxidase (PO) which catalyses reactions that lead to the production of melanin, a dark-coloured, inert pigment that is important in wound healing and the encapsulation/ nodulation responses. Another is lysozyme, an enzyme with antibacterial properties, particularly against gram-positive bacteria (Adamo,

2004). There is also a structurally diverse range of antimicrobial peptides (AMPs) (Broderick et al., 2009). In bedbugs, the activity of two humoral immune components have been observed in droplets of haemolymph *in vitro* (i) lysozyme-like substances that kill bacteria (Naylor, 2006, Otti et al., 2009) and (ii) PO (Naylor, 2006).

1.5.3 Symbiont-mediated immune effects

It is increasingly recognised that insect immunity cannot be considered in isolation of the microorganisms found inside the insect's body, known as symbionts (Haine, 2008, Gerardo et al., 2010, Parker et al., 2011). Although not without exception, there are many examples of symbionts contributing positively to host immune function. Symbionts may produce antimicrobial substances (Jarosz, 1979, Dillon and Charnley, 1995), prime the host immune system in readiness for pathogen invasion (Moreira et al., 2009) or outcompete colonising pathogens over shared resources (Sabate et al., 2009). The extent of symbiont-mediated protection has yet to be fully explored but early indications are that it is a widespread phenomenon in insects (Oliver and Moran, 2009). Bedbugs harbour variable numbers of bacterial symbionts within their bodies (Chang and Musgrave, 1973) and their effects on bedbug immunity are unknown. One of the bedbug's ubiquitous symbionts has been identified as *Wolbachia* (Hypša and Aksoy, 1997).

1.6 *Wolbachia*

To understand what is known of the effects *Wolbachia* has on bedbugs, it is useful to first summarise symbioses and the association of *Wolbachia* in arthropods and filarial nematodes.

1.6.1 Symbiosis

'Symbiosis' is a neutral term describing the lasting partnership between two or more organisms of different species that live in close association with one another, as was first defined by the plant physiologist De Bary in 1879 (Hurst and Darby, 2009). The larger partner is termed the host and the smaller partner(s) is termed the symbiont(s), in this case this is the insect and microorganism respectively. Conventionally symbiotic relationships are classified according to whether the presence of the symbiont has positive (mutualism), negative (parasitism) or no effect (commensalism) on host fitness (Table. 1.1) (Bourtzis

and Miller, 2003). Symbioses are not equitable relationships, even in mutualisms where both parties benefit, one may benefit a lot more than the other (Frean and Abraham, 2004). Although this classification is useful, the real situation is more akin to a continuum (Dillon and Dillon, 2004). The position of symbioses on this continuum depends on a range of intrinsic and extrinsic factors, such as host age, symbiont location within the host, symbiont density and environmental conditions (De Meillon and Golberg, 1947, Haine, 2008, Brownlie and Johnson, 2009)

Table 1.1 Classification of symbioses according to the effect that the symbiotic relationship has on the host's fitness.

Type of symbiosis	Symbiont	Host
Commensalism	Receive benefit	Not affected
Mutualism	Receive benefit	Receive benefit
Parasitism	Receive benefit	Harmed
Enslavement	Not affected or harmed	Receive benefit

1.6.1.1 Primary and secondary symbionts

Symbionts have long been classified as either primary or secondary. Primary symbionts have a reciprocally obligate, mutualistic relationship with their host. Within that host they are usually restricted to specialised host cells (bacteriocytes) and transmit vertically (from mother to offspring), cospeciating with the host (Hypša and Nováková, 2008). Secondary symbionts are more variable and less well defined. They tend to be facultative for the host, can invade various host cells and are often transferred horizontally between unrelated host species (Hypša and Nováková, 2008). One group of secondary symbionts known as reproductive parasites transmit vertically within the female host's eggs once a stable infection has been established. See Table 1.2 for a general summary of features of primary and secondary symbionts.

Table 1.2 Differences in the general characteristics of primary and secondary symbionts

Characteristic	Primary	Secondary
Nature of symbiosis	• Reciprocally obligate	• facultative for the host, facultative or obligate for the symbiont
Nature of symbiont	• nutritional mutualist	• mutualist, commensal or parasite
Location	• inside the host, usually intracellular	• inside or outside the host, intra or intercellular
Transmission	• vertical	• horizontal, some transfer vertically once infection has established (reproductive parasites)
Length of association	• consistent, life-long	• sporadic or consistent (some reproductive parasites)
Culture *in vitro*	• No	• Yes (facultative species) • No (obligate species)
Examples	• *Buchnera aphidicola* in pea aphids *Acyrthosiphon pisum* Harris (Dasch et al., 1984) • *Blochmannia* in carpenter ants *Camponotus fellah* Dalla Torre (De Souza et al., 2009)	• Mutualist: *Hamiltonella defensa* in pea aphids *Acyrthosiphon pisum* (Moran et al., 2005) • Reproductive parasite: *Wolbachia* in crickets *Teleogryllus taiwanemma* Ohmachi and Matsumura (Kamoda et al., 2000)

1.6.1.2 Reproductive parasites

One group of secondary symbionts known as reproductive parasites, are so called because they increase their fitness by manipulating host reproduction to favour the production or survival of infected females (Werren et al., 2008). Manipulations include: (i) killing male progeny from infected females; (ii) inducing parthenogenesis in unfertilised eggs; (iii) feminising genetic males and (iv) preventing the sperm from infected males fertilising eggs from uninfected females, by inducing cytoplasmic incompatibility (CI) (Werren et al., 2008). These manipulations have evolutionary and ecological implications for the infected host species (Duron et al., 2008) such as the rapid evolution of counter adaptive traits in hosts through natural selection (Charlat et al., 2007b), reproductive isolation (Charlat et al., 2003) and even speciation (Miller et al., 2010). Reproductive parasites include *Cardinium spp.*, *Arsenophonus spp., Spiroplasma spp.* and *Wolbachia spp*. (Duron et al., 2008). Field

studies show that *Wolbachia* have the highest incidence (infect the most species) and prevalence (infect the highest proportion of individuals within a particular species) out of all known reproductive parasites (e.g. Duron et al., 2008).

1.6.2 General biology

1.6.2.1 Characteristics and occurrence

Wolbachia are bacterial endosymbionts of many arthropod and filarial nematode species. They were first discovered in the gonads of the mosquito *Culex pipiens* by (Hertig and Wolbach, 1924), who observed the presence of "intracellular *Rickettsia*-like organisms" that were later named *Wolbachia pipientis* (Hertig, 1936). The ubiquitous nature of these bacteria was only realised following the advent of PCR techniques, when large numbers of invertebrate species were subsequently screened. *Wolbachia* are now known to infect between 40% (Zug and Hammerstein, 2012) and 65% (Stouthamer et al., 1999) of all known insect species from all major insect orders; arachnids, including spiders and mites; terrestrial crustaceans and filarial nematodes (Riegler and O'Neill, 2006, Werren et al., 2008). In most associations, the literature refers only to the genus name "*Wolbachia*" but different strains are recognised that vary either in gene sequence, host phenotype or host species infected (Charlat et al., (2002) in Riegler and O'Neill, (2006)).

Wolbachia are gram negative, α-proteobacteria belonging to the order *Rickettsiales* and are found inside vacuoles in their host's cells (Werren, 1997). The host is obligate to *Wolbachia* − they cannot grow outside cells, although *Wolbachia* can be maintained in cell-free medium for short periods of time in the laboratory (Rasgon et al., 2006). The presentation of infection in host somatic tissues varies between hosts and *Wolbachia* strain (Riegler and O'Neill, 2006). *Wolbachia* are transovarially inherited (i.e. they are passed from mother to offspring within the cytoplasm of eggs). In hosts, the female germ line is thus always infected with *Wolbachia* but the male germ line is not, *Wolbachia* are lost from sperm during spermatogenesis and are not transmitted by the male host (Riegler and O'Neill, 2006).

1.6.2.2 Host phenotypes

Wolbachia is best known as a reproductive parasite of arthropods, manipulating host reproduction to further its own dispersal, most commonly by inducing cytoplasmic incompatibility (CI - Werren, 1997). Besides costs arising from reproductive manipulations, *Wolbachia* infections generally have negligible fitness consequences for their hosts (Riegler and O'Neill, 2006), although there are some exceptions. *Wolbachia* may be (i) virulent parasites (e.g. the *w*MelPop strain in *Drosophila melanogaster* Meigen (Min and Benzer, 1997)). (ii) Facultative mutualists (increasing the host's resistance to viral parasites in fruit flies *D. melanogaster* and *Drosophila simulans* Sturtevant (Teixeira et al., 2008, Osborne et al., 2009) and confering a competitive advantage in sperm competition in flour beetles *Tribolium confusum* Jacqueline du val (Wade and Chang, 1995b) and providing non-essential metabolic provisioning in *D. melanogaster* (Brownlie et al., 2009), (iii) Obligate mutualists (essential, for example, for oogenesis in parasitoid wasps *Asobara tabida* Nees (Dedeine et al., 2001, Dedeine et al., 2005) or the biosynthesis of essential nutrients in nematodes *Brugia malayi* Brug and bedbugs *C. lectularius* (Foster et al., 2005, Hosokawa et al., 2010)). Transinfection experiments demonstrate that there is high variability in *Wolbachia*-mediated effects, and in the underlying mechanisms between different hosts and different *Wolbachia* strains (Osborne et al., 2009).

1.6.2.3 Phylogeny

Phylogenetic analyses of *Wolbachia*'s 16S r*RNA* and *FtsZ* gene sequences place *Wolbachia* in a monophyletic group (Lo et al., 2002) that is subdivided into ten taxonomic supergroups, known as supergroups A-J, (although the validity of G is disputed) (Doudoumis et al., 2012) (Fig. 1.3). Genetic exchange occurs between *Wolbachia* within supergroups more so than between supergroups (Siozios et al., 2013). Analyses of host and *Wolbachia* phylogenies provide insight into the evolution and biology of *Wolbachia* (Lo et al., 2002) and may be used to make predictions about *Wolbachia* phenotypes (e.g. Gómez-Valero et al., 2004). Supergroups C, D and J are found only in filarial nematodes and phylogenetic congruence between *Wolbachia* and hosts is high (Lo et al., 2002). By contrast, supergroups A, B, E, H, I and K which are commonly found in arthropods are phylogenetically incongruent with their hosts, suggesting *Wolbachia* have successfully transferred horizontally between species, multiple times in the past (Lo et al., 2002).

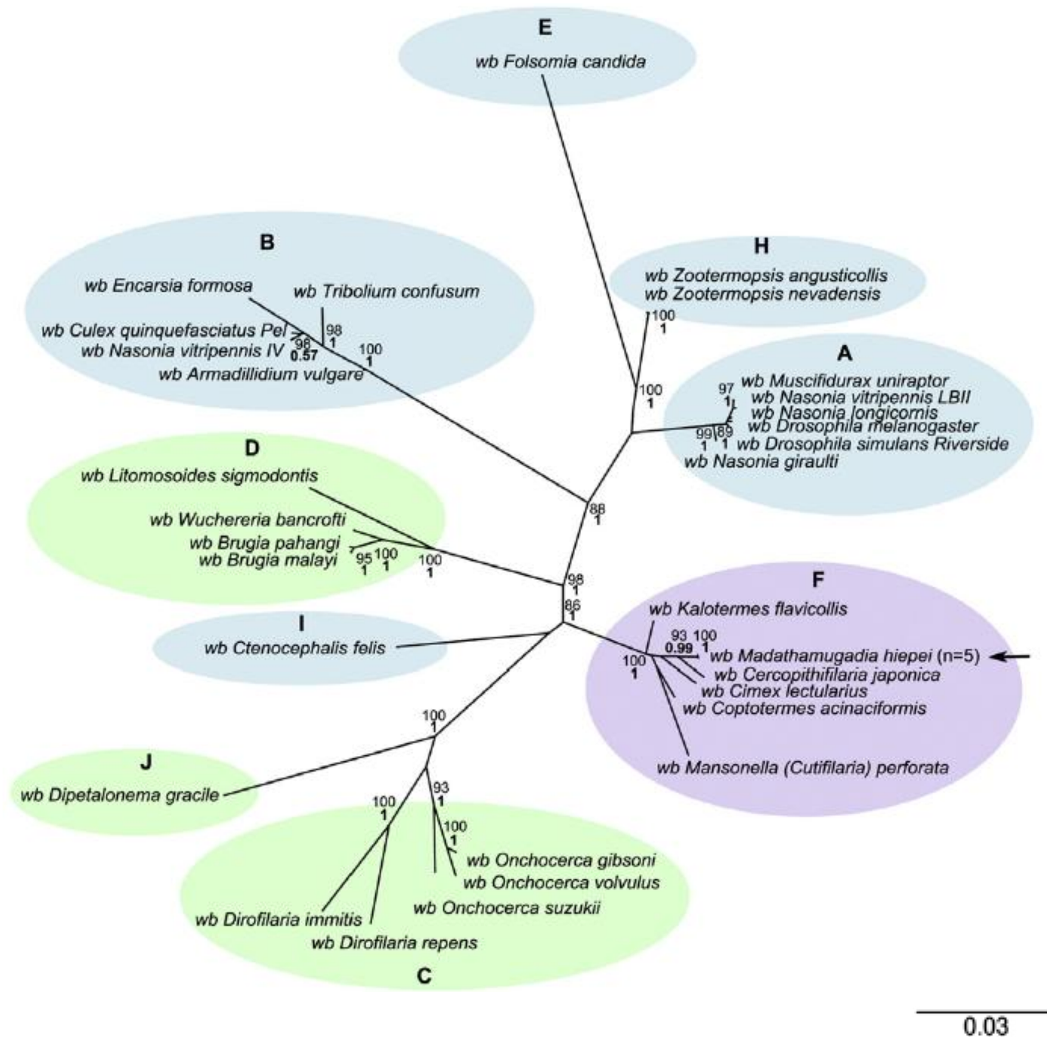


Figure 1.3 The Phylogeny of *Wolbachia*[1]. Figure is taken from Lefoulon et al., (2012). The tree is based on concatenated datasets of 16S rDNA, groEL, *FtsZ*, dnaA and coxA sequences. The sequences were obtained from GenBank except those from species identified with an arrow on the phylogram. The scale bar indicates the distance in substitutions per nucleotide, wb = *Wolbachia*.

[1] The proposed supergroup G is based on *wsp* alone, and likely represents a *wsp* recombinant clade. More sequence information is necessary to substantiate this lineage (Baldo and Werren, 2007). Lefoulon et al., (2012) therefore did not include supergroup G in their phylogeny diagram.

1.6.2.4 Application

Due to the ubiquity of *Wolbachia* in insects and the variety of effects *Wolbachia* has on its host, there is much interest in how we can apply our knowledge of *Wolbachia* for economic gain. For example recent studies show *Wolbachia* may be used to (i) suppress pest populations through mediating incompatibilities (e.g. in the medfly *Ceratitis capitata* Wiedemann (Zabalou et al., 2004)), (ii) reduce the ability of hosts to harbour and transmit disease by inducing an immune response (e.g. the mosquito *Anopheles gambiae* Giles which vectors *Plasmodium spp.* (Kambris et al., 2009, Kambris et al., 2010), (iii) directly outcompeting the disease organism from the host (e.g. the Dengue virus from mosquitoes *Aedes aegypti* L. (Moreira et al., 2009)), (iv) increase the production of insects used in biological control (e.g. the predatory bug *Macrolophus pygmaeus* Rambur used to control many greenhouse pests (Machtelinckx et al., 2009)) and (v) kill filarial nematodes (e.g. *Wuchereria bancrofti* Cobbold that causes lymphatic filariasis in humans (Taylor et al., 2005, Debrah et al., 2011)). Whether *Wolbachia* may be used to control bedbug populations is a relatively unexplored prospect. First I will review our current understanding of the ecology of *Wolbachia* and its neighbouring symbionts in bedbugs, and then I will highlight areas where knowledge is lacking.

1.7 Symbionts in bedbugs

1.7.1 Overview of previous research

Symbiotic bacteria were first observed in bedbug tissues almost a century ago using light microscopy (Arkwright et al., 1921). They were found at their highest densities in a pair of organs, known today as bacteriomes (Plate 1.1), located adjacent to the gonads in both males and females. Early researchers could distinguish 3 bacterial morphotypes which, depending on the strain of *C. lectularius*, were variably present in one or all of the gut, Malpighian tubules, spermalege, ovaries, developing eggs and testes (Arkwright et al., 1921, Hertig and Wolbach, 1924, Buchner, 1965). As microbial symbionts of obligate haematophages were thought to synthesize B vitamins absent in vertebrate blood, and necessary for their host's reproduction and development (Wigglesworth, 1936), these early investigators hypothesised that the bedbug's symbionts served a similar nutritional function.

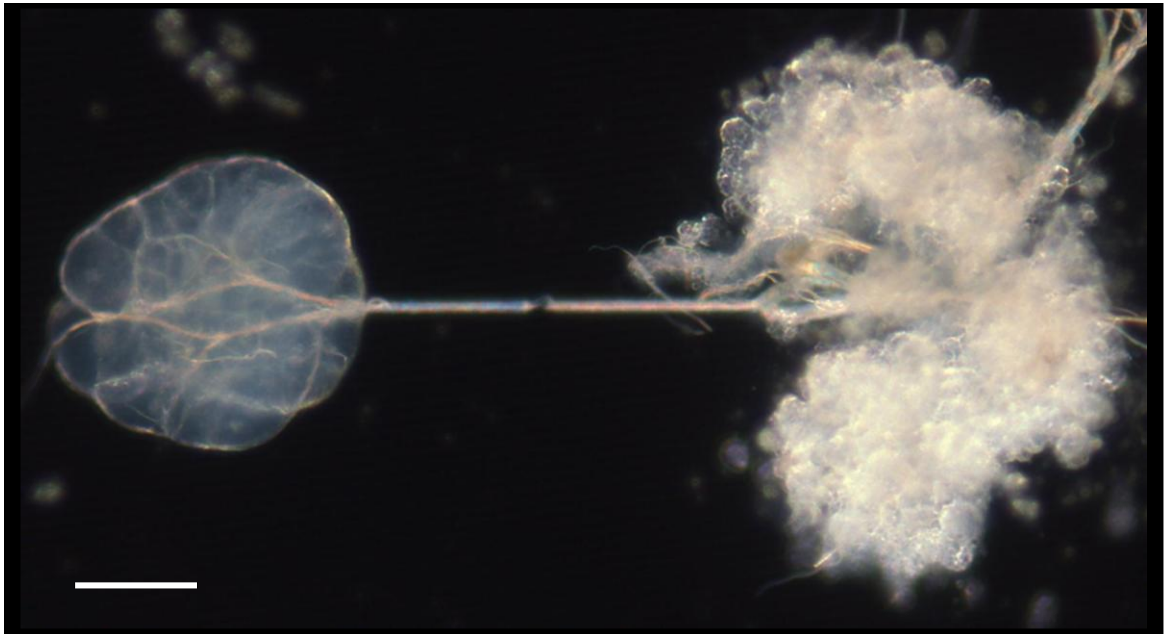


Plate 1.1 A bacteriome (grey round structure on the left) dissected from a female *C. lectularius*. Some of the fat body (white tissue on the right) within which the bacteriome was embedded whilst inside the body of the female, is also visible. Image was taken under the compound microscope using dark field phase contrast. Bar, 0.1 mm.

In 1997 two vertically transmitted symbionts of bedbugs were formally characterised based on 16S rDNA gene analysis on ovary tissue as: i) *Wolbachia* (Hypša and Aksoy, 1997) and ii) a γ-proteobacteria bearing >97% genetic similarity to a facultative parasite (Campbell and Purcell, 1993, Degnan et al., 2011) of the leaf hopper *Euscelidius variegatus* Kirschbaum known as BEV (Bacteria of *E. variegatus*); the authors thus aptly named this γ-proteobacteria in bedbugs a BEV-like symbiont (BLS) (Hypša and Aksoy, 1997).

Information on the relative proportions of these two symbiont species was later revealed by 454 titanium sequencing on wild caught individuals from infestations in Cincinnati (OH USA) (Meriweather et al., 2013). This study revealed that around 97% of the bedbug's bacterial community comprised *Wolbachia* (63%) and BLS (34%) respectively (Meriweather et al., 2013). FISH shows that both of these symbionts are largely confined to the bacteriomes, and BLS is also present in the Malpighian tubules (Hosokawa et al., 2010). *Wolbachia* are always prevalent in bedbug populations (between 83-100% of individuals sampled were infected from populations in USA, Canada and Africa (Sakamoto and Rasgon, 2006), whereas the prevalence of BLS ranges from 0-100%, (Hosokawa et al.,

2010). This has led to the suggestion that of the two, *Wolbachia* is *the* nutritional symbiont of bedbugs. Nothing is known of the role or nature of BLS and researchers' have focused their attentions on *Wolbachia*.

Analysis of *FtsZ* genes place bedbug *Wolbachia* in the F supergroup (Rasgon and Scott, 2004) along with *Wolbachia* from termites *Coptotermes acinaciformis* Froggatt and several genera of filarial nematodes (Lefoulon et al., 2012). In filarial nematodes, *Wolbachia* are thought to be mutualistic by synthesising a range of molecules for the host (Foster et al., 2005). That the bedbug's *Wolbachia* is more closely related to mutualistic *Wolbachia* strains, than for example those that manipulate host reproduction common in supergroups A and B, adds weight to the proposition that bedbug *Wolbachia* may serve a mutualistic nutritional function.

In 1974, an experiment was conducted to investigate the effect of heat treatment on (i) symbiont load and (ii) fecundity. It showed that heat treatment (i) reduced the symbiont load observed in the bacteriomes and (ii) reduced oviposition rate and hatch success (Chang, 1974). The author concluded that a normal symbiont load was required for bedbug fitness, probably due to the symbiont's nutritional role. An alternative explanation however is that these negative fecundity effects were caused by either physiological effects of heat treatment or by a lack of viable sperm, due to heat-induced sperm damage (Omori, 1941). In 2010, Hosokawa *et al* showed that a reduction in *Wolbachia* load caused by antibiotic treatment (which is not known to have the same physiological effects as heat treatment nor damage sperm) also caused a reduction in fitness due to a reduction in egg viability (proportion that hatch and reach adulthood) and slower development times. These effects could be reversed by supplementing the bedbug's blood diet with B vitamins. Although sample sizes used in experiments were small (n=3), the results suggested *Wolbachia* in bedbugs were primary symbionts that provided B vitamins (Hosokawa et al., 2010).

The role of BLS in bedbugs remains unknown. It is possible that BLS also have a nutritional role. Investigations carried out to design an artificial membrane feeding apparatus to deliver human blood to bedbugs, supplemented the blood with penicillin and streptomycin antibiotics to kill potential bacterial contaminants (Takano-Lee et al., 2003). Although *Wolbachia* replication is not affected by these antibiotics (Kambhampati, S. et

al., 1993, Fenollar et al., 2003), mated female bedbugs raised on blood delivered through this membrane feeding system did not oviposit, suggesting the antibiotic supplementation may have affected important symbionts besides *Wolbachia* (Takano-Lee et al., 2003).

1.7.2 Questions arising from the literature

1.7.2.1 What is the role of Wolbachia *in males?*

One intriguing observation in bedbugs is the persistent presence of high densities of *Wolbachia* in the bacteriomes of adult male bedbugs (Hosokawa et al., 2010). In nutritional symbioses, usually the densities of the nutritional symbionts decrease in male hosts once they have reached adulthood (Douglas, 1989, Douglas, 2009). This is presumably because the benefit provided by the symbiont is no longer required as (i) nutritionally demanding developmental processes are now complete, and (ii) sperm production is not nutritionally demanding (relative to egg production in females) (Douglas, 2009). The male may eliminate nutritional symbionts at this point as the cost of maintaining symbiont densities likely outweighs their benefit. Furthermore, the male host is surplus to the symbiont's requirements as they rely solely on the female host for their onward transmission. Studies on bedbugs to date have not attempted to separate *Wolbachia*-mediated effects in males from those in females, concomitantly manipulating the *Wolbachia* load in both sexes with either heat (Chang, 1974) or antibiotic treatment (Hosokawa et al., 2010). It is useful to discriminate sex differences, for example if the male's *Wolbachia* affects his fertility, this would inform whether a sterile insect technique (SIT) – a method of biocontrol that usually involves the mass release of sterile males (Crotti et al., 2012) − could be used as an effective control measure for bedbugs.

1.7.2.2 What are the long-term fitness consequences of Wolbachia*?*

To date, research has investigated the role of *Wolbachia* in first generation bedbugs. It is unknown whether *Wolbachia* have transgenerational effects, i.e. whether they have fitness consequences for the host's offspring. This would provide insights into whether control measures will be effective in the long-term.

1.7.2.3 *Can* Wolbachia *operate through non-nutritional mechanisms?*

Besides their nutritional role, *Wolbachia* may also mediate effects through other as yet unidentified mechanisms. One possible mechanism is through increasing host disease resistance, a symbiont-mediated phenomenon thought to be widespread in vertically transmitted symbionts (Oliver and Moran, 2009) and which has recently been identified for *Wolbachia* in a number of dipteran species (Teixeira et al., 2008, Kambris et al., 2009, Moreira et al., 2009, Osborne et al., 2009, Kambris et al., 2010). In the bedbug's natural environment, the female's fitness is compromised by infection with opportunistic microbes that are transferred during TI (Reinhardt et al., 2003). *Wolbachia* may benefit its female host by negating some of these costs directly reducing infection or indirectly through influencing the host immunity. If this was so, this would inform whether the release of pathogenic bacteria into the bedbug's environment along with techniques to reduce the bedbug's *Wolbachia* load, would be effective in biocontrol.

1.8 Summary

Our understanding of *Wolbachia* biology in bedbugs is largely limited to one study that tested the effects of antibiotic treatment to reduce *Wolbachia* load and the effects of supplementary B vitamins on sample sizes of n=3; the authors concluded that *Wolbachia* provide B vitamins in bedbugs (Hosokawa et al., 2010). It remains unknown whether (i) *Wolbachia* mediates different effects in males and females, (ii) whether *Wolbachia* can mediate effects across host generations, and (iii) if *Wolbachia* may operate through mechanisms besides nutrition. A deeper understanding of *Wolbachia*-bedbug biology may (i) be useful in developing new bedbug control strategies and (ii) provide insight into how *Wolbachia* may be manipulated in other pest insects to control them or the diseases they transmit.

1.9 Thesis outline and core questions

In chapter 2 I will describe the general methods used throughout the thesis.

In chapter 3 I will develop and test protocols to manipulate *Wolbachia* loads in male and female bedbugs. To do this, I will compare two widely used methods: heat and antibiotic treatment.

In chapter 4 I will answer the following questions about the role of *Wolbachia* in male bedbugs:

1) Where are *Wolbachia* located within males?

2) Are male *Wolbachia* loads similar to female loads?

3) Are *Wolbachia* transferred to the female in the ejaculate?

4) How do the male's *Wolbachia* affect his fitness and/or indirectly affect female fitness?

5) Do *Wolbachia* affect ejaculate characteristics (sperm numbers, seminal fluid volume and volume ejaculated)?

In chapter 5 I will answer the following questions about the role of *Wolbachia* in female bedbugs:

1) Where are *Wolbachia* located within females?

2) How do the female's *Wolbachia* affect her fitness?

3) How do the female's *Wolbachia* affect the fitness of her offspring?

In chapter 6 I will investigate whether *Wolbachia* affect host immune function by comparing the disease resistance in heat treated females with reduced *Wolbachia* loads and non-heat treated females with baseline *Wolbachia* loads. When challenged with opportunistic bacteria I will test whether they differ in terms of:

1) Longevity (survival)

2) Measures of fecundity (egg laying rate and proportion hatch success)

In chapter 7 I will investigate how *Wolbachia* affect immune function by assaying levels of humoral and cellular immune activity in heat treated females with reduced *Wolbachia* loads and non-heat treated females with baseline *Wolbachia* loads to answer the following questions:

1a) Do *Wolbachia* affect levels of lysozyme-like acitivity (LLA)?

b) Do *Wolbachia* affect constitutive or induced levels of LLA?

c) Do *Wolbachia* equally affect levels of LLA in the spermalege and haemocoel?

2a) Do *Wolbachia* affect the encapsulation response (volume of encapsulating cell mass and degree of melanisation)?

b) Do *Wolbachia* equally affect the encapsulation response in the spermalege and haemocoel?

In chapter 8 I will discuss the significance of my results, place them in a broader context and examine possible future directions for the research in this thesis.

Chapter 2

General Methods

2.1 Introduction

This chapter introduces the bedbug, *Cimex lectularius*, as a model organism and provides details of general laboratory methods used throughout the thesis. Methods covered here include: (i) bedbug husbandry, (ii) microscopy and image capture techniques, (iii) molecular techniques and (iv) statistical analysis. For lists of suppliers and catalogue numbers for reagents and selected consumables see Appendix 2.

2.1.1 Aims of chapter

The aims of this chapter are:

1. To describe the insect husbandry methods and protocols used to maintain bedbugs in the lab.
2. To describe the microscopy techniques used to examine tissues at cellular and ultracellular levels.
3. To describe the molecular techniques used to quantify the *Wolbachia* load of individuals or within specific tissues.

2.2 Insect techniques

2.2.1 Bedbug husbandry

Bedbugs used throughout this thesis were the common bedbug *C. lectularius* (Stock 1 / L1), collected in London, UK more than 40 years ago and housed at the University of Sheffield since 1999. These bedbugs are probably adapted to laboratory conditions, such as feeding protocols and are thus easy to rear, so one must bear in mind that results of experiments using these bedbugs may vary from that of bedbugs in the wild. Stock cultures were maintained in an insectory at 26±1°C, 70±5% RH in a 12:12 light:dark cycle. Mixed sex cohorts of up to 200 individuals were raised in 60 mL plastic pots with a strip of filter

paper cut to size (5 x 15 cm) and folded in concertina, to provide insects with a substrate that they can grip on to. Following the protocol of How and Lee (2010a), insects were fed weekly to promote normal development and egg production.

2.2.2 Experimental cohorts

To ensure adult insects used in experiments were of the same age and were virgin, the insects were transferred to individual 5 mL plastic tubes with a strip of filter paper (1 x 3 cm) prior to adult eclosion. After eclosion, the sex of the adult was determined, the body size was measured, the abdomen was marked so that individuals could be distinguished from the rest of the population, and in some cases females were mated (details below).

2.2.2.1 Sex determination

The sex of newly eclosed adults was determined by i) visually assessing the shape of the lower abdomen (the end of the abdomen is more pointed in males compared to females) (Plate 2.1) and ii) noting the presence/absence of the ectospermalege − a groove visible on the ventral side of the abdomen, in females only − with the aid of a stereomicroscope (Leica MZ8, Wetzlar, Germany).

Plate 2.1 To demonstrate differences in the shape of the abdomen between female and male bedbugs. The abdomen of females (left) is more rounded in shape compared to males (right) which is more pointed. Scale bar=1 mm.

2.2.2.2 Measurement of body size

The width of an individual's pronotum was recorded as a measure of body size (Plate 2.2). In bedbugs the pronotum width is a measure of skeletal body size that is not affected by how recently an individual has fed (Reinhardt et al., 2010). To measure the pronotum width, individuals were placed on a piece of filter paper and a glass microscope slide was placed on top of the insect to immobilise the individual. Individuals were photographed using a stereomicroscope (Leica MZ8, Wetzlar) with a digital camera (Micropublisher 3.3 RTV, Q Imaging, USA) using image capture software (Image-Pro Plus 5.1.2, Mediacybernetics, USA). Images were exported to open-source image analysis software Image-J 1.46r, and the width of each individual's pronotum was measured at its widest part. This method was highly repeatable (R=0.969, $F_{19,40}$=93.55, P<0.0001).

Plate 2.2 To demonstrate measuring the width of a bedbug's pronotum, indicated by the white arrow. Scale bar=1 mm.

2.2.2.3 Distinguishing individuals

In order that individuals could be identified, each bedbug was marked with two paint dots (applied with the tip of a wooden cocktail stick) of quick drying enamel paint on the 2nd abdominal segment. Different colour combinations were used to identify different individuals.

2.2.2.4 Standardised mating

All matings were carried out following the standardised mating procedure used in previous studies (Reinhardt et al., 2003, Reinhardt et al., 2009b, Reinhardt et al., 2009c). For each mating, one virgin female (fed up to 1 hour prior) was placed in a mating arena (small plastic Petri dish (50 mm DIA) lined with filter paper) at 26°C under dimmed light conditions, followed by one virgin male (fed 24 hours prior). Soft forceps were used to handle the insects. Mating duration (time interval between male intromission and genital withdrawal) was standardised to 60 seconds, by separating the male from the female at this time. This standardised the number of sperm transferred to each female (the number of sperm transferred by the male is linearly related to copulation duration (Siva-Jothy and Stutt, 2003)).

2.2.3 Feeding

Insects were fed heparinised sheep's blood through an artificial membrane feeding system. A study comparing the raising success of bedbugs on different non-human vertebrate blood sources supplemented by different chemical anticoagulants found heparinised sheep's blood to be most suitable for raising bedbug stocks long-term (more than 2 years) through an artificial membrane feeding system (Montes et al., 2002). Membrane feeding is also a useful route to administer substances such as B vitamins or antibiotics, through supplementing the blood meal. Individuals were fed in communal pots of at least n=25 (the feeding success decreases with smaller populations – pers. obs.). Individuals in the same treatment group were fed in the same pot, introducing the potential for pseudoreplication to confound results. However this was an unavoidable necessity for logistical reasons, primarily to ensure that individuals from different treatment groups did not get mixed up (for example if the distinguishing paint dots came away). It is not possible to feed bedbugs individually, as membrane feeding success decreases at lower densities, perhaps because bedbugs respond to cues from feeding individuals that stimulates them to feed.

2.2.3.1 Preparation of blood

Heparinised sheep's blood was sterilised based on the protocol of Naylor (2012a). In brief, the blood was frozen to -20°C to lyse cells, defrosted and aliquoted into 2 mL microcentrifuge tubes, and spun at 20,800 g for 1 hour at 4°C in a refrigerated centrifuge

(Eppendorf: 5417R). This forces the cell debris and bacteria out of solution, forming a pellet at the bottom of the tube. The supernatant was collected, heated in a water bath (Scientific Laboratory Supplies, Julabo TW12) for 1 hour at 60°C, aliquoted into 2 mL microcentrifuge tubes and stored at -20°C.

2.2.3.2 Preparation of feeding membrane

The feeding membrane, was prepared by spreading Sylgard® silicone elastomer over a fine nylon mesh (1089 holes per cm^{2}), as described in Naylor (2012a). To do this, the nylon mesh was first laid on top of Parafilm® 'M' laboratory film, the silicone elastomer poured on top and spread out in a thin even layer using a plastic spreader. When dry (48 h), the mesh was cut to size (circular discs of 10 cm DIA). The silicone elastomer coating ensured that when fed bedbugs withdrew their proboscises, each hole made by each proboscis sealed.

2.2.3.3 Membrane feeding apparatus

Bedbugs were fed communally at densities of *n*=20-50, in 60 mL pots with a strip of filter paper (5 x 15 cm) folded in concertina for the bedbugs to walk on (Fig. 2.1). The open end of each pot was first covered with a feeding membrane disc (the silicone coating outermost) pulled taught and secured in place by a plastic band. The exposed surface was sterilised with 90% ethanol and air dried. A 2 mL aliquot of blood was defrosted and warmed in a water bath to approximately 37°C, and 0.5 mL pipetted onto the membrane and 2 sterile glass beads (2 mm DIA) placed in the blood droplet. This was immediately covered with a strip of Parafilm®. The glass beads afforded a gap between the feeding membrane and the Parafilm®, thus preventing blood from being displaced out of the sides as the Parafilm® was stretched in place. The pot was then upturned onto a heat mat (Habistat Heat Mat 4w (5 in x 4 in), reptiles.swell.com) heated to human body temperature (37°C) and covered in aluminium foil (Fig. 2.1). The foil prevented the Parafilm® adhering to the heat mat, and allowed the heat from the heat mat to pass through to the blood. All bedbugs fed in the dark at 26°C for 50 minutes. Any unused remaining blood was thrown away.

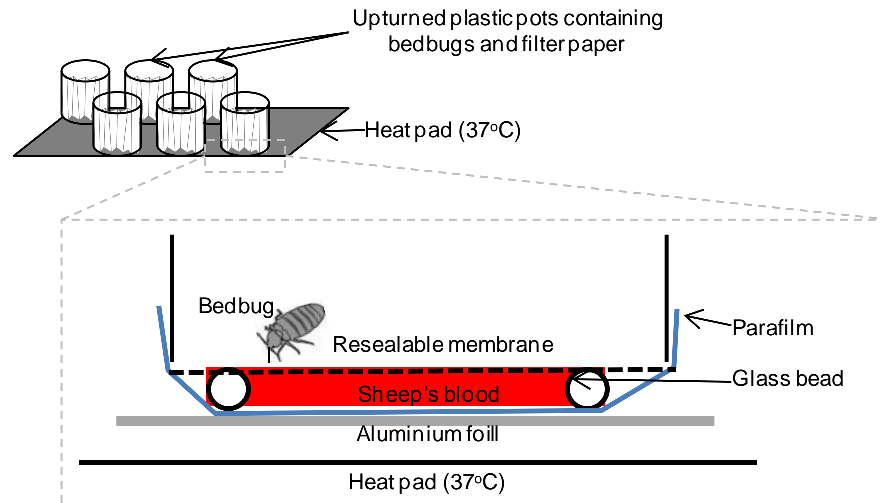


Figure 2.1 Diagram to show membrane feeding apparatus used to feed bedbugs heparinised sheep's blood.

2.2.3.4 Measuring size of blood meal

Previous investigations have revealed that the amount of blood taken up by females during a feed is directly related to measures of fecundity (Appendix 1). To determine whether there were any confounding differences in the amount of blood taken up by individuals during experiments, two feeding measures were recorded for every feed. (i) *Ad libitum* fed body mass (the mass of an individual immediately after feeding) and (ii) Mass of blood meal (the mass of blood taken up during the most recent feed, calculated as the difference between *ad libitum* fed body mass and body mass prior to feeding). The *ad libitum* fed body mass, which accounted for an individual's body mass, the mass of residual blood in the gut from previous feeds and the mass of fresh blood taken in during the most recent feed, indicated an individual's potential total available resources. The blood meal mass served to indicate if individuals had not digested their previous blood meal (the more residual blood in the gut from previous feeds, the less room there is in the gut for fresh blood). Individuals were weighed before and immediately after feeding (within 10 minutes) on a Metler Toledo classic plus AB135-S/FACT balance. The method of weighing individuals was highly repeatable (R=0.999, $F_{23,48}$=3219.52, P<0.0001).

2.3 Microscopy

2.3.1 Transmission electron microscopy

Transmission electron microscopy was used to examine the ultrastructure of bacteriocytes, the cells comprising the bacteriomes. Bacteriomes were dissected from adult bedbugs in 0.1 M sodium cacodylate buffer (Na-Cac 100mM, $CaCl_2$ 5mM, pH6.5) and pre-fixed in 0.5 mL 3% glutaraldehyde, 0.1 M sodium cacodylate buffer in a 1.5 mL microcentrifuge tube for 15 hours at 4°C. Bacteriomes were washed 2 times in 0.1 M sodium cacodylate buffer for 30 minutes at 4°C, and then post-fixed in 2% aqueous osmium tetroxide for 2 hours at room temperature. The post-fixed samples were then washed twice in 0.1 M sodium cacodylate buffer for 30 minute at 4°C, and dehydrated by being passed through a graded series of ethanol dilutions at room temperature. The ethanol series consisted of 75% ethanol for 30 min, 95% ethanol for 30 min, 100% ethanol for 2 changes of 30 minutes and 100% ethanol dried over anhydrous copper sulphate for 2 changes of 30 minutes. The specimens were then placed in propylene oxide for two changes of 30 minutes.

The specimens were then infiltrated in a 1:1 volume mixture of epoxy resin mix and propylene oxide on a rotating mixer for 15 hours at room temperature. The epoxy resin mix I used consisted of 10 mL Araldite® resin CY212, 10 mL hardener DDSA and 0.4 mL BDMA (accelerator). Samples were then transferred to epoxy resin mix for 2 changes of 4 hours at room temperature, and then embedded in fresh epoxy resin mix and cured for 48-72 hours at 60°C. Ultrathin sections of 85 nm thick, were cut on a Reichert Ultracut E ultramicrotome onto 200 mesh copper grids and stained for 30 minutes with 3% aqueous uranyl acetate followed by counter-staining with Reynold's lead citrate for 10 minutes. Sections were examined using a FEI Tecnai Transmission Electron Microscope at a voltage of 80 kv. Electron micrographs were taken using a Gatan digital camera.

2.3.2 Compound microscopy

All compound microscopy was performed on a Leitz Diaplan microscope (Wild Leitz GmbH, Germany). Images were taken on a digital camera (Micropublisher 3.3 RTV, Q Imaging, USA) with analysis software (Image-Pro Plus 5.1.2, Mediacybernetics, USA) and analysed in open-source image analysis software Image-J 1.46r.

2.3.3 Stereomicroscopy

All stereomicroscopy was performed on a Leica MZ8 microscope (Wetzlar, Germany). Images were taken on a digital camera (Micropublisher 3.3 RTV, Q Imaging, USA) with image analysis software (Image-Pro Plus 5.1.2, Mediacybernetics, USA) and analysed in open-source image analysis software Image-J 1.46r.

2.4 qPCR

2.4.1 DNA extraction

The head was removed before DNA extraction (enzymes in the salivary glands have the potential to breakdown DNA). When DNA was not extracted immediately, samples were preserved in 100% ethanol. DNA was extracted using an ammonium acetate precipitation protocol (modified from Bruford et al., (1998)). Each sample was diced with a scalpel, transferred to a 1.5 mL microcentrifuge tube with 250 µL Digsol buffer (20 mM EDTA, 120 mM NaCl, 50 mM Tris-HCl and 20% SDS, pH 8.0) and homogenized by grinding by hand with a pestle. 10 µL 10 mg mL^{-1} Proteinase K was added to the homogenate and incubated at 37°C for 12 hours.

300 µl 4 M ammonium acetate solution was added to the samples, which were vortexed for 10 seconds at 5 minute intervals over 15 minutes at room temperature, and centrifuged at 16,060 rcf for 10 minutes (Heraeus Biofuge Pico 75003235). The addition of ammonium acetate solution separated the homogenate into aqueous and organic phases: 550 µL of the upper colourless aqueous phase, containing the DNA, was transferred to a new 1.5 mL microcentrifuge tube.1 mL of 100% ethanol was added to precipitate the DNA and the samples were centrifuged for 10 minutes at 16,060 rcf to pellet the DNA. The ethanol was poured away and 500 µl 70% ethanol was added to wash the sample, and centrifuged for 10 minutes at 16,060 rcf. The ethanol was again removed by pouring away and the DNA pellet air dried for 1-2 hours.

The pellet was resuspended in 30 µL Low TE (10 mM Tris-HCl, 0.1 mM EDTA, pH 8.0) and placed in a hot block (50°C) for 30 minutes. The sample was shaken at 10 minute intervals over this time to dissolve the DNA pellet. DNA concentrations were established by spectrophotometry using NanoDrop® software at default settings (NanoDrop® ND-

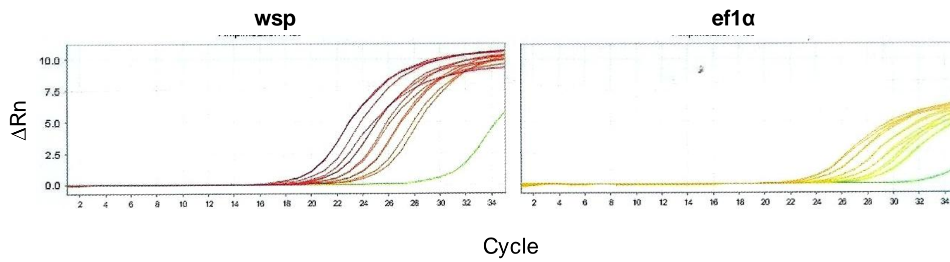
8000 v1.1.1 UV-Vis Spectrophotometer, Thermo Fisher Scientific, Wilmington, DE 19810, USA). Samples were stored at 4°C (up to 24 hours) or -20°C if longer term storage was required.

2.4.2 qPCR

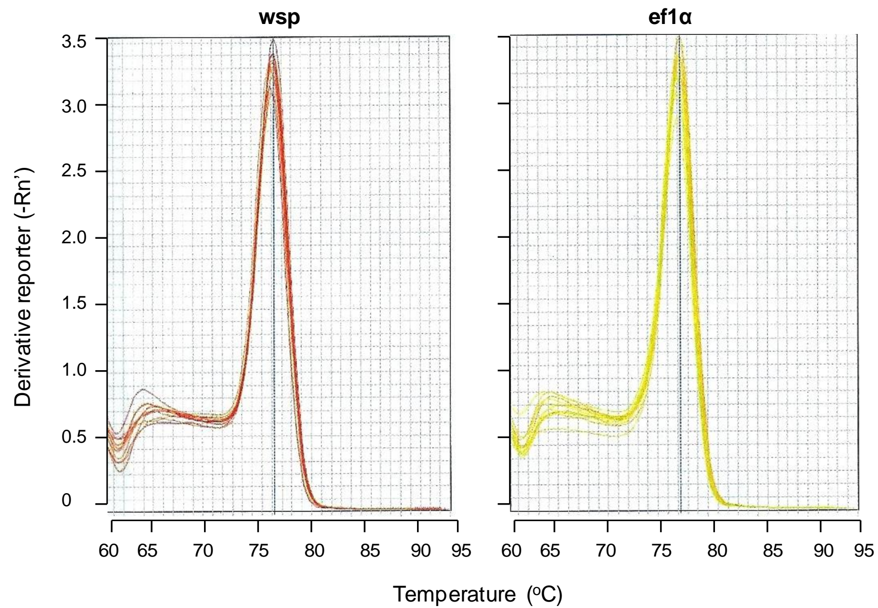
qPCR was used to measure *Wolbachia* load in bedbug individuals or their dissected tissues. *Wolbachia* load was determined as the number of *wsp* (*Wolbachia* Surface Protein) gene copies per insect ef1α (elongation factor 1-alpha) gene copy. *Wolbachia* were quantified using specific primers designed to amplify a 70-bp long section of the *Wolbachia's wsp* gene (CL*wsp*F (5′-CGG CTC TTA TGG CGC TAG C-3′) and CL*wsp*R (5′-CTT TTT GGT TGT ATC GCC AGG A-3′). Bedbug DNA was quantified using primers designed to amplify a 73-bp long section of the insect ef1α gene (CLefF (5′- GCA AAT GCC TTA TTG AAG CTC TC-3′) and CLefR (5′-GGA AGC CTA AGA GGC TTG TCA G-3′)). These primers were published and used in Hosokawa et al., (2010) with JESC, a Japanese bedbug strain. Dissociation curve analysis was performed for each primer pair to ensure all samples yielded a single sharp peak at the amplicon's melting temperature (Fig. 2.2).

qPCR was carried out using a StepOnePlus™ Real-Time PCR System (Applied Biosystems) and iTaq SYBR® Green Supermix (Bio-Rad) to monitor double-stranded DNA synthesis in combination with ROX as a passive reference dye. All qPCRs were carried out in duplicate in a 15 μL reaction volume containing 2.5 μL DNA extract [1 ng μL^{-1}], 4 μL ddH_2O, 0.5 μL forward primer [15 *p*mol μL^{-1}], 0.5 μL reverse primer [15 *p*mol μL^{-1}] and 7.5 μL SYBR®. Initial denaturisation temperature of 94°C for 1 minute was followed by 35 cycles of denaturisation at 94°C for 1 minute, annealing at 53°C for 1½ minutes and extension at 60°C for 1½ minutes. To quantify DNA, a standard curve was used to validate all primers used. The presence of *wsp* genes was then compared relative to ef1α genes. To calculate the relative expression I subtracted the mean *wsp* CT for each sample from the mean ef1α CT and then exponentially transformed this value.

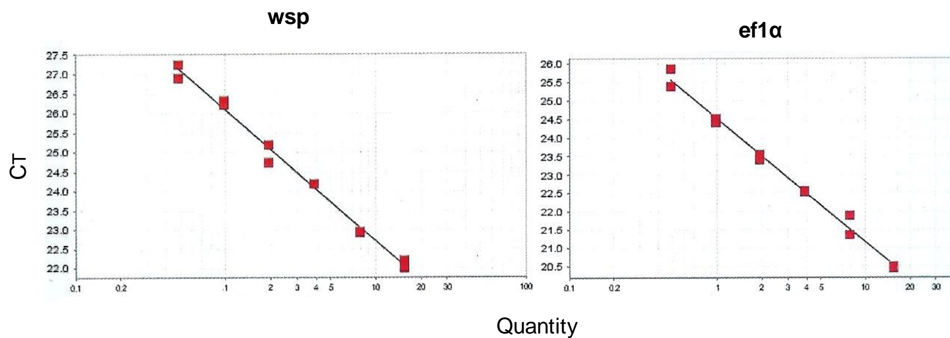
Amplification Plots



Melt Curves



Standard Curves



	wsp	ef1α
Efficiency	98.72	97.74
R^2	0.99	0.99
Y intercept	24.51	26.08
Slope	-3.35	-3.38

Figure 2.2 The melt curves, amplification plots and standard curves for *wsp* and ef1α primers. The melting temperatures of the samples peaked around ~77°C. Negative controls are shown in green.

2.5 Statistical Analysis

2.5.1 Statistical software

Statistical analyses were performed using Minitab (version 16, Minitab Company, State College, PA) except for survival analyses which were performed using the statistical package R (version 2.15.1) (R Core Team, 2012) on a Windows XP platform.

2.5.2 Check data for assumptions of statistical analyses

Checks carried out to test the data for normality included the Anderson Darling test and plots of residuals versus fits. To test for equal variances Fisher's exact tests were used. Where possible, data were analysed using parametric tests. Distributions that were significantly non-normal were transformed (as reported in results). Where even after transformation, data failed to meet the criteria necessary for parametric tests, non-parametric equivalents were used. In all cases, the means of untransformed data are presented ±1SE. Where data do not meet the assumptions for a parametric test, medians are also presented ±1CI. In all figures and tables the ranges of probabilities are coded with asterisks: * for $P<0.05$ - 0.01; ** for $P=0.01$ - 0.001; and *** for $P\leq 0.001$.

2.5.3 Adjustments for multiple comparisons tests

Analysis of variance was carried out to determine if there were differences among group means. Where a significant difference was found, a Tukey's HSD multiple comparisons post-hoc test was carried out to determine which means differed. For data that failed to meet the assumptions of an ANOVA, even after transformation, a Kruskal-Wallis test was used to determine if there were significant differences among group medians. Where a significant difference was found, a Dunn's multiple comparisons post-hoc test (which uses a generalised Bonferroni procedure) was carried out to determine differences.

2.5.4 Repeatability

To assess the accuracy of size measurements, repeatabilities (R) were calculated in MicroSoft Excel software 2007 following an ANOVA-based method (Lessells & Boag, 1987) detailed in Nakagawa & Schielzeth (2010). The value for R ranges between 0 and 1 and describes the proportion of variation in the size of the measurement that is due to

differences between individuals (within group) as opposed to differences between the repeated measures (between groups).

For each repeatability analysis, the object (e.g. pronotum, body mass, nylon implant, ejaculate in mesospermalege), was measured three times for each of $n \geq 10$ individuals and R was calculated using the *F* table of an ANOVA. R was calculated using the equation $R = S^2A/(S^2 + S^2A)$ where S^2A is the between group variance (n=3) and S^2 is the within group variance ($n \geq 10$). $S^2A = (MS_A - MS_W)/n_0$ where n_0 is the weighted average number of observations per individual (here, this is always 3).

2.6 Graphical devices

Throughout this thesis, no treatment control groups represent individuals with natural *Wolbachia* loads and are presented by grey bars. Treatment groups with reduced *Wolbachia* loads are presented by red bars if this reduction has been brought about by heat treatment, and by yellow bars if this reduction has been brought about by antibiotic treatment. Bar charts show means with error bars representing 1 standard error, box plots medians (solid lines), means (dashed lines) and whiskers represent the upper and lower quartiles.

2.7 Summary

In this chapter I have:

1. Described the bedbug-husbandry methods and protocols used to maintain bedbugs in the lab.
2. Described microscopy techniques used to examine tissues on cellular and ultracellular levels.
3. Described molecular techniques to quantify *Wolbachia* load in individuals and within specific bedbug tissues.

Chapter 3

Manipulating *Wolbachia* Load in Bedbugs: Heat and Antibiotic Treatments

"It is good to have an end to journey toward; but it is the journey that matters, in the end"

Ernest Hemingway

3.1 Introduction

Wolbachia are maternally inherited, intracellular, α-proteobacteria related to rickettsiae (Werren et al., 2008). They infect somewhere between 40% (Zug and Hammerstein, 2012, Zug et al., 2012) and 65% (e.g. Hilgenboecker et al., 2008, Werren et al., 2008) of known insect species and are best known for their ability to manipulate host sex ratios (e.g. Werren et al., 2008)). Comparisons between infected and non-infected hosts from a population reveal some *Wolbachia* species benefit their insect host via a range of mechanisms including: a competitive advantage in sperm competition (Wade and Chang, 1995b); supply of nutrients (Bandi et al., 2001, Brownlie et al., 2009, Hosokawa et al., 2010); tolerance to toxic substances (Wang et al., 2012) and resistance to viral parasites (e.g. Teixeira et al., 2008, Moreira et al., 2009, Osborne et al., 2009, Bian et al., 2010). In many cases these effects were discovered by experimentally comparing the response of *Wolbachia*-infected hosts against hosts that have had their *Wolbachia* removed (or significantly reduced).

Three techniques are widely used to remove *Wolbachia* infection from the host. These are antibiotic treatment, high temperatures and introgression (the successive backcrossing of hosts to remove the symbiont by selection) (Bordenstein and Werren, 1998, Kondo et al., 2005). Eliminating *Wolbachia* with minimal host side effects, minimal disruption to the non-target microbial community and controlling for any confounding effects of the treatment itself is a great challenge. Here I compare the effects of using heat and antibiotic

treatment for creating infected and non-infected individuals for experiments reported later in this thesis.

3.1.1 Techniques to manipulate symbiont loads

Insect hosts are most commonly treated with antibiotics which are usually fed to the host (e.g. Zeh et al., 2005, Pike and Kingcombe, 2009, Werren and Loehlin, 2009,). High temperature treatment that physiologically stresses and kills the *Wolbachia*, but is tolerated by their host, is also a popular treatment choice (e.g. van Opijnen and Breeuwer, 1999, Hurst et al., 2000, Jia et al., 2009); *Wolbachia* have a low thermal tolerance, such that natural heat curing of hosts occurs in the wild (Werren, 1997). Both antibiotic (Hosokawa et al., 2010) and heat treatment (Chang, 1974) techniques have been used to reduce *Wolbachia* loads in bedbugs. Which treatment method (heat or antibiotics) is most effective at inducing a decrease in *Wolbachia* load with minimal host side effects, remains to be determined.

3.1.2 Heat treatment

In early studies of *Wolbachia* in bedbugs, heat treatment was used to reduce symbiont densities (Chang, 1974). This was verified microscopically, through observations of the bacteriomes (Chang and Musgrave, 1973, Chang, 1975), and effects were assessed by comparing fitness measures between heat treated and non-heat treated hosts (Chang, 1974). A two-week long heat treatment of 36°C was found to deplete the symbiont load within the bacteriomes (Chang, 1974), later identified as *Wolbachia* (Hypša and Aksoy, 1997). This reduction in *Wolbachia* was thought to cause the reduced egg laying rate observed in heat treated females, and a nutritional mechanism was suggested (Chang, 1974). Because these early observations of *Wolbachia*-mediated effects were carried out whilst the bedbugs were in the heat treatment, it was not possible to separate the effects of heat treatment from the effects caused by a reduction in *Wolbachia* load (Chang, 1974). To understand how likely it is that Chang's observations were a physiological effect of the heat treatment, it is helpful to understand what the thermal tolerances of bedbugs are.

In the laboratory, bedbugs can be successfully reared at temperatures between 25°C (Benoit et al., 2009) and 28°C (Omori, 1941). Laboratory observations of the movement of bedbugs

along an artificial temperature gradient suggest individuals prefer temperatures close to 28°C (Omori, 1941). With a moderate rise in temperature egg laying ceases (32°C) whilst survival remains relatively unaffected (Omori, 1941). Once temperatures exceed 40°C survival times are reduced to <24 hours (Benoit et al., 2009, Pereira et al., 2009).

We know from studies of the effects of heat on insects that high temperatures have a multitude of physiological effects including increased metabolic and respiration rate, changes in the nervous and endocrine systems affecting insect development and behaviour, the production of heat shock proteins and beyond a critical upper limit, protein denaturation and damage to host tissues occurs (Neven, 2000). The physiological effects of a 2 week heat treatment of 36°C on bedbugs are unknown. It may be possible to remove these confounding effects by carrying out observations on the heat treated host within a lower control temperature environment. The effect of heat treatment on *Wolbachia* load in bedbugs remains to be confirmed and quantified with molecular techniques.

3.1.3 Antibiotic treatment

More recently, antibiotics have been used to reduce *Wolbachia* load in bedbugs with a reduction of over 99% achieved by rearing adults on blood supplemented with rifampicin [10 μg mL^{-1}] through an artificial membrane feeding system (Hosokawa et al., 2010). Rifampicin is a broad spectrum antibiotic that crosses cell membranes to target and kill intracellular bacteria, such as *Wolbachia* (Charlat et al., 2007a). As with heat treatment, the physiological effects of antibiotics on the host, which may include a suppressed immune response (Eleftherianos et al., 2007) and disruption to non-target microbiota (Rosengaus et al., 2011), are difficult to control. One way to control for these confounding effects would be to include an antibiotic treatment control group that are fed antibiotics that are not effective against *Wolbachia*, such as penicillin (Fenollar et al., 2003) however differences between the two compounds extend beyond their effect on *Wolbachia* and consequently this is not a robust control protocol.

In bedbugs, it is unknown what antibiotic compounds besides rifampicin may be effective against the bedbug's *Wolbachia*. Aside from rifampicin, tetracycline compounds are widely used to reduce *Wolbachia* load in a range of insect hosts (e.g. Panteleev et al., 2007,

Machtelinckx et al., 2009, Pike and Kingcombe, 2009). Both compounds target intracellular bacteria, but whereas rifampicin is bacteriocidal, killing the bacteria by inhibiting transcription (Campbell et al., 2001), tetracycline has a bacteriostatic effect, halting (but not killing) bacterial growth by preventing cell division (Chopra and Roberts, 2001). In some studies the bacteriostatic action of tetracycline compounds is not enough to stop the *Wolbachia*-mediated effects in the host, in which case a bacteriocidal compound such as rifampicin may be the antibiotic of choice (e.g. Charlat et al., 2007a).

Wolbachia in bedbugs are thought to be primary symbionts that provide B vitamins (Hosokawa et al., 2010). In the study using rifampicin, it was necessary to supplement the blood with B vitamins to compensate for the loss of *Wolbachia* (Hosokawa et al., 2010). It may be possible to use tetracycline to significantly reduce *Wolbachia* load, whilst maintaining a sufficient load to fulfil its primary role in B vitamin provisioning; such threshold effects have been observed in *Drosophila bifasciata* (Hurst et al., 2000). If this were the case, additional B vitamin supplementation of the blood meal would not be necessary. As the mechanistic basis of B vitamin provisioning is unknown, allowing the *Wolbachia* to continue its nutritional role in experiments would create a more natural situation.

3.1.4 Comparing antibiotic and heat treatment

Many authors test different treatment protocols before choosing a technique to manipulate symbiont loads in their study system, and there are a number of published studies comparing the effects of different antibiotics (e.g. Dedeine et al., 2001, Fenollar et al., 2003). These authors use qPCR or/and FISH techniques to compare the effectiveness of different antibiotics in terms of the degree of symbiont reduction, and how long the reduction remains after treatment has stopped. These studies do not compare the effects on non-target symbionts or the physiological effects of the treatment on the host. Currently there are no studies that directly compare two different modes of treatment, such as heat treatment against antibiotic treatment.

The effect of treatment on the loads of non-target symbionts, such as the BLS found in many strains of *Cimex lectularius* (Hypša & Aksoy, 1997, Meriweather et al., 2013), could

be assessed using qPCR techniques to compare BLS loads in treated and non-treated individuals. Although qPCR primers for BEV in leaf hoppers have been published (Degnan et al., 2011), there are no published qPCR primers for BLS in bedbugs. Using qPCR to assess effects on non-target symbionts in bedbugs would be both time consuming and costly, as primers would first need to be designed and tested. A suitable alternative technique would be FISH which has already been used to visualise BLS within tissues of a Japanese strain of *C. lectularius* (Hosokawa et al., 2010), however, this technique is also too costly and time consuming given the 3 year framework of my PhD. Furthermore, BLS may not be the only other non-target symbiont present within bedbugs; morphological observations suggest up to three different symbiont species may be present within the bacteriome (Chang and Musgrave, 1973, Reinhardt and Siva-Jothy, 2007).

A quicker and cheaper way to gain insight into the effect of antibiotic and heat treatment on non-target symbionts in bedbugs would be to compare TEMs of bacteriomes dissected from either heat treated or antibiotic treated individuals with bacteriomes dissected from untreated controls. This would generate data that could be used to decide whether further quantitative assessment (using qPCR or FISH) of the treatment effects on non-target symbionts was necessary.

To assess the effect of treatment on bedbug physiology, the ability to take up and process a blood meal would be suitable indicators of the bedbug's physiological health. Firstly regular blood meals are essential for development and reproduction (Reinhardt and Siva-Jothy, 2007): nymphs require a blood meal for development and eclosion, and adults require a blood meal for production of the gametes. Secondly, feeding response is unlikely to be affected by *Wolbachia* load – there have been no reports of *Wolbachia* affecting their host's feeding ability − therefore any change in feeding response is likely to be a side effect of treatment on the bedbug host. Feeding measures include the size of a blood meal and time taken to process the blood meal between feeds.

3.1.5 Aims of chapter

1. To assess the effectiveness of tetracycline treatment at reducing *Wolbachia* loads by quantifying *Wolbachia* population dynamics before, during and after tetracycline treatment in hosts over time.
2. To assess the effectiveness of heat treatment at reducing *Wolbachia* loads by quantifying *Wolbachia* population dynamics before, during and after heat treatment in hosts over time.
3. To compare the effectiveness of tetracycline and heat treatment at reducing *Wolbachia* load, using results from numbers 1 and 2 above.
4. To compare the physiological side effects of tetracycline and heat treatment on measures of 'digestion rate'.
5. To compile heat and antibiotic treatment protocols for manipulating *Wolbachia* loads, to use in the rest of the thesis.

3.2 Materials and Methods

3.2.1 Insects

The insects used in these experiments were 3 days old virgin male and female bedbugs that had been raised by the protocol in 2.2.2.1. Insects were imaged and pronotum width measured (2.2.2.2), and marked so that individuals could be distinguished (2.2.2.3). All were then mated following the protocol in 2.2.2.4.

Throughout experiments insects were maintained individually in 5 mL plastic tubes with a strip of filter paper under controlled temperature conditions of 26°C 70% RH constant controlled light conditions except during feeding when individuals were transferred to communal pots, and during heat treatment. Insects were fed weekly blood meals of heparinised sheep's blood through an artificial membrane feeding system (2.2.3) unless otherwise stated.

3.2.2 Antibiotic treatment

Tetracycline hydrochloride [150 µg mL^{-1}] was delivered in the blood meal over two feeds, one week apart to antibiotic-treated individuals. Individuals in the antibiotic treatment

control group were fed in the same way, except the blood was unsupplemented. In this way two treatment groups were generated: “+αb” and “−αb”.

3.2.3 Heat treatment

The heat treatment used throughout experiments consisted of placing individuals into an incubator at 36°C, 70% RH under constant controlled light conditions for 2 weeks (unless otherwise stated), during which individuals remained unfed. Heat treatment control individuals were treated in the same way except the incubator was maintained at (26°C 70% RH constant controlled light conditions). In this way two treatment groups were generated: “+heat” and “−heat”.

3.2.4 Effect of antibiotic and heat treatment on *Wolbachia* load

The *Wolbachia* load in “+αb” and “−αb” females (3.2.2) was assayed over a period of 5 weeks (before treatment (time 0); during treatment (weeks 1-2); and after treatment (weeks 3-5). This was done by sub-sampling six individuals from each treatment group at weekly time intervals, just prior to feeding. Individuals were chosen at random from each group and fixed in 1 mL 100% ethanol in 1.5mL microcentrifuge tubes, in preparation for DNA extraction and qPCR analysis of the *Wolbachia* load (see 2.4 for details). The *Wolbachia* load in “+heat” and “−heat” females (3.2.3) was assayed in the same way.

3.2.5 Effect of antibiotic and heat treatment on non-target symbionts

One bacteriome was removed from each of five “+αb”, five “−αb” females and from five “+heat” and five “−heat” females (3.2.2.1) at the end of the treatment period. The bacteriomes were processed for TEM (see 2.3.1 for details) and qualitative data were collected from observations of morphological differences between the electron micrographs from antibiotic/heat treated individuals and controls.

3.2.6 Physiological effects of antibiotic and heat treatment

3.2.6.1 ‘Digestion rate’

‘Digestion rate’ (the rate at which the mass of blood in the gut decreases) was measured as the decrease in body mass one week after feeding. The average over four feeds was calculated and compared between females in three treatment groups (modified from 3.2.2

and 3.2.3). (i) "+αb" (2 feeds antibiotic supplemented blood, 2 feeds unsupplemented blood, maintained at 26°C 70% RH 12:12 L:D photocycle). (ii) "+heat" (4 feeds unsupplemented blood, maintained at 36°C 70% RH 12:12 L:D photocycle for 2 weeks followed by 26°C 70% RH 12:12 L:D photocycle for 2 weeks). (iii) No-treatment control (NTC) (4 feeds unsupplemented blood, maintained at 26°C 70% RH 12:12 L:D photocycle). All individuals were weighed immediately after each feed (*ad libitum* fed body mass) and again one week later, so that 'digestion rate' could be calculated. All females were mated weekly following standard protocols (2.2.2.4).

Digestion will result in a decrease in an individual's body mass as excess water and waste products are excreted. The nutrients released by digestion will be either invested in an individual's growth and repair processes, or assimilated into eggs and lost from the individual's body during egg laying. Egg laying will therefore also account for a decrease in body mass after feeding so the total number of eggs laid by each individual per week was also recorded.

3.2.7 Data analysis

Data were analysed as detailed in 2.5. Statistical analyses were carried out on the average 'digestion rate' measured over four feeds. Statistical analyses were also carried out on the 'digestion rate' following each of the feeds separately, these analyses are provided in Appendix 3.

3.3 Results

3.3.1 Effect of antibiotic and heat treatment on *Wolbachia* load

There was a significant reduction in the female's *Wolbachia* load after two weeks of both the heat and the antibiotic treatments (relative abundance of *Wolbachia wsp* genes to host ef1α genes in the antibiotic treatment group: 1.26±1.18, and antibiotic treatment control group: 20.68±5.45, Mann-Whitney U test: U=35, n_1=6, n_2=6, P<0.01; "+heat": 1.59±0.551, "−heat": 20.03±3.86, t=7.14, 10 df, P<0.0001 on $\log_e(x+1)$ data). At this time, there was no difference in the size of these reductions in *Wolbachia* load between antibiotic and heat treated individuals (comparison of "+αb" and "+heat" groups: Mann-Whitney U

test: U=6, n_1=6, n_2=6, P>0.05; comparison of *Wolbachia* loads of "−αb" and "−heat" groups: t=0.10, 10 df, P>0.05).

In "+αb" individuals, *Wolbachia* load continued to decrease over the week after the last antibiotic feed (week 3) and remained significantly lower than antibiotic treatment control individuals for the rest of the sampling period (up to week 5) (Fig. 3.1). By contrast in "+heat" individuals, the *Wolbachia* load began to recover (increase) after heat treatment had stopped; by two weeks post heat treatment (week 4) there was no difference in the *Wolbachia* load between "+heat" (84.34±28.54) and "−heat" individuals (68.08±18.62) (t=0.23, 10 df, P>0.05 on $\log_e(x+1)$ data) (Fig. 3.2).

A comparison of the lowest *Wolbachia* load achieved for each treatment revealed that antibiotic treatment reduces the *Wolbachia* load to a level that is at least 120 times smaller than the maximum reduction achieved by heat treatment ("+αb": 0.00797±0.00109, "+heat": 1.60±0.551, t=2.89, 5 df, P<0.05 on $\log_e(x+1)$ data).

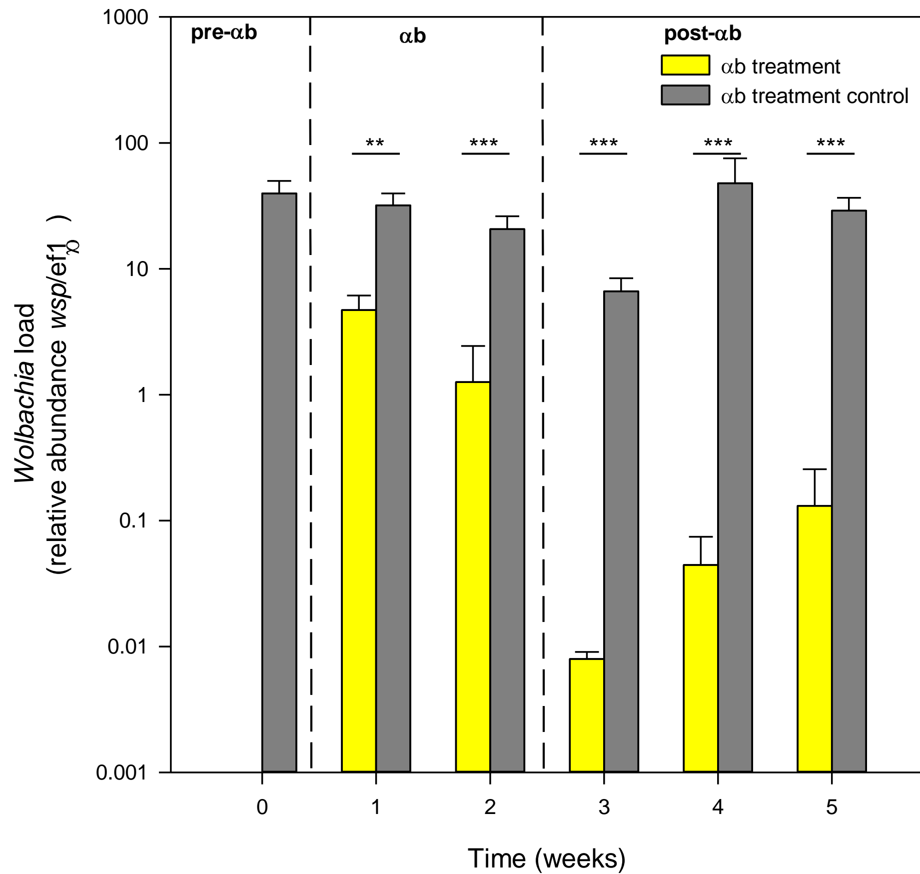


Figure 3.1 Assay of the mean *Wolbachia* load of females in response to antibiotic treatment over 5 weeks compared to antibiotic treatment controls. *N*=6 each group, bars represent 1SE, αb = antibiotics. For results of individual analyses see Table A3.1.

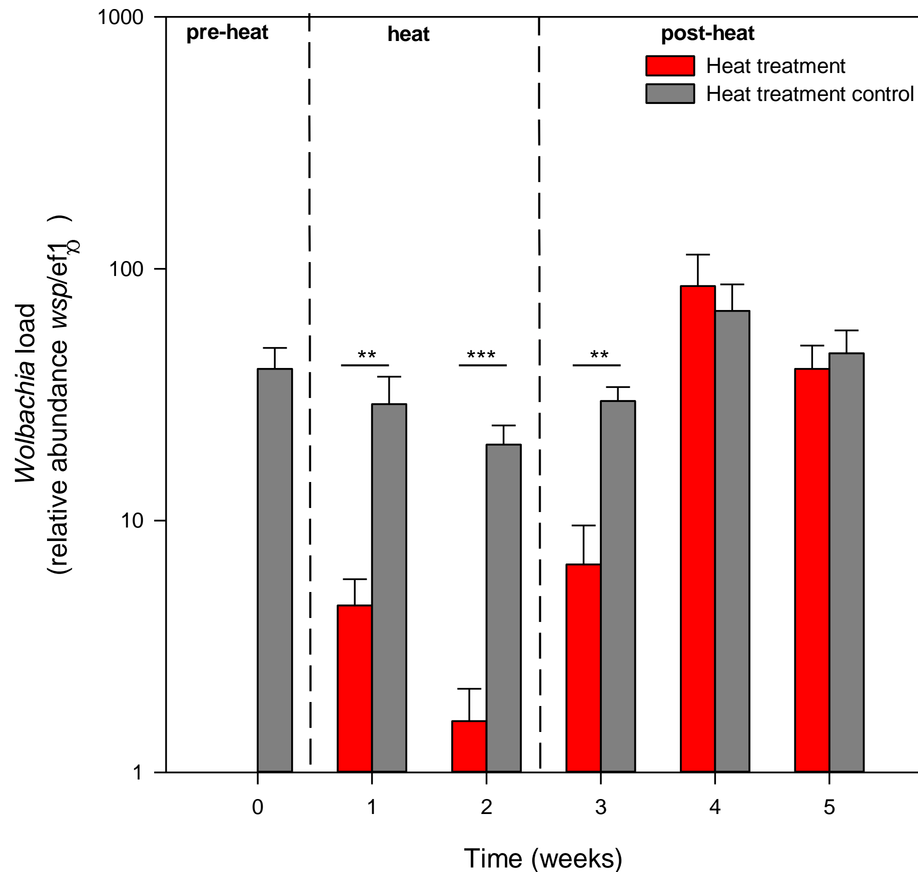


Figure 3.2 Assay of the mean *Wolbachia* load of females in response to heat treatment over 5 weeks compared to heat treatment controls. *N*=6 each group, bars represent 1SE. For results of individual analyses see Table A3.2.

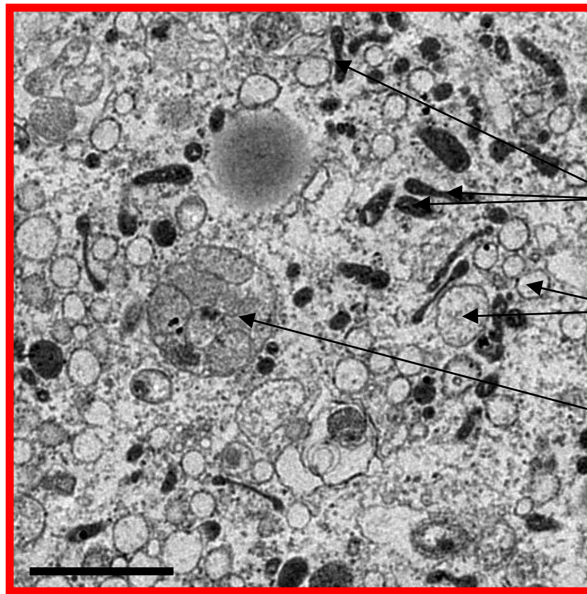
3.3.2 Effect of antibiotic and heat treatment on non-target symbionts

Bacteriomes decreased in size in response to antibiotics and heat treatment. The bacteriomes from antibiotic treated females were too small to be sampled and processed for TEM. TEM images were therefore only taken of bacteriomes from heat treated and heat treatment control females. Two morphologically distinct types of bacteria were visible in heat treatment control bacteriome TEMs. Morphological comparisons of the bacteria in *C. lectularius* bacteriomes in my electronmicrographs with and those of previous studies (Chang and Musgrave, 1973, Hosokawa et al., 2010) show that these two types resemble *Wolbachia* and a gamma proteobacteria, sometimes referred to as a *BEV*-like symbiont (BLS) (Hypša & Aksoy, 1997). The *Wolbachia*-like bacteria were the more numerous and could be identified by their characteristic double membrane, and location within cell

vacuoles (Plate 3.1 bottom). The BLS-like bacteria by contrast, were not located within the vacuoles and were electron opaque in the TEMs (Plate 3.1 top).

Comparisons of the relative or absolute abundances of *Wolbachia* versus BLS could not be made from these TEM images. Treatment was associated with a reduction in the overall size of bacteriomes which would affect the observed densities of bacteria within the bacteriome and their absolute abundance; the magnitude of the size reduction was not measured. The resolution of the images was not high enough to allow individual bacteria to be consistently and accurately counted. Therefore only qualitative comparisons were made.

Wolbachia appeared to be less abundant in the bacteriomes dissected from heat treated females compared to bacteriomes from control females. In many of the TEM images of heat treated bacteriomes, no *Wolbachia* were visible. Instead, empty vacuoles and the breakdown products of cytolysis, including protoplasts and self-assembling rings of lipo-polysaccharide membrane, were visible (e.g. Plate 3.1, top). Bacteria resembling BLS were observed in both heat treated and control bacteriomes. No obvious breakdown in the BLS-like bacteria resulting from heat treatment was visible. It was not clear whether the heat treatment induced any morphological changes in BLS or affected its abundance because it was difficult to compare the images, for example in the control bacteriomes *Wolbachia* density was very high and partly obscured the BLS.



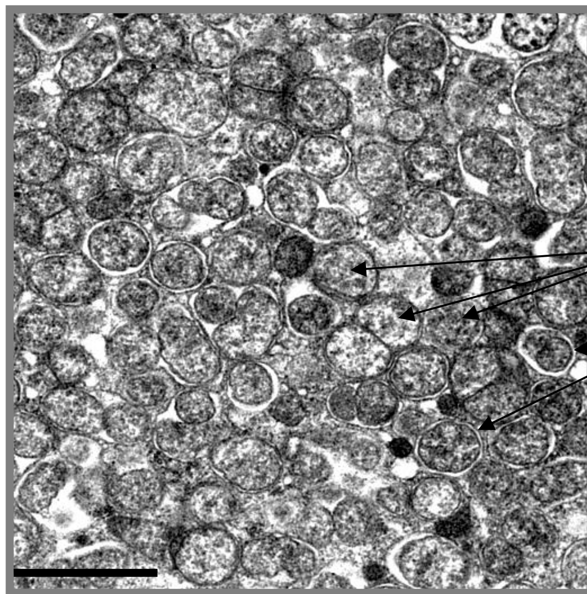


Plate 3.1 Effect of heat treatment on bacteriome symbionts. Transmission electron micrographs of bacterial symbionts within bacteriomes dissected from heat treated (36°C for 2 weeks) and non-heated (26°C for 2 weeks) females. Bar 2 μm.

3.3.3 Physiological effects of antibiotic and heat treatment

3.3.3.1 'Digestion rate'

There was a highly significant effect of treatment on 'digestion rate' (the decrease in *ad libitum* fed body mass over a one week period) (ANOVA: $F_{2,72}$=25.64, P<0.0001). 'Digestion rate' was significantly lower in "+αb" individuals, compared to "+heat" and "NTC" females (Tukey mcp test P<0.0001) (Fig. 3.3, Table A3.3). There was no difference in the *ad libitum* fed body mass of individuals after feeding (ANOVA: $F_{2,72}$=1.88, P>0.05) that could account for these results (Fig. 3.3, Table A3.4).

There were significant differences in the egg laying rate of individuals between treatment groups (Kruskal-Wallis test H=53.44, 2 df, P<0.0001). "+αb" treated individuals laid no eggs at all and "+heat" individuals had a significantly reduced egg laying rate compared to no-treatment controls (Dunn's test P<0.0001) (Fig. 3.3). Analyses of egg laying rate for each week separately revealed that "+heat" individuals laid fewer eggs compared to "NTC" during the first two weeks only (when they were being heat treated) after which there were no differences in the egg laying rates of "+heat" and "NTC" individuals (Table A3.5).

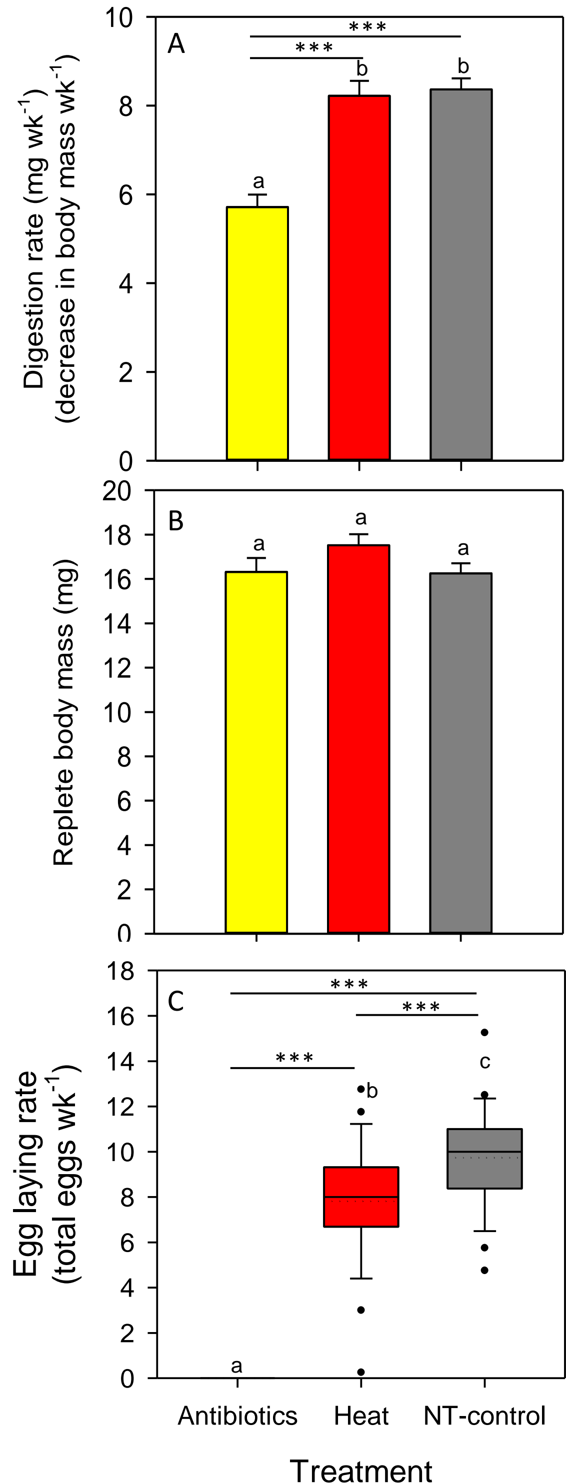


Figure 3.3 Effect of antibiotic and heat treatment on 'digestion rate'. (A) mean 'digestion rate' (the difference between *ad libitum* fed body mass and body mass one week later); means with the same letter do not differ from each other (Tukey mcp test P>0.05). (B) mean *ad libitum* fed body mass. (C) median (solid line) mean (dotted line) egg laying rate (total eggs per week); medians with the same letter do not differ from each other (Dunn's test P>0.05). Sample sizes: antibiotic treatment n=24, heat treatment n=26 and no treatment control n=25; bars represent 1SE.

3.4 Discussion

Both antibiotic and heat treatment significantly reduced the *Wolbachia* loads of bedbugs, but there were substantial differences in how that reduction in *Wolbachia* load recovered over time. Antibiotics produced a greater reduction which was longer lasting compared to heat treatment. Results suggest that the treatments also varied in their effects on non-target symbionts and host physiology ('digestion rate') (summarised in Table 3.1). These effects are important when considering which treatment method to use in bedbug-*Wolbachia* investigations. I am now going to look at the significance of the results from these investigations in more detail.

Table 3.1 Summary of the main differences between antibiotics and heat treatment to reduce *Wolbachia* load in bedbugs.

	Antibiotics	**Heat**
Maximum *Wolbachia* reduction	99%	93%
Is there recovery of *Wolbachia* load?	No recovery observed 4 weeks post treatment	Recovers to control levels by 2 weeks post treatment
Can a treatment control group be generated?	No	Yes
Use in short-term studies (≤ 2 weeks)	Yes	Yes
Use in long-term studies (≥ 2 weeks)	Yes	No
Effect on BLS densities	Possible reduction	No reduction observed
Effect on host's feeding	Reduced ability to process blood meal	No effect

3.4.1 Effect on *Wolbachia* load

Both antibiotic and heat treatment produced a reduction in *Wolbachia* load of more than 90% relative to untreated individuals, but there were differences in the pattern of *Wolbachia*'s recovery post-treatment. *Wolbachia* loads recovered to control levels by two

weeks following heat treatment, whereas in antibiotic treated individuals, *Wolbachia* loads were still more than 90% lower than control individuals 4 weeks after antibiotic treatment had stopped. The fact that *Wolbachia* loads recover limits the use of heat treatment to short-term studies that do not extent beyond 2 weeks post heat treatment. Antibiotic treatment however can, in theory, be used in both short and longer-term studies of more than 3 weeks post antibiotic treatment. However, the effect of antibiotics on feeding behaviour is likely to affect key life-history response variables in such studies. How long it takes *Wolbachia* loads to recover fully following antibiotic treatment remains to be determined.

3.4.4.1 Implications for experimental design

The effects of heat treatment are difficult to distinguish from the effects of altered *Wolbachia* load. My results show that *Wolbachia* load recovers to similar levels in non-heat treated individuals two weeks after heat treatment. One advantage in the recovery of *Wolbachia* load following heat treatment is that it is possible to use heat treated individuals in which *Wolbachia* loads have recovered ("+heat+recovery") as controls for some of the possible confounding effects of the heat treatment itself. Heat treated individuals ("+heat") will have reduced *Wolbachia* loads compared to "+heat+recovery" individuals, but both groups will have received the same heat treatment. Therefore any difference between "+heat" and "+heat+recovery" individuals can be assumed to be due to differences in their *Wolbachia* loads. However, it is also possible that another factor altered by the heat treatment may also recover to pre-heat treatment levels during this recovery period. A "+heat+recovery" group controls for the longer-term heat treatment effects. Furthermore there are differences in the number of blood meals taken up by the "+heat" or non-heat treated control ("−heat") individuals compared to the "+heat+recovery" individuals. "+Heat+recovery" individuals will have taken two blood meals as adults (during the recovery period), whereas "+heat" or "−heat" individuals will have received no blood meals. (Blood meals are an important prerequisite for *Wolbachia* load recovery.) Differences in the blood meals will confound comparisons between groups. For example, for measures of fecundity, the additional blood meals could inflate the egg laying rates of "+heat+recovery" individuals relative to "−heat" individuals. It is therefore necessary to set up a non-heat treatment control group that also has a recovery period during which they

receive two blood meals ("−heat+recovery") for comparisons with "+heat+recovery" females. As a consequence of these differences in the number of blood meals between unfed "+heat" or "−heat" individuals, and the twice fed "+heat+recovery" or "−heat+recovery" individuals, statistical comparisons should be drawn between pairs ("+heat" and "−heat") and ("+heat+recovery" and "−heat+recovery") rather than across all four groups.

Similar to the heat treatment, the effects of antibiotic treatment are also difficult to control for. I am not aware of any studies that control for the effects of antibiotic treatment. One possibility is to include a group treated with a different antibiotic that is not effective against the symbiont under study. Under this premise, it would be possible to create a tetracycline control group fed blood supplemented with an antibiotic that is not effective against *Wolbachia* such as penicillin (Fenollar et al., 2003). However, differences between the two compounds extend beyond their effect on *Wolbachia* and consequently this is not an appropriate control protocol.

3.4.2 Effect on non-target microbiota

TEMs of bacteriomes from control females revealed two morphologically distinct symbionts that were similar to the *Wolbachia* and a BEV-like symbiont (BLS) identified in TEMs of bacteriomes from previous studies (Chang and Musgrave, 1973, Hosokawa et al., 2010). BLS is a vertically transmitted γ-proteobacteria that is closely related (more than 97% genetic similarity) to a bacteria found in the leafhopper, *E. variegatus* (Hypša and Aksoy, 1997). It is important to understand the effect that heat and antibiotic treatments have on BLS as differences between treatment groups could just as well be brought about by a reduction in BLS as they could by a reduction in *Wolbachia*.

The tolerances of BLS to antibiotic and heat treatment are unknown. The tolerances of the closely related γ-proteobacteria in the leafhopper are also unknown (Almeido *pers comm.*). Individual BLS are visible in both "+heat" and "−heat" bacteriomes. Bacteriomes of "+αb" individuals were not visualised by TEM as they were much smaller than those removed from heat treated individuals and were too small for processing. It is likely that bacteriome size reduces as the density of *Wolbachia* (and possibly BLS density) diminishes. We know

at the end of heat/antibiotic treatment that *Wolbachia* densities were similar between the "+heat" and "+αb" treatment groups, and it is therefore possible that the bacteriomes from "+αb" females are smaller than those from "+heat" individuals due to lower BLS densities. Given that tetracycline is a broad spectrum antibiotic, it is possible that BLS will be reduced by antibiotic treatment. TEMs suggest the BLS densities may have not been reduced by heat treatment. BLS seemed to occur at higher densities in the "+heat" bacteriomes, but this is likely to be an artefact of the lower densities of *Wolbachia* which previously surrounded the BLS, and the smaller size of the bacteriome. My results suggest that heat treatment may be more specific than antibiotic treatment in targeting *Wolbachia* in bedbugs but quantitative techniques such as qPCR to measure BLS densities in bacteriomes from "+αb", "+heat" and "NCT" individuals are needed.

3.4.3 Effect on 'digestion rate'

Antibiotic treatment reduced 'digestion rate' and egg laying ceased completely. One explanation for this effect is that antibiotics affect the gut biota negatively and hence the host's ability to complete digestion in the normal time-frame. Nutrients normally released by digestion would therefore not be available for egg production. Egg production may also be prevented due to a B vitamin deficiency caused by a reduction in *Wolbachia* loads (Hosokawa et al., 2010). Such a drastic reduction in egg laying rate however was not observed in "+heat" individuals, yet during antibiotic and heat treatment, the reductions in *Wolbachia* load are similar (3.3.1). Antibiotic and heat treatment will have a different mode of action on the *Wolbachia*, it is possible that more *Wolbachia* are still functional in "+heat" individuals so that B vitamin deficiency is not such a problem.

Heat treatment did not affect 'digestion rate' but egg laying was significantly reduced during heat treatment; egg laying recovered to no-treatment control levels after heat treatment had stopped. These results are surprising as higher temperatures are usually associated with an increase in metabolic rate and we may therefore expect an increase in digestion and egg laying rate during heat treatment. It is possible that weekly body mass measures were not sensitive enough to detect a difference in 'digestion rate', daily measures may have been more appropriate. It is possible that the reduction in egg laying rate during heat treatment was due to limited sperm availability, if sperm were damaged by

the heat treatment. Another explanation for a reduction in egg laying rate is that heat treatment may stress the animal, causing tissue damage which the individual has to invest more nutrients in repairing rather than in egg production.

The mass of blood available in females is strongly positively related to her fecundity (total number of eggs) (Appendix 1). In fecundity experiments where *Wolbachia* loads need to be manipulated my results suggest heat treatment should be used in preference to antibiotic treatment as heat appears to have much less of an effect on the 'digestion rate'. In experiments with bedbugs that use antibiotics or heat treatment, the treatment effects on blood meal size and digestion should be controlled as far as possible. Suitable measures include: (i) "blood meal mass" (*ad libitum* fed body mass – body mass prior to feeding) which may indicate if digestion has been disrupted, as undigested residual blood in the gut from previous blood meals would reduce this measure as the mass of fresh blood that could be taken in would be limited and (ii) "*ad libitum* fed body mass" which would indicate the potential amount of blood resources available in individuals (although one must bear in mind, that just because the blood is in the gut, does not necessarily mean that the blood resources are in a state that is available to the individual, and this is why a check of both measures is important).

3.4.4 Summary

In this chapter I have:

1. Determined that tetracycline antibiotics induce a long-lasting (more than 4 weeks) significant reduction in *Wolbachia* load. There is no appropriate control for the antibiotic treatment *per se*, but the technique is easy to implement and the dose can be accurately measured provided the size of blood meal is recorded.
2. Determined that heat treatment induces a short-lived (less than or equal to 2 weeks), significant reduction in *Wolbachia* load. Heat treated individuals in which the *Wolbachia* load has recovered provide a suitable heat treatment control group. The technique is non-invasive, easy to implement and does not appear to eliminate the host's BLS symbiont.
3. Shown that one negative side effects of antibiotic treatment is that tetracycline antibiotics disrupt host digestion.

4. Established an antibiotic treatment protocol (Appendix 5).
5. Established a heat treatment protocol that is appropriate for short-term studies (up to 2 weeks) (Appendix 5).

Chapter 4

Male Fitness with Reduced *Wolbachia* Load

> *"In ...*Cimex lectularius *L. ...the paired mycetomes ... strange to say, they are slightly fused with the* vasa deferentia *of the testes in males. The testis of* Cimex lectularius *consists of seven oval chambers arranged in an arch, and the approximately oval mycetome, resembling a strayed eighth chamber, is suspended on the concave side of the testis* "
>
> Buchner 1965

4.1 Introduction

Many insects harbour symbiotic bacteria that lie within, or are closely associated with, the gut and provide missing nutrients (Douglas, 2009). These symbiotic bacteria are usually passed on to the next generation within the female's eggs (Douglas, 1989). The symbionts' fitness is thus directly correlated with that of their female hosts, and so, apparently, bears no relation to that of their male hosts. The males in effect, present an evolutionary "dead end". Once the nutritionally demanding processes of adult development are complete, symbiont densities tend to decline in male hosts of a range of insect species whilst persisting at high densities in female hosts. This is because the symbionts provide nutrition for the costly process of egg production in females, whereas in males the production of sperm is relatively cheap and is thought not to require the additional nutrients that the symbionts provide (Douglas, 1989). In these respects, bedbugs are unusual: their nutritional symbionts (*Wolbachia*) reside in organs associated with the *testes* in males (rather than the gut) (Carayon 1966; Hosokawa et al., 2010), and these organs show no reduction in size in adult males (Davis, 1956) suggesting *Wolbachia* load does not decline once males have reached adulthood. This challenges current understanding of *Wolbachia*'s function within the male bedbug suggesting a role beyond nutritional provisioning.

4.1.1 *Wolbachia* in males

Males harbour obligate intracellular *Wolbachia* in specialised cells called bacteriocytes, that constitute a pair of organs known as bacteriomes (Hosokawa et al., 2010). In some bedbug strains *Wolbachia* share the bacteriome with one or more species of γ-proteobacteria (Chang and Musgrave, 1973, Chang, 1975, Hypša and Aksoy, 1997, Hosokawa et al., 2010). The *Wolbachia* are thought to provide B vitamins absent from their hosts' blood (Hosokawa et al., 2010). Despite their nutritional role, anatomical dissections reveal that the bacteriomes are not directly associated with the male's digestive system (gut) but instead lie closely associated with the reproductive system, each bacteriome being loosely attached to the *vas deferens* at the base of each of the male's testes (Usinger, 1966, Chang and Musgrave, 1973, Hosokawa et al., 2010). This finding is not unusual for *Wolbachia* (Werren, 1997, Werren et al., 2008), but is unusual for obligate nutritional mutualists which are usually housed in bacteriomes located in the insect's body cavity (haemocoel), fat body or the epithelium of gut caeca (Douglas, 2009). The symbionts of many obligate haematophages are located inside the gut or directly associated with the gut, for example triatomine bugs (Beard et al., 2002), bat flies (Morse et al., 2013) and human lice (Sasaki-Fukatsu et al., 2006).

Wolbachia are commonly associated with the testes in host species where *Wolbachia* behave as reproductive parasites (1.6.1.2) (Werren et al., 2008), causing cytoplasmic incompatibility (CI) between infected males and uninfected females (Werren, 1997). In this situation the *Wolbachia* can be observed mixing with the spermatozoa within the testes as the sperm mature (Callaini et al., 1997, Bressac and Rousset, 1993, Kamoda et al., 2000). The *Wolbachia* are thought to alter sperm development in such a way that *Wolbachia* in the female are required (to either undo the alteration, or put back an essential substrate that the male's *Wolbachia* removed) to assure sperm viability (Callaini et al., 1997, Werren, 1997). In laboratory and naturally occurring bedbug populations, no female biased sex ratio distortion has been reported, and no C.I. has been reported.

Given that nutritional symbionts are considered surplus to requirements in adult males, since the costly processes of growth and development are complete, and the production of sperm is considered to be a relatively cheap process (by comparison to egg production in

females) this raises the question "why do male bedbugs maintain high densities of *Wolbachia* into adulthood?". Furthermore, given that there is no evidence to suggest the bedbug's *Wolbachia* functions as a reproductive parasite, this raises the further question "why are the *Wolbachia* associated with the testes?"

4.1.2 Do *Wolbachia* transfer in the ejaculate?

One hypothesis is that *Wolbachia* are associated with the testes because they are sexually transferred to the female in the ejaculate during mating (Arkwright et al., 1921). In this scenario, the male would provide a route for *Wolbachia* to spread to uninfected individuals (an estimated 0-17% of individuals in a population are uninfected (Sakamoto and Rasgon, 2006)) or to individuals infected with a different *Wolbachia* strain (it is unknown whether there is global variation in the *Wolbachia* strain harboured by bedbugs, but such variation has been reported in some host species (e.g. Werren et al., 1995). Sexual transfer from the male would reduce the *Wolbachia*'s usual tendency towards female bias (Moran and Dunbar, 2006). However, there are very few reports of sexual transmission of symbiotic bacteria in the ejaculate of insects (e.g. Moran and Dunbar, 2006). Sexual transmission of *Wolbachia* from the male germ line is only reported in two insect species, *D. melanogaster* and *D. simulans* (Bressac and Rousset, 1993, Serbus et al., 2008), and then at a very low rate (2%) (Serbus et al., 2008). The paucity of evidence suggests this transmission route for bacterial symbionts is extremely rare (Knell and Webberley, 2004). There have not been any empirical studies to date testing whether *Wolbachia* are transferred in the bedbug ejaculate.

4.1.3 *Wolbachia* may provision the ejaculate with substances that benefit the female

An alternative hypothesis is that through their association with the testes, the *Wolbachia* can influence the production or composition of the ejaculate. The ejaculate comprises two components: sperm and seminal fluid which are manufactured and stored in separate organs within the male's reproductive tract. Sperm are produced in the testes and stored in the seminal vesicles. Seminal fluid is produced by the accessory glands and stored in the seminal reservoirs (Reinhardt and Siva-Jothy, 2007) (Fig. 4.1). Of the two, seminal fluid is more costly for the male to produce (Reinhardt et al., 2011) and its availability limits the

number of successive matings a male can perform in any one time (Reinhardt et al., 2011). Hundreds of compounds have been identified in the seminal fluid (Reinhardt et al., 2009d), including micronutrients in the form of essential amino acids (Rao, 1974), antimicrobial peptides (Otti et al., 2009), antioxidants and compounds used in stress-defence (Reinhardt et al., 2009d). These types of compounds are of interest because they may benefit the female, helping to reduce her overall cost of mating (Reinhardt et al., 2009b).

Females are wounded (Stutt and Siva-Jothy, 2001) and infected (Reinhardt et al., 2005) during traumatic insemination (TI), the bedbug's mode of copulation. Mating tends to occur after the female has fed when, in her bloated state, she is less able to prevent males from mounting her and inseminating (Reinhardt et al., 2009c). Mating rate is male driven and exceeds the optimum rate for the female (Stutt and Siva-Jothy, 2001) resulting in a reduction in the female's longevity (Stutt and Siva-Jothy, 2001). Experimental evidence shows that seminal fluid (not sperm) reduces the longevity cost, and also increases the female's reproductive rate (Reinhardt et al., 2009b). Because the fitness of the female's *Wolbachia* is directly correlated with the fitness of the female host, the female's *Wolbachia* also benefit from these seminal fluid driven effects. Because these seminal fluid effects mean that the male sires more offspring, the male host gains fitness benefits from the seminal fluid, however the male's *Wolbachia* would not directly benefit, as the fitness of the male's *Wolbachia* is not correlated with that of the male host.

There are a number of ways that *Wolbachia* may influence the male's seminal fluid. First, *Wolbachia* may use its type IV secreting system (a ubiquitous feature of *Wolbachia* (Masui et al., 2000)), to secrete an, as yet unidentified, compound that is beneficial to the female, into the ejaculate. Second, the *Wolbachia* may influence the efficient production of seminal fluid by provisioning the male with, for example, nutrients such as B vitamins (Hosokawa et al., 2010).

In this chapter I will examine aspects of male fitness that may be affected by the presence of *Wolbachia*.

4.1.4 Aims of chapter

1. To identify the location of *Wolbachia* within male tissues.
2. To compare the *Wolbachia* loads (population size) between males and females.
3. To experimentally determine whether *Wolbachia* are transferred to the female in the ejaculate.
4. To examine the effect of antibiotic treatment to reduce *Wolbachia* loads on the fitness of male hosts.
5. To examine the effect of reducing the male's *Wolbachia* load using antibiotic treatment on (i) sperm numbers and (ii) seminal fluid volume within the male.
6. To examine the effect of reducing the male's *Wolbachia* load using antibiotic treatment on quantity of ejaculate transferred.

4.2 Materials and Methods

4.2.1 Insects

The insects used in these experiments were 3 days old virgin male and female bedbugs that had been raised as per protocol 2.2.2.1. All insects were imaged and pronotum width recorded (2.2.2.2) and marked with paint so that individuals could be distinguished (2.2.2.3). Insects were maintained individually in 5 mL plastic tubes with a strip of filter paper (at 26°C 70% RH constant controlled light conditions) except during feeding when individuals were transferred to communal pots. Insects were fed weekly heparinised sheep's blood via an artificial membrane feeding system (2.2.3) and the *ad libitum* fed body mass and blood meal mass of individuals were recorded (2.2.3.4).

4.2.2 Dissections

All tissue dissections were performed in PBS (NaCl 150 mM, Na_2HPO_4 10 mM, pH 6.5) under a stereomicroscope Leica MZ8 (Wetzlar, Germany). Tissues for qPCR analysis were processed as detailed in section 2.4. Tissues for size measurements were mounted on a microscope slide under a coverslip bridge of 0.1 mm depth and imaged under either a compound microscope (seminal reservoirs − as detailed in 2.3.2), or stereomicroscope (seminal vesicles and mesospermaleges − as detailed in 2.3.3). Tissue volumes were subsequently calculated from the images using the equation $V=TA$, where V is the volume of the sample (mm^3), T is the thickness of the coverslip bridge (0.1 mm) and A is the surface area of the sample (mm^2).

4.2.3 Location and load of male's *Wolbachia*

qPCR analysis of the *Wolbachia* load was carried out on the following tissues dissected from *n*=5 males: one bacteriome, two Malpighian tubules, six legs, head and foregut, anterior midgut, median midgut, posterior midgut, hindgut, fat body, a section of cuticle (half of the 3rd dorsal thoracic segment with basement membrane attached), one testis, one seminal vesicle and one seminal reservoir (Fig. 4.1). These are the same organs chosen by Hosokawa et al. (2010) to determine the presentation of *Wolbachia* in the Japanese JESC and TUA *C. lectularius* strains. In this way I can see whether the presentation of *Wolbachia* in the S1 *C. lectularius* strain is similar. Hosokawa et al. (2010) did not assess the gut for the presence of *Wolbachia*, I therefore included the gut in my samples to determine whether *Wolbachia* resided there. The gut was dissected into its distinct functional and anatomical parts (Azevedo et al., 2009). Each part of the gut is distinct in form and function; the foregut is involved in ingestion and the mechanical softening of the food, the anterior midgut is for storage, the median and posterior midgut is for digestion and absorption and the hindgut holds the waste products before they are excreted from the body (Azevedo et al., 2009). For details of qPCR methods, see 2.4.

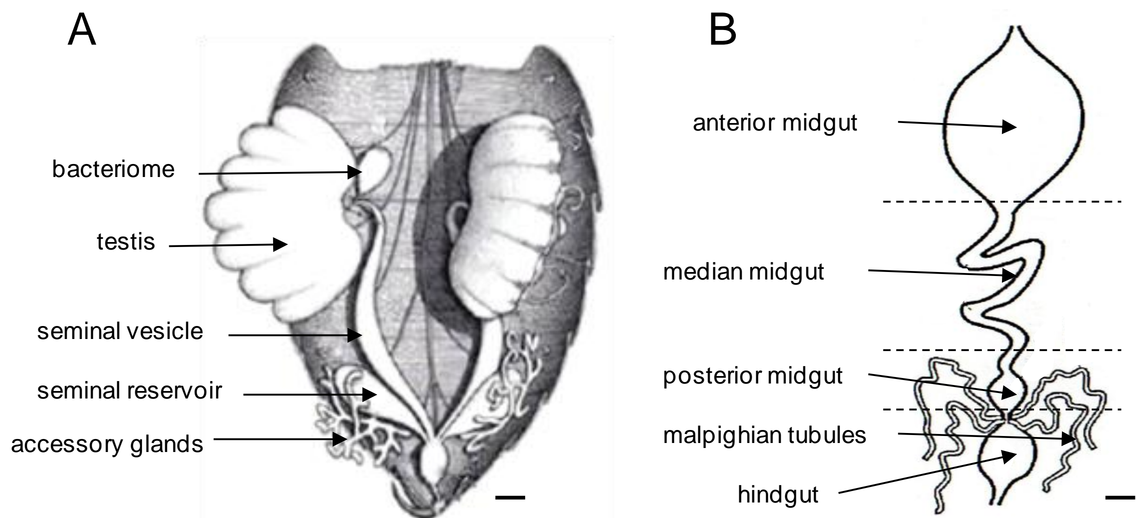


Figure 4.1 Internal anatomy of a male bedbug to show location of dissected tissues. (A) ventral view of thorax with gut removed, (modified from (Davis, 1956)). (B) the gut with different regions labelled (modified from (Azevedo et al., 2009)). Bar, 0.5 mm.

4.2.4 Comparison of *Wolbachia* load between males and females

The *Wolbachia* load in the whole bodies of n=5 males and n=5 females; and in one dissected bacteriome from n=5 males and n=5 females was determined by qPCR analysis (see 2.4 for details) and compared.

4.2.5 Sexual transmission of *Wolbachia* from male

To assess whether *Wolbachia* are transferred to the female in the male's ejaculate, the *Wolbachia* loads present in the mesospermaleges of n=10 virgin and n=10 non-virgin (just mated) females were compared. Females in the non-virgin group were mated under standard protocols (2.2.2.4). Females in the virgin group were handled in the same way, but were only exposed to males; no copulations occurred. Within 20 minutes of copulation, whilst the ejaculate was still present in the mesospermalege (sperm leave the mesospermalege at around 4 hours post copulation (Carayon, 1966)) the mesospermaleges from virgin and non-virgin females were removed by dissection and the *Wolbachia* loads compared using qPCR (2.4).

4.2.6 Fitness effects of male's *Wolbachia*

4.2.6.1 Treatment groups

To determine the effect of reducing *Wolbachia* loads on the fitness of male hosts, two treatment groups that varied in their *Wolbachia* loads were generated using antibiotic treatment as protocol (A4.1): + antibiotics (+αb) to generate males with reduced *Wolbachia* loads and – antibiotics (−αb) to generate males with higher, unmanipulated, baseline levels of *Wolbachia*. Antibiotics were used in preference to heat treatment, because previous studies suggest that heat treatment may adversely affect sperm production (Omori, 1941) and male fertility (Fig. A4.1). To control for possible differences in the availability of B vitamins caused by reducing *Wolbachia* loads, two B vitamins control groups were similarly generated except the blood was supplemented with B vitamins: antibiotic treatment + B vitamins (+αb+B) and – antibiotics + B vitamins (+αb+B). B vitamins were delivered to these groups at concentrations based on those published in Hosokawa et al., (2010) (Table 4.1).

To reduce possible confounding variation in the mass of blood meal taken up by individuals (antibiotic treatment lowers 'digestion rate' leading to a reduction in the mass

of blood meal taken up during a feed (3.3.2)) males that took up less than 1 mg of blood during any of the two feeds were excluded. One week after the final feed, the mean *Wolbachia* load of each treatment group was determined from a subsample of n=6 individuals using qPCR (2.4).

Table 4.1 List of B vitamins added to the blood meal, reproduced from Hosokawa et al., (2010).

Compound	Final concentration
Thiamine	100 µg mL^{-1}
Riboflavin	20 µg mL^{-1}
Nicotinic acid	100 µg mL^{-1}
Pantothenic acid	100 µg mL^{-1}
D-biotin	1 µg mL^{-1}
Folic acid	30 µg mL^{-1}
Cobalamin	1 µg mL^{-1}
Choline chloride	185 µg mL^{-1}
Meso-inositol	118 µg mL^{-1}

4.2.6.2 Measures of male fitness

The effect of reducing *Wolbachia* loads with antibiotic treatment on male fitness was measured indirectly through fecundity measures of the female with whom the treatment males were mated. Males from the four treatment groups were mated with the females following standard protocols (2.2.2.4). For each female, the egg laying rate (total number of eggs that hatched per week) and the proportion hatch success (proportion eggs laid that hatched per week (females that laid no eggs were excluded)) were recorded over a period of two weeks. To control for differences in amount of blood resources within females which may affect fecundity measures (Appendix 1), egg laying rate was expressed per mg *ad libitum* fed female body mass.

4.2.6.3 Ejaculate availability

Two measures of ejaculate availability were compared between the four treatment groups (4.2.6.1): (i) number of sperm and (ii) seminal fluid volume. The volume of the seminal vesicles was used as a surrogate measure of sperm numbers, and the volume of the seminal reservoirs was used as a surrogate measure of seminal fluid volume (Reinhardt et al., 2011). The tissues were fixed in 3% glutaraldehyde in 0.1 M PBS for 30 minutes (to make

the structures firmer and less prone to damage from handling), washed twice in PBS and transferred to a glass microscope slide for imaging. The seminal vesicle volume was not related to body size (pronotum width) (R^2=0.0415, $F_{1,13}$=0.563, P>0.05), however seminal reservoir volume was related to body size (R^2=0.505, $F_{1,13}$=13.29, P<0.01) (Fig. 4.2). To control for the effects of male body size, the volume of the seminal reservoirs was expressed per unit pronotum width (mm).

The volume of ejaculate transferred during a standard mating (2.2.2.4) by "+αb" and "–αb" males only. These males were mated with virgin females as standard (2.2.2.4) after which the mesospermalege was dissected from each female and placed on a microscope slide under a coverslip bridge (0.1 mm depth) and imaged down a compound microscope (2.3.2). The ejaculate could be clearly seen inside the mesospermalege (Plate 4.1). The volume of the ejaculate transferred was calculated from each image. This method was highly repeatable (R=0.976, $F_{13,28}$=120.73, P<0.0001).

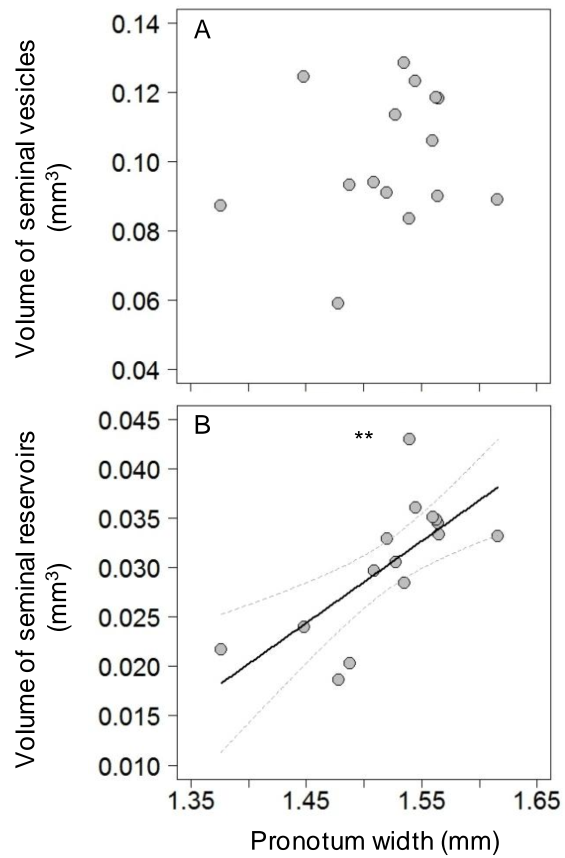


Figure 4.2 The effect of male's body size (pronotum width) on the size of his reproductive organs: (A) mean volume of seminal vesicles (R^2=0.0415, $F_{1,13}$=0.563, P>0.05); (B) mean volume of seminal reservoirs. The fitted line (±1SE) is y=- 0.0957 + 0.0829x, (R^2=0.505, $F_{1,13}$=13.29, P<0.01).

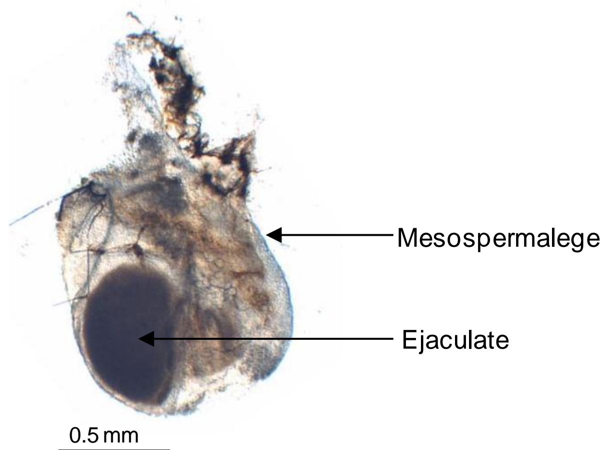


Plate 4.1 Mesospermalege from recently mated female with the ejaculate visible inside.

4.2.7 Data analysis

Data were analysed as detailed in 2.5.

4.3 Results

4.3.1 Location and load of male's *Wolbachia*

No data from the posterior mid gut from the males are presented because the amounts of DNA extracted from these samples were too small for qPCR. The majority of the male's *Wolbachia* were found in the bacteriomes, where they occurred at a density at least 100,000 times greater than in the other tissues tested (ANOVA: $F_{11,48}=38.26$, $P<0.0001$ on $\log_e(x)$ data) (Fig. 4.3). *Wolbachia* loads in the male's reproductive organs (testes, seminal vesicles, seminal reservoirs) were no different to other male tissues (except the bacteriomes).

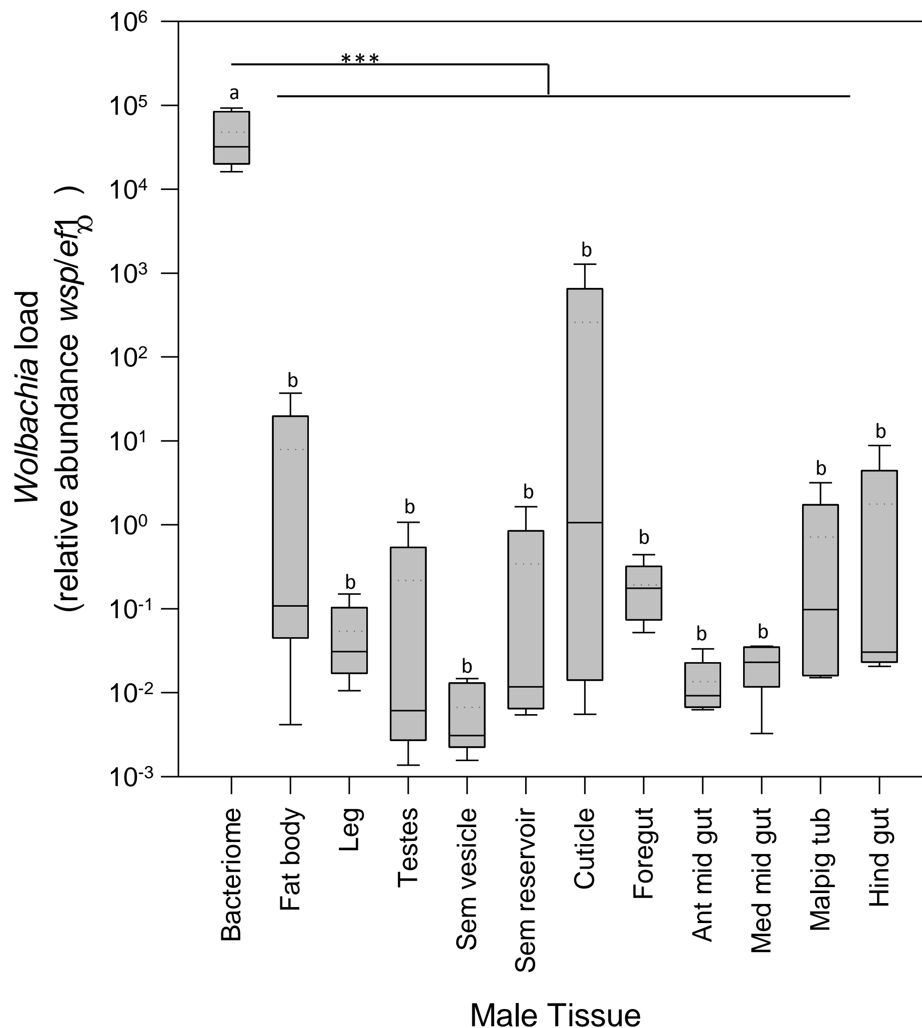


Figure 4.3 Median (solid line) and mean (dotted line) *Wolbachia* load in male tissues. Means with the same letter do not differ from each other (Tukey mcp test $P>0.05$). $N=5$ each group.

4.3.2 *Wolbachia* loads of males and females

There was no difference in the *Wolbachia* loads of males and females from qPCR analysis of either their whole bodies (t=0.610, 4.18 df, P>0.05 on $\log_e(x)$ data), or of their bacteriomes (t=1.28, 8 df, P>0.05) (Fig. 4.4).

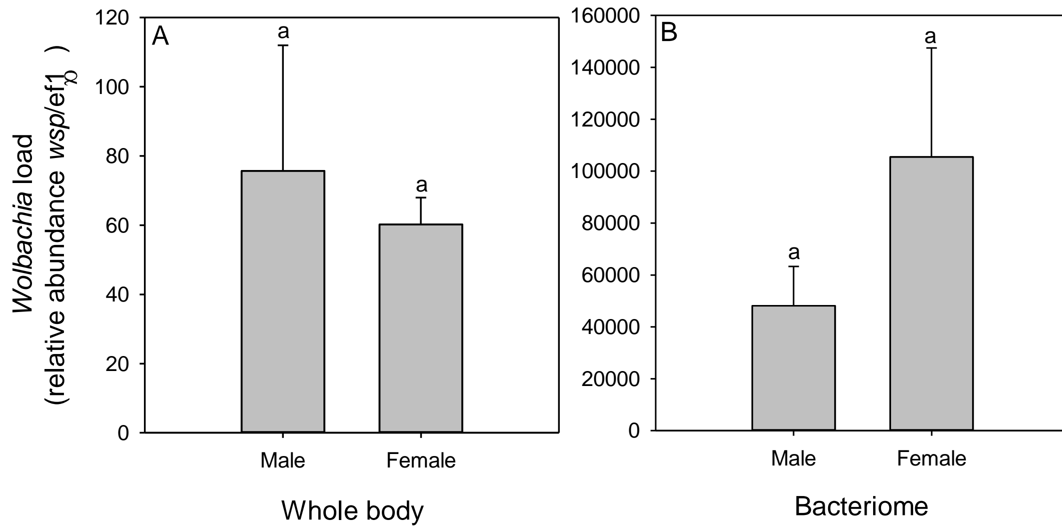


Figure 4.4 A comparison of the mean *Wolbachia* loads in male and female bedbugs revealed by qPCR analysis of (A) their whole bodies and (B) one dissected bacteriome. *N*=5 each group, bars represent 1SE.

4.3.3 Sexual transmission of *Wolbachia*

There was no difference between the mean *Wolbachia* loads in mesospermaleges dissected from virgin and mated females (Mann-Whitney U test: U=36, n_1=9, n_2=10, P>0.05) (Fig. 4.5).

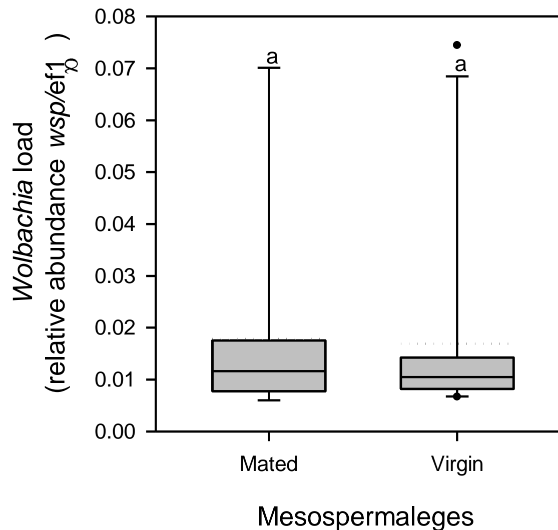


Figure 4.5 A comparison of the median *Wolbachia* loads (solid line) of the mesospermaleges from mated (n=9) and virgin (n=10) females. Means are indicated by the dotted lines.

4.3.4 Fitness effects of male's *Wolbachia*

4.3.4.1 Wolbachia *load of the treatment groups*

There were significant differences in the *Wolbachia* load between treatment groups. Antibiotic treatment caused a significant reduction in *Wolbachia* load (ANOVA: $F_{1,20=}$93.03, P<0.0001 on $\log_e(x+1)$ data), and so did B vitamin supplementation (ANOVA: $F_{1,20}$=79.77, P<0.0001 on $\log_e(x+1)$ data). There was a significant interaction between antibiotic and B vitamin treatments (ANOVA: $F_{1,20}$=77.84, P<0.0001 on $\log_e(x+1)$ data). *Wolbachia* load was higher in the absence of both antibiotics and B vitamin supplementation than in any other treatment combination (Tukey mcp test P<0.05), there was a similar, but non-significant effect of the B vitamins in the presence of antibiotics (Tukey mcp test P>0.05) (Fig.4.6).

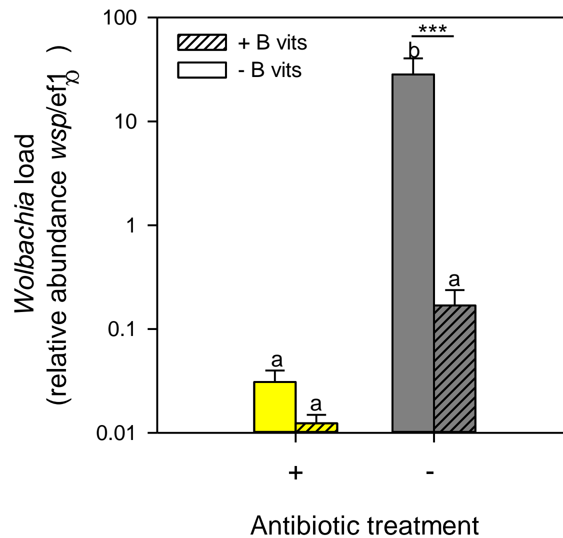


Figure 4.6 The effect of antibiotic treatment (with or without B vitamins) on male *Wolbachia* loads. Males were fed blood supplemented with (+) or without (-) antibiotics and with (+) or without (-) B vitamins in a full factorial design. N=5 each group, means with the same letter do not differ from each other (Tukey mcp test P>0.05), bars represent 1SE. There were significant main effects of antibiotics and B vitamins and a significant interaction between these treatments.

4.3.4.2 Male fitness

There was a highly significant effect of males' treatment on the egg laying rate (number of hatched eggs per week per mg) of their female partners (*Kruskal-Wallis test:* H=9.85, 3 df, P<0.05). Egg laying rate was significantly higher for "−αb" males compared to the other three treatment groups (non parametric mcp test P<0.05) (Fig. 4.7). There was no effect of the males' treatment on egg hatch success (the proportion of eggs that hatched) (*Kruskal-Wallis test:* H=7.39, 3 df, P>0.05) (Fig.4.7). There were no observable differences in the digestion and feeding of males in the four treatment groups. There were no differences in either (i) the mass of blood meal individuals had taken in (ANOVA: $F_{3,56}$=0.53, P>0.05) (Table A3.6) or (ii) in their *ad libitum* fed body masses (ANOVA: $F_{3,56}$=0.94, P>0.05) (Table A3.7).

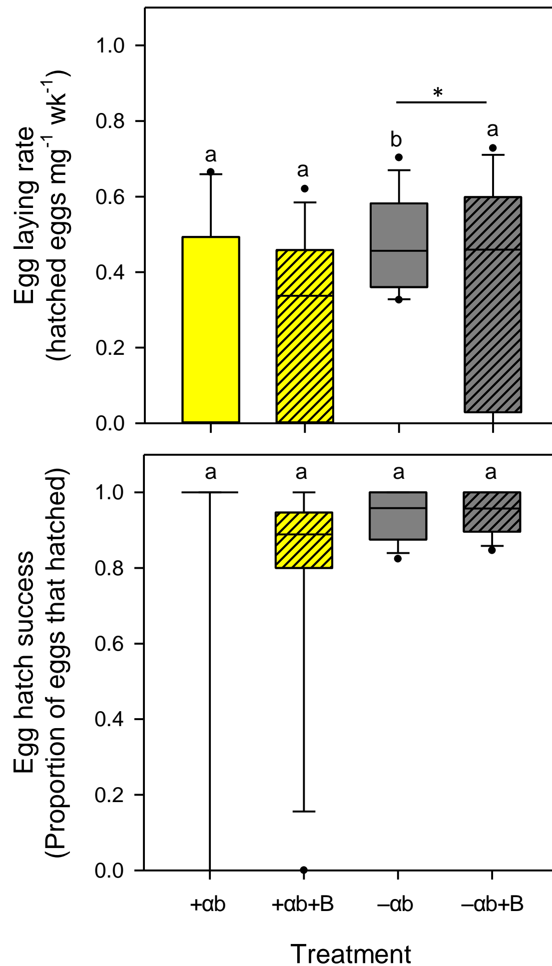


Figure 4.7 The effect of antibiotic treatment +/− supplementary B vitamins on indirect measures of male fitness (note "−αb +B" males had lower *Wolbachia* loads compared to "−αb" males). (A) Median egg laying rate (solid line) (number of eggs laid per week that hatched, expressed per mg replete female body mass), *n*=16 each group. (B) Median egg hatch success (solid line) (proportion of eggs that hatched) (sample sizes are "−αb": *n*=7; "+αb+B": *n*=11; "−αb": *n*=16; "−αb+B": *n*=12). Dotted lines indicate means, medians with the same letter do not differ from each other (Dunn's test $P>0.05$).

4.3.4.3 Ejaculate availability

Seminal fluid volume and sperm numbers were much lower in males in the "+αb" and "+αb+B" groups compared to "−αb" and "−αb+B" groups (Fig. 4.8). There was (i) a highly significant main effect of antibiotics (ANOVA: $F_{1,56}=58.49$, $P<0.0001$), (ii) no main effect of supplementary B vitamins ($F_{1,56}=0.30$, $P>0.05$) and (iii) no interaction effects of these treatments ($F_{1,56}=0.92$, $P>0.05$) on volume of seminal fluid. Similarly there was (i) a highly

significant main effect of antibiotics (ANOVA: $F_{1,56}$=40.93, P<0.0001), (ii) no main effect of supplementary B vitamins (($F_{1,56}$=0.29, P>0.05) and (iii) no interaction effects of these treatments ($F_{1,56}$=0.40, P>0.05) on sperm numbers. There were no differences in male body size in the treatment groups that could explain these results (ANOVA: $F_{3,56}$=1.21, P>0.05) (Table A3.8).

Despite differences in sperm numbers and seminal fluid volume in "+αb" and "−αb" males, there was no effect of antibiotic treatment on the volume of ejaculate transferred; there was no difference in the volume of ejaculate measured in the mesospermaleges of females mated to "+αb" and "−αb" males (t-test: t=1.53, 12 df, P>0.05) (Fig. 4.8).

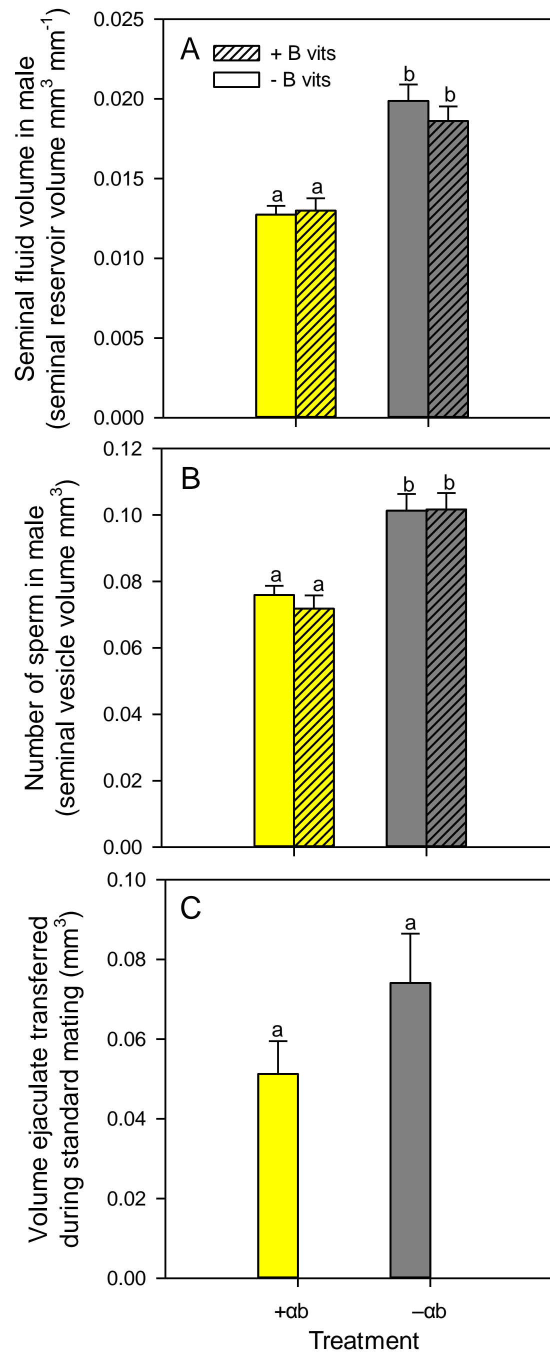


Figure 4.8 The effect of antibiotic treatment +/− B supplementary B vitamins on measures of ejaculate availability (note "−αb+B" males had lower *Wolbachia* loads compared to "−αb" males). (A) Mean volume of seminal fluid (from surrogate measures of mean volume of seminal reservoirs (mm^3) expressed per mm pronotum width), n=15 each group. (B) Mean number of sperm (from surrogate measures of mean seminal vesicle volume (mm^3)), n=15 each group. Groups with the same letter do not differ from each other (Tukey mcp test P>0.05). (C) Mean volume of ejaculate (mm^3) transferred during a standard mating for two treatment groups, measured inside the dissected mesospermaleges from mated females, n=7 each group. Groups with the same letter do not differ from each other (t-test P>0.05). Bars represent 1SE.

4.4 Discussion

My results confirm results from previous investigations that the *Wolbachia* in male bedbugs are mainly located in the bacteriomes (Hosokawa et al., 2010). My results also show that *Wolbachia* loads are similar in males and females. I found no evidence for sexual transfer of *Wolbachia* in the ejaculate, suggesting that transovarial transmission, observed by Hosokawa et al., (2010), is the main route for *Wolbachia* to pass to the next generation. I found some evidence to suggest that the male's *Wolbachia* can effect an increase in the fecundity of his female partner, at least in the short term. One possible mechanism is that *Wolbachia* directly or indirectly affect aspects of the male's ejaculate. My results suggest *Wolbachia* do not affect the quantitative aspects of the ejaculate (sperm number and seminal fluid volume) but may instead affect ejaculate quality. The underlying mechanisms are far from clear. Antibiotic treatment was associated with a reduction in the quantity of both ejaculate components, which may be a side effect of antibiotic treatment, or indicative of another symbiont in the male bedbug that may affect ejaculate quantity; one possible candidate is BLS. All these findings are discussed in more detail below.

4.4.1 The majority of *Wolbachia* are located in the bacteriomes

Wolbachia loads were quantified as the number of *wsp* gene copies per copy of the *ef1*α gene. This assumes that the *ef1*α gene copy number is stable between tissues, as variation in the *ef1*α gene copy would effect apparent variation in *Wolbachia* load. My results show that the majority of a male bedbug's *Wolbachia* reside within the bacteriomes with very few *Wolbachia* found within the reproductive organs (testes, seminal vesicles and seminal reservoirs) with which the bacteriomes are closely associated, or elsewhere within the body. By restricting the location of nutritional symbionts to bacteriomes, it is thought that hosts may better control symbiont populations, and the bacteriome may also help protect the symbiont from being attacked by the host's immune system (Douglas, 1989).

4.4.2 *Wolbachia* are found in equal densities in males and females

In most insects that harbour nutritional symbionts, their symbiont load begins to decline once the host has reached adulthood (Douglas, 1989). This decline is more rapid in adult males which, once the metabolically/nutritionally demanding processes of growth and development are complete, no longer require the nutritional benefits of the symbionts

because sperm production is 'cheap', and similarly, the symbiont is vertically transmitted through the female, so does not require the male for its passage. The situation in the female is quite different, egg production is nutritionally demanding, and symbionts depend on the female for transmission to the next generation. These differences are thought to explain why symbiont loads are usually lower in male hosts than female hosts (Douglas, 1989; Douglas, 2009).

I found no difference between the *Wolbachia* loads of males and females from whole body preparations, or from dissected bacteriomes. One explanation for my results is that the males and females, which were analysed for their *Wolbachia* loads at 14 days post-eclosion, were simply not old enough for a difference to be evident. However, this is unlikely as *Wolbachia* load has been observed to change over this time frame, as my results in section 3.2 demonstrate.

4.4.3 No sexual transfer of *Wolbachia*

I found no difference in the *Wolbachia* load in the mesospermaleges of virgin and mated females suggesting that *Wolbachia* are not transferred from males to females during TI. Males therefore appear to present a "dead-end" to *Wolbachia*. This makes the previous observation that *Wolbachia* do not decline in adult males, an intriguing one. It may be that the male has 'enslaved' the *Wolbachia* and is benefiting from its role as a nutritional mutualist.

4.4.4 Fitness effects of the male's *Wolbachia*

First I discuss the effects of manipulating the male's *Wolbachia* load using antibiotic treatment on male and female fitness. Then I discuss whether these effects are likely to be a direct consequence of antibiotic treatment or whether they are due to the manipulation of *Wolbachia* load. I then discuss some mechanistic possibilities, which are not mutually exclusive, as to how *Wolbachia* may achieve these effects including (i) *Wolbachia*'s proposed role as a B vitamin provider and (ii) *Wolbachia*'s affect on the quantity and quality of the male's ejaculate. Finally I look at the evidence to suggest a beneficial role of unidentified symbiont(s) on the production of the ejaculate.

4.4.4.1 Male's Wolbachia *may increase male fitness*

Males in the three treatments that were fed blood supplemented with antibiotic and/or B vitamins ("+αb", "+αb+B" and "-αb+B") had significantly lower *Wolbachia* loads than males fed blood alone ("-αb"). Similarly the female partners of males in the three treatments that were fed blood supplemented with antibiotic and/or B vitamins had a significantly lower egg laying rate than males fed blood alone. This is unlikely to be an effect of the direct transfer of antibiotics to the female in the male's ejaculate because egg laying rates did not differ between females mated to B vitamin-fed males only and those mated to males fed blood supplemented with antibiotics ± B vitamins. These results suggest that the male's *Wolbachia* may increase male fitness in at least the short term. These results also suggest that the female may gain fitness benefits from the male's *Wolbachia* through increased egg laying rates. No effect of supplementing the male's blood meal with antibiotics or B vitamins on the egg hatch success was found.

One explanation is that these effects were caused by the antibiotic treatment if, for example, antibiotic treatment affects sperm viability. No study to date has investigated whether antibiotic treatment affects sperm viability in bedbugs, however insights may be gained from breeder's studies which have investigated the effect of adding antibiotics to sperm stocks to remove microbial contaminants *in vitro*. Effects reported vary: for example the addition of antibiotics to honey bee sperm may ultimately increase sperm viability (Locke & Peng, 2008) or have no effect (Andere et al., 2011). Yet in vertebrates, negative effects have been observed, for example in human sperm (Hargreaves et al., 1998) and Nile Tilapia *Oreochromis niloticus* (Segovia et al., 2000). Furthermore, the effects change if the antibiotics are administered *in vitro* or *in vivo*. For example in contrast to the *in vitro* study by Hargreaves et al., (1998) above, an *in vivo* investigation into the effects of commonly prescribed antibiotics on the viability of human sperm found a positive effect brought about by a reduction in the host's immune response (in response to the lower parasite load as a result of antibiotic treatment), leading to fewer sperm being damaged by autoimmune reactions (Skau and Folstad, 2011). These examples suggest that it is possible that the fitness reduction in antibiotic treated males was caused by a reduction in sperm viability caused by the antibiotic treatment itself, however an alternative explanation is that the fitness reduction was caused by the significant reduction in *Wolbachia* load.

I found that the addition of antibiotics to the bedbug's blood meal caused a significant reduction in *Wolbachia* load. Distinguishing the possible effects of antibiotics on sperm viability from the effects mediated by a reduction in *Wolbachia* load in "+αb" and "+αb+B" males is not possible. However, I also found that the addition of B vitamins to the bedbug's blood meal caused a significant reduction in *Wolbachia* load and that similar fitness reductions were observed in B vitamin fed males ("−αb+B"). Like antibiotics, it is possible that the addition of B vitamins to the blood meal affected sperm viability, especially if B vitamins altered the environmental biochemistry. Sperm are sensitive to changes in environmental conditions including temperature, environmental biochemistry and microbes in the environment (Skau & Folstad, 2011). However, a positive effect of additional dietary B vitamins on spermatogenesis (Watanabe et al., 2003; Boxmeer et al., 2007) and sperm motility (Condorelli et al., 2012) has been observed in vertebrates. In addition if B vitamins reduced sperm viability, then why would males harbour high densities of *Wolbachia* which produce B vitamins (Hosokawa et al., 2010)? It seems more likely that in "−αb+B" males the reduction in fitness was caused by the reduction in *Wolbachia* load.

These results suggest a possible beneficial effect of *Wolbachia* on male fitness, a first for a system where C.I. does not operate. Previous examples include: (i) in the flour beetle *T. confusum*, *Wolbachia*-infected males have a paternity advantage in multiply mated females (Stevens, 1993), possibly through a *Wolbachia*-mediated competitive advantage in sperm competition (Wade and Chang, 1995a), and (ii) *Wolbachia* may mediate a higher mating rate in infected *D. simulans* and *D. melanogaster* compared to uninfected males (De Crespigny et al., 2006, Karr et al., 1998, Reynolds and Hoffmann, 2002). In my experiments, as females were singly mated and mating rate was controlled, the mechanistic explanations of the previously cited experiments cannot explain how *Wolbachia* are increasing male fitness in the bedbug system. I investigated (i) if B vitamins were involved and (ii) if *Wolbachia* affected the production of ejaculate in the male.

4.4.4.2 Role of B vitamins

From my results it is not clear whether *Wolbachia* mediate the observed fitness effects through their primary role in supplying B vitamins (Hosokawa et al., 2010). *Wolbachia*

load declined in response to B vitamin supplementation of the blood meal which may be expected if *Wolbachia* were important in providing B vitamins to the male. B vitamin supplementation of the blood meal may render *Wolbachia* surplus to the host's requirements. Symbionts are costly to maintain (Douglas, 1989), so with the host no longer needing its *Wolbachia*, the *Wolbachia* population size would be expected to be managed downwards. The negative fitness effects associated with a reduction in *Wolbachia* load however, were not ameliorated by supplying supplementary B vitamins to males via the blood meal, a method previously shown to reverse the negative effects of reduced *Wolbachia* loads in females (Hosokawa et al., 2010). A different route of administering B vitamins to males may have been effective, such as injection, and further investigations are needed to determine whether this is the case.

The next question is, why would males need B vitamins? Firstly, perhaps as in vertebrates, B vitamins promote spermatogenesis and enhance sperm viability through increased motility (e.g. Watanabe et al., 2003; Boxmeer et al., 2007; Condorelli et al., 2012). Secondly, it is possible that just as the females require B vitamins for egg production, males require B vitamins to produce high quality seminal fluid, a nutritionally costly process compared to the production of sperm (Reinhardt et al., 2011). That *Wolbachia* may affect the seminal fluid component of the ejaculate is further supported by observations that seminal fluid is associated with elevated egg laying rate in the female (Reinhardt et al., 2011). Thirdly, it is possible that the ejaculate is a means to deliver additional B vitamins to the female to increase her fitness. In this way the *Wolbachia* would be acting to provide B vitamins by way of a nuptial gift.

4.4.4.3 Quantitative effect on ejaculate components

My results show that "+αb", "+αb+B" and "−αb+B" males had significantly lower *Wolbachia* loads than "−αb" males. The size of the males' seminal vesicles and seminal reservoirs in "+αb", "+αb+B" males were significantly smaller than for "−αb" and "−αb+B" males. The groups with significantly lower *Wolbachia* loads were not the same groups as those that had smaller seminal vesicles and seminal reservoirs; "−αb+B" males had lower *Wolbachia* loads but no reduction in organ size. All groups with smaller seminal vesicles and seminal reservoirs had been antibiotic treated, suggesting that this size

reduction was associated with antibiotic treatment rather than a reduction in *Wolbachia* load.

Tetracycline effective against a range of different bacteria and it is possible that non-target symbiotic bacteria besides *Wolbachia* were reduced by antibiotic treatment. One possible candidate is the bacteria that coexist alongside *Wolbachia* within the bacteriomes, thought to be BLS (Plate 3.1). The role of BLS in bedbugs is currently unknown, however given that BLS are γ-proteobacteria and are located within a bacteriome, one possibility is that BLS serve a nutritional role (Douglas, 2009). If BLS are susceptible to tetracycline and if BLS provide nutrients needed by the males for ejaculate production, this may explain why antibiotic treated males had smaller seminal vesicles and seminal reservoirs reflective of lower quantities of sperm and seminal fluid respectively.

My results suggest that the male's *Wolbachia* increase male fitness and may increase female fitness via male effects. Further investigation is required to test whether this snapshot is reflective of the long term outcomes. For example, females with a raised reproductive rate may have a shorter reproductive lifespan, so that their overall fitness may be the same or even lower than females mated with "+αb" males with reduced *Wolbachia* loads. It is unclear how *Wolbachia* could benefit from increasing male fitness, or indirectly increasing female fitness given that the male presents a dead-end to the *Wolbachia*. Thus in male bedbugs, the *Wolbachia* symbiosis may present as a type of enslavement (Table 1.1), whereby the host benefits and the symbiont gains no benefit from the symbiosis.

4.4.5 Summary

In this chapter I have:

1. Established that bedbug males house *Wolbachia* in paired bacteriomes that are associated with the testes.
2. Determined that there is no difference in the *Wolbachia* load between males and females either in their bacteriomes, or as an average in their whole bodies.
3. Established that *Wolbachia* are not transferred to the female in the ejaculate during mating.

4. Determined that the male's *Wolbachia* load may increase both male and female lifetime reproductive success by increasing the female's egg laying rate (at least in the short-term).
5. Established that *Wolbachia* do not appear to affect sperm or seminal fluid production or the volume of ejaculate transferred. Another of the male's symbionts, possibly the BLS, may have a positive effect on ejaculate production.

Chapter 5

Effects of Reducing the Female's *Wolbachia* Load

"I would lay down my life for two brothers or eight cousins"

JBS Haldane

5.1 Introduction

Transovarially transmitted symbionts, such as *Wolbachia,* may further their own fitness by increasing that of their female host through a variety of mechanisms. It remains to be determined if they increase the fitness of the female host's offspring. Mechanisms that increase offspring fitness will benefit the *Wolbachia* compared to the host because the relatedness of *Wolbachia* will remain high through the host generations (*Wolbachia* reproduce asexually) whereas the relatedness of the host to its kin will decrease through the generations. Despite this there have been no reports of symbiont-mediated kin selection to date. Experimental evidence suggests that *Wolbachia* in bedbugs increase host fitness by providing B vitamins (Hosokawa et al., 2010) but whether these fitness benefits extend to the host's offspring is unknown. Given that bedbugs are a pest species, understanding the influence of *Wolbachia* on long-term host reproductive success may be important for informing effective control measures.

5.1.1 *Wolbachia* in females

Previous investigations show that females harbour most of their intracellular obligate *Wolbachia* in paired bacteriomes embedded in the fat body, one either side of the ovaries (Carayon 1966, Hosokawa et al., 2010). There are also substantial but lower densities found in the ovaries where *Wolbachia* infect the female's eggs, ensuring their transmission to the next generation (Hosokawa et al., 2010).

Early investigations highlighted the importance of *Wolbachia* to host fitness. Chang (1974) showed that following heat treatment, *Cimex lectularius* females suffered a 90% reduction in egg laying rate which he attributed to the heat-induced reduction in the symbiont load

within the bacteriomes. However Chang's heat experiments are confounded; mated females were heat treated and thus it is likely that the sperm inside of the mated females would have been damaged by the heat treatment itself, as observed by Omori (1941). Therefore Chang's results may have been caused by a lack of viable sperm, rather than a symbiont deficiency.

A recent investigation by Hosokawa et al., (2010) demonstrated the importance of *Wolbachia* to bedbug fitness. In this study, antibiotic treatment was used to reduce *Wolbachia* loads in *C. lectularius* which resulted in a significant decline in the female's fitness with lower egg hatch and development success rates and longer adult development times. Hosokawa et al., (2010) also showed that these negative fitness effects could be reversed by supplementing the blood meal with B vitamins, suggesting that the observed fitness decrease was caused by a deficiency in B vitamins which the *Wolbachia* were therefore thought to provide. A critical look at the experimental design used in this study however draws the robustness of these results into question.

Hosokawa et al., (2010) measured the effect of antibiotic treatment ± supplementary B vitamins on the number of eggs laid and the percentage hatch success by i) summing the total eggs laid by 3 females per week over 8 weeks and ii) recording the % of these eggs that hatched per week (Fig. 5.1). These results are confounded by pseudoreplication and small sample sizes as each data point was generated by the same 3 females. Although statistical tests are not reported (only *P* values are indicated), the presentation of results suggests an ANOVA was carried out, using weeks as the unit of replication. A more robust design would have been to count the number of eggs laid per female per week, using a sample size of at least n=10 females. The results could than have been analysed with a repeated measures ANOVA. Furthermore there is no indication that mating rate which affects the female's survival, and the size of blood meal which affects her egg laying rate were controlled (Stutt and Siva-Jothy, 2001; Fig A1.3).

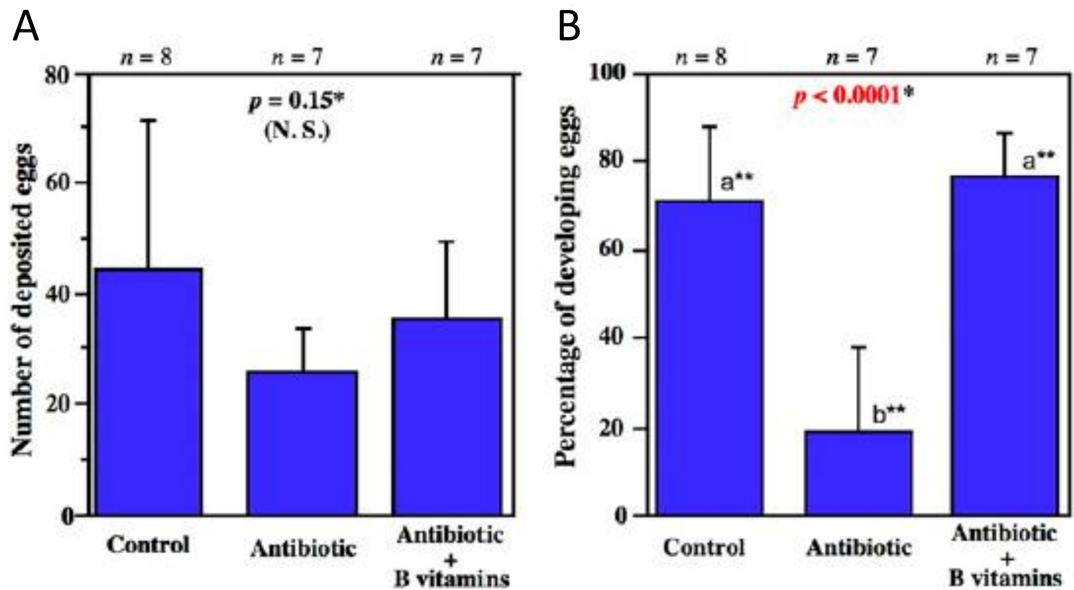


Figure 5.1 The results from a study by Hosokawa et al., (2010) showing the effect of treatment group on (A) mean sum of the number of eggs deposited per week by 3 females and (B) mean percentage of eggs laid per week by 3 females that developed into first instars. Bars represent 1SE, groups with the same letter do not differ, *n* values represent the number of weeks, (weeks when no eggs were laid were excluded). Figure reproduced and modified from Hosokawa et al., (2010).

Hosokawa et al., (2010) also investigated whether treatment affected the percentage of adult emergence and nymphal period (in other words the time taken for first instars to develop into adults) (Fig. 5.2). To determine this, first instars from eggs laid by 3 females in each of the three treatment groups were collected and subdivided into groups of n=10, and then "*randomly assigned to one of the three experimental treatment groups*" ("control", "antibiotic" and "antibiotic + B vitamins"). Although this would increase the genetic variability between nymphs within the treatment groups (as nymphs would have been born from up to 9 mothers instead of 3), this adds potentially confounding variation between nymphs in the treatment history of their mothers, differences that were not taken into account here.

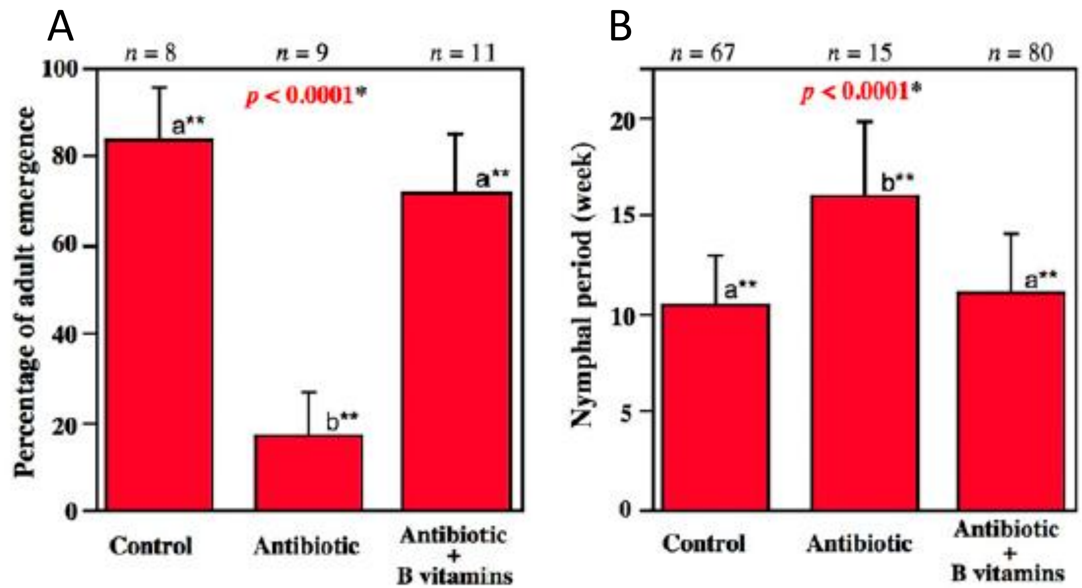


Figure 5.2 The results from a study by Hosokawa et al., (2010) showing the effect of the treatment group of first instars' on (A) percentage adult emergence and (B) nymphal period (time taken to develop into adults). Bars represent 1SE, groups with the same letter do not differ, *n* values in (A) represent the number of groups of *n*=10 first instar nymphs and in (B) represent the number of emerging adults. Figure reproduced and modified from Hosokawa et al., (2010).

Hosokawa et al., (2010) also concluded that antibiotic fed nymphs took longer to develop than antibiotic + B vitamins and control nymphs. The frequency with which nymphs feed and thus the availability of blood is intricately related to the speed of development in bedbugs (nymphs require one complete blood meal before eclosion to the next instar stage can occur (Naylor, 2012). In Hosokawa et al.'s study, the availability of blood was constrained to 20 minutes, once every 2 weeks. Blood meal availability would therefore have constrained nymph development, particularly for nymphs that failed to feed when a blood meal was offered. A better design would have been to offer the nymphs daily blood meals, so that blood meal availability was not a limiting factor.

In these experiments, Hosokawa et al., (2010) used a Japanese strain of bedbugs (JESC) which are infected only with *Wolbachia*; this strain lacks coexisting symbiont species commonly present in bedbugs, such as BLS (Meriweather et al., 2013). On the one hand this is good because the results of Hosokawa et al., (2010) will not be confounded by affects caused by the manipulation of non-target symbionts as a result of antibiotic treatment (Hosokawa et al., 2010). On the other hand though, given that the vast majority

of bedbug strains are co-infected with BLS (Meriweather et al., 2013)), this raises the question whether Hosokawa et al.'s results reflect the common situation. *Wolbachia* may function differently in the presence of BLS.

Given the drawbacks with the design of Hosokawa et al.'s study and that their results may be specific to the JESC strain, it is worthwhile verifying some of Hosokawa et al.'s findings with the S1 strain that is used throughout this thesis. Furthermore, it would be interesting to discriminate *Wolbachia* mediated effects between host sexes, something which no previous studies have attempted to do. Given that the presentation of *Wolbachia* in males and females is very different – in males the bacteriomes are attached to the testes and in females the bacteriomes are embedded in the fat body – it would be interesting to determine whether the observed negative fitness effects in the female were caused by a reduction in the male's or the female's *Wolbachia* load.

5.1.2 Long-term fitness consequences of *Wolbachia* in bedbugs

There are many studies that investigate the consequences of *Wolbachia* infection on measures of their host's fitness (e.g. Zchori-Fein et al., 2000, Fry et al., 2004, Montenegro et al., 2006, Chiel et al., 2009) but there are no studies that investigate the longer term effects of *Wolbachia* infection on the fitness of the host's offspring. Given that *Wolbachia* reproduce asexually and will thus be genetically identical to *Wolbachia* in the next host generation, it would be selectively advantageous to *Wolbachia* to enhance the fitness of the host's offspring. The female host would also benefit because increasing her offspring's fitness will increase her inclusive fitness.

Nutritional symbionts may directly contribute to the nutrient stores within the egg or provide additional nutrition to the female. Either way they may enable her to invest more of her resources to produce higher quality (in insects usually larger) eggs: in many insects, offspring that receive a higher investment during development, have a higher fitness as adults and often have a larger body size (e.g. Fox and Czesak, 2000). In many insects, larger females are more fecund (Honek, 1993), and larger males may have a higher mating success either due to a size advantage in male-male competition or because females tend to choose to mate with larger males (e.g. Savalli and Fox, 1998). In bedbugs, the female's

body size is positively correlated with her fecundity (A1.3.3, A1.3.4); it is unknown whether the male's body size is related to his fitness. Offspring body size may therefore be a suitable surrogate measure of offspring quality, indicative of their expected lifetime reproductive success.

5.1.3 Aims of chapter

- To quantify the distribution of *Wolbachia* in the bodies of female bedbugs
- To examine the effect of reducing *Wolbachia* load using heat treatment on the fitness of female hosts.
- To examine the long-term effects of reducing the female's *Wolbachia* load using heat treatment on the fitness of her offspring.

5.2 Materials and Methods

5.2.1 Insects

The insects used in these experiments were 3 days old virgin male and female bedbugs that had been raised as described in protocol 2.2.2.1. Insects were imaged so that pronotum width measurements could be recorded (2.2.2.2), marked with paint so that individuals could be distinguished (2.2.2.3) and mated following a standard mating protocol (2.2.2.4).

Insects were maintained individually in 5 mL plastic tubes with a strip of filter paper under controlled temperature conditions of 26°C 70% RH 24 hours darkness except during feeding when individuals were transferred to communal pots, and during heat treatment. Insects were fed weekly blood meals of heparinised sheep's blood through an artificial membrane feeding system (2.2.3).

5.2.2 Location and load of female's *Wolbachia*

qPCR analysis of *Wolbachia* load was carried out on the following tissues dissected from *n*=5 females: one bacteriome, two Malpighian tubules, six legs, head and foregut, anterior midgut, median midgut, posterior midgut, hindgut, fat body, cuticle (half of the 3rd dorsal thoracic segment with basement membrane attached), mesospermalege, and one set of ovaries (Fig. 5.3). Howokawa et al. (2010) used these same organs to assess the presentation of *Wolbachia* in the Japanese JESC and TUA *C. lectularius* strains. I can

therefore assess whether the presentation of *Wolbachia* in the S1 *C. lecturlarius* strain is similar. Hosokawa et al. (2010) did not analyse the gut for the presence of *Wolbachia*, I therefore included the gut in my samples to determine if any *Wolbachia* occurred here. The gut was dissected into its distinct functional and anatomical parts (Azevedo et al., 2009). Each part of the gut is quite distinct in both form and function; the foregut is involved in ingestion and the mechanical softening of the food, the anterior midgut is for storage, the median and posterior midgut is for digestion and absorption and the hindgut holds the waste products before they are excreted from the body (Azevedo et al., 2009). All tissue dissections were performed in PBS (NaCl 150 mM, Na_2HPO_4 10 mM, pH 6.5) under a stereomicroscope Leica MZ8 (Wetzlar, Germany). Tissues for qPCR analysis were processed as detailed in 2.4.

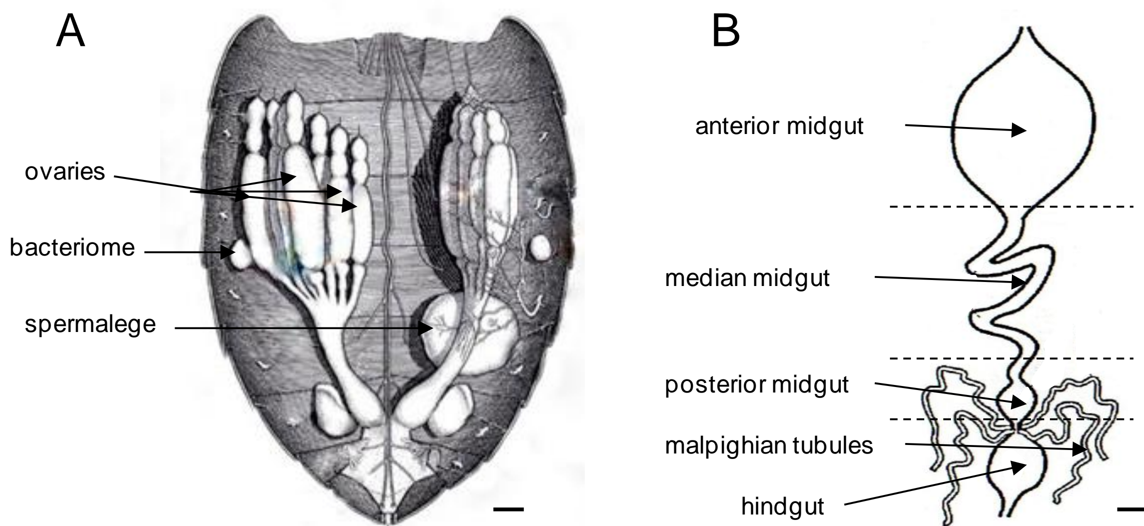


Figure 5.3 Internal anatomy of a female bedbug to show location of dissected tissues. (A) ventral view of thorax with gut removed, (modified from (Davis, 1956)). (B) the gut with different regions labelled (modified from (Azevedo et al., 2009)). Bar, 0.5 mm.

5.2.3 Fitness effects of female's *Wolbachia*

5.2.3.1 Treatment groups

Two treatment groups that differed in their *Wolbachia* loads were generated following a heat treatment protocol (A4.1.2): "+heat" (36°C for 2 weeks) and "−heat" (26°C for 2 weeks). To check that heat treatment had been effective, the *Wolbachia* loads for each

treatment group was determined from *n*=6 individuals taken from each treatment group using qPCR.

5.2.3.2 Female's fitness – survival assay

To assess the effect of reduced *Wolbachia* loads on survival, "+heat" and "−heat" females (*n*=12) were fed once following standard feeding protocols (2.2.3), housed individually in 5 mL plastic tubes with a strip of filter paper and placed in a controlled temperature environment (26°C, 70% RH, 12:12 light:dark cycle). Weekly survival checks were carried out over 6 months. Bedbugs are well known for surviving long periods of starvation which, depending on the temperature of the environment, can range from 6 months to 2 years. The survival assay was stopped after 6 months.

5.2.3.3 Female's fitness – fecundity

To assess the effect of reduced *Wolbachia* loads on fecundity, "+heat" and "−heat" females (*n*=50 in each) were fed and mated following the protocols in 2.2.3 and 2.2.2.4 and individually housed in 5 mL plastic tubes with a piece of filter paper (1 x 3 cm), on which females could lay their eggs. Three measures of fecundity were compared over 2 weeks: (i) egg laying rate (number of eggs laid that hatched per week), (ii) egg hatch success (proportion of total eggs laid that hatched) and (iii) mean egg size (volume (mm^3)) of eggs from *n*=20 females. The time-frame for these measures is constrained because *Wolbachia* loads recover in heat treated insects after *ca.* 14 days. Egg laying rate was expressed per mg *ad libitum* fed body mass to control for differences in blood resource availability between individuals. To measure egg volume, eggs were imaged under stereomicroscope with a digital camera (detailed in 2.3.3). As eggs are prolate-spheroid in shape, egg volume was calculated using the equation: $V=1/6 \, Pi \, W^2 \, L$ where W is the egg width and L is the egg length (Berrigan, 1991).

5.2.4 Fitness effects of female's *Wolbachia* on offspring fitness

5.2.4.1 Treatment groups

The first instars from "+heat" and "−heat" females (5.2.3.1) were collected and housed communally (*n*≈100 individuals) in 50 mL pots with a strip of filter paper (5 x 15 cm) folded in concertina. I raised the nymphs in communal pots for logistical reasons – nymphs

feed with greater success in higher density pots of between 25-50 individuals compared to pots of <20 individuals (pers obs). The disadvantage of this though is that it is not possible to identify which nymphs have originated from which mother, thus it is not possible to control for possible confounding differences in the size of mothers, or the clutch size from which the individual offspring originated.

The *Wolbachia* load of some first instars was determined by qPCR analysis of the whole bodies of first instar nymphs (*n*=6 samples, each sample consisted of *n*=3 nymphs originating from the same mother which was chosen at random). The remaining first instars were raised to adulthood in a controlled temperature environment of 26°C, 70% RH, 12:12 light:dark cycle on weekly blood meals over 6 weeks, using standard feeding protocols (2.2.3). The adult development success (proportion of first instars that developed to adulthood) was recorded. The *Wolbachia* load of newly eclosed adults (*n*=6 males and *n*=6 females) was determined by qPCR analysis of their whole bodies. Body size measurements (pronotum widths) were made from images of newly eclosed adult males and females (*n*=35 each group). The fitness of male and female offspring was then empirically tested, as detailed below.

5.2.4.2 Measures of offspring fitness

Adult female offspring (*n*=10) from "+heat" and "−heat" females were fed and mated with virgin males following standard procedures (2.2.3 and 2.2.2.4). Eggs laid were collected over a 2 week egg laying period. Two measures of fecundity were recorded: (i) egg laying rate (number of eggs laid that hatched per week per mg replete female body mass) and (ii) egg hatch success (proportion of all eggs laid that hatched).

Adult male offspring (*n*=20) from "+heat" and "−heat" females were fed and mated with virgin females following standard procedures 2.2.3 and 2.2.2.4). Indirect measures of the fitness of male offspring were made from fecundity measures of the mated females. Eggs laid were collected over a 7 week egg laying period, during which females were fed weekly (but not re-mated). Three measures of fecundity were recorded each week: (i) egg laying rate (number of eggs laid that hatched per week per mg replete female body mass), (ii) egg hatch success (proportion of all eggs laid that hatched) and (iii) mean egg size (volume

(mm^3)) of all eggs laid during the first week only by a subsample of females (n=12) from each group.

5.2.5 Data analysis

Data were analysed as detailed in 2.6. For the measures of fecundity, data collected each week were pooled to give an average for the period of data collection. For individual analyses for each week of data collection, see results Tables A3.10-A3.11.

5.3 Results

5.3.1 Location and load of female's *Wolbachia*

The majority of the female's *Wolbachia* load was found in the bacteriomes, where they occurred at a density more than 800 times greater than in the ovaries, and more than 300,000 times greater than in the other tissues tested (ANOVA: $F_{11,48}$=59.10, P<0.0001 on $\log_e(x)$ data) (Fig. 5.4).

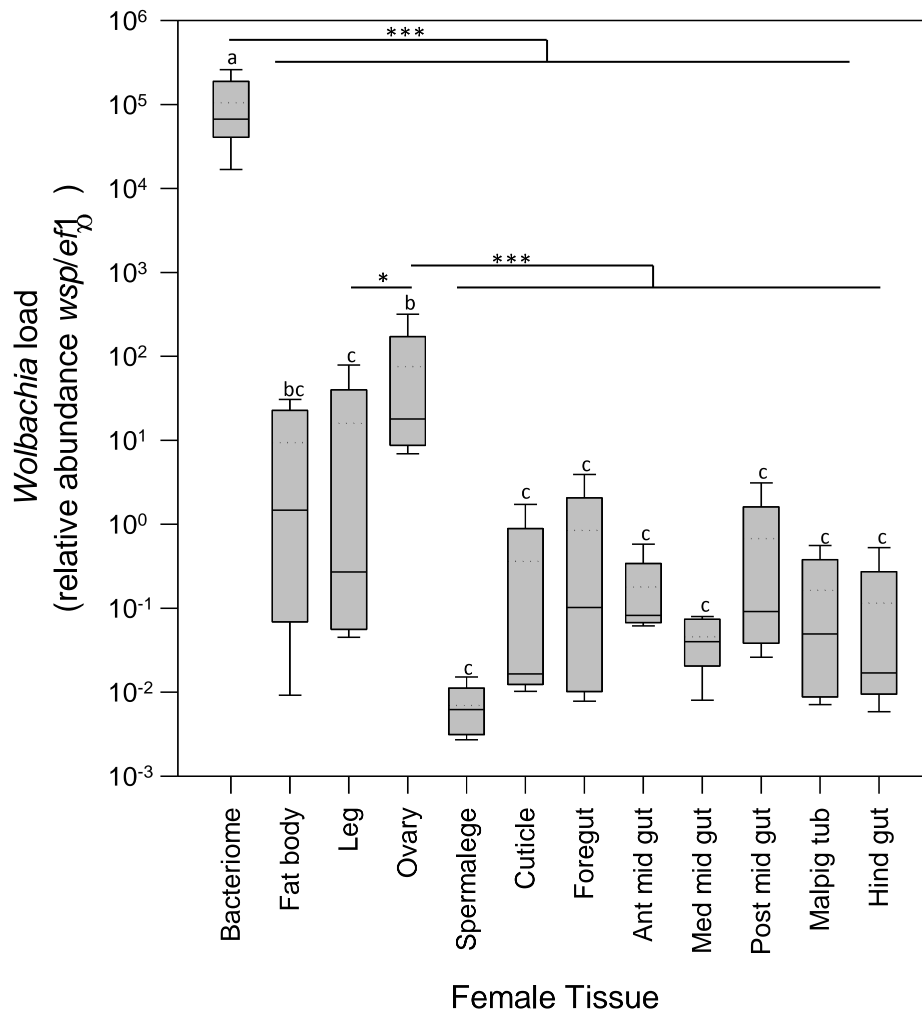


Figure 5.4 Median (solid line) and mean (dotted line) *Wolbachia* load in female tissues. Means with the same letter do not differ from each other (Tukey mcp test P>0.05). N=5 each group.

5.3.2 Effects of female's *Wolbachia* on host fitness

5.3.2.1 Wolbachia *load of treatment groups*

qPCR results confirmed that the *Wolbachia* loads in "+heat" females (0.897±0.385) were significantly lower than in "−heat" females (18.99±4.06) (t=7.84, 10 df, P<0.0001 on $\log_e(x+1)$ transformed data).

5.3.2.2 Female's fitness – survival

No females had died by the end of the 6 month observation period.

5.3.2.3 Female's fitness – fecundity

There was no difference in the egg laying rate between "+heat" and "−heat" females (Mann-Whitney *U* test: U=1388, n_1=50, n_2=50, P>0.05) (Fig. 5.3). There were however significant differences in other measures of fecundity. "−Heat" females with baseline *Wolbachia* loads (i) laid larger eggs (t=2.34, 38 df, P<0.05) (Plate 5.1), (ii) had a higher proportion egg hatch success (Mann-Whitney *U* test: U=-1567, n_1=50, n_2=50, P<0.01) and (iii) had a higher proportion adult development success (χ^2=39.77, 1 df, P<0.0001) (Fig. 5.5). There were no differences in the body size (pronotum width) (t=0.99, 98 df, P>0.05) or mass of blood meal taken up (t=0.63, 98 df, P>0.05) for "+heat" and "−heat" females.

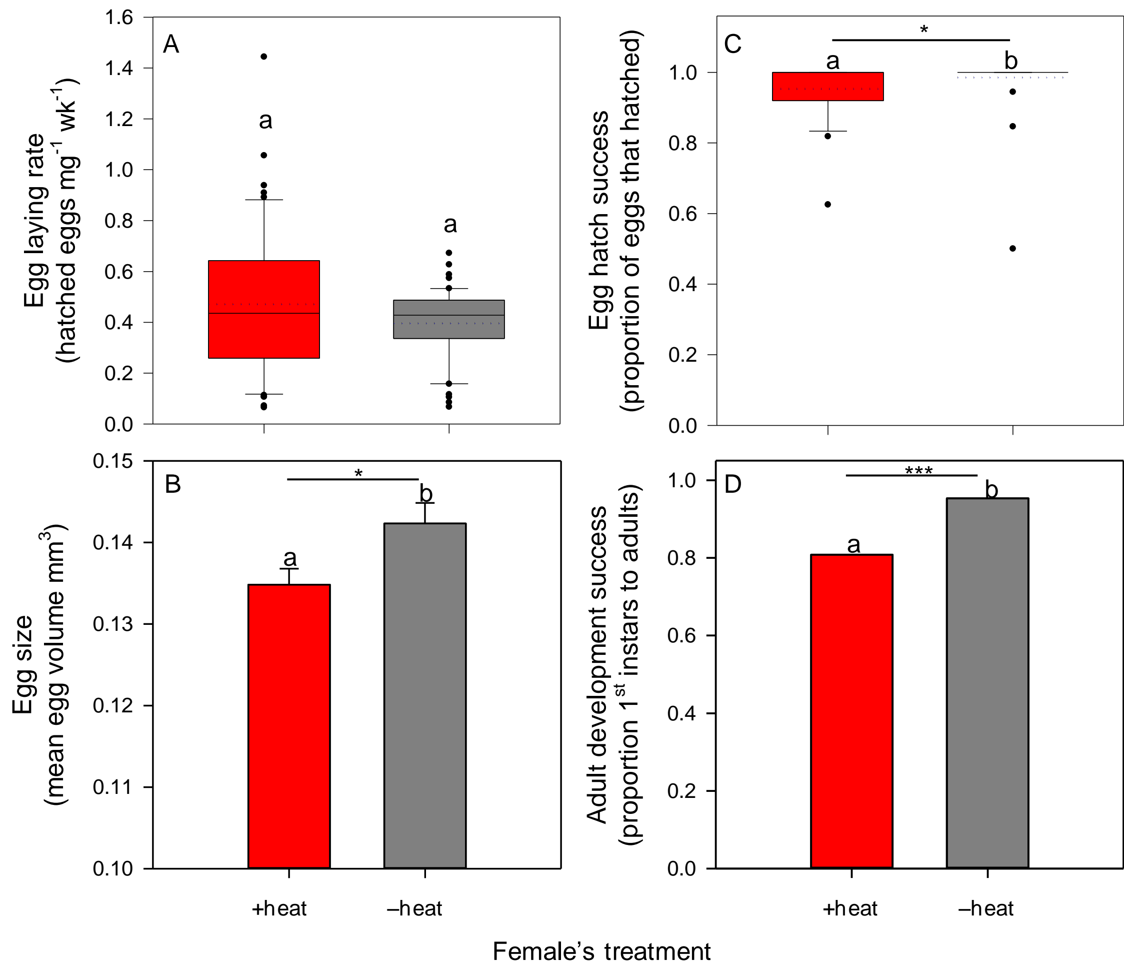


Figure 5.5 The effect of heat treatment on fecundity. (A) Egg laying rate (number of eggs laid per week that hatched, expressed per mg replete female body mass), median (solid line), mean (dotted line), *n*=50 each group. (B) Mean size of eggs (volume (mm^3)) from *n*=20 females each group. (C) Egg hatch success (proportion of eggs that hatched), median (solid line), mean (dotted line), *n*=50 each group. (D) Mean adult development success (the proportion of *n*≥380 first instars that developed into adults, from two samples consisting of *n*=50 females), *n*=1 each treatment group. Groups with the same letter do not differ from each other. Bars represent 1SE.

Plate 5.1 Effect of heat treatment on egg size. Eggs laid by heat treated females (red border) are smaller in volume on account of their being shorter in length, compared to those laid by heat control females (grey border). Colours on the images have been artificially enhanced so that the egg can be seen more clearly. Bar, 1 mm.

5.3.3 Effects of a female's *Wolbachia* on her offspring's fitness

5.3.3.1 Offspring's Wolbachia *load*

The first instar offspring from "+heat" females had significantly lower *Wolbachia* loads compared to the first instar offspring from "−heat" females (t=3.99, 10 df, P<0.01 on $\log_e(x+1)$. By the time offspring had reached adulthood there was no difference in *Wolbachia* load between the two treatment groups (adult males: t=1.68, 5 df, P>0.05; adult females t=1.80, 10 df, P>0.05) (Fig. 5.6).

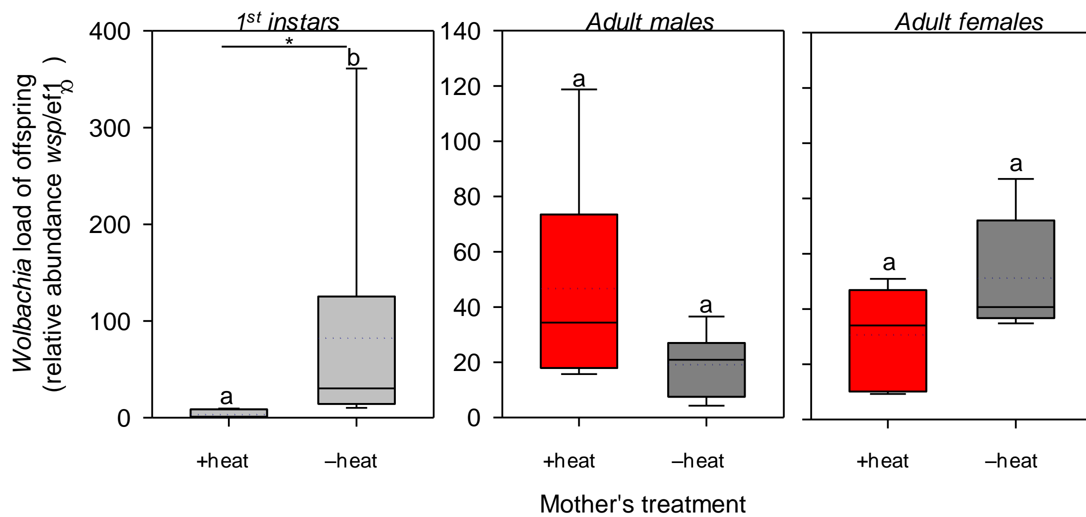


Figure 5.6 Effect of heat treating mothers on the *Wolbachia* load of her offspring as (A) first instars, (B) adult males and (C) adult females. *N*=6 each group, median (solid line), mean (dotted line), groups with the same letter do not differ from each other.

5.3.3.2 Offspring's body size

Heat treating females had a highly significant effect on the body size (pronotum width) of her adult offspring. The male and female offspring of "+heat" females were significantly smaller than those from "−heat" females (t=5.36, 74 df, P<0.0001 and t=4.15, 67 df, P<0.0001 respectively) (Fig. 5.7).

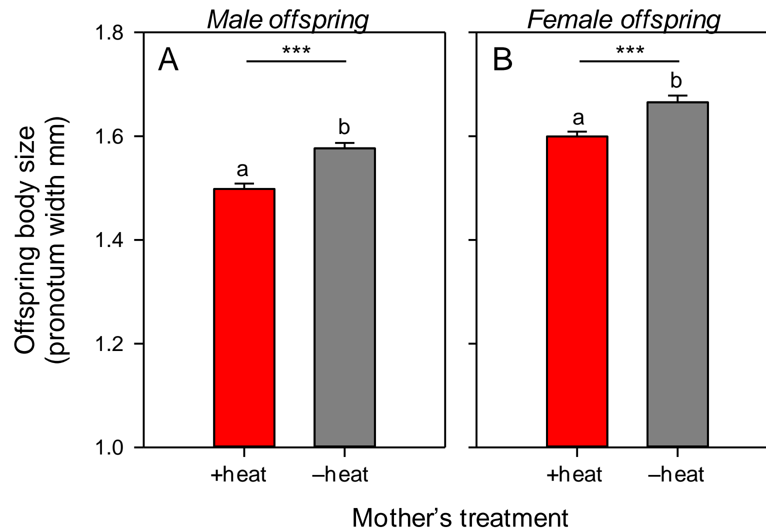


Figure 5.7 The effect of heat treating females on the mean body size (pronotum width (mm)) of her adult offspring ((A) males and (B) females). N=35 each group, groups with the same letter do not differ from each other, bars represent 1SE.

5.3.3.3. Offspring's fitness

There were no differences in (i) the egg laying rate (t=1.53, 17 df, P>0.05) or (ii) the egg hatch success (Mann-Whitney U-test U=54, n_1=9, n_2=10, P>0.05) for female offspring of "+heat" and "−heat" females (Fig. 5.8).

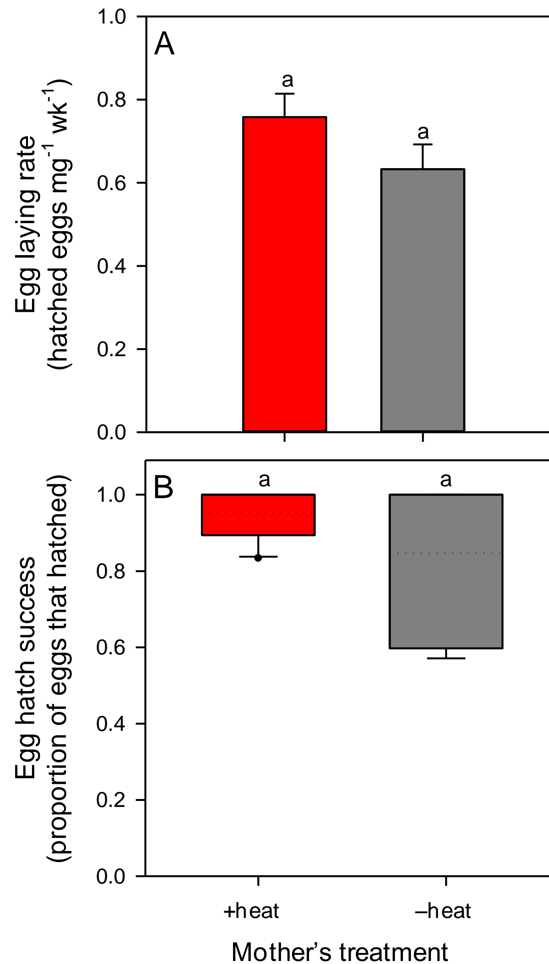


Figure 5.8 The effect heat treating females ("+heat" (n=10) and "−heat" (n=9)) on measures of her offspring's fecundity. (A) Mean egg laying rate (number of eggs laid per week that hatched expressed per mg replete female body mass). (B) Egg hatch success (proportion of eggs laid that hatched), median (solid line), mean (dotted line). Groups with the same letter do not differ from each other, bars represent 1SE.

There were no differences in (i) the egg laying rate (t=0.99, 38 df, P>0.05) (Table A3.10, Fig. A3.1), (ii) the egg hatch success (t=1.01, 38 df, P>0.05) (Table A3.11 and Fig. A3.1), or (iii) mean egg size (Mann-Whitney U-test U=11067, n_1=12, n_2=12, P>0.05) of females mated to the male offspring from "+heat" and "−heat" females (Fig. 5.9). Egg laying had

ceased in all females that were mated to male offspring by the end of week 7 post mating (Fig. A3.1).

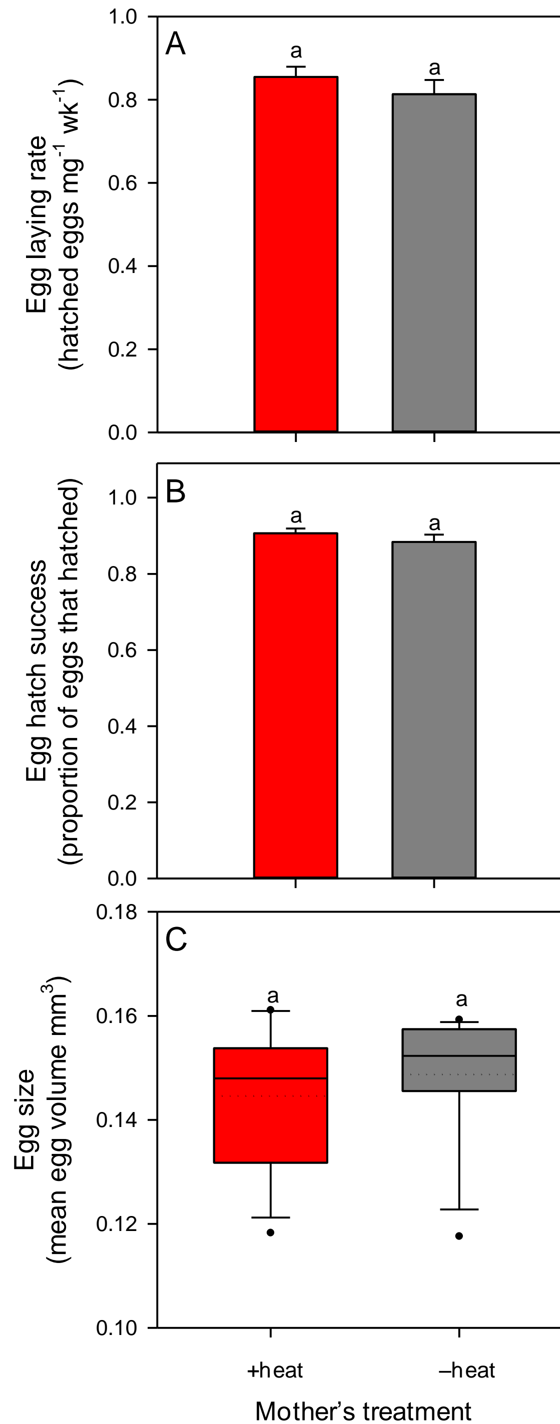


Figure 5.9 Effect of heat treating females on indirect measures of her male offspring's fitness. (A) Mean egg laying rate (number of eggs laid per week that hatched, expressed per mg replete female body mass), *n*=20 each group. (B) Mean egg hatch success (proportion of eggs that hatched), *n*=20 each group. (C) Egg size (volume (mm^3)), median (solid line), mean (dotted line), *n*=12 females each group. Groups with the same letter do not differ from each other, bars represent 1SE.

5.4 Discussion

In line with findings by Hosokawa et al., (2010), my results show that the female's *Wolbachia* are located mainly in the bacteriomes, with lower but substantial densities in the ovaries. To determine the fitness consequences of the female's *Wolbachia*, heat treatment was used to manipulate *Wolbachia* loads to create two groups with different *Wolbachia* loads: i) Non-heat treated control females ("−heat") which had baseline *Wolbachia* loads and ii) heat treated females ("+heat") which had significantly reduced *Wolbachia* loads relative to the "−heat" females. I found that "+heat" females had lower fitness than "−heat" females due to lower offspring developmental success. This finding supports the results of Hosokawa et al., (2010), suggesting that *Wolbachia* may increase female host fitness. I also found that the offspring from "−heat" mothers were significantly larger in body size compared to offspring from "+heat" mothers. Given the fitness benefits associated with a larger female body size (A1.3.3 and A1.3.4), the female's *Wolbachia* may have long-term benefits through increasing the fitness of the host's offspring.

It is difficult to separate the effects of the heat treatment used to reduce *Wolbachia* loads, from the effects caused by the reduction in *Wolbachia* load. Effects were measured in the two week period after heat treatment had finished, whilst a difference in the *Wolbachia* loads are known to persist between "+heat" and "−heat" females (Chapter 3). It is possible that other effects of heat treatment also persist during this timeframe, therefore one must exercise caution when drawing conclusions about the effects of *Wolbachia* on female hosts and their offspring, discussed below.

5.4.1 *Wolbachia* in females

Wolbachia loads in different tissues were determined as the number of *wsp* gene copies relative to the number of *ef1*α gene copies. Numbers of *ef1*α gene copies must be stable across different tissues as changes would affect the result falsely suggesting variation in *Wolbachia* load. I found the presentation of *Wolbachia* in S1 females was very similar to that found in the JESC and TUA *C. lectularius* strains used by Hosokawa et al., (2010). I found *Wolbachia* at high densities within the bacteriomes, some *Wolbachia* in the ovaries, and few *Wolbachia* elsewhere in the body. From their location in the ovaries, the

Wolbachia have been shown to infect the developing eggs and in this way pass on to the next generation (Hosokawa et al., 2010). I discuss the fitness effects of *Wolbachia* for the female host and her offspring separately below.

5.4.2 Effect of heat induced reduction of *Wolbachia* loads on female's fitness

"+Heat" females were found to have a lower overall fitness over the temporal span tested here (the time frame of fecundity measures was constrained to the two weeks following the end of the heat treatment, because *Wolbachia* load recovers after this time (chapter 3)). I found no difference in egg laying rate between "+heat" and "−heat" females, but a greater proportion of eggs hatched successfully and a greater proportion of offspring reached adulthood for "−heat" females.

Contrary to my findings, a previous study using heat treatment reported a reduction in egg laying rate associated with a reduction in bedbug symbionts (Chang, 1974) later identified as *Wolbachia* (Hypša and Aksoy, 1997). In my experiments mated females were returned to a cooler, optimal, environment for egg laying, whereas in the experiments conducted by Chang (1974), mated females remained in elevated thermal conditions. Consequently it is possible that Chang's observed reduction in egg laying resulted from a lack of viable sperm, damaged by the elevated temperatures (Omori, 1941). As previously explained, the fitness effects that I observed could be a side effect of the heat treatment, or could have been caused by differences in the *Wolbachia* loads between "+heat" and "−heat" treatment groups. If heat treatment is stressful, the female may need to invest some her resources in tissue repair processes rather than egg production which may affect egg hatch and development success. If *Wolbachia* were responsible, the reduction in fitness that I observed in the "+heat" females could have been mediated by a reduction in B vitamin availability, as *Wolbachia* are thought to supply B vitamins (Hosokawa et al., 2010). Similar findings have been found in other haematophages that rely on symbionts for B vitamins (Nogge, 1976, Beard et al., 2002, Sasaki-Fukatsu et al., 2006) and may be reversed by supplementing the blood meal B vitamins (Nogge, 1981, Hosokawa et al., 2010).

Life-time reproduction success depends on both fecundity and survival. I detected no difference in the longevity of "−heat" and "+heat" females over a 6 month starvation period. This is in line with previous studies that have investigated the effects of reducing the load of nutritional symbionts on host survival and found no effect (Lehane, 2005). It is possible that survival differences between "+heat" and "−heat" treated groups may become apparent under more stressful conditions, such as in face of disease challenge or extreme environmental conditions.

5.4.3 Effect of heat induced reduction of mothers' *Wolbachia* loads on offspring fitness

The male and female offspring from "−heat" females were significantly larger compared to the offspring from "+heat" females. There are a number of non-exclusive explanations for this observation. "+Heat" females may have fewer resources available for egg production if: i) they need to divert some of their blood resources away from egg production to repair tissues damaged by the stressful heat treatment; ii) there is a reduction in the availability of B vitamins necessary for normal egg production, an effect of the reduction in *Wolbachia* load and iii) the female's *Wolbachia* load is an resource in itself and there is less *Wolbachia* available to seed each egg (significant differences in the *Wolbachia* load of first instar nymphs was observed suggesting that less *Wolbachia* seeded each egg in the "+heat" females compared to the "−heat" females). It could be that the amount of *Wolbachia* inside offspring during the early developmental stage determines the body size of adult offspring. The underlying mechanisms could be nutritional if *Wolbachia* provide B vitamins in the eggs. As the eggs did not develop under heat conditions (because eggs do not begin to be manufactured until after the first blood meal as an adult female which was after the heat treatment had finished) differences in egg size were not due to direct effects of heat treatment on the eggs *per se*.

Previous studies with insects have demonstrated that high environmental temperature can affect offspring body size, causing offspring to be either larger (e.g. Beckwith, 1982) or smaller (e.g. Brittain et al., 1984). Unlike in my experiments, in these cases, the developing eggs were subjected to high temperatures. It is unclear to what extent the observed

reduction in offspring body size was caused by the heat treatment or the reduction in *Wolbachia* load.

Another possible explanation for the smaller body size of offspring from "+heat" mothers is that this is a maternal effect in response to either the heat treatment or to reduced resource availability. "+Heat" mothers may invest in larger clutch sizes of eggs. My results suggest this may be the case because there was no difference in the number of viable eggs laid by "+heat" and "−heat" mothers, but egg hatch success was significantly lower for "+heat" mothers suggesting a larger overall clutch size to begin with. Alternatively, mothers may produce smaller offspring in response to a high temperature environment if smaller offspring are better suited to these high temperature environmental conditions. This is unlikely to be the case here as smaller individuals have a lower survival rate compared to larger individuals when maintained under constant raised temperature conditions of 36 ^{o}C (pers obs). Females with depleted *Wolbachia* loads as a result of heat treatment may have fewer micro-nutritional resources (e.g. B vitamins) available for egg production. If in response to this, the mother allocates her limited resources to maintain egg laying rate at the expense of egg size then smaller eggs would result as a maternal effect. Previous studies have reported similar reductions in offspring size from mothers with reduced nutritional symbiont loads, particularly if the symbionts synthesise amino acids, e.g. a reduction in *Buchnera aphidicola* in pea aphids *Acyrthosiphon pisum* (Koga et al., 2007) and a reduction in *Candidatus Ishikawaella capsulata* in stinkbugs *Megacopta punctatissima* Montandon (Hosokawa et al., 2007, Hosokawa et al., 2008).

In insects, smaller offspring are usually considered of a lower quality. Smaller females have lower fecundity (Honek, 1993) and smaller males are more likely to lose in competitive interactions and are more likely to be shunned by choosy females (e.g. Savalli and Fox, 1998). In female bedbugs there is a significant relationship between female body size and fecundity (egg laying rate) (Fig. A1.4). This appears to be driven by larger females having a larger blood-feeding capacity: when the size of blood meal was controlled, I found no difference in the fecundity of the smaller female offspring from "+heat" mothers and the larger female offspring from "−heat" mothers (Fig. 5.6 Top). Nonetheless because of the relationships between body size, fecundity and blood meal size, I expect the fecundity of smaller offspring to be lower than the larger offspring.

The effects of body size on male bedbug fitness have not been directly tested. Like the female, a larger body size would allow males to take a larger blood meal and thus larger males may benefit from having more resources available for ejaculate production. Larger males may thus produce more ejaculate. However, studies of mating (Siva-Jothy and Stutt, 2003, Reinhardt et al., 2011) show that ejaculate size is relatively constant: larger males may therefore benefit by garnering more mating opportunities. Another disadvantage for male bedbugs of having a small body size is that when trying to mate with large females, the body size discrepancy may mean the male is unable to reach the ectospermalege for insemination (pers obs). This will both reduce the proportion of females in a population available for small males to mate with, and restrict these smaller males to mating with smaller females, which have a lower fitness.

Offspring body size is an important trait that affects the lifetime reproductive success of both male and female individuals. It is unclear whether the heat treatment, change in *Wolbachia* load or both caused the observed reduction in body size in the offspring from "+heat" females. Repeating the experiments using a different method to manipulate *Wolbachia* load may be helpful in determining this.

5.4.4 Summary

In this chapter I have:

1. Established that bedbug females house the majority of their *Wolbachia* in paired bacteriomes.
2. Determined that *Wolbachia* may increase the female's fitness (at least in the short-term) by increasing egg hatch and development success. No effect of manipulating *Wolbachia* load on female longevity was detected in the 6 month data collection period. Survival effects may become apparent within this time-frame under stressful conditions, such as when the female is challenged with disease.
3. Determined that the female's *Wolbachia* may increase the body size of her offspring and are thus likely to increase the fitness of her offspring.

It is difficult to separate the effects of heat treatment from the effects of a change in *Wolbachia* load, the thus the above conclusions should be taken with caution. Since

Wolbachia provide B vitamins in bedbugs, *Wolbachia* may mediate these fitness effects, at least in part, through a nutritional mechanism. Ultimately, *Wolbachia*-mediated host-fitness benefits will be selected because they increase *Wolbachia* fitness.

Chapter 6

Effect of *Wolbachia* on Female's Fitness after Microbial Challenge

"*At the time of mating, the male punctures the body wall of the female with his copulatory organ and injects an abundance of sperm into her abdomen but outside of the usual reproductive tract. Insemination is thus "extragenital" and "traumatic", because it always begins with an integumental wound.*"

(Carayon, 1966)

6.1 Introduction

Bedbug mating is termed "traumatic insemination" (TI), because it is associated with wounding and infection (Morrow and Arnqvist, 2003, Reinhardt et al., 2003) which lower the female's longevity (Stutt and Siva-Jothy, 2001). The female has physical adaptations to reduce these costs and uses innate immune mechanisms to combat infection, but questions remain as to whether her symbionts are important in this process. Recent work with other insect systems has revealed that symbionts can protect their hosts by increasing resistance or tolerance to disease and potentially reducing the costs of wound healing (Oliver and Moran, 2009). This raises the question, "do the *Wolbachia* in female bedbugs serve a protective function besides their role as a nutritional mutualist?"

6.1.1 Traumatic insemination

TI is a mode of reproduction where the male stabs his intromittent organ through the abdominal wall of the female, inseminating into her haemocoel, rather than using her genital tract. In bedbugs this is used only for oviposition (Carayon, 1966). TI is rare in gonochorists and is mainly restricted to two insect orders: Hemiptera and Strepsiptera. Exactly why it evolved in these groups is unclear (Stutt and Siva-Jothy, 2001, Arnqvist and Rowe, 2005).

In bedbugs, males habitually remate with females, probably in part because sperm precedence appears to favour the last male to mate (Stutt and Siva-Jothy, 2001). Females that are repeatedly mated have a reduced lifespan compared to females mated at lower frequencies (Stutt and Siva-Jothy, 2001) probably due to the higher costs of wounding and infection associated with multiple TIs (Stutt and Siva-Jothy, 2001, Morrow and Arnqvist, 2003, Reinhardt et al., 2003). However recent work suggests that ejaculate components may compensate for this effect (Reinhardt et al., 2011).

6.1.1 1 Costs of TI

The process of TI creates a wound through which opportunistic microbes may be transferred into the female's haemocoel via the male's intromittent organ (Reinhardt et al., 2005). Copulatory wounding is probably a relatively common phenomenon (Siva-Jothy, 2009) and may underpin mating costs in a range of taxa (e.g. Crudgington and Siva-Jothy, 2000). Sexually transmitted disease is also a pervasive cost of mating (Hurst et al., 1995, Lockhart et al., 1996). It is likely that the subsequent wound healing and immune response divert limited resources away from reproduction and somatic maintenance resulting in the observed reduction in the host's longevity (Stutt and Siva-Jothy, 2001, Reinhardt et al., 2003, Morrow and Arnqvist, 2003, Lange et al., 2013).

6.1.1.2 Reducing the costs of TI

Insects rely on innate immune mechanisms for wound healing and to clear infection. A female evolutionary response to TI in bedbugs is the evolution of an immune organ, the spermalege (Reinhardt et al., 2003), located at the site of TI and into which sperm are deposited (Carayon, 1966). Externally, the spermalege is manifest as a grove in the female's cuticle (the ectospermalege), which guides the male's intromittent organ into the mesospermalege where the male ejaculates. The mesospermalege contains high densities of immune cells (haemocytes) that are involved in phagocytosis and wound healing (Siva-Jothy, 2006). Older studies report that these haemocytes also phagocytise sperm (Usinger, 1966), an observation which led Eberhard (1996) to hypothesise that the spermalege functioned in cryptic female choice. It has since however been shown experimentally that the spermalege functions to reduce the costs of wounding and infection associated with TI

(Reinhardt et al., 2003, Morrow and Arnqvist, 2003) and has no apparent function in cryptic female choice (Stutt, 1999).

6.1.2 Protective role of symbionts

Recent studies show that the vertically transmitted symbionts of insects perform important protective roles in their hosts - termed symbiont-mediated protection (SMP). SMP includes resistance, tolerance and wound healing benefits against pathogens (Brownlie and Johnson, 2009). SMP can also operate against predators but I am not going to discuss these mechanisms here. Although not ubiquitous (there are cases where symbionts appear to have no protective role (Bourtzis et al., 2000), or even increase the host's susceptibility to disease (Fytrou et al., 2006, Eleftherianos et al., 2007)), SMP is thought to be widespread. It has been demonstrated for a range of different symbionts including gut microbiota (Dillon et al., 2005, Hernandez-Martinez et al., 2010) and reproductive parasites (Hansen et al., 2007), across a range of insect taxa including Hemiptera (Oliver et al., 2005), Diptera (Moreira et al., 2009, Jaenike et al., 2010, Weiss et al., 2011), Hymenoptera (Sabate et al., 2009), Lepidoptera (Hernandez-Martinez et al., 2010) and Orthoptera (Dillon et al., 2005) and against a spectrum of pathogens from multicellular parasites (Oliver et al., 2005, Jaenike et al., 2010) to viruses (Hedges et al., 2008, Teixeira et al., 2008, Osborne et al., 2009).

There are three, non exclusive, hypotheses to explain the evolution of a protective role for a host's symbionts. The symbiont protects the host (i) to increase host fitness and further the symbiont's own propagation (Ewald and Scubert, 1989, Oliver et al., 2005), (ii) to reduce conflict with the microbial opportunists, which through their immediate requirement for host resources, would reduce host fitness and thus reduce the symbiont's fitness as well (Jones et al., 2007, Lipsitch et al., 1996, Lively, 2005) and (iii) incidentally by reducing pathogen loads through competition for shared, limited resources (Moreira et al., 2009). Because TI associated wounding and microbial infection potentially reduces female fitness (Stutt and Siva-Jothy, 2001), I predict a protective role for a female's symbiotic *Wolbachia* which should reduce these costs.

6.1.3 Aims of chapter

I will quantify the effect of *Wolbachia* load on fitness traits following the experimentally controlled infection and wounding (TI) of heat treated females with reduced *Wolbachia* and non-heat treated females with baseline *Wolbachia* loads.

6.2 Materials and methods

6.2.1 Insects

All insects used in these experiments were 3 days old virgin adults that had been raised via the protocol described in 2.2.2.1. Insects were imaged so that pronotum width measurements could be recorded (see 2.2.2.2).

6.2.2 Experimental reduction, recovery and measurement of *Wolbachia* load

Four treatment groups were generated using the heat treatment protocol (A4.1.2). (i) "+heat" (high temperature environment of 36°C for 2 weeks to lower their *Wolbachia* loads). (ii) "−heat" (control environment of 26°C for 2 weeks to maintain normal *Wolbachia* loads, significantly higher than the "+heat" group). (iii) "+heat+recovery" (high temperature environment of 36°C for 2 weeks, followed by 2 weeks at 26°C with weekly blood meals to allow *Wolbachia* loads to recover; this group serves as a heat treatment control group). (iv) "−heat+recovery" (control environment of 26°C for 2 weeks, followed by 2 weeks at 26°C with weekly blood meals; this group serves to control for differences in the feeding status between groups (prior to being assigned to immune challenge groups, "+heat" and "−heat" groups were unfed whilst "+heat+recovery" and "−heat+recovery" groups were twice fed). The *Wolbachia* loads were determined from 6 individual hosts taken from each treatment group using qPCR analysis (details in 2.4).

6.2.3 The effect of heat induced reduction of *Wolbachia* loads on fitness after immune insult

6.2.3.1 Challenge techniques

The immune challenge techniques used here were based on the methods of Reinhardt et al., (2003). An immune challenge was elicited by piercing the female's abdomen with a needle

that had been dipped for 3 seconds in a suspension of microbes in PBS (150 mM NaCl, 10 mM Na_2HPO_4, pH 6.5). The needle pierced into the spermalege thus mimicking the natural trauma and infection route that occurs during TI (Reinhardt et al., 2003). The challenge I used however was larger than would be naturally encountered during TI. This was necessary to observe a response. In their natural environment, bedbugs copulate multiple times over a lifetime, each copulation being associated with a low level challenge to the female's immune system (Stutt and Siva-Jothy, 2001). It was not possible to carry out multiple, low level challenges over an extended time period, as *Wolbachia* loads in the "+heat" group recover to the same levels as the "−heat" group within 2 weeks of the end of heat treatment (Fig. 3.2). I therefore opted to carry out a larger than natural challenge due to a limited timeframe of 2 weeks. Needles were single-use and consisted of glass microcapillaries (1 x 900 mm) that had been pulled and ground into a fine needle of the same dimensions as the adeagus (tip diameter approximately 100 μm) using a Narishige microelectrode puller (model PC-10) and microelectric grinder (model EG-44), sterilised in ethanol and air-dried.

The microbial suspension was cultured using soiled substrate from a bedbug refuge, following the protocol of Reinhardt et al., (2003). Male bed bugs have the same microbial species on their genitalia as are found in their refugia (Reinhardt et al., 2005). In brief, one strip of filter paper (5 cm x 15 cm) on which the bedbugs had been raised for several generations, was incubated in 400 mL LB broth (4 g tryptone, 2 g yeast extract, 4 g NaCl, 400 mL dH_2O) at 30^oC for 24 hours. The suspension was aliquoted to 50 mL falcon tubes and centrifuged (Eppendorf centrifuge 5810R) for 25 minutes, 3250 rcf at 4^oC. The upper solutions were poured away and the remaining microbial pellets were collected into one microcentrifuge tube and gently vortexed. From this, 0.05 mL of the microbial pellet was diluted in 9.95 mL PBS and gently vortexed. The suspension was aliquoted (0.75 mL) into 1 mL microcentrifuge tubes and stored at -80^oC. Control preparations of sterile PBS aliquots were also stored at -80^oC.

6.2.3.2 Immune treatment groups

Females from the four heat treatment groups (6.2.2) were allocated to three immune treatment groups: "microbial insult", "sterile insult" and "no treatment control". Females in the "microbial insult" group were pierced at the site of the spermalege with a contaminated

needle (as described above). The microbial insult involved both wounding and a microbial insult. To control for the effects of wounding, a “sterile insult” group was generated by wounding females in the same way (by piercing the spermalege with a needle) except the needle was dipped in sterile PBS. The “no treatment control” group consisted of handled, but not wounded, females. Insects from all treatments were then maintained in individual 5 mL tubes (see section 2.2.2) and surrogate measures of fitness (see below) were recorded.

I measured three fitness surrogates: (i) survival (days), over 6 weeks, (ii) egg laying rate over a period of up to10 days following the start of the experiment (data are presented as number of eggs laid day^{-1} that hatched, if the female died before the end of the 10 day period her egg laying rate was calculated as the number of eggs laid day^{-1} whilst she was alive) and (iii) egg hatch success (the proportion of all eggs laid that hatched, females that laid no eggs were excluded). Egg laying rate data were collected from females that were fed once and mated once following standard protocols (2.2.3 and 2.2.2.4). The time-frame for these measures was constrained because the heat-treated insects recover their *Wolbachia* load after *ca.* 14 days (Fig. 3.2).

6.2.4 Data analysis

Data were analysed between groups of similar feeding status prior to immune treatment. The “+heat” group was compared with the “−heat” group, these groups differed in their *Wolbachia* loads and heat treatment, and were both unfed. The “+heat+recovery” group was compared with the “−heat+recovery” group, these groups differed in their heat treatment, had similar *Wolbachia* loads and were both fed. To aid interpretation, the data for all four groups are presented on single graphs, but the results of analyses are only shown between the pairs that were compared. The statistical analyses used are detailed in 2.5. Survival estimates were assessed using an accelerated failure time model with Weibull distribution. To check the Weibull was the most appropriate distribution, I assessed plots of the residuals against different survival models (Weibull, log-normal, log-logistic and gamma) and used the model where the residuals fitted best.

6.3 Results

6.3.1 Comparing *Wolbachia* loads across heat treatment groups

Heat treatment caused a significant reduction in the *Wolbachia* load of females ("+heat" group) compared to heat-treatment controls ("−heat" group) (t=7.71, 5 df, P=0.001) on ($\log_e(x+1)$ data). Following a recovery period, *Wolbachia* loads recovered in the heat treated individuals ("+heat+recovery" group) to match those in the corresponding heat-treatment controls ("−heat+recovery" group) (t=1.04, 10 df, P>0.05) ($\log_e(x+1)$ data) Fig. 6.1.

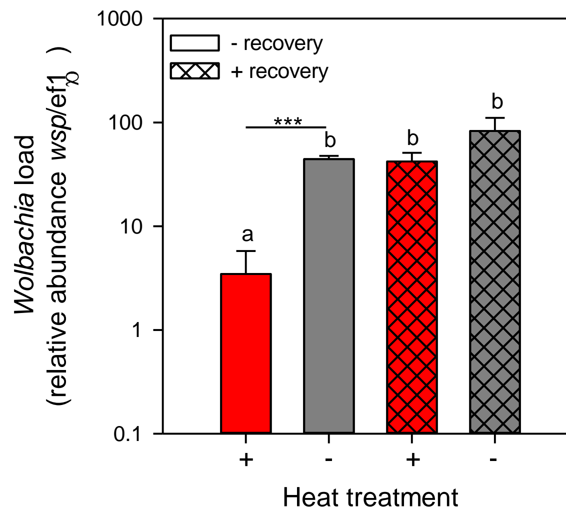


Figure 6.1 The effect of heat treatment (with or without a recovery period) on female *Wolbachia* loads. Females were maintained in a high (+) or normal (-) temperature environment for two weeks, followed by (+) or not (-) a two week recovery period. N=6 each group, means with the same letter do not differ from each other (t-test on $\log_e(x+1)$ data, P>0.05), bars represent 1SE.

6.3.2 Effect heat induced reduction of *Wolbachia* loads on host fitness across immune treatments

6.3.2.1 Immune treatment: microbial insult

The survival of "+heat" females was significantly reduced compared to "−heat" females (χ^2=4.22, 1 df, P<0.05). There was no difference in the survival of "+heat+recovery" and "−heat+recovery" females (χ^2=0.70, 1 df, P>0.05) (Fig. 6.2 A). There were no differences in the egg laying rate or egg hatch success between "+heat" and "−heat" females (Mann-Whitney U-tests: U=83, n_1=15, n_2=15, P>0.05; U=74.5, n_1=14, n_2=11, P>0.05 respectively). There was no difference in the egg laying rate or egg hatch success between

"+heat+recovery" and "−heat+recovery" females (Mann-Whitney U-test: U=52.5, n_1=10, n_2=10, P>0.05; U=10, n_1=4, n_2=4, P>0.05) (Figs. 6.3 A and 6.4 A).

6.3.2.2 Immune treatment: sterile insult

There were no differences in the survival of "+heat" females compared to "−heat" females (χ^2=0.06, 1 df, P<0.05), or of "+heat+recovery" females compared to "−heat+recovery" females (χ^2=0.61, 1 df, P>0.05). There were no differences in the egg laying rate or the proportion egg hatch success between "+heat" and "−heat" females (Mann-Whitney U-tests: U=137, n_1=15, n_2=15, P>0.05; U=99.5, n_1=10, n_2=11, P>0.05 respectively). There were also no differences in the egg laying rate or egg hatch success between "+heat+recovery" and "−heat+recovery" females (Mann-Whitney U-test: U=48.5, n_1=10, n_2=10, P>0.05; U=74.5, n_1=9, n_2=7, P>0.05) (Fig. 6.4 B).

6.3.2.3 Immune treatment: no treatment control

There were no differences in the survival of "+heat" females compared to "−heat" females (χ^2=2.33, 1 df, P>0.05), or of "+heat+recovery" females compared to "−heat+recovery" females (χ^2=0.00, 1 df, P>0.05) (Fig 6.2 C). "+Heat" females had a significantly higher egg laying rate compared to "−heat" females (Mann-Whitney U-tests: U=172.5, n_1=15, n_2=15, P<0.05) but no difference in their proportion egg hatch success (Mann-Whitney U-tests: U=98.5, n_1=11, n_2=12, P>0.05).

I observed that "−heat" females appeared to take in a smaller blood meal compared to "+heat" females, their bodies were less rotund after feeding. This appeared to be due to a faulty feeding membrane – the sylgard layer used to seal the membrane was much thicker on this particular membrane, and the bedbugs appeared to have difficulty piercing though the membrane to reach the blood below. Measures of *ad libitum* fed body mass confirmed a significant difference in the amount of blood taken up by the two groups (mean±1SE *ad libitum* fed body masses of "+heat" females (10.72±0.587 mg) and "−heat" females 6.81±0.219 mg) (t=5.48, 15 df, P<0.001 on squared data). When these differences were controlled by expressing egg laying rate per mg *ad libitum* fed body mass, there was no difference in the egg laying rate between the "+heat" and "−heat" no treatment control groups (t=0.47, 28 df, P>0.05).

There were no differences in the egg laying rate or the proportion egg hatch success between "+heat+recovery" and "−heat+recovery" females (Mann-Whitney *U*-test: U=117, n_1=10, n_2=10, P>0.05; U=75.5, n_1=9, n_2=10, P>0.05) (Fig. 6.4 B).

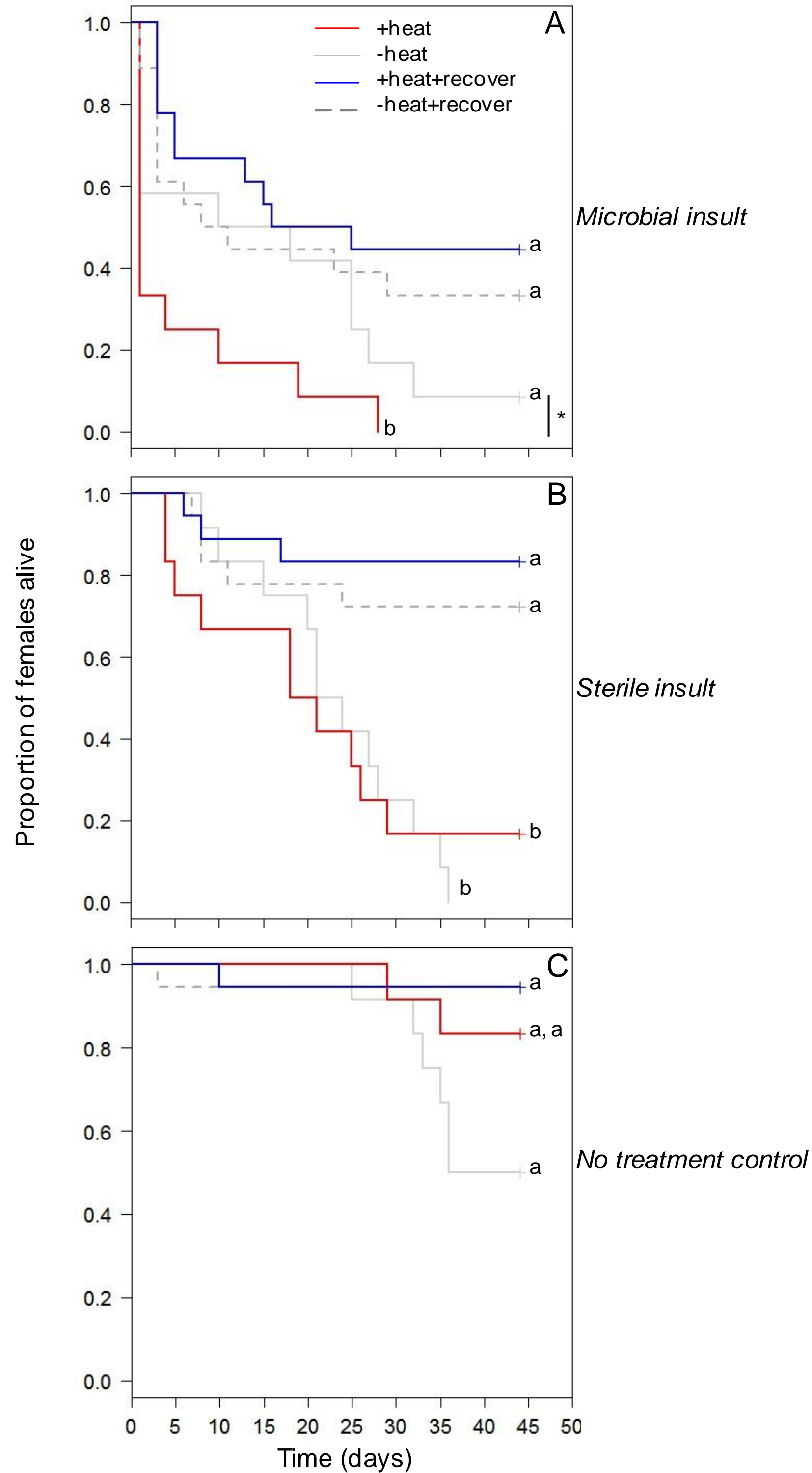


Figure 6.2 The effect of heat treatment on female survival following an immune insult. The survival

of females in heat treatment groups ("+heat", "−heat", "+heat+recovery" and "−heat+recovery") was assayed following an immune treatment ((A) microbial insult, (B) sterile insult and (C) no treatment control). In all immune treatments, *n*=12 in "+heat" and "−heat" groups and *n*=18 in "+heat+recovery" and "−heat+recovery" groups. Groups with the same letter do not differ.

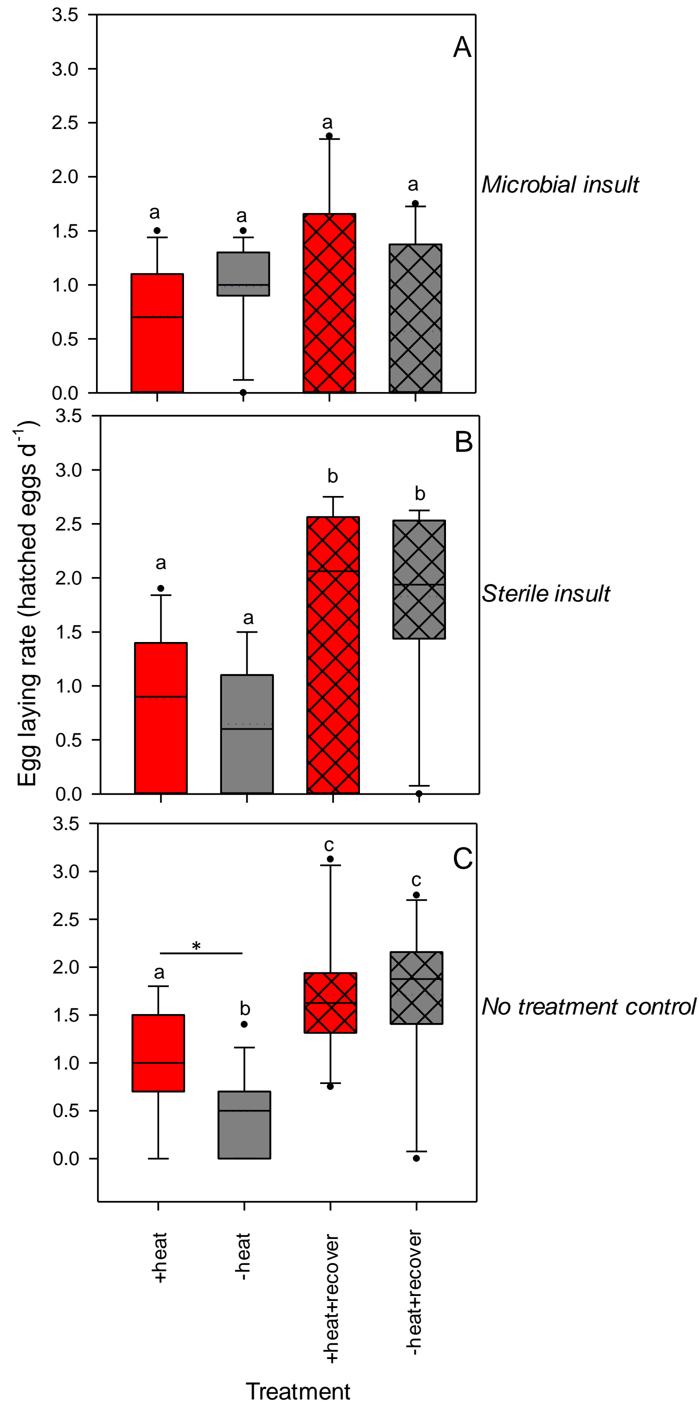


Figure 6.3 The effect of heat treatment on egg laying rate (number of eggs laid per day that hatched) following an immune insult, median (solid line), mean (dotted line). Egg laying rate in four

heat treatment groups (“+heat”, “−heat”, “+heat+recovery” and “−heat+recovery”) was measured following an immune treatment ((A) microbial insult, (B) sterile insult and (C) no treatment control). In all immune treatments, n=15 in “+heat” and “−heat” groups and n=10 in “+heat+recovery” and “−heat+recovery” groups. Groups with the same letter do not differ.

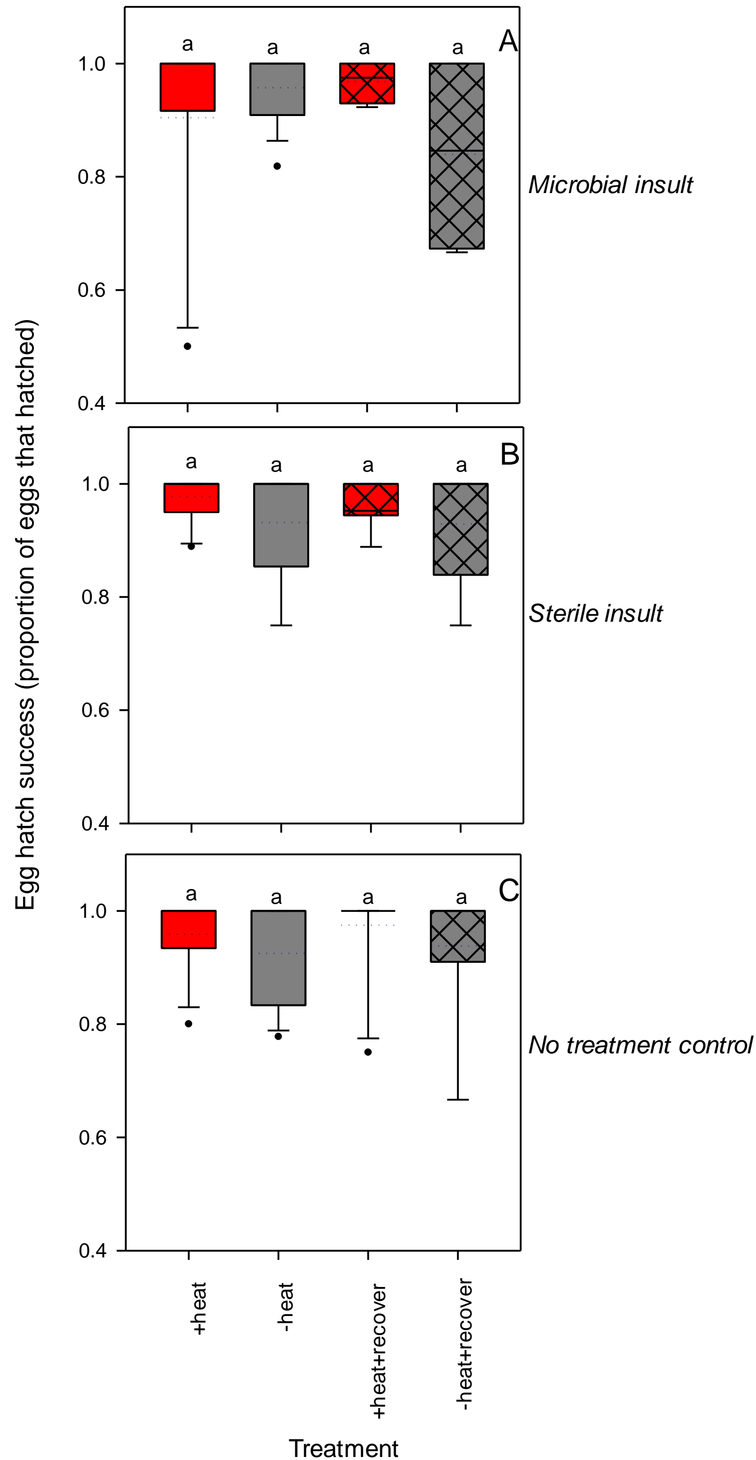


Figure 6.4 The effect of heat treatment on egg hatch success (the proportion of all eggs laid that hatched) following an immune insult, median (solid line), mean (dotted line). Egg hatch success in

heat treatment groups ("+heat", "−heat", "+heat+recovery" and "−heat+recovery") was measured following an immune treatment ((A) microbial insult, (B) sterile insult and (C) no treatment control). The sample sizes for each immune treatment, as shown from left to right are: (A): 11, 14, 4, 4; (B): 11, 10, 7, 9; and (C): 12, 11, 10, 9. Groups with the same letter do not differ.

6.4 Discussion

"+Heat" females had significantly lower *Wolbachia* loads compared to the other three treatment groups ("−heat", "+heat+recovery" and "−heat+recovery"). "+Heat" females also had significantly reduced survival rate following microbial challenge compared to "−heat" females. This difference disappeared when heat treated females were allowed to recover their *Wolbachia* loads suggesting *Wolbachia* may reduce costs associated with opportunistic infection associated with TI. This would ultimately increase host fitness. However, caution must be exercised when drawing these conclusions as the effects of heat treatment and *Wolbachia* mediated effects cannot be separated with certainty. For example heat-mediated effects may also recover during the 2 week recovery period post heat treatment. Manipulating *Wolbachia* load using heat treatment had no effect on egg laying rate or egg hatch success over the course of the experiment.

6.4.1 Effect of heat induced reduction of *Wolbachia* loads on wounding costs

I found that heat treatment to reduce *Wolbachia* loads had no effect on the survival costs associated with wounding. Differences in survival between the "+heat", "−heat" groups and the "+heat+recovery", "−heat+recovery" groups were evident, which likely reflects differences in the feeding status of these groups at the time of wounding ("+heat", "−heat" groups were unfed, whereas "+heat+recovery", "−heat+recovery" groups had received two blood meals, this is because these blood meals are needed in order for the *Wolbachia* load to recover). There are no published reports of *Wolbachia* affecting wound healing in other hosts.

6.4.2 Effect of heat induced reduction of *Wolbachia* loads on microbial infection costs

I found that females which had been heat treated to reduce *Wolbachia* loads had lower survival following microbial challenge compared to "−heat" females (there was no difference in egg laying rates). These results suggest that in the short term, the fitness of "+heat" females is lower than "−heat" females following an immune challenge. The longer-term effects of reducing *Wolbachia* loads using heat treatment are unknown

(recovery of *Wolbachia* loads post heat treatment meant that egg laying rate was only measured over the short-term (period of 10 days)) and may be different. For example, in the long-term "+heat" females may have a higher fitness than "−heat" females if they show terminal investment. Survival differences between "+heat" and "−heat" females after an immune challenge may be due to differences in their *Wolbachia* loads as no survival differences were observed between the "+heat+recovery" and "−heat+recovery" groups. However, one cannot rule out that another effect of heat treatment that may also recovery during the recovery period, was responsible. My results provide evidence that suggests *Wolbachia* in *Cimex lectularius* may be important for their host's immune function.

6.4.3 *Wolbachia* mediated protection

My results suggest heat treatment caused a reduction in host protection against opportunistic microbes. One possibility is that *Wolbachia* mediates some protection in bedbugs so that protection is lower when *Wolbachia* loads are reduced by the heat treatment. There are a number of examples of *Wolbachia* mediating protection in nature. It is likely that the microbial challenge used here consisted of both bacteria and fungi (Reinhardt et al., 2003, Reinhardt et al., 2005). There are no published examples of *Wolbachia* increasing protection against bacterial pathogens; two studies that have investigated this by challenging *D. melanogaster* and *D. simulans*, extracellularly (Wong et al., 2011) or intracellularly (Rottschaefer and Lazzaro, 2012) with three bacterial pathogens: there was no effect of *Wolbachia* infection on resistance (survival). There is one example of *Wolbachia* increasing resistance (survival) against a fungal pathogen, *Blauveria bassiana* Bals. in its *D. melanogaster* host (Panteleev et al., 2007), but the majority of evidence for *Wolbachia*-mediated protection focuses on virus protection.

Reduced mortality from infection with RNA viruses as a consequence of *Wolbachia* infection has been reported in *D. melanogaster* against Drosophila C virus, Nora virus and cricket paralysis virus (Teixeira et al., 2008, Hedges et al., 2008), and in *D. simulans* against Flock House Virus and Drosophila C virus (Osborne et al., 2009). There is variability in the protection conferred between different *Wolbachia* strains with some increasing the host's tolerance to viral infection, some reducing virus replication and increasing the host's resistance, and others having no effect at all (Osborne et al., 2009).

One study has also found a positive relationship between *Wolbachia* density and the degree of resistance conferred (Osborne et al., 2009). The mechanisms of virus protection in *D. melanogaster* and *D. simulans* are not yet fully understood, however some insights may be gained from *Wolbachia*-mediated protection in the mosquito *Aedes aegypti*.

Wolbachia is not naturally found in *A. aegypti*, which is a major vector of human disease. To discover new methods of controlling the spread of these diseases researchers have experimented with infecting *A. aegypti* with *Wolbachia* from either *D. melanogaster* (Teixeira et al., 2008), or the mosquito *A. albopictus* (Bian et al., 2010). *Wolbachia* infection of *A. aegypti* reduced viral loads and virus transmission rates (Teixeira et al., 2008, Bian et al., 2010). Infection with *Wolbachia* from *D. melanogaster* has also been found to reduce the loads of *Plasmodium falciparum* in *A. aegypti*, (Moreira et al., 2009), and of filarial nematodes *Brugia pahangi* in *A. gambiae* (Kambris et al., 2009) suggesting a general immune mechanism.

Wolbachia infection is associated with an increased basal expression of immune genes and it is thought *Wolbachia* increases resistance through priming general aspects of immunity that are effective against a range of pathogens (Moreira et al., 2009). The melanisation response was found to be higher in *Wolbachia* infected *D. melanogaster*, *D. simulans* and *A. aegypti* suggesting this may be a candidate mechanism (Thomas et al., 2011). Melanisation is an important component of wound healing and the encapsulation response (Siva-Jothy et al., 2005). *Wolbachia* in *A. aegypti* also increases the production of the antimicrobial peptides defensins and cecropins (Pan et al., 2012).

My results suggest the host's immune function returns to normal by 2 weeks post heat treatment, and coincides with the recovery of *Wolbachia*. Although this supports the suggestion that *Wolbachia* are mediating protection, it is possible that an unknown factor is altered by heat treatment leaving the host more susceptible to microbial infection, and that this factor also recovers within the recovery timeframe. I therefore cannot be certain that it is the *Wolbachia* that are mediating these effects. The underlying mechanisms are worth exploring further and may provide some insight into what factor is mediating these differences. It is unknown how *Wolbachia* may increase resistance in bedbugs and given that studies to date suggest symbiont-mediated protection mechanisms vary widely

between different systems, such mechanisms in the bedbug are difficult to predict. Given that bedbugs have a spermalege, a unique immune organ that has evolved to offset the costs of TI (Reinhardt et al., 2003, Morrow & Arnqvistn, 2003), it is possible that *Wolbachia* may affect the immune functioning of this organ to reduce the costs of infection.

6.4.4 Summary

In this chapter I have:

1. Determined that heat treatment results in increased survival costs in response to TI-associated microbial infection. This is associated with reduced *Wolbachia* loads. I showed this using a protocol that reflects what happens during natural infection events, using a natural route of infection and microbes isolated from the host's natural environment and cuticle.
2. Determined that the effects of heat treatment are evident over short time scales. It is unknown how this relates to the female's long term fitness.

These findings raise the question: "How does *Wolbachia* protect against microbial infection in their female hosts?"

Chapter 7

Effect of *Wolbachia* on Female Immunity

7.1 Introduction

I have shown that *Wolbachia* protect their female hosts against the microbial infection (chapter 6) that is associated with TI (Reinhardt et al., 2005). The mechanistic basis for this protection is unknown. In this chapter I ask: "how do *Wolbachia* protect their host from TI-associated microbial infection?"

7.1.1 *Wolbachia*-mediated protection

Previous studies have shown that *Wolbachia* may protect their host from a range of immune insults from (i) RNA viruses (in *Drosophila melanogaster* (Hedges et al., 2008, Teixeira et al., 2008)), (ii) naturally occuring parasites such as protozoa (e.g. *Plasmodium gallinaceum* in artificially infected *Aedes aegypti* (Moreira et al., 2009), and *Anopheles gambiae* (Kambris et al., 2010)), (iii) filarial nematodes and gram-negative bacteria in artificially infected *A. aegypti* (Kambris et al., 2009)) and (iv) sexually transferred opportunistic microbes in bedbugs *Cimex lectularius* (chapter 6).

Few studies have explored the mechanistic basis of *Wolbachia*-mediated protection, and those that have, suggest mechanisms may be direct or indirect and may also vary between different hosts, *Wolbachia* strains and parasites. For example in fruit flies, the *w*Mel strain of *Wolbachia* in *D. melanogaster* increases host resistance to *Drosophila* C virus (DCV) by interfering with viral replication (Teixiera et al., 2008), whereas in *D. simulans* infected with DCV, the *w*Au stain of *Wolbachia* increases the host's tolerance to high virus loads and associated pathogenesis (Osborne et al., 2009). In mosquitoes, resistance to *Plasmodium sp.* is conferred by artificially infecting the host with *Wolbachia*. In *A. aegypti*, the wMel-Pop-CLA strain of *Wolbachia* confers resistance against *P. gallinaceum* through competition for shared resources (Moreira et al., 2009); whereas in *Anopheles stephensi* Liston, the *w*AlbB strain of *Wolbachia* confers resistance against *P. falciparum*

through the production of reactive oxygen species (Bian et al., 2013). In *D. melanogaster*, *Wolbachia* increase host resistance to the fungal pathogen *Blauveria bassiana* Bals. by up-regulating a general immune response within the host (Panteleev et al., 2007), such as melanisation (Thomas et al., 2011). The variety of mechanisms demonstrated by these examples makes it difficult to predict what *Wolbachia*-mediated mechanisms are operating in bedbugs. I will begin my investigation by examining whether *Wolbachia* have an effect on two easily-assayed aspects of bedbug immunity.

7.1.2 Bedbug immunity

Bedbugs have an innate immune system which is conventionally subdivided into cellular mechanisms (those involving cells) and humoral mechanisms (involving soluble immune effectors) (See 1.5 for a general introduction to bedbug immunity). Previous investigations in bedbugs have identified lysozyme-like enzymes (Naylor, 2006, Otti et al., 2009, Bai et al., 2011) and phenoloxidase (PO) (Naylor, 2006) as important humoral effectors, and phagocytosis and clotting (e.g. Siva-Jothy, 2006) as important cellular processes. These effectors and processes vary in their constitutive levels and inducibility and in their expression between the spermalege and the haemocoel. I ask whether *Wolbachia* affect (i) constitutive or induced levels of lysozyme-like activity in the spermalege and haemocoel and (ii) the cellular encapsulation response within the spermalege and haemocoel. Details about these aspects of bedbug immunity are given below.

7.1.2.1 Lysozyme-like activity

Lysozyme-like enzymes, manufactured in the fat body (Gillespie et al., 1997), are present constitutively in the bedbug's haemolymph and are up-regulated in response to bacterial infection (Naylor, 2006). In males, lysozyme-like activity (LLA) has also been observed in the ejaculate and is thought to protect the sperm from microbes (Otti et al., 2009). Lysozyme has been found in all insect orders (Gillespie et al., 1997), and in some (e.g. Lepidoptera and Diptera) it is known to work in synergy with two other groups of antibacterial proteins: attacins and cecropins (Boman and Hultmark, 1987). Because these protein(s) are detected by a lytic-zone assay that uses purified lysozyme as a standard, and because the exact nature of the "cocktail" of proteins is not known, researchers refer to

antibacterial action showing the characteristic feature of lysozyme digestion as "lysozyme-like activity" (LLA) (Otti et al., 2009, Naylor, 2006).

Lysozyme is predominantly effective against gram positive bacteria (Adamo, 2004) which are killed by hydrolysis of the peptidoglycan cell wall, causing cell lysis (Kurtz et al., 2000). LLA of bedbugs can be assayed *in vitro* (Naylor, 2006, Haine et al., 2007, Otti et al., 2009): because LLA titre is directly related to the insect's ability to resist some bacteria, a LLA assay allows investment in this aspect of immune function to be compared between individuals (Adamo, 2004).

7.1.2.2 Encapsulation

When levels of microbial infection are high, bedbugs respond with a process known as nodulation (Siva-Jothy and Tsubaki, 1998) whereby clusters of microbes which are too large to be individually phagocytosed are externalised *in situ* within impermeable, unreactive, nodules of melanised haemocytes. Melanin is the end product of a protein cascade catalysed by PO, which yields toxic by-products (free oxygen species, phenols and quinines) that may kill the microbes early in the nodulation process. The melanin coating is also thought to prevent further microbial growth and reduces the toxic by-products' potential to damage host tissues in the vicinity.

In immune studies it is more usual to assay encapsulation rather than nodulation. Encapsulation is believed to be the same process as nodulation but occurs around multicellular parasites such as parasitoid wasp eggs or larvae (e.g. Siva-Jothy and Tsubaki, 1998). Phenotypically similar to nodulation, encapsulation involves the formation of a multi-cell capsule surrounding the multicellular parasite and a humoral melanisation response whereby the parasite and cell capsule are melanised. The encapsulation response is considered a good general indicator of an insect's immune function, as it involves the coordinated action of both humoral and cellular immunity (Siva-Jothy and Tsubaki, 1998).

7.1.2.3 Immunity in the spermalege and haemocoel

In bedbugs, the spermalege and the haemocoel are immunologically distinct; for example constitutive levels of LLA measure significantly higher in the haemocoel compared to the spermalege (Naylor, 2006) and haemocyte densities are substantially greater in the

spermalege compared to the haemocoel (Siva-Jothy, 2006). This reflects differences in the risk of infection to these areas. Infection risk in the spermalege is high, being the site of TI where opportunistic microbes will first enter the host through copulatory wounds. As the infection will have to pass through the spermalege before entering the haemocoel, infection risk in the haemocoel will be much lower by comparison. This raises the question, "does *Wolbachia* differentially affect the immune response in these compartments?"

7.1.3 Aims of chapter

1. To quantify the effect of reducing *Wolbachia* loads using heat treatment on constitutive levels of LLA inside the spermalege and haemocoel.
2. To quantify the effect of reducing *Wolbachia* loads using heat treatment on induced LLA inside the spermalege and haemocoel.
3. To quantify the effect of reducing *Wolbachia* loads using heat treatment on the melanotic and cellular encapsulation response in the spermalege.
4. To quantify the effect of reducing *Wolbachia* loads using heat treatment on the melanotic and cellular encapsulation response in the haemocoel.

7.2 Materials and Methods

7.2.1. Insects

The insects used in these experiments were once-fed, 3 days old virgin adults that had been raised using protocols 2.2.2.1 and 2.2.3. Insects were imaged so that pronotum width measurements could be recorded as a measure of individual body size (2.2.2.2). The size of each individual's blood meal was measured following standard protocols (2.2.3.4). Between treatments, all insects were housed individually in 5 mL plastic tubes with a strip of filter paper (1 x 3 cm) under controlled environmental conditions of 26°C±2°C, 70% RH in a 12:12 light:dark cycle.

7.2.2 The effect of heat treatment to reduce *Wolbachia* loads on LLA

7.2.2.1 Experimental reduction and measurement of Wolbachia *load*

Two groups with different *Wolbachia* loads were generated using the heat treatment protocol (A4.1.2): "+heat" (heat treated at 36°C for 2 weeks to lower their *Wolbachia* loads) and "−heat" (maintained at 26°C for 2 weeks, a temperature that does not

compromise *Wolbachia* loads and thus loads are higher in this group relative to "+heat" females). The mean *Wolbachia* load of each treatment group was determined from subsamples of 6 individuals using qPCR analysis (details in 2.4).

7.2.2.2 Immune treatment groups

Females from the two groups with altered *Wolbachia* load ("+heat" and "−heat") were allocated to each of three immune treatment groups ("microbial insult", "sterile insult" and "no treatment control"). The microbial insult consisted of piercing the female's spermalege with a needle that had been dipped for 3 seconds in a suspension of microbes in PBS (details in 6.2.3.1). To distinguish the effects of microbial insult from the effects of wound repair, sterile insult groups were generated by wounding the female in the spermalege with a needle dipped in sterile PBS. The no treatment control group consisted of handled, but not wounded, females.

48 hours after the immune treatment, a 2 μL sample of haemolymph was taken from each female from a superficial wound made into the end of her abdomen using a 2 uL pipette, and immediately loaded onto a *Micrococcus luteus* agar plate to assay LLA in the haemocoel. The mesospermalege was also dissected at this time from each female and loaded onto a *M. luteus* agar plate to assay LLA in the spermalege. LLA levels in microbially insulted females provided a measure of induced immunity, and in the NTC group provided a measure of constitutive immunity.

7.2.2.3 LLA assay

LLA was measured *in vitro*, as its ability to clear lyophilised bacteria suspended in an agar plate, following the protocol of Naylor (2006). In brief the agar plates consisted of a suspension of lyophilised *Micrococcus luteus* in agar (*M. luteus* 5 mg mL^{-1}, agar 10 mg mL^{-1}, streptomycin sulphate 0.1 mg mL^{-1} and 2% triton X-100) poured out into a Nunclon™ 12 well culture plate lid, with 12 x 1.5 mm diameter wells into which the haemolymph or mesospermalege samples were loaded. After the samples had been loaded, the plates were incubated for 72 hours at 30°C (Naylor, 2006). The action of the lysozyme in the samples on the suspended bacteria, produces a clear zone around the point where the sample has been loaded (Plate 7.1). The area of this zone of clearance is proportional to the amount of LLA in the sample (Naylor, 2006). The zones of clearance surrounding the holes

were imaged using a stereomicroscope and digital camera and processed as described in 2.3.3.

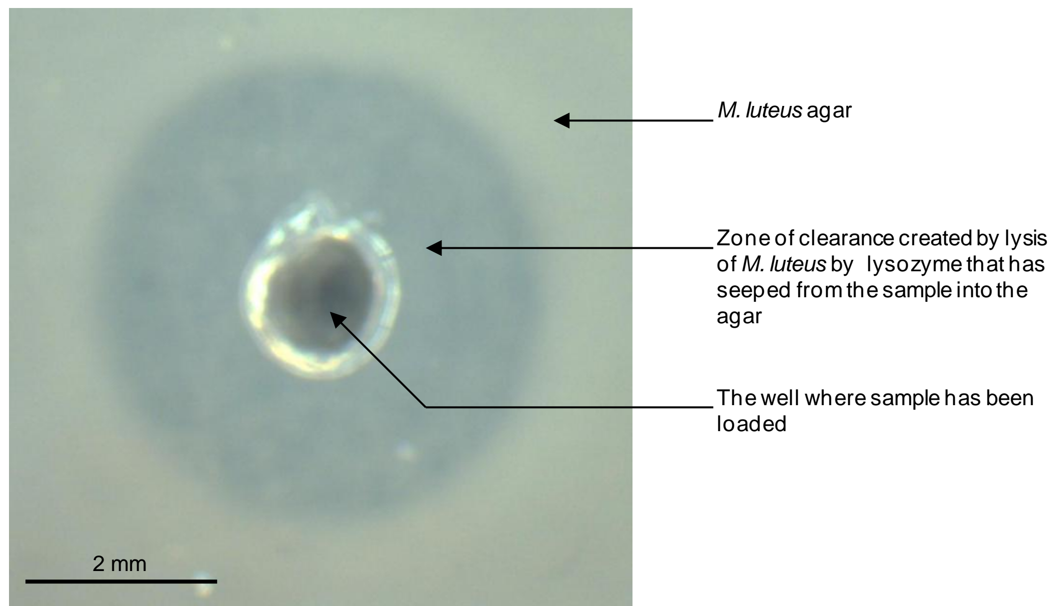


Plate 7.1 *Micrococcus luteus*-agar plate to demonstrate a zone of clearance. When the plate is put on a dark surface, the zone of clearance is visibly darker than the rest of the *M. luteus*-agar plate.

Areas of clearance were converted to units of lysozyme-like activity based on a standard curve of 72-hour incubation created from serial dilutions of lysozyme (from chicken egg-white) to concentrations of 0.001, 0.002, 0.004, 0.006, 0.01, 0.02, 0.04, 0.06, 0.1, 0.2, 0.4 and 0.6 mg mL^{-1} (n=10) in (Naylor, 2006) (Fig. 7.1). The lysozyme concentration was expressed as an absolute number of units in each well, enabling the activity in the mesospermalege samples to be expressed as units of lysozyme equivalents.

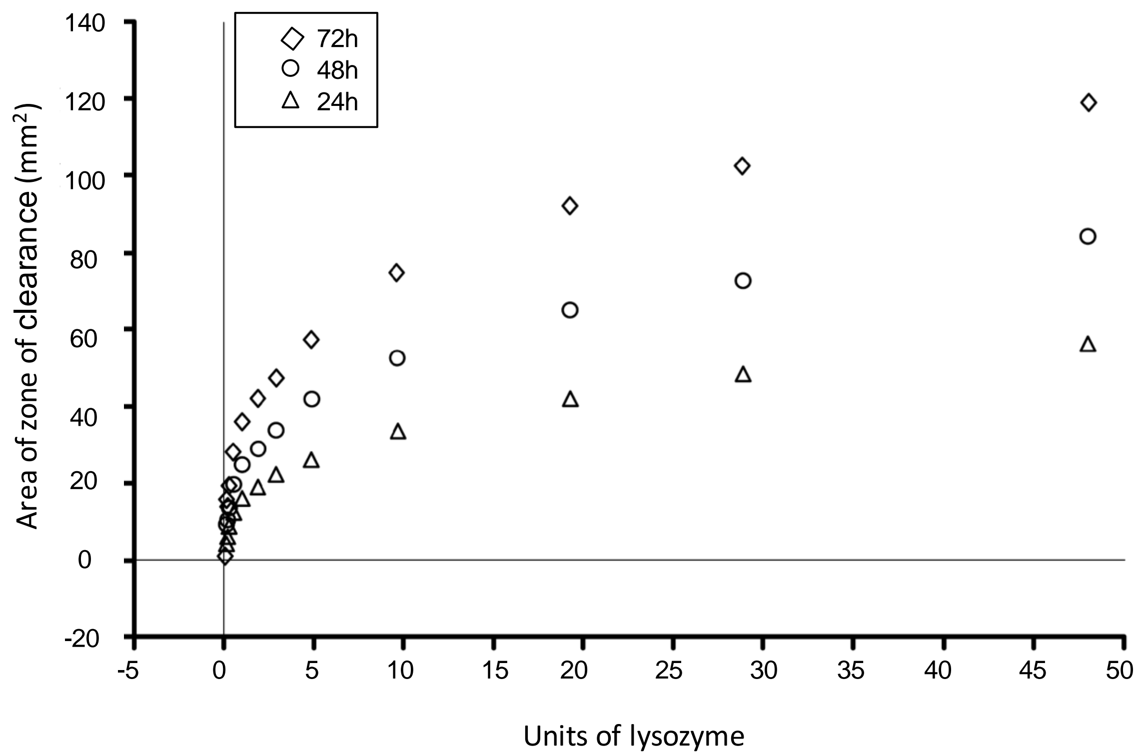


Figure 7.1 The relationship between the zone of clearance and lysozyme activity for three incubation times of 24, 48 and 72 hours. For each time, an equation describing the relationship between units of lysozyme (U) and area of the zone of clearance (a) was produced using regression ($U=e^{(a-b)/c}$). At 24 hours $U=e^{(a-18.631)/7.634}$ (r^2=0.943, P<0.0001); at 48 hours $U=e^{(a-29.599)/11.396}$ (r^2=0.952, P<0.0001) and at 72 hours $U=e^{(a-41.873)/16.04}$ (r^2=0.949, P<0.0001). Modified from Naylor (2006).

7.2.3 The effect a heat induced reduction in *Wolbachia* loads on the encapsulation response

7.2.3.1 Experimental reduction, recovery and measurement of *Wolbachia* load

Four treatment groups were generated using the heat treatment protocol (A4.1.2) as before (6.2.2): "+heat" (36°C for 2 weeks to lower *Wolbachia* loads)*;* "−heat" (26°C for 2 weeks to maintain normal *Wolbachia* loads) "+heat+recovery" (36°C for 2 weeks then 26°C for 2 weeks with weekly feeds) and "−heat+recovery" (26°C for 2 weeks then 26°C for 2 weeks with weekly feeds). The *Wolbachia* loads for each treatment group was determined from 6 individual hosts taken from each treatment group using qPCR analysis (details in 2.4).

7.2.3.2 Immune treatment groups

The melanotic and cellular encapsulation response was measured in the spermalege and haemocoel of the four treatment groups. The encapsulation response can be triggered by inserting an inert antigen, such as a nylon filament. I inserted a 1 mm long (±0.162 SD (n=113)) x 0.1 mm diameter piece of silicon-free nylon monofilament through a puncture wound into the female's spermalege or her haemocoel (Fig. 7.2). The implant was left *in situ* for 24 hours (de Souza et al., 2009) after which the encapsulation process was halted by storing (and killing) the females at -80°C. After defrosting, the implants were removed from the female and mounted on a glass slide in PBS under a cover-slip bridge so that the degree of melanisation and size of cellular encapsulation could be measured.

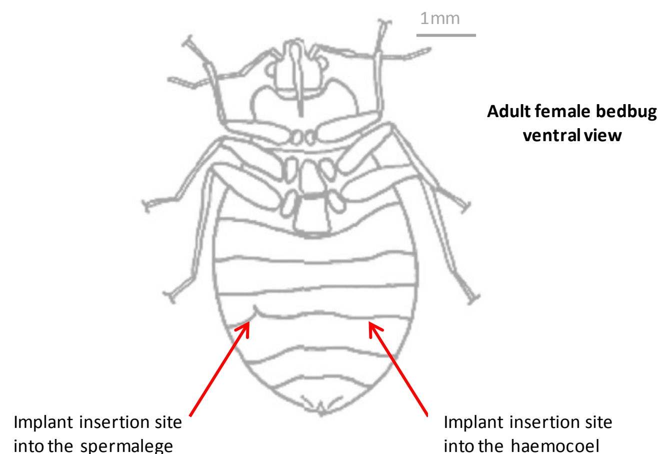


Figure 7.2 Diagram to show two sites where the nylon implant was inserted: through the ectospermalege (and the mesospermalege), or through the abdomen between the 4th and 5th abdominal sternites, parallel to the ectospermalege, into the haemocoel. Diagram modified from Reinhardt et al., (2003).

7.2.3.3 Measurement of melanisation

The melanisation (darkness) of the filament was examined under a stereomicroscope and both upper and lower sides were imaged (2.3.3) (Plate 7.2). An average melanisation value was calculated from the two images using methods detailed below. Images were analysed in ImageJ 1.46r which produces a frequency distribution of light intensities in a user-defined region of interest and gives a weighted average luminance (WAL) on a greyscale between between 0 and 255 (0 darkest, 255 lightest). For each image, the grey value of the nylon was subtracted from the grey value of the background to correct for variation in the

light intensity between images. Because a smaller grey value indicates higher melanisation rate, data were inverted and counter-intuitive. The data were therefore transformed so that the highest grey values corresponded with the highest degree of melanisation. The transformation was done by i) subtracting the observed grey values from the control grey value (clear implant (220)) ii) 220 divided by the value gained in i). To measure the repeatability of this method I chose 12 images at random and analyzed them as above. The repeatability (R) of this method was high (R=0.998; $F_{11,24}$=1740.61, P<0.0001).

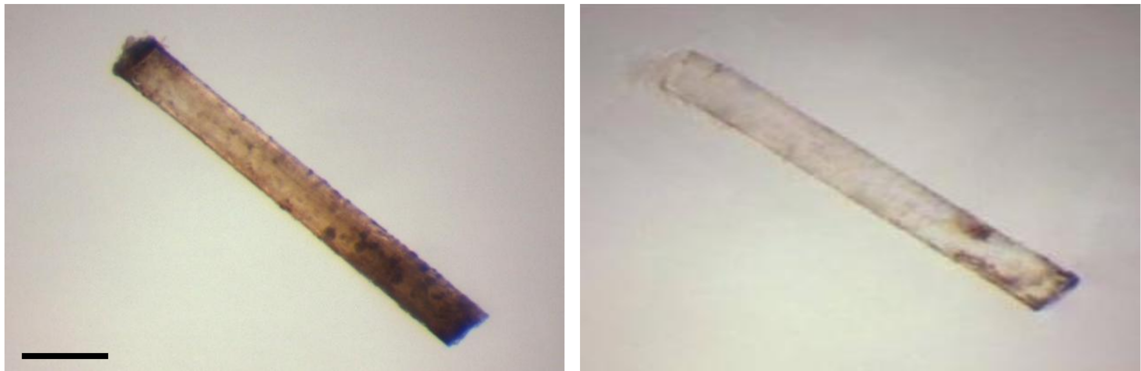


Plate 7.2 Nylon implants to demonstrate different degrees of melanisation (darkness): high melanisation on the left and low melanisation on the right. Bar, 0.2 mm.

7.2.3.3 Measurement of the number of encapsulating cells

Capsule volume was used as a surrogate measure for the number of encapsulating cells using methods modified from (Siva-Jothy and Tsubaki, 1998). In order to measure the volume of the encapsulating cells the filament was placed on a microscope slide under a coverslip bridge (0.1 mm depth) and imaged down a compound microscope (2.3.2) (Plate 7.3). The microscope slide was flipped over and another image taken so that both sides of the capsule were imaged. An average capsule volume was calculated from the two images. To measure the repeatability of this method I chose 10 images at random and analysed them as above. The repeatability (R) of this method was high (R=0.996, $F_{9,20}$=694.21, P<0.0001).

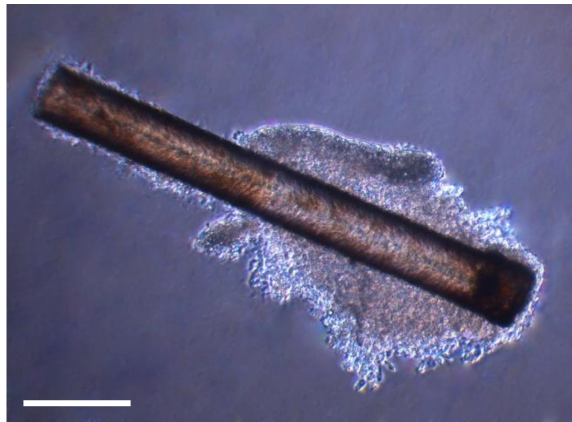


Plate 7.3 A nylon implant to show the cell capsule. Image was taken down a compound microscope in dark-field phase contrast, Bar, 0.2 mm.

7.2.4 Data Analysis

Units of LLA are expressed per unit pronotum width to control for individual variation in body size (LLA is positively related to body size, (Fig. A3.2)). To control for possible confounding variation in the body size of individuals and in the size of nylon implants, measurements of melanisation and capsule volume were analysed with ANCOVAs, with pronotum width and size of nylon implants as co-variates. The statistical analyses used are detailed in 2.5.

7.3 Results

7.3.1 Effect of a heat induced reduction in *Wolbachia* loads on LLA

The *Wolbachia* load of individuals in the "+heat" groups (1.168±0.297) were significantly lower than for individuals in the "−heat" groups (20.06± 3.88) (*t*-test: t=8.40, 10 df, P<0.0001 on $\log_e(x+1)$). There was no effect of heat treatment (ANOVA: $F_{1,30}$=0.83, P>0.05); a significant effect of immune treatment ($F_{2,30}$=7.21, P<0.01), and no interaction effect between these treatments ($F_{2,30}$=1.39, P>0.05) on the units of LLA measured in the mesospermalege (Fig. 7.3). There was no effect of heat treatment ($F_{1,25}$=0.527, P>0.05), immune treatment (ANOVA: $F_{2,25}$=0.770, P>0.05) or interaction between these treatments ($F_{2,25}$=1.91, P>0.05) on the units of LLA measured in the haemocoel (Fig. 7.3).

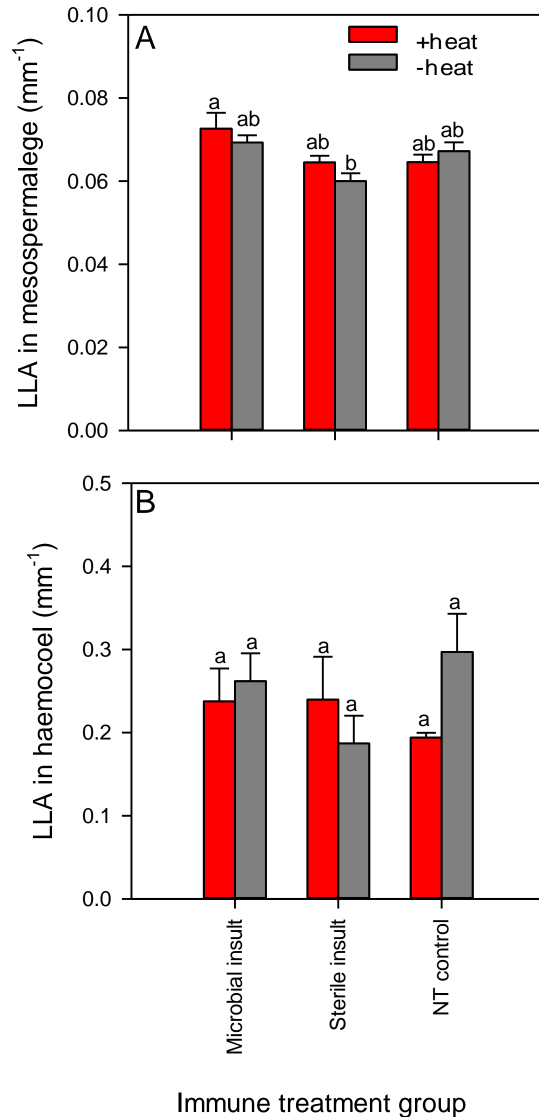


Figure 7.3 Effect of heat treatment (+heat and −heat) on mean units of lysozyme-like activity (LLA) in (A) the mesospermalege and (B) the haemocoel, in response to three immune treatments (microbial insult, sterile insult and no treatment control). LLA is expressed per unit pronotum width (mm) a measure of body size. *N*=6 each group except for measures of LLA in the haemocoel for "+heat" females (microbial insult *n*=4; sterile insult *n*=5; no treatment control *n*=4) groups. Groups having the same letter code do not differ (Tukey mcp test *P*>0.05).

7.3.2 Effect of a heat induced reduction in *Wolbachia* loads on the encapsulation response

7.3.2.1 Comparing Wolbachia *loads across heat treatment groups*

There was a significant difference in the *Wolbachia* load between the four heat treatment groups (ANOVA, $F_{3,20}$=50.26, P<0.0001) on $\log_e(x+1)$ data. *Wolbachia* loads in the "+heat" group were significantly lower than all the other groups (Tukey mcp test P<0.0001) Fig. 6.1.

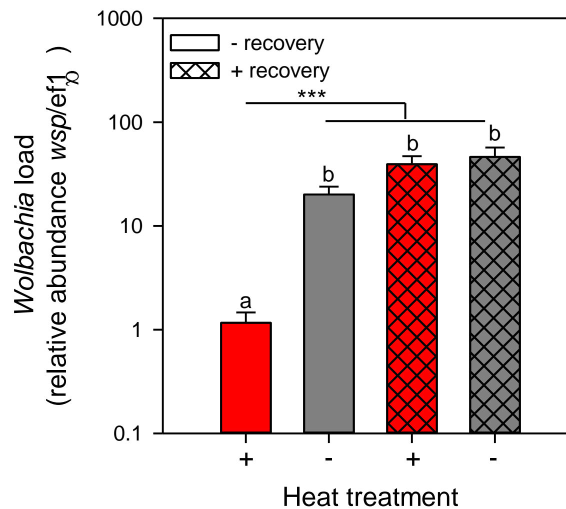


Figure 7.4 The effect of heat treatment (with or without a recovery period) on female *Wolbachia* loads. Females were maintained in a high (+) or normal (-) temperature environment for two weeks, followed by (+) or not (-) a two week recovery period. N=6 each group, means with the same letter do not differ from each other (Tukey mcp on $\log_e(x+1)$ data, P>0.05), bars represent 1SE.

7.3.2.2 Encapsulation response in the mesospermalege

There was a significant effect of heat treatment on the degree of melanisation (ANCOVA $F_{3,49}$=5.75, P<0.01 on $\log_e(x)$ data), and no effect of pronotum width ($F_{1,49}$=0.72, P>0.05) or size (surface area) of the nylon implant ($F_{1,49}$=0.43, P>0.05). The degree of melanisation was significantly lower in the "+heat" group compared to other groups (Tukey mcp test P<0.05) (Fig. 7.5). There was no effect of heat treatment to reduce *Wolbachia* loads on the volume of the encapsulating cell mass (ANCOVA $F_{3,50}$=1.99, P>0.05 on $\log_e(x+1)$ data); nor an effect of the pronotum width ($F_{1,50}$=1.75, P>0.05) or size of nylon implant ($F_{1,50}$=4.00, P>0.05) (Fig. 7.5).

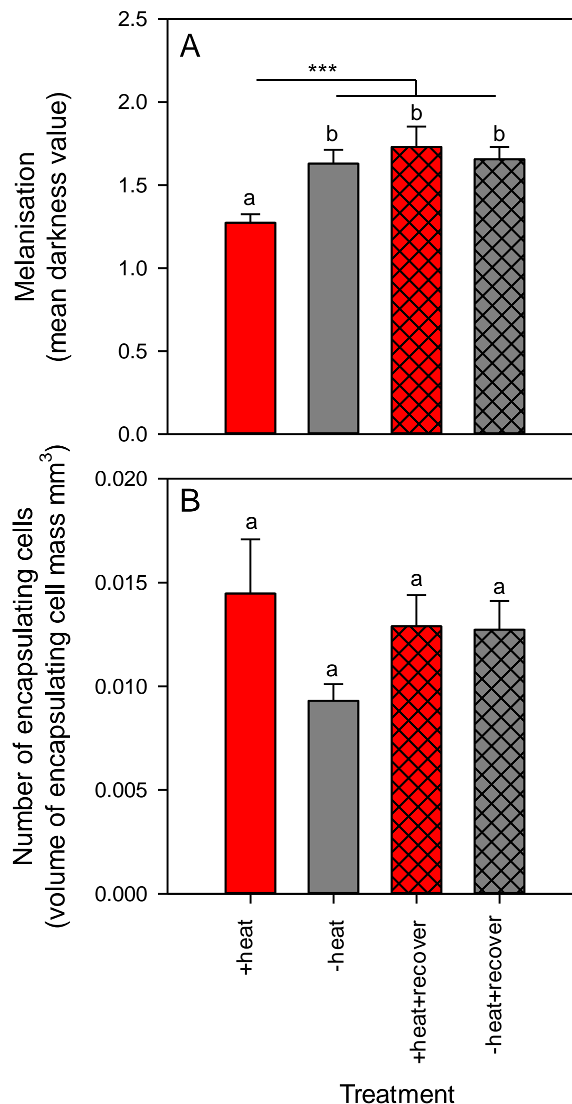


Figure 7.5 The effect of heat treatment on the encapsulation response in the mesospermalege. (A) Mean degree of melanisation (mean darkness value). (B) Number of encapsulating cells (mean volume of encapsulating cell mass (mm^3)). *N*=14 each group except for the melanisation response in "+heat" group (*n*=13). Means having the same letter code do not differ (Tukey mcp test *P*>0.05), bars represent 1SE.

7.3.2.3 Encapsulation response in the haemocoel

There was no effect of heat treatment on the degree of melanisation (ANCOVA $F_{3,45}$=0.65, *P*>0.05 on $\log_e x$ data) nor an effect of pronotum width ($F_{1,45}$=0.06, *P*>0.05) or size of nylon implant ($F_{1,45}$=0.18, *P*>0.05) (Fig. 7.6). There was no effect of heat treatment to reduce *Wolbachia* loads on the volume of the cellular capsule (ANCOVA $F_{3,45}$=1.42, *P*>0.05 on

$\log_e(x+1)$ data) nor an effect of the covariates pronotum width ($F_{1,45}$=0.94, P>0.05) or size of nylon implant ($F_{1,45}$=3.59, P>0.05) (Fig. 7.6).

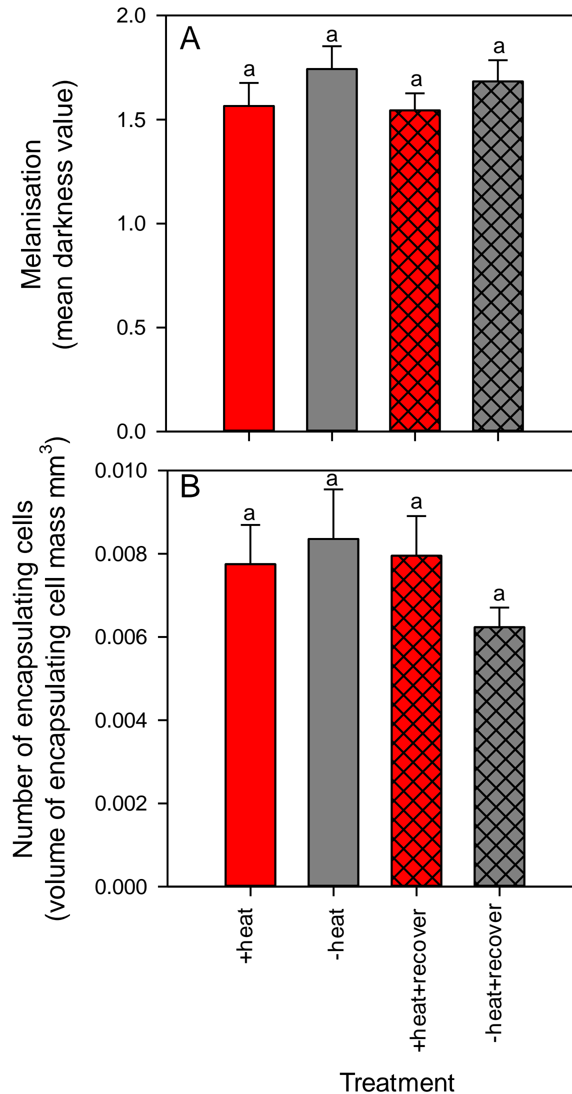


Figure 7.6 The effect of heat treatment ("+heat" n=10, "−heat" n=13, "+heat+recovery" n=13, "−heat+recovery" n=15) on the encapsulation response in the haemocoel. (A) Mean degree of melanisation (mean darkness value). (B) Number of encapsulating cells (mean volume of encapsulating cell mass (mm^3)). Means having the same letter code do not differ (Tukey mcp test P>0.05), bars represent 1SE.

7.4 Discussion

I tested the effect of reducing *Wolbachia* load using heat treatment on three aspects of host immunity: (i) LLA (ii) volume of encapsulating cells and (iii) degree of melanisation in the capsule. LLA in the spermalege and haemocoel showed no measureable differences across treatments. However, there was an effect of heat treatment on the ability of bedbugs to melanise encapsulating cells in the spermalege. These results suggest that *Wolbachia* may protect their host from sexually transferred microbial infection by increasing the melanisation rate in the spermalege. The results are discussed in detail below.

7.4.1 Lysozyme-like activity (LLA)

I found no differences in constitutive or induced levels of LLA between "+heat" and "−heat" females suggesting that *Wolbachia* do not affect LLA in bedbugs.

7.4.2 Encapsulation response

Heat treatment to reduce *Wolbachia* loads showed no measurable effect on the cellular encapsulation response in the spermalege, but showed a significant reduction in the melanisation response. Heat treatment corresponded with a reduction in the degree to which the nylon insert was melanised in the spermalege but there was no effect of heat treatment on the degree of melanisation observed in the haemocoel. Furthermore the difference in melanisation observed in the spermalege disappeared when heat treated individuals were allowed to recover. As *Wolbachia* loads would recover during this time, this suggests that a difference in the *Wolbachia* load of "+heat" and "−heat" females may be responsible for the difference in melanisation observed in the spermalege. However, it is possible that another, identified factor, altered by the heat treatment which also recovered during the recovery period, is responsible for this effect although elevated temperatures had no measurable effects on these aspects of melanisation in *Tenebrio molitor* or *Acheta domesticus* (Siva-Jothy pers com).

The deposition of melanin as an immune response occurs during the encapsulation of multicellular organisms and around clusters of microbes within cellular nodules and at wound sites (Tang, 2009). Melanisation is a fast-acting response which generates quinines and other reactive oxygen intermediates which are thought to be directly toxic to

microorganisms and other parasites (Tang, 2009). As part of the wound healing response, any introduced microbes are melanised and sequestered at the wound site (Tang, 2009). By increasing the melanisation response in the spermalege, *Wolbachia* may reduce the wounding and infection costs associated with TI (Stutt and Siva-Jothy, 2001, Reinhardt et al., 2005). This may explain why microbially challenged "+heat" females had a lower survival compared to "−heat" females (chapter 6). If *Wolbachia* can affect an increase in the melanisation this would be beneficial to the *Wolbachia* because this would increase the fitness of the female host with whom *Wolbachia*'s fitness is directly related.

My results suggest that the heat treatment protocol, reduced melanisation in the spermalege, but not in the haemocoel. The spermalege is the natural entry point for microbes associated with TI, not the haemocoel, therefore the risk of infection is greater in the spermalege. The production of melanin also produces cytotoxic substances which are thought to kill parasites, but can also cause collateral damage to host tissues (Tang, 2009). By limiting an increased melanisation response to the spermalege, damage to the host's tissues elsewhere in the haemocoel may be prevented or at least reduced. If *Wolbachia* are mediating an increase in the melanisation response, it makes sense that they should do so in the spermalege, since this is the main arena for controlling haemocoelic infection of the host. These findings raise the question, what could be the proximate mechanisms of *Wolbachia*- mediated melanisation?

It is possible that *Wolbachia* could affect the melanisation response indirectly through its supply of B vitamins to the host. The melanisation response may be reduced in hosts with lower *Wolbachia* loads as a result of heat treatment, if the low *Wolbachia* loads cause a B vitamin deficiency which the host compensates for by trading-off investment in the production of melanin's inactive precursor molecules for a life-history trait such as egg production.

7.4.3 Summary

In this chapter my results suggest that:

1. Manipulating the *Wolbachia* loads of a female host using heat treatment does not affect induced levels of LLA inside the spermalege and haemocoel in response to microbial challenge directed through the spermalege.
2. A heat-induced reduction of a female's *Wolbachia* load is associated with a decrease in the rate of melanotic encapsulation, but does not affect the cellular encapsulation response in the spermalege.
3. A heat-induced reduction of a female's *Wolbachia* load does not affect the rate of melanotic encapsulation or the cellular encapsulation response in the haemocoel.

Chapter 8

General Discussion

8.1 Introduction

The global resurgence of bedbugs is proving difficult to control due to widespread resistance to most common classes of insecticide; developing non-chemical control strategies is therefore a matter of urgency. It has been suggested that manipulating the symbiotic *Wolbachia* loads within bedbugs may be a suitable alternative; because it has potential to control other pest species (e.g. McMeniman et al., 2009) or the diseases they transmit (e.g. Walker et al., 2011, Bian et al., 2013). When *Wolbachia* is used in biocontrol it is usually based on its ability to induce C.I. in the host, or to drive 'control' genes into a population. However, *Wolbachia* in bedbugs are nutritional mutualists (Hosokawa et al., 2010) (they do not induce C.I. and already occur at high infection levels (>83% (Sakamoto & Rasgon, 2006)) within populations). Whether *Wolbachia* manipulation could be used in biocontrol is unclear.

In this thesis I set out to increase understanding of *Wolbachia*'s role on bedbug biology in order to assess if and how *Wolbachia* manipulation could be effective to control bedbugs. I specifically looked at whether *Wolbachia* mediate any other effects in their hosts (besides nutritional provisioning), whether these effects manifest differently between the genders, and whether effects are transgenerational. To achieve this I developed methods to manipulate levels of *Wolbachia* infection in bedbugs, and used these to experimentally investigate the fitness effects and underlying mechanisms of *Wolbachia*'s symbiotic relationship with males, females and their offspring. The implications of these findings for bedbug control strategies and future studies are discussed.

8.2 Thesis overview

Two methods to manipulate *Wolbachia* load in bedbugs: antibiotic and heat treatment

In chapter 3, I compared the effectiveness of antibiotic and heat treatments at reducing the bedbug's *Wolbachia* load. I also wanted to see which treatment method had the least side effects in the host. Heat treatment produces a temporary reduction in *Wolbachia* load with no apparent disruption to host digestion. Antibiotic treatment produces a long-lasting reduction in *Wolbachia* load but appears to disrupt host digestion. Measures of the host's blood meal mass or body mass can be used to retrospectively adjust for side effects on host digestion. Other possible side effects of heat and antibiotic treatment however are more difficult to control for (3.4.1.1). Overall when using these protocols, one must be cautious about assigning the observed effects solely to the role of *Wolbachia*.

Whilst some of the side effects of heat treatment can be controlled by a heat treatment control group other factor(s) altered by the heat treatment may also recover during this period and confound results. One way of examining this is to adjust the *Wolbachia* load using different treatments (e.g. heat and a parallel treatment of antibiotics). Whilst each treatment will have its own associated side-effects, the focal effect is the same – reduction in *Wolbachia*. If the response variable tracks the *Wolbachia* density in the same way in both treatments it would be unlikely that this was caused by similar unobserved side effects, since the two treatments are very different.

Unwanted side effects of antibiotic treatment are also difficult to control for, and a suitable control group could not be generated (3.4.1.1). No recovery of *Wolbachia* after antibiotic treatment was observed, so using an antibiotic treated group that had recovered its *Wolbachia* load to control for antibiotic effects was not possible.

My comparisons between antibiotic and heat treatments have a wider significance for symbiosis studies that involve manipulating symbiont infection levels in the host study system. My results demonstrate the value of investigating the effects of different methods to manipulate symbiont load, on both symbiont load and the host. This is particularly important in studies of primary symbionts, when 1st generation hosts are often used in

experiments rather than their untreated offspring. Investigation of the side effects of treatments used to reduce symbiont loads reduce the risk of misinterpreting results, such as likely occurred for Chang (1974) who attributed a reduced bedbug fecundity to reduced bacteriome symbionts rather than the more likely sperm damage caused by heat treatment. My results also highlight a novel way of controlling for at least some of the treatment effects, by using treated individuals that have recovered their *Wolbachia* loads. It may be possible to apply this principle to other treatment methods besides heat treatment, from which the symbiont loads can recover.

Fitness effects of reducing male's *Wolbachia* load

In chapter 4, I conducted experiments to determine the fitness consequences of *Wolbachia* infection in the male host. Using antibiotic treatment to manipulate *Wolbachia* loads, results suggest that the male's *Wolbachia* may increase the fitness of their male hosts by increasing the egg laying rate of the females with whom the males have mated. It is possible that *Wolbachia* mediate this effect through increasing the quality of some aspect of the male's ejaculate; *Wolbachia* were not detected in the ejaculate, and no effects of manipulating *Wolbachia* load on ejaculate quantity were observed.

My results demonstrate that *Wolbachia* may benefit male bedbug fitness making this example particularly unusual as *Wolbachia*-mediated fitness effects normally only benefit the female host. Further work is needed to identify how or if *Wolbachia* are increasing ejaculate quality (8.4).

Fitness effects of reducing female's *Wolbachia* load for the female and her offspring

In chapter 5, I conducted experiments to determine the fitness consequences of *Wolbachia* infection in the female host. Results supported previous work by Hosokawa et al., (2010) that *Wolbachia* may increase female fitness by increasing egg hatch and development success. My results also suggest *Wolbachia* may have positive longer-term fitness consequences for the female by increasing the body size and thus fitness of her offspring.

These results demonstrate that *Wolbachia* may increase its propagation in a population by increasing the fitness potential of the next generation of infected hosts. Transgenerational effects are well known with *Wolbachia*, such as the feminisation of male offspring, but this is the first time to my knowledge that evidence has been found to suggest *Wolbachia* may affect a life-history trait of the next generation, in this case body size. Further investigations on transgenerational *Wolbachia*-mediated effects on life history traits are needed, for example to determine: (i) whether these effects are caused by the heat treatment used to manipulate *Wolbachia* loads, or due to the change in *Wolbachia* load itself; (ii) whether these effects are common in other study systems and (iii) whether similar effects are always observed between male and female offspring.

Effect of reducing a female's *Wolbachia* load on her fitness after microbial challenge

In chapter 6, I conducted experiments to determine whether the female's *Wolbachia* affected the wounding and infection fitness costs associated with TI. The results of these experiments suggested that the female's *Wolbachia* may reduce the survival costs of TI-associated infection.

These results join the growing body of research demonstrating the occurrence of *Wolbachia*-mediated protection. *Wolbachia*-mediated protection has been demonstrated in predominantly *Drosophila spp.* and mosquitoes against a range of parasites including viruses (e.g. Teixeira et al., 2008), protozoa (e.g. Kambris et al., 2010), fungi (e.g. Panteleev et al., 2007) and nematodes (e.g. Kambris et al., 2009). Here I demonstrate *Wolbachia*-mediated protection may also occur against opportunistic microbes (bacteria and fungi) in the bedbug.

Effect of reducing the female's *Wolbachia* load on immunity

In chapter 7, I conducted experiments to investigate how *Wolbachia* may reduce the survival costs of TI-associated infection (chapter 6) by investigating the effect of manipulating the female's *Wolbachia* loads on levels of her immunity. An assay of the encapsulation response suggested that the female's *Wolbachia* may increase the rate of melanin formation in the spermalege. Melanin formation is important in wound healing and

nodulation, by-products of its production kills microbes, and layers of melanin help sequester and externalise microbes at wound sites and in cell nodules. Increasing the melanisation rate in the spermalege is one mechanism through which *Wolbachia* could reduce the female's wounding and infection costs associated with TI.

These results support previous research demonstrating that *Wolbachia* infection may mediate protection through increasing the host's melanisation response (Thomas et al., 2011). However, the general up-regulation in melanisation in the haemolymph of artificially infected *D. melanogaster*, *D. simulans* and *A. aegypti* observed in Thomas et al.'s (2011) study could have been an artefact of *Wolbachia* infection rather than a *Wolbachia*-mediated effect. The increase in melanisation reported here is therefore important, because it is more likely to be a *Wolbachia*-mediated response since: (i) the *Wolbachia* infection is naturally occurring in the bedbug (so the bedbug host is not reacting to *Wolbachia* infection); (ii) the increase in melanisation response is not general in the bedbug, but specific to the spermalege; and (iii) in experiments, it is unlikely that the host is responding to the reduction in *Wolbachia* load by lowering the melanisation response in only the spermalege – if the host had to trade-off the melanisation response anywhere, it would make more sense to trade-off in the haemocoel where infection risk is low, rather than the spermalege.

8.3 Significance of results for bedbug control

Bedbugs are a pest species of increasing concern. Reports suggest that their numbers are rising unabated across the globe, particularly in the USA, Australia and the UK ((Boase, 2004, Boase, 2007, Doggett et al., 2004, Doggett and Russell, 2008). Chemical control strategies are proving ineffective in the long-term, as bedbugs are becoming increasingly resistant to most common insecticides (Doggett et al., 2004). New non-chemical strategies, such as biocontrol, are being urgently sought after.

Most biocontrol strategies operate by increasing the levels of predation or parasitism that naturally regulate pest populations. Bedbugs have some natural predators including spiders (particularly *Thanatus flavidus* Simon), ants, mites, centipedes and cockroaches (Usinger, 1966); but their natural levels of predation are too low to regulate bedbug populations.

Furthermore these predators themselves are considered pest species and are a target of pest control, ruling out their use in biocontrol. Bedbugs are also known to be susceptible to some disease pathogens including the fungus *Aspergillus flavus* (Cockbain & Hastie, 1961) and *Serratia* spp. of bacteria (Strand, 1977), however these are also known to cause illness in humans so could not be used in biocontrol. The results from my thesis suggest that the bedbug problem maybe being exacerbated by the beneficial effects mediated by the bedbug's *Wolbachia* which may increase the bedbug's capability of rapid population growth. This raises the question: "could biocontrol treatments that reduce the natural *Wolbachia* infection levels in bedbugs be used to reduce the size of bedbug populations?"

Like many pest species, bedbugs are capable of rapid population growth. In the laboratory, mated females with *ad libitum* access to blood, lay on average 20 eggs week^{-1} (*pers obs*), a high proportion of which will be sexually mature adults within 6 weeks. The results of my investigations show that *Wolbachia* may have multiple beneficial fitness effects on their host which will help bedbugs achieve this high reproduction rate. *Wolbachia* may increase the female's egg laying rate through possibly increasing ejaculate quality (chapter 4), increase the female's egg hatch and development success (chapter 5), help females to offset the survival costs associated with opportunistic infection during mating (chapter 6) through increasing the rate of melanisation (chapter 7) and increase the fitness of the next generation through increasing offspring body size (chapter 5). These are strategies that will ultimately increase the propagation of *Wolbachia*; unfortunately these strategies also increase the propagation of bedbugs. It therefore seems to stand to reason that reducing *Wolbachia* infection in bedbugs could help control their numbers. If we can lower *Wolbachia* infection levels, then this may reduce the reproductive success of bedbugs potentially making infestations smaller and easier to control.

Ongoing investigations are exploring how the range of *Wolbachia*-mediated effects can be used in the control of pest species or the diseases that they vector. For example, the introduction of parasitic life-shortening *Wolbachia* has been investigated to control mosquito populations (McMeniman et al., 2009), and the introduction of *Wolbachia* that outcompete or indirectly reduce disease pathogens vectored by mosquitoes (Kambris et al., 2009, Hughes et al., 2011, Bian et al., 2013) are being explored. Understanding the nuances of *Wolbachia*'s relationship with their host, particular to each system, can help to tailor

control strategies to a particular system. For example, *Wolbachia* in filarial nematodes function as mutualists, probably serving a nutritional function in their hosts (Foster et al., 2005). Now this is known, filariasis patients infected with filarial nematodes are routinely treated with antibiotics that target the nematode's symbiotic *Wolbachia* leading to nematode death (Taylor et al., 2005, Debrah et al., 2011). This raises the question: "is there any feature particular to the bedbug-*Wolbachia* system highlighted in my thesis that may reveal whether *Wolbachia* could be used in biocontrol?"

Firstly, there is a logistical difficulty in affecting a permanent decrease in the *Wolbachia* load of all individuals within an infestation. Although antibiotic treatment effects a long-lived significant decrease in *Wolbachia* load (chapter 3), in the field, it is likely that many insects would be reluctant to feed on antibiotic-supplemented blood through an artificial membrane feeding setup. (Delivering high doses of antibiotics through the human host would not be medically advisable). This is not a problem in the laboratory as the bedbug populations are selected for those that survive and reproduce under laboratory conditions, including feeding through an artificial membrane feeding setup. An alternative to antibiotic treatment is a long duration, low grade heat treatment. However my results show that although the heat-induced decrease in *Wolbachia* load is significant, it is short-lived as the *Wolbachia* recover once heat treatment has stopped (chapter 3). Furthermore in the field, bedbugs tend to hide in crevices and within furniture, which may make it easy for the insects to avoid the heat treatment. Furthermore heat treatment may encourage insects to disperse which would exacerbate the problem if the infestation was not properly sealed off.

Secondly, my results suggest that a decrease in the *Wolbachia* load of individuals that comprise an infestation may not produce a drastic enough fitness reduction to be useful as a method of bedbug control. Although egg laying completely stopped in females after being fed blood supplemented with antibiotics (Fig. 3.3), males fed antibiotics remain fertile, as do males and females after low grade heat treatment. Given the nature of bedbug infestations, control methods need to be fast acting and to kill all of the bedbugs in the infestation.

An alternative to trying to decrease the *Wolbachia* load of a whole infestation *in situ*, is to reduce the *Wolbachia* loads of laboratory reared individuals and then release them into an

infestation. For example, sterile insect techniques (SIT) and control based on CI, rely on releases of large numbers of sterile, or incompatible males, which overwhelm the numbers of males in the natural pest population. SITs and C.I. based strategies work when the mating system of the pest species is such that the female mates once (with the sterile/incompatible male), oviposits (infertile eggs) and then dies. I found a 4 week long heat treatment rendered males ‘infertile’ likely caused by heat sperm damage (Appendix 4). Although this effect is not likely to be permanent, it does raise the possibility of using sterile males to control an established bedbug infestation. Unfortunately the mating system of bedbugs is probably not suitable for such a strategy: females mate multiple times and lay eggs continuously through their lives. Without also removing the fertile males from the infestation, such an SIT would be ineffective. Releasing large numbers of sterile males would also add to the problem because they can still bite.

For these reasons, manipulating *Wolbachia* in bedbugs as a stand-alone biocontrol strategy is unlikely to be effective.

8.4 Future Directions

8.4.1 How do *Wolbachia* in male bedbugs increase female fitness?

In chapter 4 I showed that *Wolbachia* in male bedbugs may increase the egg laying rate of mated females. The mechanisms are unclear and demand further investigation. Understanding how microorganisms can affect male ‘fertility’ may have wider implications for the rest of the animal kingdom, including humans. I have shown that *Wolbachia* are not transferred in the ejaculate and my results suggest that *Wolbachia* do not affect the quantity of the ejaculate components. One possibility is that *Wolbachia* may directly or indirectly affect ejaculate quality.

To investigate whether *Wolbachia* effects a qualitative change to ejaculate components, the following could be compared between males with different *Wolbachia* loads: (i) the composition of the ejaculate within mesospermaleges of mated females and (ii) the composition of the ejaculate during its production within the male reproductive organs. The location of *Wolbachia* within these dissected tissues could be verified using fluorescence *in*

situ hybridisation (FISH). This involves the use of fluorescently labelled probes that locate *Wolbachia* within the tissue, and fluoresce when viewed under a confocal microscope to create an image. MALDI-TOF (matrix-assisted laser desorption/ionization time-of-flight) mass spectrometry, could also be used to determine where differences in the chemical composition of these tissues occurs. During MALDI-TOF, the tissue is ionised and the weight of the constituent ions determines how fast they travel over a fixed distance; an image of the tissue is created and the location of ions of different speeds are presented by different colours in the image. To identify the compounds involved, proteomic analyses would be necessary.

8.4.2 What is the role of BLS in bedbugs?

In chapter 3, I distinguished two bacterial symbionts in bedbug bacteriomes: *Wolbachia* and BLS. Currently the role of BLS in bedbugs is unknown. As *Wolbachia* does not appear to be a suitable candidate for biocontrol, other candidate bacteria, such as BLS must be explored. As BLS are located in the bacteriomes, one possibility is that BLS serve a nutritional role and work synergistically with *Wolbachia* to increase female fitness.

Genetically similar bacteria to BLS are found in other insects including leafhoppers (Hypša & Aksoy, 1997), ant lions (Dunn & Stabb, 2005) and tick flies (Reeves 2005), suggesting this group may be a widespread clade of insect endosymbiotic bacteria (Sakamoto and Rasgon, 2006). Very little is known of its role within any of these insects. No qPCR primers for BLS have been published, so these would first need to be designed so that the presence of BLS, and manipulations of BLS loads in bedbugs, could be quantified. Two important questions to address are: “what is the fitness advantage of having BLS?” and “can bedbugs survive and reproduce when infected with only BLS and not *Wolbachia*?”

To answer the first of these questions, a bedbug strain infected with only *Wolbachia* could be used. Measures of fitness could be compared between individuals that have or have not been doubly infected with BLS. (BLS can be cultured axenically (Purcell et al., 1986) and experiments with leaf hoppers show that hosts can be readily infected via their food (Degnan et al., 2011)). To answer the second question, bedbugs could be antibiotic treated to remove their *Wolbachia*, infected with BLS and a BLS-only infected population raised.

qPCR would be necessary to determine that these bedbugs were infected with BLS and not infected with *Wolbachia*.

8.5 Final conclusions

The results from my thesis serve to highlight the diverse effects *Wolbachia* may have on its host. Multiple effects may be experienced in one host; effects may be responsive to changing situations; effects may vary between different genders and life stages, and effects may cross generations. As the introduction or manipulation of *Wolbachia* infection is currently being explored for the control of several insect pests, this thesis highlights the importance of thorough investigation of the consequences of the *Wolbachia* manipulation for host biology before wide-scale releases of infected insects take place.

Appendix 1

Relationship Between Female's Fecundity, Body Size and Blood Meal

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A1.1 Introduction

There is considerable variation in the body size of individual adult bedbugs and sometimes variation in how successfully individuals feed – most feed to completion, but some may stop feeding before they are full (pers obs). In many of my experiments I am interested in the effect of manipulating an individual's *Wolbachia* load on measures of the individual's fecundity (egg number and egg size). Variation in other traits that may affect fitness such as body size and resource availability (size of blood meal) must be controlled. Here I characterise the relationships between female body size, size of blood meal and fecundity (number of eggs laid); and the relationship between female body size and egg size.

A1.1.1 Aims of investigation

1. To characterise variation in the body size of females in a population.
2. To characterise the relationship between size of blood meal and fecundity (number eggs laid that hatch).
3. To characterise the relationship between female body size and fecundity (number of eggs laid that hatch).
4. To characterise the relationship between body size and size of blood meal when individuals feed to completion.

A1.2 Materials and Methods

A1.2.1 Insects

3 days old virgin female bedbugs were raised following standard protocols (2.2.2.1). Insects were imaged and the width each individual's pronotum (a measure of body size) was measured (2.2.2.2). Throughout the experiments insects were maintained individually

in 5 mL plastic tubes with a strip of filter paper (1 x 3 cm) under controlled environmental conditions of 26°C 70% RH constant controlled light conditions.

A1.2.2 Effect of size of blood meal on egg laying rate

Females (*n*=55) were fed using standard feeding protocols (2.2.3) and the amount of blood taken up by each female was manipulated by interrupting feeding after a set time, so that females differed in their feeding duration. Feeding duration is the time interval from when the female pierces the feeding membrane with her proboscis and begins to take up blood until she stops feeding and her proboscis is lifted free of the membrane. Females were equally assigned to eleven feeding duration treatment groups (0, ¾, 1½, 2, 2½, 3, 3½, 4, 5, 5½ and more than 5½ minutes). Females that fed for more than 5½ minutes group were allowed to feed to natural completion and the feeding duration was recorded. After feeding, two measures of size of blood meal were recorded: (i) the difference in the female's body weight before and after feeding (a measure of the mass of blood taken up during the feed) and (ii) female's body mass after feeding (the sum mass of the female's body, undigested blood in the gut from previous feeds and her most recent blood meal).

Females were then mated with virgin males as standard (2.2.2.4) and the total number of eggs laid by each female that hatched, were counted. Recording stopped after 14 days by which time egg laying had ceased because the blood resources had been used up.

A1.2.3 Effect of body size on number of eggs laid

To determine the effect of a female's body size on the number of eggs laid that hatched, the relationship between an individual's pronotum width and the individual's residual value in the relationship between total number of viable eggs and mass of blood meal (difference in the female's body weight before and after feeding) was determined. In this way individual variation in the size of blood meal was controlled.

A1.2.4 Effect of body size on maximum size of blood meal

Females (*n*=54) were allowed to feed uninterrupted to natural completion (2.2.3). The relationship between the body size of individuals (pronotum width) and two measures of

the size of the individual's blood meal ((i) the difference in the female's body weight before and after feeding and (ii) female's body weight after feeding were analysed.

A1.2.5 Effect of body size on egg size

Females (*n*=31) were mated as standard (2.2.2.4) and egg laying was monitored daily. All eggs were imaged within 24 hours of being laid using a stereomicroscope and a digital camera (2.3.3). The size of eggs (width, length and volume) was calculated from the images. Analyses were carried out to test whether there was a relationship between female's body size (pronotum width) and (i) egg width, (ii) egg length and (iii) egg volume. Eggs are prolate-spheroid in shape and the volume was calculated using the equation: $V=1/6 \, Pi \, W^2 \, L$ where W is the egg width and L is the egg length (Berrigan, 1991).

A1.2.6 Data analysis

Data were analysed as detailed in 2.5.

A1.3 Results

A1.3.1 Variation in body size in the insect population

There was substantial variation in female body size (1.56±0.0550 mm, mean ± SD; 1.451-1.691 mm, range; *n*=54) (Fig. A1.1).

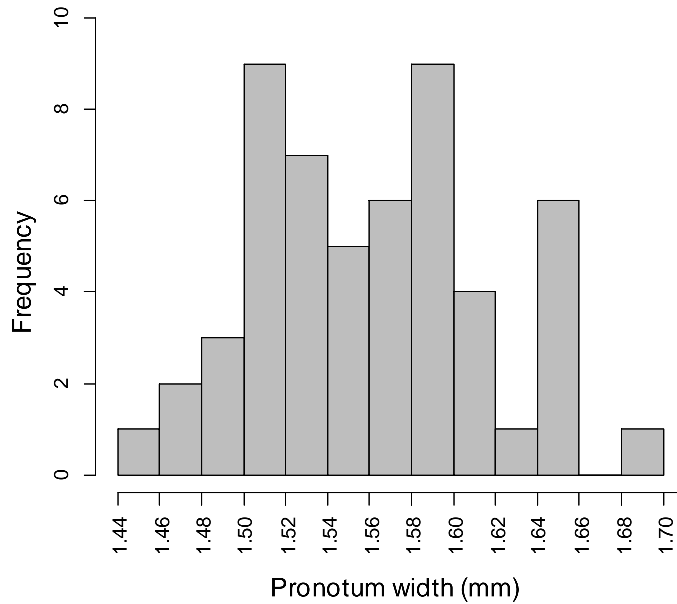

Figure A1.1 Frequency histogram of pronotum widths of females in the sample population (1.56±0.0550 mm, mean ± SD; 1.451-1.691 mm, range; *n*=54).

A1.3.2 Effect of size of blood meal on egg number

One female failed to lay any eggs at all despite a large blood meal (10.26 mg). This was likely due to an unsuccessful mating, perhaps caused by a genetic incompatibility between the male and female, therefore this individual was excluded from analyses. The size of blood meal was successfully manipulated through altering the feeding duration; the mass of blood meal is positively related to the duration of feed, (R^2=0.775, $F_{1,52}$=178.8, P<0.0001). The body size of individuals does not affect this relationship (R^2=0.0431, $F_{1,52}$=2.34, P>0.05) (Fig. A1.2).

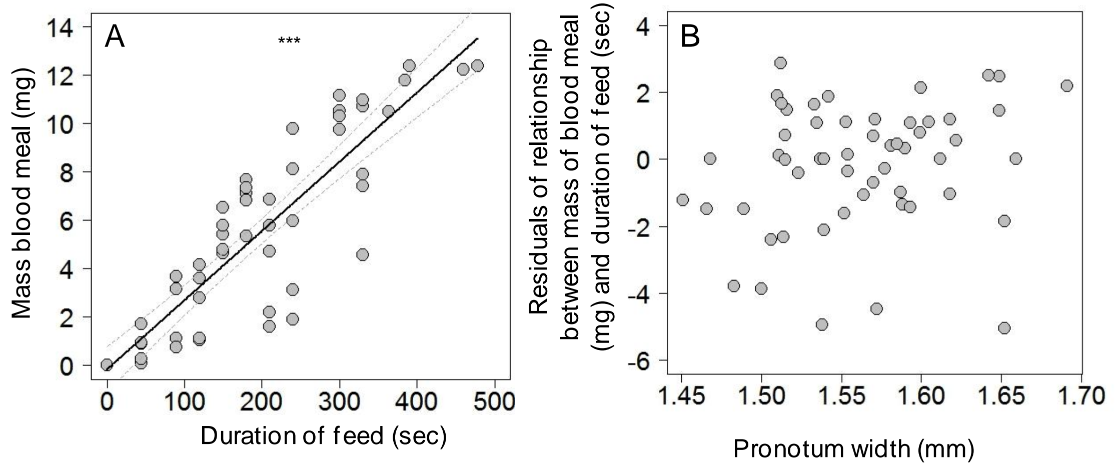

Figure A1.2 (A) The relationship between feeding duration and the mass of blood meal. The fitted line (±1SE) is *y*=-0.157 + 0.0286*x* (R^2=0.775, $F_{1,52}$=178.8, P<0.0001). (B) The relationship between pronotum width (mm) (a measure of body size) and the size of the residuals for the relationship between mass of blood meal (mg) feeding duration (R^2=0.0431, $F_{1,52}$=2.34, P>0.05).

The number of eggs laid that hatched by each female is highly positively related to both measures of her blood meal: mass of blood meal taken up (R^2=0.909, $F_{1,52}$=521.3, P<0.0001) and body mass after feeding (R^2=0.953, $F_{1,52}$=1049, P<0.0001) (Fig. A1.3).

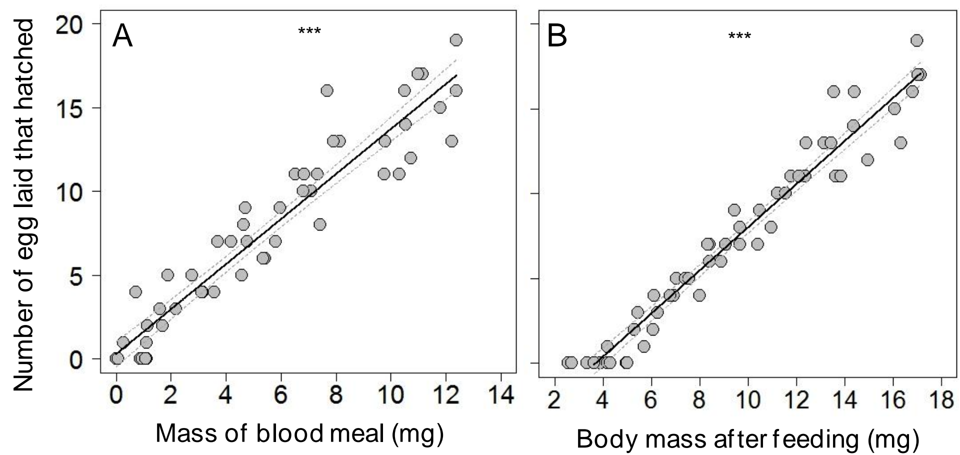

Figure A1.3 (A) The relationship between the mass of a female's blood meal and the number of eggs laid that hatched. The fitted line (±1SE) is *y*=0.289 + 1.34*x* (R^2=0.909, $F_{1,52}$=521.3, P<0.0001). (B) The relationship between the female's body mass measured immediately after feeding and the number of eggs laid that hatched. The fitted line (±1SE) is *y*=-4.70 + 1.27*x* (R^2=0.953, $F_{1,52}$=1049, P<0.0001). Sample size: N=54.

A1.3.3 Effect of body size on egg number

The number of eggs laid was related to the female's body size (with size of blood meal controlled) (R^2=0.291, $F_{1,52}$=21.35, P<0.0001) (Fig. A1.4).

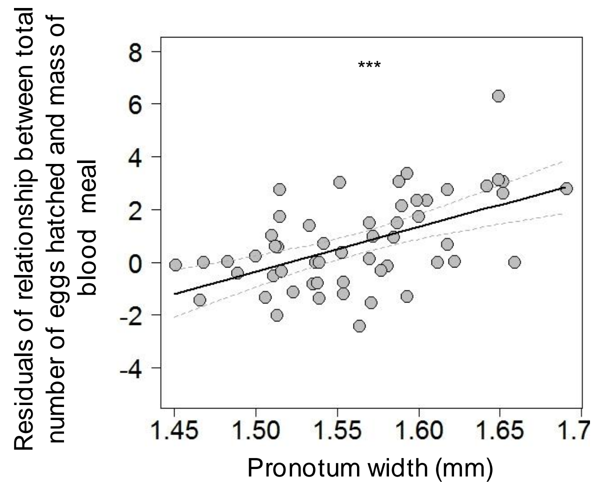

Figure A1.4 Relationship between female body size (pronotum width (mm)) and the size of the residuals for the relationship between total number of eggs that hatched and mass of blood meal for individual females (Fig. A. 1.3). The fitted line (±1SE) is y=-25.65 + 16.86x ($R^{2=}$0.291, $F_{1,52}$=21.35, P<0.0001), n=54, error bars are 1SE.

A1.3.4 Effect of body size on size of blood meal

When left to feed to natural completion, larger females tend to take in a larger blood meal; mass of blood meal is positively related to pronotum width (R^2=0.217, $F_{1,53}$=14.66, P<0.001) (Fig. A1.5).

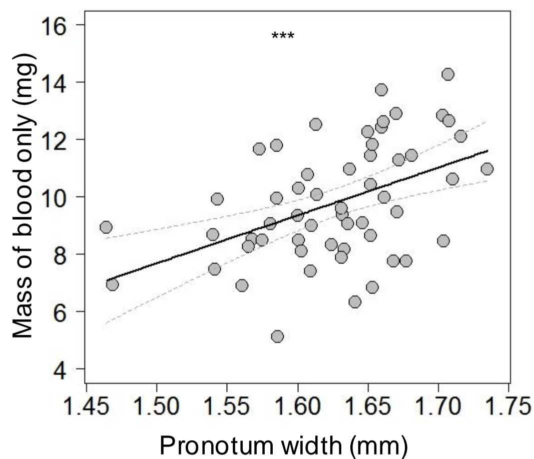

Figure A1.5 The relationship between body size (pronotum width (mm)) and mass of blood meal for females that fed to natural completion. The fitted line (±1SE) is y=-17.33 + 16.68x (R^2=0.217, $F_{1,53}$=14.66, P<0.001).

A1.3.5 Effect of body size on egg size

No relationship was found between female's body size and egg size. This was true for all three measures of eggs size recorded: egg volume (R^2=0.0471, $F_{1,30}$=1.48, P>0.05), egg length (R^2=0.00488, $F_{1,30=}$0.147, P>0.05) and egg width (R^2=0.0558, $F_{1,30=}$1.77, P>0.05) (Fig. A1.6).

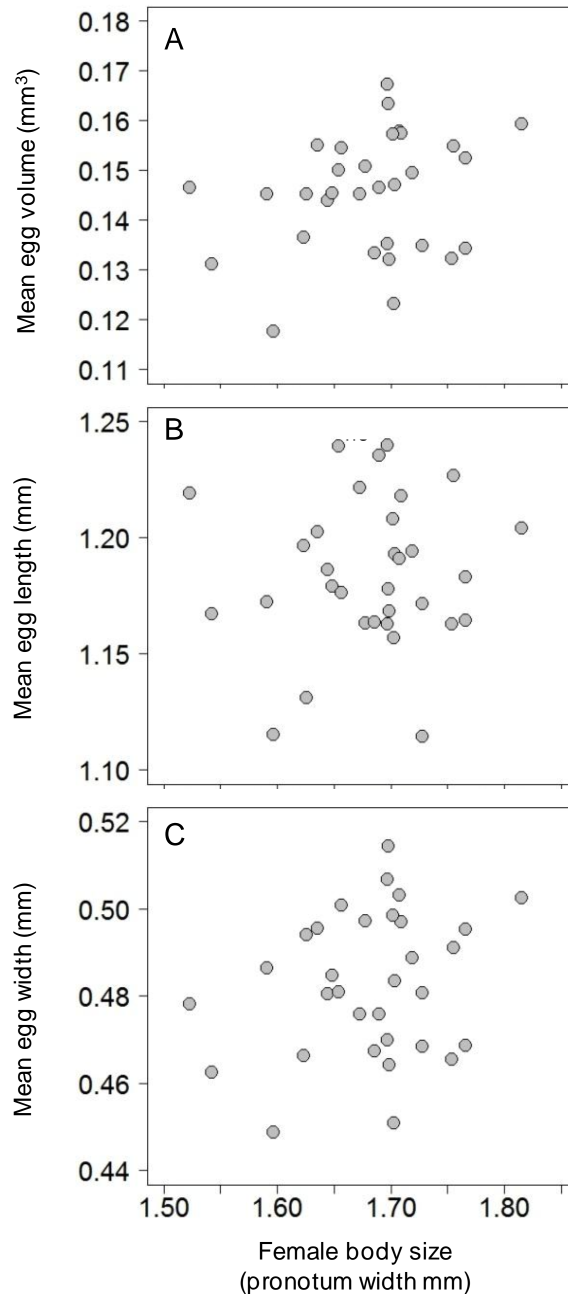

Figure A1.6 The relationship between mother's body size (pronotum width (mm)) and three measures of egg size: (A) egg volume (R^2=0.0471, $F_{1,30=}$1.48, P>0.05); (B) egg length (R^2=0.00488, $F_{1,30}$=0.147, P>0.05); (C) egg width (R^2=0.0558, $F_{1,30=}$1.77, P>0.05). Error bars are 1SE. At least 10 eggs were measured for each female (n=31).

A1.4 Discussion

My results show that there is substantial variation in body size between individuals in a laboratory population. Body size is positively related to fecundity (number of eggs laid that hatchged) but not related to egg size. Body size limits the maximum size of blood meal that an individual can take up during a feed. Size of blood meal is positively related to fecundity and has a greater effect on fecundity than body size. The mass of an individual after feeding, (the sum mass of an individual's body, residual blood remaining in the individual's gut from previous feeds and blood taken up during the most recent blood meal), is the more accurate predictor of a female's fecundity.

Appendix 2

Lists of Reagents and Consumables

A2.1 Reagents list (selected)

Chemical compound	Company name	Catalogue number
Ammonium acetate	Sigma Aldrich	A1542
Anhydrous copper sulphate	Sigma Aldrich	451657
Araldite® resin CY212	Agar Scientific	AGR1030
BDMA	Agar Scientific	AGR1030
Calcium chloride	Sigma Aldrich	C1016
Choline chloride	Sigma Aldrich	C7527
Cobalamin	Sigma Aldrich	V6629
D-biotin	Sigma Aldrich	47868
DDSA	Agar Scientific	AGR1030
Ethanol	Sigma Aldrich	E7023
Folic acid	Sigma Aldrich	F8758
Glutaraldehyde	Sigma Aldrich	G6403
Go-Taq® polymerase	Promega	M829
Hydrochloric acid	Sigma Aldrich	H1758
Lyophilised *Micrococcus luteus*	Sigma Aldrich	M3770
Lysozyme from chicken egg white	Sigma Aldrich	62970-1G-F
Meso-inositol	Sigma Aldrich	I7808
Osmium tetroxide	Sigma Aldrich	75633
Nicotinic acid	Sigma Aldrich	N0761
Pantothenic acid	Sigma Aldrich	P5155
Propylene oxide	Sigma Aldrich	82320
Proteinase K	Sigma Aldrich	P2308-25MG

Reynold's lead citrate	Sigma Aldrich	15326
Riboflavin	Sigma Aldrich	47861
SDS	Sigma Aldrich	L3771
Sodium Cacodylate	Sigma Aldrich	C0125
Sodium chloride	Sigma Aldrich	S9888
Sodium hydrogen phosphate	Sigma Aldrich	S7907
Streptomycin suplhate	Sigma Aldrich	S6501
SYBR® green	Sigma Aldrich	QR0100
Tetracycline hydrochloride	Sigma Aldrich	T7660
Thiamine hydrochloride	Sigma Aldrich	T1270
Tris	Sigma Aldrich	252859
Triton X-100	Sigma Aldrich	X100
Tryptone	Sigma Aldrich	T7293
Uranyl acetate	Sigma Aldrich	73943
Yeast extract	Sigma Aldrich	Y1625

A2.2 Consumables list (selected)

Product	Company name	Catalogue number
1.5 mL microcentrifuge tubes	Biostores	100326
2 mL microcentrifuge tubes	Sigma Aldrich	Z666513
5 mL plastic tubes	Elkay Precison Laboratory Consumables	500-1000-407
50 mL falcon tubes	SLS	SLS8106
60 mL plastic pots	ThermoFisher	11723194
Aspirator tube assembly	Sigma Aldrich	A5177-5EA
Enamel paint	(Humbrol Enamel, Hornby Hobbies Ltd., UK)	NA
Filter paper	Scientific Laboratory Supplies	1001-932
Glass beads (2 mm DIA)	Sigma Aldrich	Z273627
Glass coverslip (0.1 mm thick)	Electron Microscopy Sciences	72198-10
Glass microcapillaries (1 mm outer DIA, 90 mm length)	Narishige Japan	G-1
Heparinised sheep's blood	TCS Biosciences	TCS: SB 075
Microscope slides	ThermoFisher	10144633A
Nunclon™ 24 well culture plates	SLS	143982
Parafilm® 'M' laboratory film	Sigma Aldrich	P7793-1EA
Pasteur pipette	ThermoFisher	13-678-20A
Plastic Petri dishes (60 mm DIA)	ThermoFisher	FB0875713A
Sylgard® silicone elastomer	Dow Corning: Kit 184	NA

Appendix 3

Additional Results

This appendix chapter provides full results for individual analyses. Tables are divided up into relevant chapters, and are ordered as they appear in each chapter.

Chapter 3

Table A3.1 *Wolbachia* load in antibiotic treated and control groups over 5 weeks, means ±1SE of original data. Relates to Fig. 3.1 p.40

Week	Antibiotic treatment	Control	Results
0	39.7±10.20	NA	NA
1	24.70±1.43	31.96±7.69	U=34, n_1=6, n_2=6, *
2	1.26±1.18	20.68±5.45	U=35, n_1=6, n_2=6, **
3	0.00797±0.00109	6.63±1.78	U=36, n_1=6, n_2=6, **
4	0.0445±0.0301	47.8±27.7	U=36, n_1=6, n_2=6, **
5	0.131±0.125	28.94±7.72	U=1, n_1=6, n_2=6, **

Table A3.2 *Wolbachia* load in heat treated and control groups over 5 weeks, means ±1SE of original data. Relates to Fig. 3.2 p.41

Week	Heat treatment	Control	Results
0	40.07±8.41	NA	NA
1	4.61±1.25	29.04±8.26	t=4.41, 10 df, *** on $\log_e(x+1)$ data
2	1.60±0.551	20.03±3.86	t=7.14, 10 df, *** on $\log_e(x+1)$ data
3	6.70±2.88	29.85±4.09	t=4.65, 10 df, *** on $\log_e(x+1)$ data
4	85.34±28.5	68.08±18.62	t=0.23, 10 df, *ns* on $\log_e(x+1)$ data
5	40.07±9.44	46.16±10.74	t=0.33, 10 df, *ns* on $\log_e(x+1)$ data

Table A3.3 *Ad libitum* fed body mass for females in three treatment groups: antibiotic fed (n=24); heat treated (n=26) and controls (n=25), means ±1SE on original data. Means with the same letter do not differ from each other (Tukey mcp test P>0.05). Relates to Fig. 3.3 part (B) p.45

Week	Antibiotics	Heat	Control	ANOVA
1	14.83±0.610	16.70±0.624	15.02±0.516	$F_{2,72}$=3.12 *ns*
2	15.62±0.875	16.14±0.739	15.65±0.556	$F_{2,72}$=0.16 *ns*
3	17.47±0.905	18.34±0.561	17.76±0.547	$F_{2,72}$=0.42 *ns*
4	17.37±1.03	18.91±0.778	16.58±0.828	$F_{2,72}$=1.87 *ns*

Table A3.4 'Digestion rate' (difference in *ad libitum* fed body mass and body mass one week later) for females from three treatment groups: antibiotic fed (n=24); heat treated (n=26) and controls (n=25), means ±1SE on original data. Means with the same letter do not differ from each other (Tukey mcp test P>0.05). Relates to Fig. 3.3 p.45

Week	Antibiotics	Heat	Control	ANOVA
1-2	6.82±0.394 (A)	9.73±0.532 (B)	8.95±0.406 (B)	$F_{2,72}$=10.95 ***
2-3	5.41±0.544 (A)	7.34±0.553 (B)	7.61±0.444 (B)	$F_{2,72}$=5.28 **
3-4	4.93±0.530 (A)	7.62±0.417 (B)	8.53±0.331 (B)	$F_{2,72}$=18.48 ***

Table A3.5 Egg laying rate (total eggs wk^{-1}) for females from three treatment groups: antibiotic fed (n=24); heat treated (n=26) and no treatment controls (n=25), means ±1SE on original data. Means with the same letter do not differ from each other (Non-parametric mcp test P>0.05). Relates to Fig. 3.3 part (C) p.45

Week	Antibiotics	Heat	Control	Kruskal-Wallis
1	0.000±0.000 (A)	6.54±0.682 (B)	10.32±0.702 (C)	H=55.43, 2 df, ***
2	0.000±0.000 (A)	1.54±0.509 (B)	5.84±0.427 (C)	H=49.76, 2 df, ***
3	0.000±0.000 (A)	12.46±0.847 (B)	13.00±0.619 (B)	H=48.49, 2 df, ***
4	0.000±0.000 (A)	10.73±0.878 (B)	9.80±0.911 (B)	H=46.78, 2 df, ***

Chapter 4

Table A3.6 Means ±1SE body of size of males in the four treatment groups (*n*=15) from which measures of ejaculate availability were taken (4.3.3.3) (ANOVA: $F_{3,56}$=1.21, P>0.05).

Treatments group	Pronotum width (mm)
+αb	1.522±0.0149
+ αb+B	1.510±0.0184
−αb	1.550±0.0114
−αb+B	1.541±0.0198

Table A3.7 Means ±1SE mass of blood taken up by individuals in the four treatment groups (*n*=15), prior to measures of ejaculate availability being taken (4.3.3.3) (ANOVA: $F_{3,56}$=0.53, P>0.05). Mass of blood meal was calculated from the difference in the weight of individuals before and after feeding.

Treatments group	Mass of blood meal (mg)
+αb	6.103±0.325
+αb+B	5.809±0.389
−αb	5.706±0.385
−αb+B	5.809±0.389

Table A3.8 Means ±1SE *ad libitum* fed body mass of individuals in the four treatment groups (*n*=15), prior to measures of ejaculate availability being taken (4.3.3.3) (ANOVA: $F_{3,56}$=0.94, P>0.05).

Treatments group	Mass of blood meal (mg)
+αb	16.646±0.646
+αb+B	17.371±0.709
−αb	16.482±0.535
−αb+B	15.816±0.719

Table A3.9 Means±1SE body of size of males in the two treatment groups (*n*=6) used in measures of ejaculate transferred (4.3.3.4) (*t*-test: t=1.53, 12 df, P>0.05).

Treatments group	Pronotum width (mm)
+αb	1.577±0.0186
−αb	1.529±0.0256

Chapter 5

Table A3.10 Weekly analyses of effect of heat treatment to reduce the mother's *Wolbachia* load (+heat or −heat) on male offspring 'fertility' (egg laying rate (hatched eggs per mg replete body weight of female)). Data are means ±1SE See also Fig. A3.1.

Week	−heat	+heat	Statistical test
1	1.18±0.0425	1.21±0.0281	t=0.658, 32.92 df, *ns*
2	0.845±0.0344	0.808±0.0287	t=0.832, 38 df, *ns*
3	1.18±0.0496	1.23±0.0262	U=207.0, n_1=20, n_2=20, *ns*
4	0.850±0.0863	0.912±0.0919	U=196.0, n_1=20, n_2=20, *ns*
5	0.457±0.0590	0.347±0.0667	U=154.0, n_1=20, n_2=20, *ns*
6	0.507±0.0978	0.385±0.0885	U=160.0, n_1=20, n_2=20, *ns*
7	0.125±0.0564	0.0897±0.0379	U=194.5, n_1=20, n_2=20, *ns*

Table A3.11 Mean ±1SE proportion of all eggs that hatched, from females mated to the male offspring of "−heat" and "+heat" mothers over 7 weeks. Females that laid no eggs were excluded. Relates to Fig. A3.1.

Week	−heat (n_1)	+heat (n_2)	Mann-Whitney U-tests
1	0.971±0.0202	0.996±0.00435	U =220.5, n_1=20, n_2=20, *ns*
2	0.997±0.00333	0.997±0.00278	U=200.5, n_1=20, n_2=20, *ns*
3	0.979±0.0106	0.987±0.00498	U=197.0, n_1=20, n_2=20, *ns*
4	0.874±0.0674	0.946±0.0581	U=226.5, n_1=20, n_2=20, *ns*
5	0.882±0.0517	0.765±0.0889	U=111.0, n_1=17, n_2=16, *ns*
6	0.546±0.0845	0.534±0.0983	U=143.5, n_1=18, n_2=16, *ns*
7	0.267±0.107	0.306±0.0907	U=67.0, n_1=10, n_2=7, *ns*

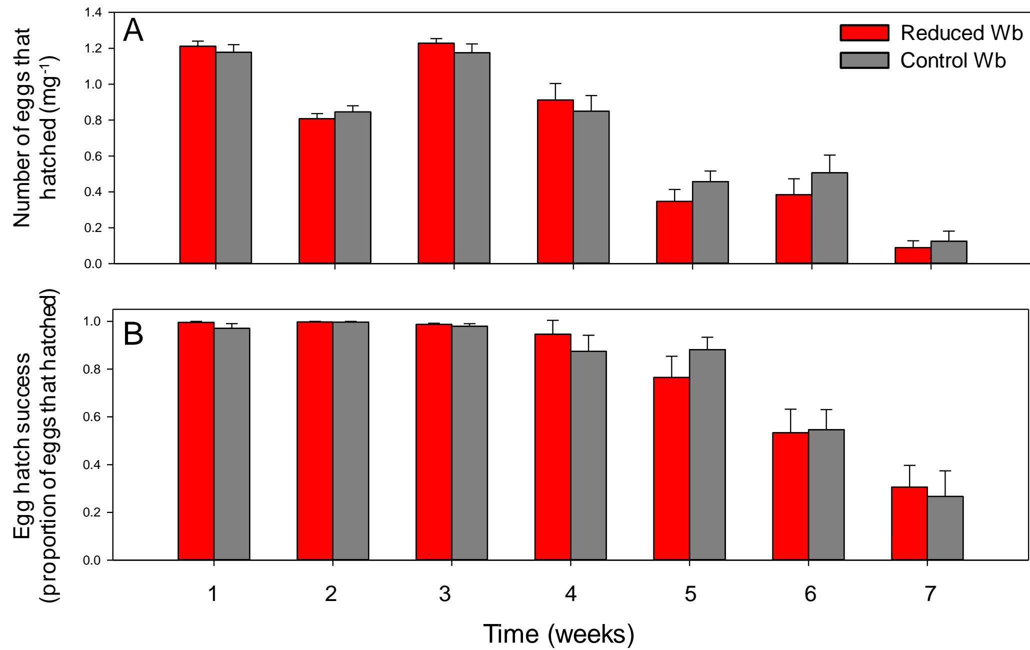

Figure A3.1 Effect of heat treating the female (+heat or −heat) on indirect fitness measures of her male offspring over 7 weeks. (A) Mean number of laid that hatched (expressed per mg *ad libitum* fed female body mass) *n*=20. (B) Mean egg hatch success (proportion of all eggs laid that hatched), females that laid no eggs were excluded, *n*=20 each group except for week 5: +heat *n*=16, −heat *n*=17, week 6: +heat *n*=16, −heat *n*=16 and week 7: +heat *n*=7, −heat *n*=10. Bars represent 1SE. Results of statistical tests in Tables A3.10 and A3.11.

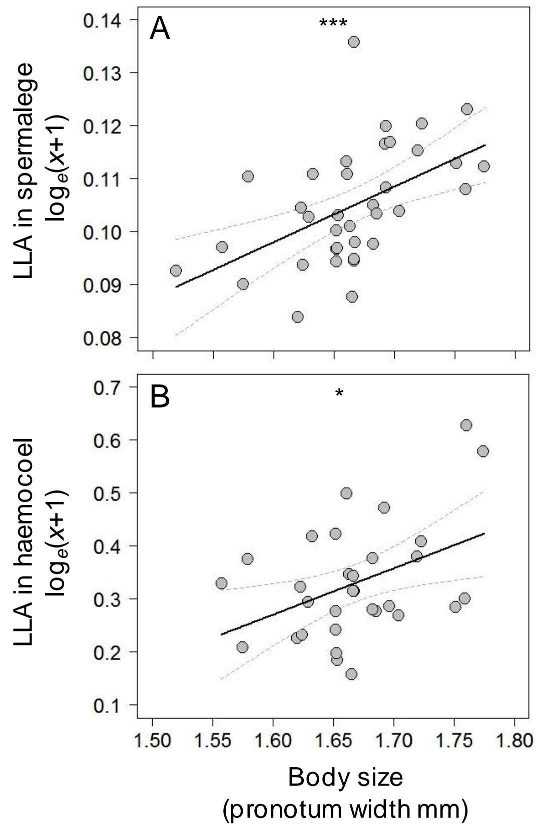

Figure A3.2 (A) The relationship between body size (pronotum width (mm)) and LLA in the mesospermalege (n=36). The fitted line (±1SE) is y=-0.0697 + 0.105x (R^2=0.269, $F_{1,34}$=12.54, P=0.0012 on $\log_e(x+1)$ data). (B) The relationship between body size (pronotum width (mm)) and LLA in the haemocoel (n=31). The fitted line (±1SE) is y=-1.13 + 0.875x (R^2=17.81, $F_{1,29}$=6.28, P=0.018 on $\log_e(x+1)$ data). Data were collected from individuals in the immune treatment groups (7.2.3.2).

Appendix 4

Effect of heat treatment to reduce male's *Wolbachia* load on sperm viability

A4.1 Introduction

Heat treatment may be used to manipulate *Wolbachia* loads in insect hosts in experiments to determine the role of *Wolbachia* within that host. There are a number of side effects of heat treatment including increased metabolic rate and the production of heat shock proteins. These side effects disappear once the host has been returned to a its preferred environmental temperatures. However, there are some side effects that may not ameliorate so quickly. For example, Omori (1941) observed a reduction in sperm numbers and morphological aberrations in sperm from male *Cimex lectularius* following continuous heat treatment at 37°C. Males can produce some sperm after adult eclosion, from residual resources left over from their last blood meal as 5th instar nymphs. If the viability of sperm is adversely affected by heat treatment as Omori's observations suggest, then an alternative method to heat treatment should be used to manipulate *Wolbachia* loads in male hosts to avoid this confound.

A4.1.1 Aims of investigation

1. To determine the effect of heat treatment on male 'fertility'

A4.2 Materials and Methods

A4.2.1 Insects

3 days old virgin female bedbugs were raised following standard protocols (2.2.2.1). Throughout experiments insects were maintained individually in 5 mL plastic tubes with a strip of filter paper (1 x 3 cm) under controlled environmental conditions of 26°C 70% RH constant controlled light conditions.

A4.2.2 Effect of duration of heat treatment on male ‘fertility’

Male ‘fertility’ was measured indirectly through fecundity measures in mated females. Three fecundity measures were recorded: (i) egg laying rate (number of eggs laid per week), (ii) egg laying rate (number of eggs laid that hatched per week) and (iii) egg hatch success (the proportion of all eggs laid that hatched). The effect of heat treatment was assessed by generating three treatment groups: (i) two weeks heat treatment (males were maintained under normal temperature conditions (26°C) for two weeks, and then heat treated for two weeks); (ii) four weeks heat treatment (males were heat treated for four weeks) and (iii) no-treatment control (males were maintained under normal temperature conditions for four weeks).

Following standard mating protocols (2.2.2.4), virgin, fed females were mated with males from the three treatment groups. Females were then housed individually in 5 mL plastic tubes with a strip of filter paper and maintained under normal temperature conditions. Females were fed weekly and fecundity measures were collected over 3 weeks. Any females that died before the end of the 3 weeks were excluded from analyses.

A4.2.3 Data analysis

Data were analysed as detailed in 2.5.

A4.3 Results

One female from each group died before the end of the 3 weeks and were excluded from analyses. There was a highly significant negative effect of heat treatment on male ‘fertility’ (number of hatched eggs per female per mg blood) (*Kruskal-Wallis test*: H=26.48, 2 df, P<0.0001) (Fig. A4.1, Table A4.1). Heat treatment control males had a significantly higher ‘fertility’ compared to 2 weeks heat treated males, which in turn had significantly higher ‘fertility’ compared to 4 weeks heat treated males (Dunn’s test P<0.05). This difference was brought about by differences in the total number of eggs laid (*Kruskal-Wallis* test: H=24.50, 2 df, P<0.0001) and the proportion egg hatch success (Mann-Whitney U test: U=0, n_1=11, n_2=8, P<0.01) (Fig. A4.1, Tables A4.2-A4.3).

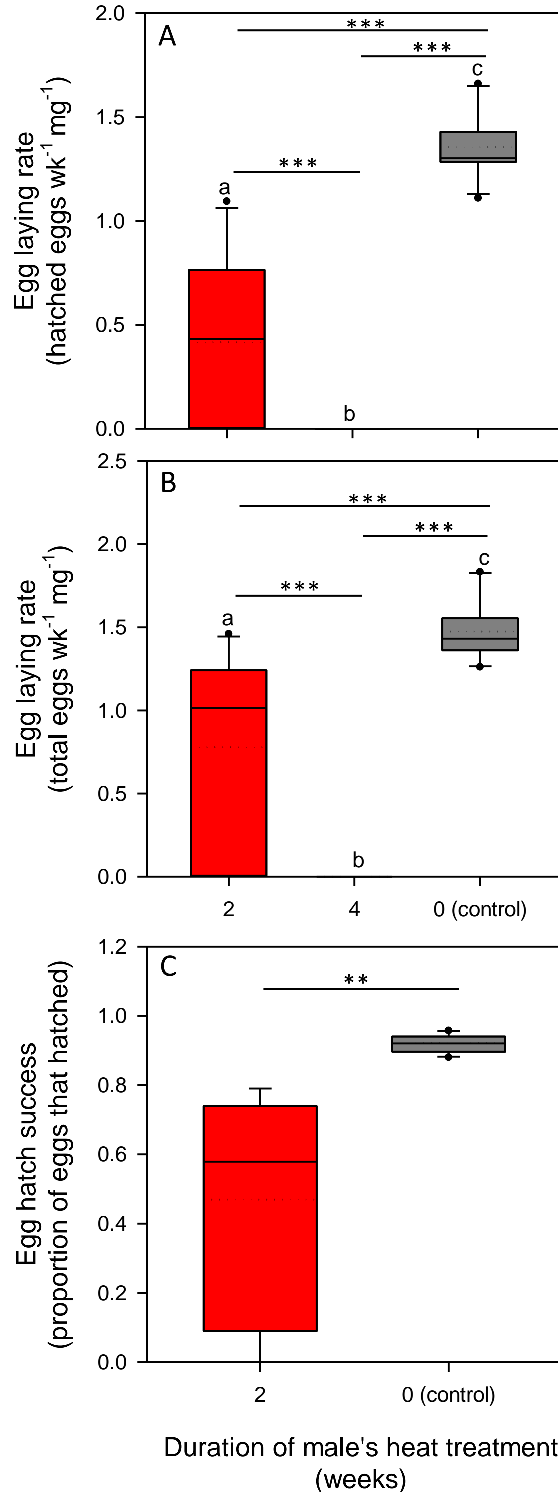

Figure A4.1 Effect of heat treating males for different durations (2, 4 and 0 weeks) on indirect measures of male 'fertility': (A) median (solid line) egg laying rate (number of eggs laid per week that hatched); (B) median (solid line) egg laying rate (total number of eggs laid per week) and (C) median (solid line) egg hatch success (proportion of eggs that hatched). Egg laying rates are expressed per mg replete female body mass. *N*=11 for each group except control group for egg

hatch success n=8. Dotted lines indicate means. Groups with the same letter do not differ from each other (Dunn's test P>0.05).

Table A4.1 Effect of heat treatment on male 'fertility' (hatched eggs per mg *ad libitum* fed female body mass) means ±1SE on original data (n=11). Relates to Fig. A3.1.

Week	2 weeks heat treated males	4 weeks heat treated males	control males (0 days)	*Kruskal-Wallis* test
1	0.313±0.0976	0.000±0.000	1.383±0.221	H=25.79, 2 df, ***
2	0.684 ±0.201	0.000±0.000	1.070±0.0535	H=15.67, 2 df ***
3	0.261±0.160	0.000±0.000	1.703±0.0302	H=27.03, 2 df, ***

Table A4.2 Effect of heat treatment on male 'fertility' (total eggs (hatched and unhatched) per mg *ad libitum* fed female body mass) means ± SE on original data (n=11). Relates to Fig. A3.1

Week	2 week heat treated males	4 week heat treated males	Control males	*Kruskal-Wallis* test
1	0.557±0.125	0.000±0.000	1.523±0.248	H=25.79, 2 df, ***
2	0.985±0.224	0.000±0.000	1.269±0.0481	H=17.24, 2 df, ***
3	0.822±0.222	0.000±0.000	1.743±0.0322	H=23.11, 2 df, ***

Table A4.3 Effect of heat treatment on male 'fertility' (proportion of eggs laid that hatched) means ±1SE on original data. Females that laid no eggs were excluded. Relates to Fig. A3.1

Week	14 days heat treated males	28 days heat treated males	control males 0 days	Mann-Whitney U test
1	0.550± 0.127 (n=8)	NA (n=0)	0.914±0.0254 (n=11)	U=8.5, $n_{1=}$11, n_2=8, **
2	0.613± 0.140 (n=8)	NA (n=0)	0.841± 0.0211 (n=11)	U=28, $n_{1=}$11, n_2=8, *ns*
3	0.270± 0.146 (n=7)	NA (n=0)	0.978± 0.00889 (n=11)	U=0.5, $n_{1=}$11, n_2=7, ***

A4.4 Discussion

Heat treatment reduced male 'fertility' with egg laying rate being significantly reduced in females mated to heat treated males compared to control males. This reduction in 'fertility' increased with duration of heat treatment, with the matings of 4 week heat treated males

failing to produce any offspring at all during the time of the experiment. It is not clear how heat treatment has caused this reduced ‘fertility’. One possibility is that heat treatment is detrimental to spermatogenesis.

Appendix 5

Two Protocols to Reduce *Wolbachia* Load

A5.1 Antibiotic Treatment

This protocol describes reducing the load of *Wolbachia* symbionts in *Cimex lectularius* males.

A5.1.1 Materials

Tetracycline hydrochloride
60 mL plastic pots
1.5 mL microcentrifuge tubes
Filter paper
Bedbug Cultures
Membrane feeding apparatus (2.2.3.3)
Heparinised sheep's blood

1. Prepare 0.10% solution of tetracycline hydrochloride in heparinised sheep's blood, vortex for 20-30 seconds and aliquot 0.5 mL into 1 mL tubes. Store at -20°C. At the same time, prepare 0.5 mL aliquots of unsupplemented heparinised sheep's blood to serve as an antibiotic control. 0.5 mL of blood will feed 50 insects.

2. Take a single cohort of unfed, recently eclosed (2-5 day old) males and house the males in communal 60 mL pots of *n*=25-50 individuals, with a strip of filter paper (4.5 cm x 10 cm) that has been folded into a concertina. Randomly assign each pot of males to two treatment groups: "+αb" and "−αb".

3. Feed the "+αb" males blood that has been antibiotic supplemented and feed the "−αb" males unsupplemented blood through an artificial membrane feeding system (see 2.2.3.3). Exclude males that do not feed.

4. One week later, repeat step 3.

5. One week later feed both treatment groups unsupplemented heparinised sheep's blood. Exclude males that do not feed.
6. To check that the antibiotic treatment has been effective take a subsample of individuals (*n*≈6) chosen at random from the two treatment groups and analyse the *Wolbachia* loads of these individuals using qPCR (e.g. Hosokawa et al., 2010).

The treatment groups are now ready to be used in experiments. The "+αb" group will have significantly lower *Wolbachia* loads compared to the "−αb" group. It is unknown whether *Wolbachia* loads recover in the "+αb" group over time, however no recovery is known to occur over 4 weeks.

A5.2 Heat Treatment

This protocol describes reducing the load of *Wolbachia* symbionts in *Cimex lectularius* females. This protocol is not suitable to treat male *C. lectularius*, as heat causes sperm damage.

A5.2.1 Materials

Incubator (36°C, 70% RH, constant controlled light conditions)
Digital thermometer (VWR 620-0915)
Sodium chloride solution
Plastic boxes (L x W x D: 10 cm x 15 cm x 5 cm)
5 mL tubes
Filter paper
Bedbug Cultures
Membrane feeding apparatus (2.2.3.3)
Heparinised sheep's blood

A 5.2.2 Procedure

1. Prepare two different temperature environments: (i) a high temperature incubator (36°C 70% RH constant controlled light conditions) and (ii) a control temperature incubator (26°C 70% RH constant controlled light conditions). To maintain a RH of 70%, place several open plastic containers containing ≈ 10% saline solution on the

top shelf of the incubator. Monitor the conditions within the incubators with a digital thermometer.

2. Take a single cohort of unfed, recently eclosed (2-5 day old) females and house each female individually in a 5 mL tube with a piece of filter paper (1 cm x 3 cm) throughout the protocol. Randomly assign the females to four treatment groups: "+heat", "−heat", "+heat+recovery" and "−heat+recovery".
3. Place the "+heat" and "+heat+recovery" groups into the centre of the 36°C incubator to be heat treated and the "−heat" and "−heat+recovery" groups into the 26°C control temperature incubator.
4. After two weeks, move the "+heat" and "+heat+recovery" females into the 26°C control temperature incubator.
5. After 24 hours, feed all groups heparinised sheep's blood through an artificial membrane feeding system.
6. The "+heat" and "−heat" groups can now be used in experiments. Individuals in the "+heat" will have lower *Wolbachia* loads relative to "−heat" individuals. To check that heat treatment has been successful, measure the *Wolbachia* loads from a subsample from each group (e.g. of n=6) using qPCR (e.g. Hosokawa *et al* 2010). Data collection for experiments is restricted to 2 weeks because the *Wolbachia* load is likely to recover in the "+heat" group after this time.
7. Place the "+heat+recovery" and "−heat+recovery" groups into the 26°C control temperature incubator for two weeks, feeding them after one week.
8. The "+heat+recovery" and "−heat+recovery" groups can now be used in experiments. *Wolbachia* loads will have recovered to match levels in the control group. These groups therefore serve to control the effects of heat treatment. To check that *Wolbachia* loads have recovered, measure the *Wolbachia* loads from a subsample from each group (e.g. of n=6) using qPCR (e.g. Hosokawa *et al* 2010).

References

Adamo, S. A. (2004) Estimating disease resistance in insects: Phenoloxidase and lysozyme-like activity and disease resistance in the cricket *Gryllus texensis*. *Journal of Insect Physiology*. **50**(2-3), pp. 209-216.

Andere, C. I., Monteavaro, C., Palacio, M. A., Catena, M., Rodríguez, E. M. & Collins, A. M. (2011) *Apis mellifera* semen: bacterial contamination and susceptibility to antibiotics. *Apidologie*. **42**(5), pp. 551-559.

Arkwright, J. A., Atkin, E. E. & Bacot, A. (1921) An hereditary *Rickettsia*-like parasite of the bed bug (*Cimex lectularius*). *Parasitology*. **13**(01), pp. 27-36.

Arnqvist, G. & Rowe, L. (Eds.) (2005) *Sexual conflict.* Princeton, New Jersey, Princeton University Press.

Augustinos, A. A., Santos-Garcia, D., Dionyssopoulou, E., Moreira, M., Papapanagiotou, A., Scarvelakis, M., Doudoumis, V., Ramos, S., Aguiar, A. F., Borges, P. A., Khadem, M., Latorre, A., Tsiamis, G. & Bourtzis, K. (2011). Detection and characterization of Wolbachia infections in natural populations of aphids: is the hidden diversity fully unraveled? *PLoS One*, **6**(12), pp. e28695.

Azevedo, D. O., Neves, C. A., Mallet, J. R., Goncalves, T. C., Zanuncio, J. C. & Serrao, J. E. (2009) Notes on midgut ultrastructure of *Cimex hemipterus* (Hemiptera: Cimicidae). *Journal of Medical Entomology*. **46**(3), pp. 435-441.

Bai, X., Mamidala, P., Rajarapu, S. P., Jones, S. C. & Mittapalli, O. (2011). Transciptomics of the bed bug (*Cimex lectularius*). *PLoS One*, **6**(1), pp. e16336.

Baldo, L. & Werren, J. H. (2007) Revisiting *Wolbachia* supergroup typing based on WSP: spurious lineages and discordance with MLST. *Current Microbiology*. **55**(1), pp. 81-87.

Bandi, C., Trees, A. J. & Brattig, N. W. (2001) *Wolbachia* in filarial nematodes: evolutionary aspects and implications for the pathogenesis and treatment of filarial diseases. *Veterinary Parasitology*. **98**(1-3), pp. 215-238.

Beard, C. B., Cordon-Rosales, C. & Durvasula, R. V. (2002) Bacterial symbionts of the triatominae and their potential use in control of chagas disease transmission. *Annual Review of Entomology*. **47,** pp. 123-141.

Beckwith, R. C. (1982) Effects of constant laboratory temperatures on the douglas-fir tussock moth (Lepidoptera: Lymantriidae). *Environmental Entomology*. **11**(6), pp. 1159-1163.

Benoit, J. B., Lopez-Martinez, G., Teets, N. M., Phillips, S. A. & Denlinger, D. L. (2009) Responses of the bed bug, *Cimex lectularius*, to temperature extremes and dehydration: levels of tolerance, rapid cold hardening and expression of heat shock proteins. *Medical and Veterinary Entomology*. **23**(4), pp. 418-425.

Berrigan, D. (1991) Allometry of egg size and number in insects. *Oikos*. **60**(3), pp. 313-321.

Bian, G., Joshi, D., Dong, Y., Lu, P., Zhou, G., Pan, X., Xu, Y., Dimopoulos, G. & Xi, Y. (2013) *Wolbachia* invades *Anopheles stephensi* populations and induces refractoriness to *Plasmodium* infection. *Science*. **340**(6133), pp. 748-751.

Bian, G., Xu, Y., Lu, P., Xie, Y. & Xi, Z. (2010). The endosymbiotic bacterium *Wolbachia* induces resistance to dengue virus in *Aedes aegypti*. *PloS Pathogens* [Online], **6**(4), pp. e1000833. Available from: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=20368968 [Accessed 1st April 2013].

Boase, C. (2004) Bed-bugs -- reclaiming our cities. *Biologist*. **51,** pp. 9-12.

Boase, C. J. (2007). Bed bugs: research and resurgence. In*:* W. Takken & B. J. Knols (Eds.) *Emerging pests and vector borne diseases in Europe.* Wageningen Academic Publishers. pp. 261-281.

Boman, H. G. & Hultmark, D. (1987) Cell-free immunity in insects. *Annual Review of Microbiology*. **41,** pp. 103-126.

Bordenstein, S. R. & Werren, J. H. (1998) Effects of A and B *Wolbachia* and host genotype on interspecies cytoplasmic incompatibility in *Nasonia*. *Genetics.* **148**(4), pp. 1833-1844.

Bourtzis, K. & Miller, T. A. (2003). *Insect Symbiosis.* 1st edition. Boca Raton, CRC Press.

Bourtzis, K., Pettigrew, M. M. & O'Neill, S. L. (2000) *Wolbachia* neither induces nor suppresses transcripts encoding antimicrobial peptides. *Insect Molecular Biology.* **9**(6), pp. 635-639.

Boxmeer, J. C., Smit, M., Weber, R. F., Lindermans, J., Romijn, J. C., Eijkemans, M. J., Macklon, N. S. & Steegers-Therunissen, R. P. (2007) Seminal plasma cobalamin significantly correlates with sperm concentration in men undergoing IVF or ICSI procedures. *Journal of Andrology*. **28**(4), pp. 521-527.

Bressac, C. & Rousset, F. (1993) The reproductive incompatibility system in Drosophila simulans: DAPI-staining analysis of the *Wolbachia* symbionts in sperm cysts. *Journal of Invertebrate Pathology.* **61**(3), pp. 226-230.

Brittain, J. E., Lillehammer, A. & Saltveit, S. T. (1984) The effect of temperature on intraspecific variation in egg biology and nymphal size in the stonefly Capnia atra (Plecoptera). *Journal of Animal Ecology.* **53**(1), pp. 161-169.

Broderick, N. A., Welchman, D. P. & Lemaitre, B. (2009). Recognition and response to microbial infection in *Drosophila*. In*:* J. Rolff & S. E. Reynolds (Eds.) *Insect Infection and Immunity.* Oxford, Oxford University Press. pp. 13-33.

Brownlie, J. C., Cass, B. N., Riegler, M., Witsenburg, J. J., Iturbe-Ormaetxe, I., McGraw, E. A. & O'Neill, S. L. (2009). Evidence for metabolic provisioning by a common invertebrate endosymbiont, *Wolbachia pipientis*, during periods of nutritional stress. *PloS Pathogens* [Online], **5**(4), pp. e1000368. Available from: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=19343208 [Accessed 1st April 2013].

Brownlie, J. C. & Johnson, K. N. (2009) Symbiont-mediated protection in insect hosts. *Trends in Microbiology.* **17**(8), pp. 348-354.

Bruford, M. W., Hanotte, O., Brookfield, J. F. Y. & Burke, T. (1998). Multilocus and single-locus DNA fingerprinting. In*:* A. R. Hoelzel (Ed.) *Molecular Genetic Analysis of Populations: A practical Approach.* 2nd Edition ed. Oxford, IRL Press. pp. 287-336.

Buchner, P. (1965). *Endosymbiosis of animals with plant microorganisms.* London, Interscience Publishers, Wiley and Co.

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

Campbell, B. C. & Purcell, A. H. (1993) Phylogenetic affiliation of BEV, a bacterial parasite of the leafhopper *Euscelidius variegatus*, on the basis of 16S rDNA sequences. *Current Microbiology.* **26**(1), pp. 37-41.

Campbell, E. A., Korzheva, N., Mustaev, A., Murakami, K., Nair, S., Goldfarb, A. & Darst, S. A. (2001) Structural mechanism for rifampicin inhibition of bacterial rna polymerase. *Cell.* **104**(6), pp. 901-912.

Carayon, J. (1966). Traumatic insemination and the paragentical system. In*:* R. L. Usinger (Ed.) *Monograph of the Cimicidae.* Philadelphia, Entomological Society of America. pp. 81-178.

Chang, K. P. (1974) Effects of elevated temperature on the mycetome and symbiotes of the bed bug *Cimex lectularius* (Heteroptera). *Journal of Invertebrate Pathology.* **23**(3), pp. 333-340.

Chang, K. P. (1975) Haematophagous insect and haemoflagellate as hosts for prokaryotic endosymbionts. *Symposia of the Society for Experimental Biology*. **29,** pp. 407-428.

Chang, K. P. & Musgrave, A. J. (1973) Morphology, histochemistry, and ultrastructure of mycetome and its rickettsial symbiotes in *Cimex lectularius* L. *Canadian Journal of Microbiology.* **19**(9), pp. 1075-1081.

Charlat, S., Bourtzis, K. & Merot, H. (2002). *Wolbachia* induced cytoplasmic incompatibility. In*:* J. Seckbach, S. (Ed.) *Symbiosis:Mechanisms and Model Systems.* Dordrecht, Kluwer Academic Press. pp. 621-644.

Charlat, S., Davies, N., Roderick, G. K. & Hurst, G. D. D. (2007a) Disrupting the timing of *Wolbachia*-induced male-killing. *Biology Letters.* **3**(2), pp. 154-156.

Charlat, S., Hornett, E. A., Fullard, J. H., Davies, N., Roderick, G. K., Wedell, N. & Hurst, G. D. (2007b) Extraordinary flux in sex ratio. *Science.* **317**(5835), pp. 214.

Charlat, S., Hurst, G. D. & Mercot, H. (2003) Evolutionary consequences of *Wolbachia* infections. *Trends in Genetics.* **19**(4), pp. 217-223.

Chiel, E., Inbar, M., Mozes-Daube, N., White, J. A., Hunter, M. S. & Zchori-Fein, E. (2009) Assessments of fitness effects by the facultative symbiont *Rickettsia* in the sweetpotato whitefly (Hemiptera: Aleyrodidae). *Annals of the Entomological Society of America.* **102**(3), pp. 413-418.

Chopra, I. & Roberts, M. (2001) Tetracycline antibiotics: mode of action, applications, molecular biology, and epidemiology of bacterial resistance. *Microbiology and Molecular Biology Reviews.* **65**(2), pp. 232-60.

Cockbain, A. J. & Hastie, A. C. (1961) Susceptibility of the bed bug, *Cimex lectularius* Linnaeus, to *Apergillus flavus* Link. *Journal of Invertebrate Pathology*. **3**(1) pp. 95-97.

Condorelli, R. A., La Vignera, S., Bellanca, S., Vicari, E. & Calogero, A. E. (2012) Myoinositol: Does it improve sperm mitochondrialfunction and sperm motility? *Urology*. **79**(6) pp.1290-1295.

Cook, P. E. & McGraw, E. A. (2010) *Wolbachia pipientis*: an expanding bag of tricks to explore for disease control. *Trends in Parasitology.* **26**(8), pp. 373-375.

Crudgington, H. S. & Siva-Jothy, M. T. (2000) Genital damage, kicking and early death - The battle of the sexes takes a sinister turn in the bean weevil. *Nature.* **407**(6806), pp. 855-856.

Dasch, G. A., Weiss, E. & Chang, K.-P. (1984). Endosymbionts of insects. In*:* N. R. Krieg & J. G. Holt (Eds.) *Bergey's Manual of Sytematic Bacteriology.* London, Williams and Wilkins. pp.

Davis, N. (1956) The morphology and functional anatomy of the male and female reproductive systems of *Cimex lectularius* L. (Heteroptera, cimicidae). *Annals of the Entomological Society of America.* **49**(5), pp. 466-493.

De Crespigny, F. E., Pitt, T. D. & Wedell, N. (2006) Increased male mating rate in *Drosophila* is associated with Wolbachia infection. *Journal of Evolutionary Biology.* **19**(6), pp. 1964-1972.

De Meillon, B. & Golberg, L. (1947) Preliminary studies on the nutritional requirements of the bedbug (*Cimex lectularius* L.) and the tick *Ornithodorus moubata* Murray. *Journal of Experimental Biology.* **24**(1), pp. 41-61.

De Souza, D. J., Bezier, A., Depoix, D., Drezen, J. M. & Lenoir, A. (2009) *Blochmannia* endosymbionts improve colony growth and immune defence in the ant *Camponotus fellah*. *BMC Microbiology.* 9(1) pp.29

Debrah, A. Y., Mand, S., Marfo-Debrekyei, Y., Batsa, L., Albers, A., Specht, S., Klarmann, U., Pfarr, K., Adjei, O. & Hoerauf, A. (2011). Macrofilaricidal activity in *Wuchereria bancrofti* after 2 weeks treatment with a combination of rifampicin plus doxycycline. *Journal of Parasitology Research* [Online], 2011(pp. 201617. Available from: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=21687646 [Accessed 10th August 2013].

Dedeine, F., Bouletreau, M. & Vavre, F. (2005) *Wolbachia* requirement for oogenesis: occurrence within the genus *Asobara* (Hymenoptera, Braconidae) and evidence for intraspecific variation in *A. tabida*. *Heredity.* **95**(5), pp. 394-400.

Dedeine, F., Vavre, F., Fleury, F., Loppin, B., Hochberg, M. E. & Bouletreau, M. (2001) Removing symbiotic *Wolbachia* bacteria specifically inhibits oogenesis in a parasitic wasp. *Proceedings of the National Academy of Sciences of the United States of America.* **98**(11), pp. 6247-6252.

Degnan, P. H., Bittleston, L. S., Hansen, A. K., Sabree, Z. L., Moran, N. A. & Almeida, R. P. (2011) Origin and examination of a leafhopper facultative endosymbiont. *Current Microbiology.* **62**(5), pp. 1565-1572.

Dillon, R. J. & Charnley, A. K. (1995) Chemical barriers to gut infection in the desert locust - *in vivo* production of antimicrobial phenols associated with the *Pantoea agglomerans*. *Journal of Invertebrate Pathology.* **66**(1), pp. 72-75.

Dillon, R. J. & Dillon, V. M. (2004) The gut bacteria of insects: Nonpathogenic interactions. *Annual Review of Entomology*. **49,** pp. 71-92.

Dillon, R. J., Vennard, C. T., Buckling, A. & Charnley, A. K. (2005) Diversity of locust gut bacteria protects against pathogen invasion. *Ecology Letters.* **8**(12), pp. 1291-1298.

Doggett, S. L., Geary, M. J. & Russell, R. C. (2004) The resurgence of bed bugs in Australia: With notes on their ecology and control. *Environmental Health Journal.* **4**(2), pp. 30-38.

Doggett, S. L. & Russell, R. C. Year. The resurgence of bed bugs, *Cimex spp*. (Hemiptera: Cimicidae) in Australia *In:* W. H. Robinson & D. Bajomi, eds. Proceedings of the Sixth International Conference on Urban Pests, 2008 Veszprém, Hungary. OOK-Press Kft.

Doudoumis, V., Tsiamis, G., Wamwiri, F., Brelsfoard, C., Alam, U., Aksoy, E., Dalaperas, S., Abd-Alla, A., Ouma, J., Takac, P., Aksoy, S. & Bourtzis, K. (2012). Detection and characterization of *Wolbachia* infections in laboratory and natural populations of different species of tsetse flies (genus *Glossina*). *BMC Microbiology* [Online], **12**(1), pp. S3. Available from:

http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=22376025 [Accessed 18th January 2013].

Douglas, A. E. (1989) Mycetocyte symbiosis in insects. *Biological Reviews of the Cambridge Philosophical Society.* **64**(4), pp. 409-434.

Douglas, A. E. (2009) The microbial dimension in insect nutritional ecology. *Functional Ecology.* **23**(1), pp. 38-47.

Dunn, A. K. & Stabb, E. V. (2005). Culture-independent characterization of the microbiota of the ant lion *Myrmeleon mobilis* (Neuroptera: Myrmeleontidae). *Applied Environmental Biology*. **71**(12) pp. 8784-8794.

Duron, O., Bouchon, D., Boutin, S., Bellamy, L., Zhou, L., Engelstadter, J. & Hurst, G. D. (2008). The diversity of reproductive parasites among arthropods: *Wolbachia* do not walk alone. *BMC Biology* [Online], **6**(1), pp. 27. Available from: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=18577218 [Accessed 5th May 2013].

Eberhard, W. G. (1996). *Female control: Sexual selection through cryptic female choice.* Princeton, NJ, Princeton Univ. Press

Eleftherianos, I., Boundy, S., Joyce, S. A., Aslam, S., Marshall, J. W., Cox, R. J., Simpson, T. J., Clarke, D. J., ffrench-Constant, R. H. & Reynolds, S. E. (2007) An antibiotic produced by an insect-pathogenic bacterium suppresses host defenses through phenoloxidase inhibition. *Proceedings of the National Academy of Sciences of the United States of America.* **104**(7), pp. 2419-2424.

Ewald, P. W. & Scubert, J. (1989). Vertical and vector-borne tranmission of insect endocytobionts and the evolution of benignity. In*:* W. Schemmler & G. Gassner (Eds.) *Insect Endocytobiosis: Morphology, Physiology, Genetics, Evolution.* Florida, CRC Press Inc.

Fenollar, F., Maurin, M. & Raoult, D. (2003) *Wolbachia pipientis* growth kinetics and susceptibilities to 13 antibiotics determined by immunofluorescence staining and real-time PCR. *Antimicrobial Agents and Chemotherapy.* **47**(5), pp. 1665-1671.

Foster, J., Ganatra, M., Kamal, I., Ware, J., Makarova, K., Ivanova, N., Bhattacharyya, A., Kapatral, V., Kumar, S., Posfai, J., Vincze, T., Ingram, J., Moran, L., Lapidus, A., Omelchenko, M., Kyrpides, N., Ghedin, E., Wang, S., Goltsman, E., Joukov, V., Ostrovskaya, O., Tsukerman, K., Mazur, M., Comb, D., Koonin, E. & B., S. (2005). The *Wolbachia* genome of *Brugia malayi*: Endosymbiont evolution within a human pathogenic nematode. *PLoS Biology* [Online], **3**(4), pp. e121. Available from: http://www.ncbi.nlm.nih.gov/pubmed/15780005 [Accessed 1st July 2013].

Fox, C. W. & Czesak, M. E. (2000) Evolutionary ecology of progeny size in arthropods. *Annual Review of Entomology*. **45,** pp. 341-369.

Frean, M. R. & Abraham, E. R. (2004) Adaptation and enslavement in endosymbiont-host associations. *Physical review. E, Statistical, nonlinear, and soft matter physics* **69**(5), pp. 051913.

Frentiu, F. D., Robinson, J., Young, P. R., McGraw, E. A. & O'Neill, S. L. (2010). *Wolbachia*-mediated resistance to dengue virus infection and death at the cellular level. *PLoS ONE*, **5**(10), pp. e13398

Fry, A. J., Palmer, M. R. & Rand, D. M. (2004) Variable fitness effects of *Wolbachia* infection in *Drosophila melanogaster*. *Heredity.* **93**(4), pp. 379-389.

Fytrou, A., Schofield, P. G., Kraaijeveld, A. R. & Hubbard, S. F. (2006) *Wolbachia* infection suppresses both host defence and parasitoid counter-defence. *Proceedings of the Royal Society of London Series B-Biological Sciences.* **273**(1588), pp. 791-796.

Gerardo, N. M., Altincicek, B., Anselme, C., Atamian, H., Barribeau, S. M., de Vos, M., Duncan, E., Evans, J. D., Gabaldon, T., Ghanim, M., Heddi, A., Kaloshian, I., Latorre, A., Moya, A., Nakabachi, A., Parker, B. J., Perez-Brocal, V., Pignatelli, M., Rhabe, Y., Ramsey, J. S., Spragg, C., Tamames, J., Tamarit, D., Tamborindeguy, C., Vincent-Monegat, C. & Vilcinskas, A. (2010) Immunity and other defenses in pea aphids, *Acyrthosiphon pisum*. *Genome Biology.* **11**(2), pp. R21.

Gillespie, J. P., Kanost, M. R. & Trenczek, T. (1997) Biological mediators of insect immunity. *Annual Review of Entomology*. **42,** pp. 611-643.

Gómez-Valero, L., Soriano-Navarro, M., Perez-Brocal, V., Heddi, A., Moya, A., Garcia-Verdugo, J. M. & Latorre, A. (2004) Coexistence of *Wolbachia* with *Buchnera aphidicola* and a secondary symbiont in the aphid *Cinara cedri*. *Journal of Bacteriology.* **186**(19), pp. 6626-6633.

Haine, E. R. (2008) Symbiont-mediated protection. *Proceedings of the Royal Society of London Series B-Biological Sciences.* **275**(1633), pp. 353-361.

Haine, E. R., Rolff, J. & Siva-Jothy, M. T. (2007) Functional consequences of blood clotting in insects. *Developmental and Comparative Immunology.* **31**(5), pp. 456-464.

Hansen, A. K., Jeong, G., Paine, T. D. & Stouthamer, R. (2007) Frequency of secondary symbiont infection in an invasive psyllid relates to parasitism pressure on a geographic scale in California. *Applied and Environmental Microbiology.* **73**(23), pp. 7531-7535.

Hargreaves, C. A., Rogers, S., Hills, F., Rahman, F., Howell, R. J. & Homa, S. T. (1998) Effects of co-trimoxazole, erythromycin, amoxycillin, tetracycline and chloroquine on sperm function *in vitro*. *Human Reproduction*. **13**(7), pp. 1878-1886.

Hedges, L. M., Brownlie, J. C., O'Neill, S. L. & Johnson, K. N. (2008) *Wolbachia* and virus protection in insects. *Science.* **322**(5902), pp. 702.

Hernandez-Martinez, P., Naseri, B., Navarro-Cerrillo, G., Escriche, B., Ferre, J. & Herrero, S. (2010) Increase in midgut microbiota load induces an apparent immune priming and increases tolerance to *Bacillus thuringiensis*. *Environmental Microbiology.* **12**(10), pp. 2730-2737.

Hertig, M. (1936) The rickettsia, *Wolbachia pipientis* (gen. et. sp. n.) and associated inclusions of the mosquito *Culex pipiens*. *Parasitology.* **28**(04), pp. 453-486.

Hertig, M. & Wolbach, S. B. (1924) Studies on rickettsia-like microorganisms in insects. *Journal of Medical Research.* **44**(3), pp. 328.

Hilgenboecker, K., Hammerstein, P., Schlattmann, P., Telschow, A. & Werren, J. H. (2008) How many species are infected with *Wolbachia*? — a statistical analysis of current data. *FEMS Microbiology Letters.* **281**(2), pp. 215-220.

Hoffmann, J. A., Reichhart, J. M. & Hetru, C. (1996) Innate immunity in higher insects. *Current Opinion Immunology.* **8**(1), pp. 8-13.

Honek, A. (1993) Intraspecific variation in body size and fecundity in insects: a general relationship. *Oikos.* **66**(3), pp. 483-492.

Hosokawa, T., Kikuchi, Y., Shimada, M. & Fukatsu, T. (2007) Obligate symbiont involved in pest status of host insect. *Proceedings of the Royal Society of London Series B-Biological Sciences.* **274**(1621), pp. 1979-1984.

Hosokawa, T., Kikuchi, Y., Shimada, M. & Fukatsu, T. (2008) Symbiont acquisition alters behaviour of stinkbug nymphs. *Biology Letters.* **4**(1), pp. 45-48.

Hosokawa, T., Koga, R., Kikuchi, Y., Meng, X.-Y. & Fukatsu, T. (2010) *Wolbachia* as a bacteriocyte-associated nutritional mutualist. *Proceedings of the National Academy of Sciences of the United States of America.* **107**(2), pp. 769-774.

How, Y.-F. & Lee, C. Y. (2010a) Effects of life stages and feeding regimes on active movement behavior of the tropical bed bug, *Cimex hemipterus* (Hemiptera: Cimicidae). *Journal of Medical Entomology.* **47**(3), pp. 305-312.

How, Y. F. & Lee, C. Y. (2010b) Fecundity, nymphal development and longevity of field-collected tropical bedbugs, *Cimex hemipterus*. *Medical and Veterinary Entomology.* **24**(2), pp. 108-116.

Hughes, G. L., Koga, R., Xue, P., Fukatsu, T. & Rasgon, J. L. (2011). *Wolbachia* infections are virulent and inhibit the human malaria parasite *Plasmodium falciparum* in *Anopheles gambiae*. *PloS Pathogens* [Online], **7**(5), pp. e1002043. Available from: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=21625582 [Accessed 5th May 2013].

Hurst, G. D. & Darby, A. C. (2009). The inherited microbiota of arthropods, and their importance in understanding resistance and immunity. In*:* J. Rolff & S. E. Reynolds (Eds.) *Insect Infection and Immunity Evolution, Ecology, and Mechanisms.* Oxford, Oxford University Press. pp. 119-136.

Hurst, G. D., Johnson, A. P., Schulenburg, J. H. & Fuyama, Y. (2000) Male-killing *Wolbachia* in *Drosophila*: a temperature-sensitive trait with a threshold bacterial density. *Genetics.* **156**(2), pp. 699-709.

Hurst, G. D. D., Sharpe, R. G., Broomfield, A. H., Walker, L. E., Majerus, T. M. O., Zakharov, I. A. & Majerus, M. E. N. (1995) Sexually-transmitted disease in a promiscuous insect, *Adalia bipunctata*. *Ecological Entomology.* **20**(3), pp. 230-236.

Hypša, V. & Aksoy, S. (1997) Phylogenetic characterization of two transovarially transmitted endosymbionts of the bedbug *Cimex lectularius* (Heteroptera: Cimicidae). *Insect Molecular Biology.* **6**(3), pp. 301-304.

Hypša, V. & Nováková, A. (2008). Insect symbionts and molecular phylogenetics. In*:* K. Bourtzis & T. A. MIller (Eds.) *Insect Symbiosis.* CRC Press. pp. 384.

Jaenike, J., Unckless, R., Cockburn, S. N., Boelio, L. M. & Perlman, S. J. (2010) Adaptation via symbiosis: recent spread of a *Drosophila* defensive symbiont. *Science.* **329**(5988), pp. 212-215.

Jarosz, J. (1979) Gut flora of *Galleria mellonella* suppressing ingested bacteria. *Journal of Invertebrate Pathology.* **34**(2), pp. 192-198.

Jia, F.-X., Yang, M.-S., Yang, W.-J. & Wang, J.-J. (2009) Influence of Continuous High Temperature Conditions on *Wolbachia* Infection Frequency and the Fitness of *Liposcelis tricolor* (Psocoptera: Liposcelididae). *Environmental Entomology.* **38**(5), pp. 1365-1372.

Jones, E. O., White, A. & Boots, M. (2007) Interference and the persistence of vertically transmitted parasites. *Journal of Theoretical Biology.* **246**(1), pp. 10-17.

Kambhampati, S., Rai, K. & Burgun, S. (1993). Unidirectional cytoplasmic incompatibility in the mosquito, *Aedes albopictus*. *Evolution*. **47**(2), pp. 673-677.

Kambris, Z., Blagborough, A. M., Pinto, S. B., Blagrove, M. S., Godfray, H. C., Sinden, R. E. & Sinkins, S. P. (2010). *Wolbachia* stimulates immune gene expression and inhibits *Plasmodium* development in *Anopheles gambiae*. *PloS Pathogens* [Online], **6**(10), pp. e1001143. Available from: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=20949079 [Accessed 1st July 2013].

Kambris, Z., Cook, P. E., Phuc, H. K. & Sinkins, S. P. (2009) Immune activation by life-shortening *Wolbachia* and reduced filarial competence in mosquitoes. *Science.* **326**(5949), pp. 134-136.

Kamoda, S., Masui, S., Ishikawa, H. & Sasaki, T. (2000) *Wolbachia* infection and cytoplasmic incompatibility in the cricket *Teleogryllus taiwanemma*. *Journal of Experimental Biology.* **203**(16), pp. 2503-2509.

Karr, T. L., Yang, W. & Feder, M. E. (1998) Overcoming cytoplasmic incompatibility in *Drosophila*. *Proceedings of the Royal Society of London Series B-Biological Sciences.* **265**(1394), pp. 391-395.

Klein, C. & Kallenborn, H. (2000) Ultrastrukturelle Untersuchungen zum Spermientransfer bei der Bettwanze *Cimex lectularius*, (Cimicidae, Heteroptera). *Heteropteron Köln*, **8**(1) pp. 11–12.

Knell, R. J. & Webberley, K. M. (2004) Sexually transmitted diseases of insects: distribution, evolution, ecolgy and host behaviour. *Biological Reviews of the Cambridge Philosophical Society.* **79**(3), pp. 557-581.

Koga, R., Tsuchida, T., Sakurai, M. & Fukatsu, T. (2007) Selective elimination of aphid endosymbionts: effects of antibiotic dose and host genotype, and fitness consequences. *FEMS Microbiology Ecology.* **60**(2), pp. 229-39.

Kondo, N., Shimada, M. & Fukatsu, T. (2005) Infection density of *Wolbachia* endosymbiont affected by co-infection and host genotype. *Biology Letters.* **1**(4), pp. 488-491.

Kurtz, J., Wiesner, A., Gotz, P. & Sauer, K. P. (2000) Gender differences and individual variation in the immune system of the scorpionfly *Panorpa vulgaris* (Insecta: Mecoptera). *Developmental and Comparative Immunology.* **24**(1), pp. 1-12.

Lange, R., Reinhardt, K., Michiels, N. K. & Anthes, N. (2013) Functions, diversity, and evolution of traumatic mating. *Biological Reviews of the Cambridge Philosophical Society*. **88**, pp. 585-601.

Lefoulon, E., Gavotte, L., Junker, K., Barbuto, M., Uni, S., Landmann, F., Laaksonen, S., Saari, S., Nikander, S., de Souza Lima, S., Casiraghi, M., Bain, O. & Martin, C. (2012) A new type F *Wolbachia* from Splendidofilariinae (Onchocercidae) supports the recent emergence of this supergroup. *International Journal for Parasitology.* **42**(11), pp. 1025-1036.

Lehane, M. J. (2005). *The Biology of Blood-Sucking in Insects.* Cambridge, Cambridge University Press.

Lessells, C. M. & Boag, P. T. (1987). Unrepeatable repeatabilities: a common mistake. *The Auk*. **104**, pp. 116–121.

Lipsitch, M., Siller, S. & Nowak, M. A. (1996) The evolution of virulence in pathogens with vertical and horizontal transmission. *Evolution.* **50**(5), pp. 1729-1741.

Lively, C. M. (2005) Competitive co-existence of vertically and horizontally transmitted parasites. *Evolutionary Ecology Research.* **7**(8), pp. 1183-1190.

Lo, N., Casiraghi, M., Salati, E., Bazzocchi, C. & Bandi, C. (2002) How many *Wolbachia* supergroups exist? *Molecular Biology and Evolution.* **19**(3), pp. 341-346.

Locke, S. J. & Peng, Y-S. (1993) The effects of drone age, semen storage and contamination on semen quality in the honey bee (*Apis mellifera*). *Physiological Entomology*. **18**(2), pp. 144-148.

Lockhart, A. B., Thrall, P. H. & Antonovics, J. (1996) Sexually transmitted diseases in animals: Ecological and evolutionary implications. *Biological Reviews of the Cambridge Philosophical Society.* **71**(3), pp. 415-471.

Machtelinckx, T., Van Leeuwen, T., Vanholme, B., Gehesquiere, B., Dermauw, W., Vandekerkhove, B., Gheysen, G. & De Clercq, P. (2009) *Wolbachia* induces strong cytoplasmic incompatibility in the predatory bug *Macrolophus pygmaeus*. *Insect Molecular Biology.* **18**(3), pp. 373-381.

Masui, S., Sasaki, T. & Ishikawa, H. (2000) Genes for the type IV secretion system in an intracellular symbiont, *Wolbachia*, a causative agent of various sexual alterations in arthropods. *Journal of Bacteriology.* **182**(22), pp. 6529-6531.

McMeniman, C. J., Lane, R. V., Cass, B. N., Fong, A. W., Sidhu, M., Wang, Y. F. & O'Neill, S. L. (2009) Stable introduction of a life-shortening *Wolbachia* infection into the mosquito *Aedes aegypti*. *Science.* **323**(5910), pp. 141-144.

Meriweather, M., Matthews, S., Rio, R. & Baucom, R. S. (2013). A 454 survey reveals the community composition and core microbiome of the common bed bug (*Cimex lectularius*) across an Urban Landscape. *PLoS One*, **8**(4), pp. e61465.

Miller, W. J., Ehrman, L. & Schneider, D. (2010). Infectious speciation revisited: impact of symbiont-depletion on female fitness and mating behavior of *Drosophila paulistorum*. *PLoS Pathogens* [Online], **6**(12), pp. e1001214. Available from: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=21151959 [Accessed 1st July 2013].

Min, K. T. & Benzer, S. (1997) *Wolbachia*, normally a symbiont of *Drosophila*, can be virulent, causing degeneration and early death. *Proceedings of the National Academy of Sciences of the United States of America.* **94**(20), pp. 10792-10796.

Montenegro, H., Petherwick, A. S., Hurst, G. D. & Klaczko, L. B. (2006) Fitness effects of *Wolbachia* and *Spiroplasma* in *Drosophila melanogaster*. *Genetica.* **127**(1-3), pp. 207-215.

Montes, C., Cuadrillero, C. & Vilella, D. (2002) Maintenance of a laboratory colony or *Cimexlectularius* (Hemiptera: Cimicidae) using an artificial feeding technique. *Journal of Medical Entomology.* **39**(4), pp. 675-679.

Moran, N. A., Degnan, P. H., Santos, S. R., Dunbar, H. E. & Ochman, H. (2005) The players in a mutualistic symbiosis: Insects, bacteria, viruses, and virulence genes. *Proceedings of the National Academy of Sciences of the United States of America.* **102**(47), pp. 16919-16926.

Moran, N. A. & Dunbar, H. E. (2006) Sexual acquisition of beneficial symbionts in aphids. *Proceedings of the National Academy of Sciences of the United States of America.* **103**(34), pp. 12803-12806.

Moreira, L. A., Iturbe-Ormaetxe, I., Jeffery, J. A., Lu, G., Pyke, A. T., Hedges, L. M., Rocha, B. C., Hall-Mendelin, S., Day, A., Riegler, M., Hugo, L. E., Johnson, K. N., Kay, B. H., McGraw, E. A., van den Hurk, A. F., Ryan, P. A. & O'Neill, S. L. (2009) A *Wolbachia* symbiont in *Aedes aegypti* limits infection with dengue, *Chikungunya*, and *Plasmodium*. *Cell.* **139**(7), pp. 1268-1278.

Moriyama, M., Koga, R., Hosokawa, T., Nikoh, N., Futahashi, R. & Fukatsu, T. (2012) Comparative transcriptomics of the bacteriome and the spermalege of the bedbug *Cimex lectularius* (Hemiptera Cimicidae). *Applied Entomology and Zoology.* **47**(3), pp. 233-243.

Morrow, E. H. & Arnqvist, G. (2003) Costly traumatic insemination and a female counter-adaptation in bed bugs. *Proceedings of the Royal Society of London Series B-Biological Sciences.* **270**(1531), pp. 2377-2381.

Morse, S. F., Bush, S. E., Patterson, B. D., Dick, C. W., Gruwell, M. E. & Dittmar, K. (2013) Evolution, multiple acquisition, and localization of endosymbionts in bat

flies (Diptera: hippoboscoidea: streblidae and nycteribiidae). *Applied and Environmental Microbiology*. **79**(9), pp. 2952-2961.

Nakagawa, S. & Schielzeth, H. (2010). Repeatability for Gaussian and non-Gaussian data: a practical guide for biologists. *Biological Reviews of the Cambridge Philosophical Society*. **85**(4), pp.935-956.

Naylor, R. (2006). *Development of an assay for lysozyme and lysozyme-like activity in Measuring the costs of traumatic insemination in the bed bug Cimex lectularius.* Masters Thesis, University of Sheffield.

Naylor, R. (2012a). *Ecology and dispersal of the bedbug.* Doctor of Philosophy, University of Sheffield.

Naylor, R. (2013). *CimexStore* [Online]. Available from: http://www.cimexstore.co.uk [Accessed 9th April 2013].

Naylor, R. A. (2012b). *Ecology and dispersal of the bedbug.* PhD Thesis, University of Sheffield.

Neven, L. G. (2000) Physiological responses of insects to heat. *Postharvest Biology and Technology.* **21**(1), pp. 103-111.

Nogge, G. (1976) Sterility in tsetse flies (*Glossina morsitans* Westwood) caused by loss of symbionts. *Experientia.* **32**(8), pp. 995-996.

Nogge, G. (1981) Significance of symbionts for the maintenance of an optimal nutritional state for successful reproduction in haematophagous arthropods. *Parasitology.* **82**(4), pp. 101-104.

Oliver, K. M. & Moran, N. A. (2009). Defensive symbionts in aphids and other insects. In*:* J. F. White & M. S. Torres (Eds.) *Defensive Mutualism in Microbial Symbiosis.* London, CRC Press.

Oliver, K. M., Moran, N. A. & Hunter, M. S. (2005) Variation in resistance to parasitism in aphids is due to symbionts not host genotype. *Proceedings of the National Academy of Sciences of the United States of America.* **102**(36), pp. 12795-12800.

Omori, N. (1941) Comparative studies on the ecology and physiology of common and tropical bed bugs, with special references to the reactions to temperature and moisture. *Journal of the Formosan Medical Association.* **60**(4), pp. 555-729.

Osborne, S. E., Leong, Y. S., O'Neill, S. L. & Johnson, K. N. (2009). Variation in antiviral protection mediated by different *Wolbachia* strains in *Drosophila simulans*. *PLoS Pathogens* [Online], **5**(11), pp. e1000656. Available from: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=19911047 [Accessed 11th November 2013].

Otti, O., Naylor, R. A., Siva-Jothy, M. T. & Reinhardt, K. (2009) Bacteriolytic activity in the ejaculate of an insect. *American Naturalist.* **174**(2), pp. 292-295.

Pan, X., Zhou, G., Wu, J., Bian, G., Lu, P., Raikhel, A. S. & Xi, Z. (2012) *Wolbachia* induces reactive oxygen species (ROS)-dependent activation of the Toll pathway to control dengue virus in the mosquito *Aedes aegypti*. *Proceedings of the National Academy of Sciences of the United States of America.* **109**(1), pp. E23-31.

Panteleev, D. Y., Goryacheva, I. I., Andrianov, B. V., Reznik, N. L., Lazebny, O. E. & Kulikov, A. M. (2007) The endosymbiotic bacterium *Wolbachia* enhances the nonspecific resistance to insect pathogens and alters behavior of *Drosophila melanogaster*. *Russian Journal of Genetics.* **43**(9), pp. 1066-1069.

Parker, B. J., Barribeau, S. M., Laughton, A. M., de Roode, J. C. & Gerardo, N. M. (2011) Non-immunological defense in an evolutionary framework. *Trends in Ecology & Evolution.* **26**(5), pp. 242-248.

Pereira, R. M., Koehler, P. G., Pfiester, M. & Walker, W. (2009) Lethal effects of heat and use of localized heat treatment for control of bed bug infestations. *Journal of Economic Entomology.* **102**(3), pp. 1182-1188.

Pike, N. & Kingcombe, R. (2009). Antibiotic treatment leads to the elimination of *Wolbachia* endosymbionts and sterility in the diplodiploid collembolan *Folsomia candida*. *BMC Biology* [Online], **7**(1), pp. 54. Available from: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=19698188 [Accessed 1st July 2013].

Potter, M. F. (2009). *Your guide to bed bugs completely revised and updated understanding and control.* Richfield, Ohio, GIE Media.

Purcell, A. H., Steiner, T., Mégraud, F. & Bové, J. (1986) *In vitro* isolation of a transovarially transmitted bacterium from the leafhopper *Euscelidius variegatus* (Hemiptera: Cicadellidae). *Journal of Invertebrate Pathology*. **48**(1), pp. 66-73.

Rao, H. V. (1974) Free amino acids in seminal fluid & haemolymph of Cimex lectularious L. & their possible role in sperm metabolism. *Indian J Exp Biol.* **12**(4), pp. 346-8.

Rasgon, J. L., Gamston, C. E. & Ren, X. (2006) Survival of *Wolbachia pipientis* in cell-free medium. *Applied and Environmental Microbiology.* **72**(11), pp. 6934-6937.

Rasgon, J. L. & Scott, T. W. (2004) Phylogenetic characterization of *Wolbachia* symbionts infecting *Cimex lectularius* L. and *Oeciacus vicarius* Horvath (Hemiptera: Cimicidae). *Journal of Medical Entomology.* **41**(6), pp. 1175-1178.

Reinhardt, K., Isaac, D. & Naylor, R. (2010) Estimating the feeding rate of the bedbug *Cimex lectularius* in an infested room: an inexpensive method and a case study. *Medical and Veterinary Entomology.* **24**(1), pp. 46-54.

Reinhardt, K., Kempke, D., Naylor, R. A. & Siva-Jothy, M. T. (2009a) Sensitivity to bites by the bedbug, *Cimex lectularius*. *Medical and Veterinary Entomology.* **23**(2), pp. 163-166.

Reinhardt, K., Naylor, R. & Siva-Jothy, M. T. (2003) Reducing a cost of traumatic insemination: female bedbugs evolve a unique organ. *Proceedings of the Royal Society of London Series B-Biological Sciences.* **270**(1531), pp. 2371-2375.

Reinhardt, K., Naylor, R. A. & Siva-Jothy, M. T. (2005) Potential sexual transmission of environmental microbes in a traumatically inseminating insect. *Ecological Entomology.* **30**(5), pp. 607-611.

Reinhardt, K., Naylor, R. A. & Siva-Jothy, M. T. (2009b) Ejaculate components delay reproductive senescence while elevating female reproductive rate in an insect. *Proceedings of the National Academy of Sciences of the United States of America.* **106**(51), pp. 21743-21747.

Reinhardt, K., Naylor, R. A. & Siva-Jothy, M. T. (2009c) Situation exploitation: higher male mating success when female resistance is reduced by feeding. *Evolution.* **63**(1), pp. 29-39.

Reinhardt, K., Naylor, R. & Siva-Jothy, M. (2011). Male mating rate is constrained by seminal fluid availability in bedbugs, *Cimex lectularius*. *PLoS One*, **6**(7), pp. e22082.

Reinhardt, K. & Siva-Jothy, M. T. (2007) Biology of the bed bugs (Cimicidae). *Annual Review of Entomology*. **52,** pp. 351-374.

Reinhardt, K., Wong, C. H. & Georgiou, A. S. (2009d) Detection of seminal fluid proteins in the bed bug, *Cimex lectularius*, using two-dimensional gel electrophoresis and mass spectrometry. *Parasitology.* **136**(3), pp. 283-292.

Reynolds, K. T. & Hoffmann, A. A. (2002) Male age, host effects and the weak expression or non-expression of cytoplasmic incompatibility in *Drosophila* strains infected by maternally transmitted *Wolbachia*. *Genetics Research.* **80**(2), pp. 79-87.

Riegler, M. & O'Neill, S. L. (2006). The Genus *Wolbachia*. In*:* M. Dworkin, S. Falkow, E. Rosenberg, K-H. Schleifer & E. Stackebrandt (Eds.) *The Prokaryotes: a handbook on the biology of bacteria.* 3rd edition ed. New York, Springer. pp. 547-561.

Rolff, J. & Reynolds, S. E. (Eds.) (2009) *Insect Infection and Immunity.* New York, Oxford University Press Inc.

Rolff, J. & Siva-Jothy, M. T. (2003) Invertebrate ecological immunology. *Science.* **301**(5632), pp. 472-475.

Rosengaus, R. B., Zecher, C. N., Schultheis, K. F., Brucker, R. M. & Bordenstein, S. R. (2011) Disruption of the termite gut microbiota and its prolonged consequences for fitness. *Applied and Environmental Microbiology.* **77**(13), pp. 4303-4312.

Rottschaefer, S. M. & Lazzaro, B. P. (2012). No effect of *Wolbachia* on resistance to intracellular infection by pathogenic bacteria in *Drosophila melanogaster*. *PLoS One*, **7**(7), pp. e40500.

Sabate, D. C., Carrillo, L. & Audisio, M. C. (2009) Inhibition of *Paenibacillus larvae* and *Ascosphaera apis* by *Bacillus subtilis* isolated from honeybee gut and honey samples. *Research in Microbiology.* **160**(3), pp. 193-199.

Sakamoto, J. M. & Rasgon, J. L. (2006) Geographic distribution of *Wolbachia* infections in *Cimex lectularius* (Heteroptera: Cimicidae). *Journal of Medical Entomology.* **43**(4), pp. 696-700.

Sasaki-Fukatsu, K., Koga, R., Nikoh, N., Yoshizawa, K., Kasai, S., Mihara, M., Kobayashi, M., Tomita, T. & Fukatsu, T. (2006) Symbiotic bacteria associated with stomach discs of human lice. *Applied and Environmental Microbiology.* **72**(11), pp. 7349-7352.

Savalli, U. M. & Fox, C. W. (1998) Sexual selection and the fitness consequences of male body size in the seed beetle *Stator limbatus*. *Animal Behaviour.* **55**(2), pp. 473-483.

Segovia, M., Jenkins, J. A., Paniagua-Chavez, C. & Tiersch, T. R. (2000) Flow cytometric evaluation of antibiotic effects on viability and mitochondrial function of refrigerated spermatozoa of Nile tilapia. *Theriogenology*. **53**(7), pp. 1489-1499.

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

Siljander, E., Gries, R., Khaskin, G. & Gries, G. (2008) Identification of the airborne aggregation pheromone of the common bed bug, *Cimex lectularius*. *Journal of Chemical Ecology.* **34**(6), pp. 708-718.

Siozios, S., Ioannidis, P., Klasson, L., Andersson, S. G., Braig, H. R. & Bourtzis, K. (2013). The diversity and evolution of *Wolbachia* ankyrin repeat domain genes. *PLoS One*, **8**(2), pp. e55390.

Siva-Jothy, M. T. (1997) Odonate ejaculate structure and mating systems." *Odonatologica* **26(**4), pp. 415-437.

Siva-Jothy, M. T. (2006) Trauma, disease and collateral damage: Conflict in cimicids. *Philosophical Transactions of the Royal Society of London Series B-Biological Sciences.* **361**(1466), pp. 269-275.

Siva-Jothy, M. T. (2009). Reproductive Immunity. In*:* J. Rolff & S. E. Reynolds (Eds.) *Insect Infection and Immunity.* Oxford University Press. pp. 241-250.

Siva-Jothy, M. T., Moret, Y. & Rolff, J. (2005) Insect immunity: an evolutionary ecology perspective. *Advances in Insect Physiology*. **32,** pp. 1-48.

Siva-Jothy, M. T. & Stutt, A. D. (2003) A matter of taste: direct detection of female mating status in the bedbug. *Proceedings of the Royal Society of London Series B-Biological Sciences.* **270**(1515), pp. 649-652.

Siva-Jothy, M. T. & Thompson, J. J. W. (2002) Short-term nutrient deprivation affects immune function. *Physiological Entomology.* **27**(3), pp. 206-212.

Siva-Jothy, M. & Tsubaki, Y. (1998) Decreased immune response as a proximate cost of copulation and oviposition in a damselfly. *Physiological Entomology.* **23**(3), pp. 274-277.

Skau, P. A. & Folstad, I. (2005) Does immunity regulate ejaculate quality and fertility in humans? *Behavioral Ecology*. **16**(2), pp. 410-416.

Slatko, B. E., Taylor, M. J. & Foster, J. M. (2010) The *Wolbachia* endosymbiont as an anti-filarial nematode target. *Symbiosis.* **51**(1), pp. 55-65.

Sonawane, Y. S. & More, N. K. (1993) The circulating hemocytes of the bed bug, *Cimex rotundatus* (Sign.) (Heteroptera: Cimicidae). *Journal of Animal Morphology and Physiology*. **40,** pp. 79–86.

Steigner, A. (2001) *Licht-und elektronenmikroskopische Untersuchungen am inneren Genitalapparat weiblicher Bettwanzen (Cimex lectularius Linnaeus 1758, Cimicidae, Heteroptera).* Ph.D. Thesis, University of Saarbrucken, Germany.

Stevens, L. (1993) Cytoplasmically inherited parasites and reproductive success in *Tribolium* flour beetles. *Animal Behaviour.* **46**(2), pp. 305.

Stouthamer, R., Breeuwer, J. A. & Hurst, G. D. (1999) *Wolbachia pipientis*: microbial manipulator of arthropod reproduction. *Annual Review of Microbiology*. **53,** pp. 71-102.

Strand, M. A. (1977) Pathogens of cimicidae (bedbugs). *Bulletin of the World Health Organisation*. **55**(1) pp. 313-315.

Stutt, A. D. (1999). *Reproductive strategies and sexual conflict in the bed bug Cimex lectularius.* Doctor of Philosophy, University of Sheffield.

Stutt, A. D. & Siva-Jothy, M. T. (2001) Traumatic insemination and sexual conflict in the bed bug *Cimex lectularius*. *Proceedings of the National Academy of Sciences of the United States of America.* **98**(10), pp. 5683-5687.

Takano-Lee, M., Velten, R. K., Edman, J. D., Mullens, B. A. & Clark, J. M. (2003) An automated feeding apparatus for *in vitro* maintenance of the human head louse, *Pediculus capitis* (Anoplura: Pediculidae). *Journal of Medical Entomology.* **40**(6), pp. 795-799.

Tang, H. (2009) Regulation and function of the melanization reaction in *Drosophila*. *Fly (Austin).* **3**(1), pp. 105-111.

Taylor, M. J., Makunde, W. H., McGarry, H. F., Turner, J. D., Mand, S. & Hoerauf, A. (2005) Macrofilaricidal activity after doxycycline treatment of *Wuchereria bancrofti*: a double-blind, randomised placebo-controlled trial. *Lancet.* **365**(9477), pp. 2116-2121.

R Core Team (2012). *R: A Language and Environment for Statistical Computing*, Vienna, Austria, Available from: http://www.R-project.org/

Teixeira, L., Ferreira, A. & Ashburner, M. (2008). The bacterial symbiont *Wolbachia* induces resistance to RNA viral infections in *Drosophila melanogaster*. *PLoS Biology* [Online], **6**(12), pp. e2. Available from: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=19222304 [Accessed 23rd December 2012].

Thomas, P., Kenny, N., Eyles, D., Moreira, L. A., O'Neill, S. L. & Asgari, S. (2011) Infection with the wMel and wMelPop strains of *Wolbachia* leads to higher levels

of melanization in the hemolymph of *Drosophila melanogaster*, *Drosophila simulans* and *Aedes aegypti*. *Developmental and Comparative Immunology.* **35**(3), pp. 360-365.

Usinger, R. L. (1966). *Monograph of the Cimicidae (Hemiptera-Heteroptera).* The Thomas Say Foundation Vol. VII. College Park, Maryland, USA, Entomological Society of America.

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

Wade, M. J. & Chang, N. W. (1995a) Increased male fertility in Tribolium confusum beetles after infection with the intracellular parasite Wolbachia. *Nature.* **373**(6509), pp. 72-4.

Wade, M. J. & Chang, N. W. (1995b) Increased male fertility in *Tribolium confusum* beetles after infection with the intracellular parasite *Wolbachia*. *Nature.* **373**(6509), pp. 72-74.

Walker, T., Johnson, P. H., Moreira, L. A., Iturbe-Ormaetxe, I., Frentiu, F. D., McMeniman, C. J., Leong, Y. S., Dong, Y., Axford, J., Kriesner, P., Lloyd, A. L., Ritchie, S. A., O'Neill, S. L. & Hoffmann, A. A. (2011) The *w*Mel *Wolbachia* strain blocks dengue and invades caged *Aedes aegypti* populations. *Nature.* **476**(7361), pp. 450-453.

Wang, J., Wu, Y., Yang, G. & Aksoy, S. (2009) Interactions between mutualist *Wigglesworthia* and tsetse peptidoglycan recognition protein (PGRP-LB) influence trypanosome transmission. *Proceedings of the National Academy of Sciences of the United States of America.* **106**(29), pp. 12133-12138.

Wang, L., Zhou, C., He, Z., Wang, Z. G., Wang, J. L. & Wang, Y. F. (2012). *Wolbachia* infection decreased the resistance of Drosophila to lead. *PLoS One*, **7**(3), pp. e32643.

Weeks, E. N., Logan, J. G., Gezan, S. A., Woodcock, C. M., Birkett, M. A., Pickett, J. A. & Cameron, M. M. (2010) A bioassay for studying behavioural responses of the common bed bug, *Cimex lectularius* (Hemiptera: Cimicidae) to bed bug-derived volatiles. *Bull Entomol Res.* pp. 1-8.

Weiss, B. L., Wang, J. & Aksoy, S. (2011). Tsetse immune system maturation requires the presence of obligate symbionts in larvae. *PLoS Biol* [Online], **9**(5), pp. e1000619. Available from: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=21655301 [Accessed 5th May 2013].

Werren, J. H. (1997) Biology of *Wolbachia*. *Annual Review of Entomology*. **42,** pp. 587-609.

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

Werren, J. H. & Loehlin, D. W. (2009) Curing *Wolbachia* infections in *Nasonia* (parasitoid wasp). *Cold Spring Harbor Protocols.* **2009**(10), pp. pdb prot5312.

Werren, J. H., Zhang, W. & Guo, L. R. (1995) Evolution and phylogeny of *Wolbachia*: reproductive parasites of arthropods. *Proceedings of the Royal Society of London Series B-Biological Sciences*. **261**(1360), pp. 55-63.

Wigglesworth, V. B. (1936) Symbiotic bacteria in a blood-sucking insect, *Rhodnius prolixus* Stål. (Hemiptera, Triatomidae). *Parasitology.* **28**(2), pp. 284-289.

Wiwatanaratanabutr, S. & Kittayapong, P. (2006) Effects of temephos and temperature on *Wolbachia* load and life history traits of *Aedes aliblopictus*. **20**(3), pp. 301-307.

Wong, Z. S., Hedges, L. M., Brownlie, J. C. & Johnson, K. N. (2011). *Wolbachia*-mediated antibacterial protection and immune gene regulation in *Drosophila*. *PLoS One*, **6**(9), pp. e25430.

Zabalou, S., Riegler, M., Theodorakopoulou, M., Stauffer, C., Savakis, C. & Bourtzis, K. (2004) *Wolbachia*-induced cytoplasmic incompatibility as a means for insect pest population control *Proceedings of the National Academy of America.* **101**(42), pp. 15042-15045.

Zchori-Fein, E., Gottlieb, Y. & Coll, M. (2000) *Wolbachia* density and host fitness components in *Muscidifurax uniraptor* (Hymenoptera: pteromalidae). *Journal of Invertebrate Pathology.* **75**(4), pp. 267-272.

Zeh, D. W., Zeh, J. A. & Bonilla, M. M. (2005) *Wolbachia*, sex ratio bias and apparent male killing in the harlequin beetle riding pseudoscorpion. *Heredity.* **95**(1), pp. 41-49.

Zhou, W., Rousset, F. & O'Neill, S. (1998) Phylogeny and PCR–based classification of *Wolbachia* strains using *wsp* gene sequences. *Proceedings of the Royal Society of London Series B-Biological Sciences.* **265**(1395), pp. 509-515.

Zug, R. & Hammerstein, P. (2012). Still a host of hosts for *Wolbachia*: analysis of recent data suggests that 40% of terrestrial arthropod species are infected. *PLoS One*, **7**(6), pp. e38544.

Zug, R., Koehncke, A. & Hammerstein, P. (2012) Epidemiology in evolutionary time: the case of *Wolbachia* horizontal transmission between arthropod host species. *Journal of Evolutionary Biology.* **25**(11), pp. 2149-2160.