ENVIRONMENTAL IMPACTS ON THE USE OF MODIFIED MOSQUITOES FOR ARBOVIRUS CONTROL

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ABSTRACT

*Aedes aegypti* mosquitoes infected with the bacterium *Wolbachia* are being released throughout the tropics to control arbovirus transmission. This approach has faced challenges in some locations due to poor-performing mosquitoes and loss of *Wolbachia* infections. I investigated the effects of different environmental conditions on *Wolbachia*’s ability to manipulate host reproduction and affect mosquito performance. *Wolbachia* infections reduce the ability of mosquito larvae to survive under nutritionally poor and competitive conditions, but their effects on the host otherwise remain stable. In contrast, *Wolbachia* infections are susceptible to cyclical heat stress, which could compromise their ability to invade mosquito populations in some tropical environments. *Wolbachia*-infected mosquitoes selected for increased thermal tolerance responded negatively, exhibiting a reduced density of *Wolbachia* and weakened cytoplasmic incompatibility in males. I also investigated the effects of laboratory rearing and inbreeding on mosquito fitness. I found that maintaining mosquitoes at small population sizes greatly reduces their performance, but I found limited evidence for adaptation to artificial rearing conditions. My findings will inform future disease control efforts using modified mosquitoes.
DECLARATION

This is to certify that:

1. This thesis comprises only my original work towards the PhD except where indicated in the Preface,

2. Due acknowledgement has been made in the text to all other material used,

3. This thesis is fewer than 100,000 words in length, exclusive of tables, maps, bibliographies and appendices.

_____________________________________

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This thesis contains two chapters that were previously published in peer reviewed journals and one chapter that is currently under review. These studies appear in the thesis with minor modifications from their published or submitted format. Modifications are largely restricted to additional data that were not included in the original publications. One other results chapter is unpublished. Five additional papers that Perran A. Ross (PAR) wrote or contributed to during the PhD are included as appendices.

For Chapter 2, the study was conceived and designed by PAR, Nancy M. Endersby-Harshman (NMEH) and Ary A. Hoffmann (AAH). PAR performed all experiments, analysed the data and wrote the first draft of the manuscript. NMEH and AAH provided supervision and assisted in writing the paper. PAR performed 85% of the work. This chapter was published in PLoS Neglected Tropical Diseases 10 (1), e0004320. The supplementary information from this paper is included as appendix A.

For Chapter 3, JKA performed the maternal transmission experiment and Vanessa L. White (VLW) performed the molecular work for this experiment. Itsanun Wiwatanaratanabutr (IW) assisted PAR in rearing larvae and estimating Wolbachia density. NMEH and AAH provided supervision and all authors provided feedback on the manuscript. PAR performed all other experiments, analysed the data and wrote the paper, comprising 65% of the work. This chapter was published in PLoS Pathogens 13 (1), e1006006, and supplementary information is included as appendix B.

For chapter 4, NMEH and AAH assisted with experimental design and editing the chapter. PAR performed all experiments, analysed the data and drafted the chapter, comprising 90% of the work. Data from this chapter will be combined with other experiments from the Hoffmann laboratory for publication. The supplementary information from this chapter is available in appendix C.

For chapter 5, NMEH and AAH provided supervision and assisted with designing experiments and editing the paper. PAR performed all experiments, analysed the data and drafted the paper, comprising 85% of the work. This paper has been submitted to Evolutionary Applications for peer review. The supplementary information from this paper is included as appendix D.

The paper “Larval competition extends developmental time and decreases adult size of υMelPop Wolbachia-infected Aedes aegypti” published in the American Journal of Tropical Medicine and Hygiene 91 (1), 198-205 was part of a MSc project by PAR. This paper was revised and resubmitted during PAR’s first year of PhD, and has been included in the thesis as appendix E.

The paper “Wolbachia strains for disease control: ecological and evolutionary considerations” published in Evolutionary Applications 8 (8), 751-768 is a review written by AAH, PAR and Gordana
Rašić (GR). PAR compiled the supplementary table and contributed to writing the manuscript. Approximately 25% of the work was undertaken by PAR. This paper is included as appendix F.

The paper “Fitness of wAlbB *Wolbachia* infection in *Aedes aegypti*: parameter estimates in an outcrossed background and potential for population invasion” published in *The American Journal of Tropical Medicine and Hygiene* 94 (3), 507-516 was written by Jason K. Axford (JKA), PAR, Heng Lin Yeap (HLY), Ashley G. Callahan (AGC) and AAH. PAR performed, analysed and wrote the larval development, body size and maternal transmission experiment sections of the paper, and contributed to writing and editing of the rest of the manuscript, comprising 25% of the work. This paper is included as appendix G.

The paper “Maintaining *Aedes aegypti* mosquitoes infected with *Wolbachia*” is a video methods paper published in *the Journal of Visualized Experiments* 126. PAR wrote the paper, prepared the mosquitoes and equipment for filming, reviewed the script and demonstrated the procedure on video. JKA, Kelly M. Richardson (KMR), NMEH and AAH assisted in editing the paper and provided input into the protocol. The methods for maintaining mosquitoes were developed previously. Approximately 75% of the work for the paper was undertaken by PAR. The written component of this paper is included as appendix H, and the accompanying video can be found at [https://www.jove.com/video/56124](https://www.jove.com/video/56124).

The review “Rates and patterns of laboratory adaptation in (mostly) insects” is in press in *the Journal of Economic Entomology* at the time of writing. PAR contributed to locating relevant studies, analysing the data, making figures and writing the paper. Approximately 40% of the work for the paper was undertaken by PAR. This paper is included as appendix I.

The results of many scientific experiments never leave the lab book. Research projects change direction, experiments fail, and sometimes the results never find a place where they belong. I have included a few of these odds and ends in appendix J in the hope that they will be of interest to other researchers in the field.
ACKNOWLEDGEMENTS

I would like to thank Ary Hoffmann and Nancy Endersby-Harshman for supporting me throughout my PhD and providing the perfect balance of structure and freedom with my project. I had a wonderful time in the lab and I couldn’t have hoped for a better pair of supervisors. I also thank my committee members Gordana Rasic and Penny Hancock who also provided guidance throughout my PhD.

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I would like to thank all the volunteers who assisted me with some of my more unwieldy experiments, especially Elizabeth Valerie, Fionna Zhu and Isabelle Foo. I can’t bear to think of how many less experimental replicates I would have had without your help.

I thank Scott Ritchie and his group at James Cook University (Chris, Mick, Tamara, Kyran and others) for providing the mosquitoes for many of my experiments, and for being so welcoming when I visited Cairns at the end of my PhD.

To my family; my wife Jess, my parents and my brother. Thank you for supporting me.

And lastly, I thank the mosquitoes. *Wolbachia*-infected, uninfected, inbred, outbred, starved and heat-stressed; without them this thesis would not be possible. I hope that their contribution to my research will aid in the effort to eradicate the diseases they transmit.

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# TABLE OF CONTENTS

ABSTRACT ................................................................................................................................................ i
DECLARATION .......................................................................................................................................... ii
PREFACE ............................................................................................................................................... iii
ACKNOWLEDGEMENTS ........................................................................................................................... v
FUNDING ................................................................................................................................................. v
TABLE OF CONTENTS .............................................................................................................................. vi
LIST OF FIGURES .................................................................................................................................... x
LIST OF TABLES ..................................................................................................................................... xiii

## CHAPTER 1 – INTRODUCTION ............................................................................................................ 1
1.1 *Wolbachia* as a biological control agent for arboviruses ........................................................... 1
1.2 Experimental *Wolbachia* infections in mosquitoes and their field release ............................... 6
1.3 Environmental effects on *Wolbachia* infections ...................................................................... 16
  1.3.1 Nutrition .............................................................................................................................. 17
  1.3.1 Temperature ....................................................................................................................... 18
  1.3.1 Diapause, quiescence and age .......................................................................................... 20
  1.3.1 Environmental antibiotics .............................................................................................. 20
  1.3.1 Genetic background ......................................................................................................... 21
1.4 Stability of *Wolbachia* infections after field deployment .......................................................... 22
1.5 Conclusions and research aims ................................................................................................. 24

## CHAPTER 2 – COSTS OF THREE *WOLBACHIA* INFECTIONS ON THE SURVIVAL OF *AEDES AEGYPTI* LARVAE UNDER STARVATION CONDITIONS ........................................................................................... 25
2.1 Abstract ......................................................................................................................................... 25
2.2 Author summary ............................................................................................................................ 25
2.3 Introduction .................................................................................................................................. 26
2.4 Materials and methods ............................................................................................................... 29
  2.4.1 Colony maintenance and mosquito strains ...................................................................... 29
  2.4.2 Rearing regime .................................................................................................................... 29
  2.4.3 Survival of isolated larvae under starvation conditions ....................................................... 30
  2.4.4 Survival and development of larvae held in groups under starvation conditions ............ 30
  2.4.5 Recovery from food deprivation ....................................................................................... 31
  2.4.6 Cytoplasmic incompatibility when larvae are food-deprived then re-fed ......................... 32
  2.4.7 Maternal transmission and fecundity when larvae are food-deprived then re-fed .......... 32
2.4.8 Wing length measurements ................................................................. 33
2.4.9 DNA extraction and Wolbachia detection ........................................... 33
2.4.10 Statistical analysis ........................................................................... 34
2.5 Results ................................................................................................. 34
2.5.1 Survival of isolated larvae under starvation conditions .................... 34
2.5.2 Survival and development of larvae held in groups under starvation conditions .... 36
2.5.3 Recovery from food deprivation ....................................................... 42
2.5.4 Cytoplasmic incompatibility, maternal transmission and fecundity when larvae are food-deprived then re-fed ............................................. 45
2.6 Discussion .......................................................................................... 47
2.7 Acknowledgements ............................................................................. 50

CHAPTER 3 – WOLBACHIA INFECTIONS IN Aedes aegypti DIFFER MARKEDLY IN THEIR RESPONSE TO CYCICAL HEAT STRESS ........................................................................................................................ 51
3.1 Abstract ............................................................................................. 51
3.2 Author summary .................................................................................. 51
3.3 Introduction ......................................................................................... 52
3.4 Results ................................................................................................. 55
3.4.1 Maximum daily temperatures of 37°C during development reduce the hatch rate of wMel-infected eggs ........................................................ 55
3.4.2 Wolbachia density is reduced in wMel and wMelPop-CLA, but not wAlbB-infected adults reared under cyclical temperatures of 26-37°C .......... 56
3.4.3 Cytoplasmic incompatibility is partially lost in wMel and wMelPop-CLA, but not wAlbB-infected adults reared under cyclical temperatures of 26-37°C ........................................................................ 58
3.4.4 The wMel and wMelPop-CLA infections are not maternally transmitted, and wAlbB exhibits incomplete maternal transmission fidelity at 26-37°C ........................................ 60
3.5 Discussion .......................................................................................... 61
3.6 Materials and methods ....................................................................... 64
3.6.1 Ethics statement ............................................................................. 64
3.6.2 Colony maintenance and Wolbachia infections ............................... 64
3.6.3 Rearing at cyclical temperatures ................................................... 65
3.6.4 Hatch rate and cytoplasmic incompatibility .................................... 65
3.6.5 Wolbachia quantification ................................................................. 66
3.6.6 Maternal transmission of Wolbachia ............................................. 66
3.6.7 Statistical analysis ......................................................................... 67
3.7 Acknowledgements ............................................................................. 67
CHAPTER 4 — NEGATIVE RESPONSE OF WOLBACHIA STRAIN wMel TO SELECTION FOR INCREASED THERMAL TOLERANCE ................................................................. 68

4.1 Abstract ......................................................................................................................... 68
4.2 Introduction .................................................................................................................... 68
4.3 Methods ......................................................................................................................... 71
  4.3.1 Ethics statement ....................................................................................................... 71
  4.3.2 Mosquito strains and colony maintenance .............................................................. 71
  4.3.3 Selection regime ...................................................................................................... 72
  4.3.4 Cytoplasmic incompatibility and restoration of compatibility ................................ 73
  4.3.5 Wolbachia quantification ....................................................................................... 73
  4.3.6 Statistical analysis .................................................................................................. 74
4.4 Results ............................................................................................................................ 74
  4.4.1 Egg hatch proportions during selection ................................................................. 74
  4.4.2 Cytoplasmic incompatibility in males reared under heat stress .............................. 76
  4.4.3 Restoration of compatibility by females reared under heat stress .......................... 78
  4.4.4 Wolbachia density .................................................................................................. 80
4.5 Discussion ....................................................................................................................... 80

CHAPTER 5 — A COMPREHENSIVE ASSESSMENT OF INBREEDING AND LABORATORY ADAPTATION IN Aedes aegypti MOSQUITOES .............................................................................................. 83

5.1 Abstract ........................................................................................................................ 83
5.2 Introduction ................................................................................................................... 83
5.3 Materials and methods ............................................................................................... 86
  5.3.1 Replicate population establishment ...................................................................... 86
  5.3.2 Colony maintenance .............................................................................................. 88
  5.3.3 Fitness comparisons between Townsville F13 populations ................................... 89
  5.3.4 Male mating competitiveness ................................................................................ 90
  5.3.5 Pooled double-digest RADseq library preparation .............................................. 90
  5.3.6 Data processing and effective population size estimates ....................................... 91
  5.3.7 Statistics on life history traits ............................................................................... 91
5.4 Results ............................................................................................................................ 92
  5.4.1 Preliminary fitness comparisons ............................................................................ 92
  5.4.2 Larval development time ....................................................................................... 92
  5.4.3 Survival to adulthood and sex ratio ...................................................................... 94
  5.4.4 Fecundity and egg hatch rate ............................................................................... 96
  5.4.5 Adult body size ...................................................................................................... 99
  5.4.6 Overall performance ............................................................................................ 100
  5.4.7 Effective population size ..................................................................................... 101
## LIST OF FIGURES

| FIGURE 1.1 | Effects of three different *Wolbachia* strains on the fitness of *Aedes aegypti* | 8 |
| FIGURE 2.1 | Survival of *Ae. aegypti* larvae when isolated under starvation conditions | 35 |
| FIGURE 2.2 | Survival of *Ae. aegypti* larvae under starvation conditions in groups of 50 per container | 37 |
| FIGURE 2.3 | Proportion of *Ae. aegypti* larvae developing when fed *ad libitum* after extended food deprivation | 43 |
| FIGURE 3.1 | Proportion of eggs hatched from *Wolbachia*-infected *Ae. aegypti* reared at cyclical temperatures | 55 |
| FIGURE 3.2 | Relative density of *Wolbachia* in *Ae. aegypti* reared at cyclical temperatures | 57 |
| FIGURE 3.3 | Effect of cyclical temperatures on cytoplasmic incompatibility in *Wolbachia*-infected *Ae. aegypti* | 59 |
| FIGURE 4.1 | Proportion of eggs hatching from crosses between *w*Mel-infected *Ae. aegypti* females reared under heat stress and *w*Mel-infected males reared at 26°C during the selection experiment | 75 |
| FIGURE 4.2 | Egg hatch proportions of uninfected female *Ae. aegypti* when crossed to males from the *w*Mel, *w*Mel-HS and *w*AlbB lines reared under different temperature conditions | 77 |
| FIGURE 4.3 | Egg hatch proportions of female *Ae. aegypti* from the *w*Mel, *w*Mel-HS and *w*AlbB lines when reared under different temperature conditions and then crossed to infected males reared at 26°C | 79 |
| FIGURE 4.4 | Relative *Wolbachia* density of *Ae. aegypti* from the *w*Mel and *w*Mel-HS lines reared at either a constant 26°C or one of two cyclical temperature conditions | 80 |
| FIGURE 5.1 | Maintenance scheme for replicate *Ae. aegypti* laboratory populations | 88 |
| FIGURE 5.2 | Development time of *Ae. aegypti* F13 laboratory populations maintained at different census sizes | 93 |
| FIGURE 5.3 | Survival to adulthood of *Ae. aegypti* F13 laboratory populations maintained at different census sizes | 95 |
| FIGURE 5.4 | Fecundity of *Ae. aegypti* F13 laboratory populations maintained at different census sizes | 97 |
| FIGURE 5.5 | Egg hatch proportions of *Ae. aegypti* F13 laboratory populations maintained at different census sizes | 98 |
| FIGURE 5.6 | Wing length of *Ae. aegypti* F13 laboratory populations maintained at different census sizes | 100 |
| FIGURE 5.7 | Relative performance of *Ae. aegypti* F13 laboratory populations maintained at different census sizes | 101 |
FIGURE 5.8 Relative mating success of *Ae. aegypti* males maintained in the laboratory for different numbers of generations

FIGURE A.1 Survival of *Ae. aegypti* larvae under starvation conditions during the recovery from food deprivation experiment

FIGURE A.2 Comparison of larval survival under starvation conditions between experiments

FIGURE A.3 Larval mortality and dead larvae observed when *Ae. aegypti* larvae were held in groups under starvation conditions

FIGURE A.4 Larval mortality of *Ae. aegypti* and the number of larvae consumed when held in groups under starvation conditions

FIGURE A.5 Pupae and adults of *Ae. aegypti* observed when larvae were held under starvation conditions in groups of 50

FIGURE B.1 Diurnal temperature fluctuations in incubators

FIGURE B.2 Wing length of *Ae. aegypti* adults reared at a constant 26°C, cycling 26-32°C and cycling 26-37°C

FIGURE B.3 Relative density of *Wolbachia* in *Ae. aegypti* reared at cyclical temperatures and different levels of nutrition

FIGURE B.4 Wing lengths of *Wolbachia*-infected *Ae. aegypti* reared at cyclical temperatures and different levels of nutrition

FIGURE C.1 Temperatures experienced in larval rearing trays during the cytoplasmic incompatibility experiment on *Ae. aegypti*

FIGURE C.2 Hatch proportions of eggs from *wMel*-infected female *Ae. aegypti* reared under cyclical heat stress and held at 26°C for 1, 4 or 7 days before being crossed to *wMel*-infected males reared at 26°C

FIGURE C.3 Fecundity of *wMel*, *wMel-HS* and *wAlbB* female *Ae. aegypti* reared under different temperature conditions

FIGURE D.1 Fitness comparisons between Townsville F2 and Cairns F11 populations of *Ae. aegypti* in the laboratory

FIGURE D.2 Fitness comparisons between large populations and inbred lines of *Ae. aegypti* at F5

FIGURE D.3 Proportional changes in life history traits in the large populations of *Ae. aegypti* at F13 relative to the ancestral population (Townsville F6/5)

FIGURE D.4 Proportional changes in life history traits in laboratory populations of *Ae. aegypti* from Townsville, Cairns and Innisfail relative to their respective ancestral or field populations

FIGURE D.5 The effective population size (*N_e*) of *Ae. aegypti* populations at F13 versus their index of performance
FIGURE D.6 Outcrossing inbred *Ae. aegypti* mosquitoes dramatically improves their fitness 158

FIGURE D.7 Relative larval competitive ability of the Cairns F₂, F₇, F₂7 and inbred F₁₈ (Inbred A) populations of *Ae. aegypti versus Wolbachia*-infected larvae 160
LIST OF TABLES

<table>
<thead>
<tr>
<th>TABLE 1.1</th>
<th>Open field releases of <em>Wolbachia</em>-infected mosquitoes for population replacement and suppression strategies around the world</th>
<th>5</th>
</tr>
</thead>
<tbody>
<tr>
<td>TABLE 1.2</td>
<td>Effects of experimental <em>Wolbachia</em> infections on fitness traits of <em>Aedes aegypti</em> and <em>Aedes albopictus</em></td>
<td>10</td>
</tr>
<tr>
<td>TABLE 2.1</td>
<td>Pupation and adult emergence from <em>Ae. aegypti</em> larvae held under starvation conditions in groups of 50</td>
<td>39</td>
</tr>
<tr>
<td>TABLE 2.2</td>
<td><em>Wolbachia</em> infection frequencies in surviving <em>Ae. aegypti</em> larvae when held at different initial proportions under starvation conditions</td>
<td>40</td>
</tr>
<tr>
<td>TABLE 2.3</td>
<td>Wing lengths of <em>Ae. aegypti</em> adults emerging from groups of larvae held under starvation conditions</td>
<td>41</td>
</tr>
<tr>
<td>TABLE 2.4</td>
<td>Mean development time and wing length of <em>Ae. aegypti</em> when fed <em>ad libitum</em> after extended food deprivation</td>
<td>44</td>
</tr>
<tr>
<td>TABLE 2.5</td>
<td>Percentage of hatching eggs from crosses between <em>Wolbachia</em>-infected males and uninfected female <em>Ae. aegypti</em></td>
<td>45</td>
</tr>
<tr>
<td>TABLE 2.6</td>
<td>Average wing length and fecundity of isolated female <em>Ae. aegypti</em> tested for their maternal transmission fidelity</td>
<td>46</td>
</tr>
<tr>
<td>TABLE 3.1</td>
<td>Maternal transmission of <em>Wolbachia</em> by <em>Ae. aegypti</em> under cyclical temperatures</td>
<td>60</td>
</tr>
<tr>
<td>TABLE 4.1</td>
<td>Average maximum temperatures and egg hatch rates of wMel-infected <em>Ae. aegypti</em> females each generation during the selection experiment</td>
<td>74</td>
</tr>
<tr>
<td>TABLE 5.1</td>
<td>Effective population sizes (<em>N</em>) of <em>Ae. aegypti</em> F13 laboratory populations maintained at different census sizes, calculated using three temporal methods</td>
<td>102</td>
</tr>
<tr>
<td>TABLE B.1</td>
<td>Primers used in qPCR for detection of <em>Wolbachia</em> in <em>Ae. aegypti</em></td>
<td>140</td>
</tr>
<tr>
<td>TABLE D.1</td>
<td>Studies of laboratory adaptation in mosquitoes</td>
<td>144</td>
</tr>
<tr>
<td>TABLE D.2</td>
<td>Maintenance and loss of inbred <em>Ae. aegypti</em> populations during successive generations of full-sib mating.</td>
<td>148</td>
</tr>
</tbody>
</table>
CHAPTER 1 – INTRODUCTION

1.1 *Wolbachia* as a biological control agent for arboviruses

Arboviruses such as dengue, Zika and chikungunya virus are becoming an increasingly problematic threat to global health. Over half of the world’s population lives in areas at risk of arbovirus transmission and hundreds of millions of infections occur each year from dengue alone (BHATT *et al.* 2013). *Aedes aegypti* mosquitoes are the principal vectors of dengue (SALAZAR *et al.* 2007). These mosquitoes are widespread in the tropics (KRAEMER *et al.* 2015) and thrive in urban environments where they have adapted to breed in artificial containers (CHEONG 1967). *Aedes albopictus* is also an important arbovirus vector that is rapidly expanding its global range (BONIZZONI *et al.* 2013; KRAEMER *et al.* 2015). Due to a lack of effective vaccines and specific treatments for many arboviruses, the most effective way to reduce disease incidence is by controlling mosquito populations. Chemical insecticides are often used during outbreaks (VAZQUEZ-PROKOPEC *et al.* 2017) but this approach is unlikely to be sustainable as insecticide resistance is widespread in many parts of the world (ENDERSBY-HARSHMAN *et al.* 2017; MOYES *et al.* 2017). Public engagement and education, source reduction (GUBLER AND CLARK 1996) and trapping (JOHNSON *et al.* 2017) are often used to curb mosquito populations, but these approaches are unable to eliminate mosquitoes completely. Alternative control methods such as the use of pathogenic fungi (SCHOLTE *et al.* 2004) and the release of modified mosquitoes (BENEDICT AND ROBINSON 2003; HARRIS *et al.* 2012) are also effective at reducing mosquito populations in the short term. However, these approaches can require continuous effort as mosquito elimination is unlikely to be permanent outside isolated populations.

Several genetic and biological techniques that reduce the capacity for *Aedes* mosquitoes to transmit viruses are under development (FRANZ *et al.* 2015). These approaches would not require the eradication of mosquitoes or any further control efforts once mosquitoes refractory to disease transmission are established in the population. By far the most developed approach is the use of *Wolbachia*; intracellular bacteria that infect a wide range of insect species (HILGENBOECKER *et al.* 2008; ZUG AND HAMMERSTEIN 2012). *Wolbachia* have been introduced experimentally into several disease vectors including *Ae. aegypti*, where they are now being implemented to reduce the transmission of arboviruses around the world (KAMITCHUM-TATUENE *et al.* 2017).

*Wolbachia* are notable for the diversity of effects they impose on their hosts which aid their spread throughout populations (HOFFMANN *et al.* 2015). *Wolbachia* infections are transmitted from mother to offspring (HOFFMANN *et al.* 1990), and often manipulate the reproduction of their hosts to provide an advantage to infected females (WERREN *et al.* 2008). *Wolbachia* may increase the proportion of offspring that are female by killing or feminizing males (HURST AND JIGGINS 2000), by inducing parthenogenesis to minimize the need for males in reproduction (HUIGENS AND STOUTHAMER 2003;
Wolbachia infections were originally introduced into disease vector mosquitoes with the intent of establishing antiviral genes in natural populations (Curtis and Sinkins 1998; Xi et al. 2005), but they have several other applications. Cytoplasmic incompatibility induced by Wolbachia can be utilized to reduce the reproductive output of wild-type females leading to mosquito population suppression (O’Connor et al. 2012). Wolbachia infections can also reduce the transmission of arboviruses. A virulent strain of Wolbachia called wMelPop (Min and Benzer 1997) that arose in a laboratory line of Drosophila melanogaster was introduced into Ae. aegypti where it substantially shortened adult lifespan (McMeniman et al. 2009) and reduced the blood feeding success of older females (Moreira et al. 2009b; Turley et al. 2009). Only females older than at least a week are capable of transmitting dengue because the virus must undergo an extrinsic incubation period of 8 to 12 days in a blood-fed mosquito before it can be transmitted to a new human host (Gubler 1998; Salazar et al. 2007). Therefore, the deleterious effects of wMelPop on older females could be harnessed to reduce the likelihood of transmission occurring (Brownstein et al. 2003; Ranson et al. 2003; Cook et al. 2008). wMelPop also greatly reduces the viability of quiescent Ae. aegypti eggs (McMeniman and O’Neill 2010; Yeap et al. 2011), a trait which could be used to eliminate populations of mosquitoes during the dry season following the establishment of wMelPop during the wet season (Ritchie et al. 2015).

Strains of Wolbachia provide protection against some pathogenic fungi and viruses in their natural hosts (Panteleev et al. 2007; Hedges et al. 2008; Teixeira et al. 2008). This protection encompasses a whole suite of pathogens, hosts and Wolbachia strains, including both natural and experimental infections (Martinez et al. 2014; Hoffmann et al. 2015), though not all strains block pathogens and some even enhance infection (Hughes et al. 2012; Dodson et al. 2014). Experimental infections of Wolbachia in Ae. aegypti and Ae. albopictus can suppress the transmission of dengue (Bлагаrove et al. 2012; Ferguson et al. 2015), Zika (Aliota et al. 2016; Dutra et al. 2016b), chikungunya (Van den Hurk et al. 2012; Blagrove et al. 2013), yellow fever (Van den Hurk et al. 2012) and West Nile (Hussain et al. 2013) viruses, and can protect against other pathogens including Plasmodium (Moreira et al. 2009a; Caragata et al. 2016), filarial nematodes (Kambris et al. 2009) and other bacteria (Kambris et al. 2009; Ye et al. 2013). The direct suppression of arboviruses by Wolbachia strains is a particularly desirable phenotype, as Wolbachia could be used to transform wild-type Aedes populations into populations that are refractory to arbovirus transmission.
*Wolbachia* infections can be implemented in three main ways to reduce the transmission of arboviruses by mosquitoes:

(1) **Replacing populations with mosquitoes that have a reduced ability to transmit arboviruses.** *Wolbachia* infections that induce cytoplasmic incompatibility or other reproductive manipulations could invade natural mosquito populations and reduce their vector competence. This could be achieved by *Wolbachia* strains that directly interfere with viral transmission, shorten mosquito lifespan to reduce their transmission potential, or are coupled with a transgene that reduces vector competence.

(2) **Suppressing populations through the release of incompatible males.** Cytoplasmic incompatibility induced by *Wolbachia*-infected males could be harnessed to reduce the reproduction of wild-type females. Mass-releases of *Wolbachia*-infected males into a population with no *Wolbachia* infection (or with a different *Wolbachia* infection type) could result in the sterility of wild-type females that mate with released males. This suppression approach could be followed by the replacement strategy in (1) which would be aided by a reduced population size.

(3) **Replacing populations followed by contextual suppression.** Following the invasion of a population driven by cytoplasmic incompatibility, the deleterious effects of certain *Wolbachia* strains could be utilized to suppress populations under certain conditions. For instance, the *w*MelPop infection reduces the desiccation tolerance of *Ae. aegypti* eggs which could lead to population extinction under dry conditions.

Strains of *Wolbachia* are now being harnessed to reduce the incidence of dengue and other arboviruses by *Aedes* mosquitoes using the above strategies (Table 1.1). The population replacement approach has already met with some success, particularly with the establishment of the *w*Mel strain in Australian populations of *Ae. aegypti* (HOFFMANN *et al.* 2011; SCHMIDT *et al.* 2017). However, subsequent releases in other countries and with other strains have faced challenges (NGUYEN *et al.* 2015). Recent releases of *Wolbachia*-infected males for population suppression also show promise, but have failed to achieve rates of cytoplasmic incompatibility equivalent to the ratio of released males (MAINS *et al.* 2016). *Wolbachia* infections are often characterized under laboratory conditions, but the ability of *Wolbachia* to invade or suppress natural populations of insects and reduce viral transmission depends on a wide array of variables. In this thesis introduction, I discuss the influence of environmental conditions on *Wolbachia* infections in insect hosts, particularly in the context of harnessing *Wolbachia* to reduce arbovirus transmission by mosquitoes. *Wolbachia* infections can be adversely affected by the environment, which can modulate their effects on host fitness, their capacity for viral blockage, and their ability to manipulate host reproduction. However, *Wolbachia* infections are exceptionally diverse in their effects, which can differ distinctly under different environmental conditions and in different hosts. Experimental *Wolbachia* infections for arbovirus control should
therefore be characterized under a range of environmental conditions before deploying strains in the field.
Table 1.1 Open field releases of *Wolbachia*-infected mosquitoes for population replacement and suppression strategies around the world. Releases are listed in order of date commenced.

<table>
<thead>
<tr>
<th>Mosquito species</th>
<th>Wolbachia strain</th>
<th>Objective</th>
<th>Location</th>
<th>Date commenced</th>
<th>Outcome</th>
<th>Reference(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Culex pipiens</em> fatigans</td>
<td>Unknown</td>
<td>Suppression</td>
<td>Okpo, Burma</td>
<td>February 1967</td>
<td>Eradication of mosquitoes in the trial area through the release of <em>Wolbachia</em>-infected males</td>
<td>(LAVEN 1967)</td>
</tr>
<tr>
<td><em>Culex quinquefasciatus</em></td>
<td>Unknown</td>
<td>Suppression</td>
<td>Near Delhi, India</td>
<td>August 1973</td>
<td>Reduction in population size and egg hatch rates during releases of <em>Wolbachia</em>-infected males</td>
<td>(CURTIS et al. 1982)</td>
</tr>
<tr>
<td><em>Aedes polynesiensis</em></td>
<td>wRivB</td>
<td>Suppression</td>
<td>French Polynesia</td>
<td>December 2009</td>
<td>Reduction in population size and female fertility during the release of wRivB-infected males</td>
<td>(O’CONNOR et al. 2012)</td>
</tr>
<tr>
<td><em>Aedes aegypti</em></td>
<td>wMel</td>
<td>Replacement</td>
<td>Cairns, Australia</td>
<td>January 2011</td>
<td>wMel was established in two populations and remains at a frequency above 80% in both populations over two years after releases ceased</td>
<td>(HOFFMANN et al. 2011; HOFFMANN et al. 2014a)</td>
</tr>
<tr>
<td><em>Aedes aegypti</em></td>
<td>wMelPop</td>
<td>Replacement</td>
<td>Cairns, Australia</td>
<td>January 2012</td>
<td>wMelPop reached high frequencies in two populations during active releases, but the infection later dropped out after releases ceased</td>
<td>(NGUYEN et al. 2015)</td>
</tr>
<tr>
<td><em>Aedes aegypti</em></td>
<td>wMel</td>
<td>Replacement</td>
<td>Cairns, Australia</td>
<td>January 2013</td>
<td>wMel was established in three suburban populations and exhibited slow spatial spread outside the release area in two of the three populations</td>
<td>(SCHMIDT et al. 2017)</td>
</tr>
<tr>
<td><em>Aedes aegypti</em></td>
<td>wMelPop</td>
<td>Replacement</td>
<td>Tri Nguyen Island, Vietnam</td>
<td>April 2013</td>
<td>wMelPop reached high frequencies in the population during active releases, but the infection later dropped out after releases ceased</td>
<td>(NGUYEN et al. 2015)</td>
</tr>
<tr>
<td><em>Aedes aegypti</em></td>
<td>wMel</td>
<td>Replacement</td>
<td>Cairns, Australia</td>
<td>January 2014</td>
<td>wMel has persisted for at least one year in two release areas</td>
<td>(SCHMIDT et al. 2018)</td>
</tr>
<tr>
<td><em>Aedes albopictus</em></td>
<td>wPip</td>
<td>Suppression</td>
<td>Kentucky, USA</td>
<td>June 2014</td>
<td>Reduction in population size and female fertility during the release of wPip-infected males</td>
<td>(MAINS et al. 2016)</td>
</tr>
<tr>
<td><em>Aedes aegypti</em></td>
<td>wMel</td>
<td>Population size estimate</td>
<td>Rio de Janeiro, Brazil</td>
<td>September 2014</td>
<td>The wMel infection was used as a marker in mark, release and recapture experiments to estimate population size and daily survival rates</td>
<td>(GARCIA GDE et al. 2016)</td>
</tr>
</tbody>
</table>
1.2 Experimental *Wolbachia* infections in mosquitoes and their field release

Mosquitoes with *Wolbachia* infections have been released into the field to suppress populations on several occasions since the 1960s (Table 1.1). In two of the first studies to use this approach, male *Culex* mosquitoes naturally infected with *Wolbachia* were released into areas where the native mosquitoes were uninfected (LAVEN 1967; CURTIS et al. 1982). A later study released a strain of *Aedes polynesiensis* males generated by introgression of *Wolbachia* from a closely related species (O’CONNOR et al. 2012). These experiments relied on the induction of cytoplasmic incompatibility from crosses between released males and wild females to reduce egg hatch rates, leading to reductions in population size or extinction. Though these earlier studies demonstrated the potential for the release of *Wolbachia*-infected males to achieve population suppression, released mosquitoes must possess a *Wolbachia* infection type that is incompatible with mosquitoes in the target release area (MAINS et al. 2016). Since many mosquito species do not possess natural *Wolbachia* infections (KITTAYAPONG et al. 2000) and some species are nearly completely infected (ARMBRUSTER et al. 2003), novel *Wolbachia* infections must first be generated for this approach to be effective.

Artificial *Wolbachia* infections can be generated experimentally using the microinjection technique, where cytoplasm from an infected donor egg is transferred to an embryo of the target species (BOYLE et al. 1993; BRAIG et al. 1994). *Ae. aegypti* do not generally harbour *Wolbachia* naturally (KITTAYAPONG et al. 2000; POPOVICI et al. 2010) though infected mosquitoes were reported in a recent survey in Florida, USA (COON et al. 2016b). *Ae. albopictus* are naturally infected with two strains of *Wolbachia*: *wAlbA* and *wAlbB* (ARMBRUSTER et al. 2003). Experimental infections in this species can be generated by clearing the natural infections with antibiotics (SUH et al. 2009; CALVITTI et al. 2010; BLAGROVE et al. 2012) or by generating superinfections, where both the original strains and a novel strain infect the mosquito simultaneously (ZHANG et al. 2015a; SUH et al. 2016). Naturally occurring *Wolbachia* infections in field populations could impede suppression or transformation by experimental *Wolbachia* infections; the recent detection of *Wolbachia* in *Ae. aegypti* highlights the need to conduct surveys in the target population before releases occur (COON et al. 2016b).

Several experimental *Wolbachia* infections have now been generated in *Aedes* mosquitoes through microinjection (Table 1.2, reviewed in HOFFMANN et al. 2015), and a handful of these infections have now been implemented in field trials to suppress or replace natural populations (Table 1.1). The *wMel* infection originating from *D. melanogaster* is currently the only strain being used by the World Mosquito Program (formerly Eliminate Dengue) to replace populations of uninfected *Ae. aegypti* in several countries (http://www.eliminatedengue.com/project). This strain was chosen due to its relative lack of deleterious effects on mosquito life-history traits (Figure 1.1) and its ability to inhibit dengue replication and impose complete cytoplasmic incompatibility (WALKER et al. 2011). *wMel*-infected
Ae. aegypti have successfully invaded caged populations (WALKER et al. 2011) and are now established in multiple field populations in Queensland, Australia (HOFFMANN et al. 2011; SCHMIDT et al. 2017). After releases ceased, infected mosquitoes have persisted at a high frequency in these locations (HOFFMANN et al. 2014a; SCHMIDT et al. 2017), and have retained their ability to inhibit dengue replication (FRENTIU et al. 2014) and induce complete cytoplasmic incompatibility (HOFFMANN et al. 2014a). wMelPop is the only other strain that has been tested in field trials for population replacement (Table 1.1). wMelPop-infected mosquitoes were released in Australia and Vietnam in 2012 and 2013 respectively, but the infection failed to sustain high frequencies in the release areas and declined to extinction after releases ceased (NGUYEN et al. 2015). The failure of wMelPop to invade natural populations can largely be attributed to the costly effects of this infection on Ae. aegypti life-history traits (Figure 1.1, Table 1.2).
Figure 1.1 Effects of three different Wolbachia strains on the fitness of Aedes aegypti. Data were extracted from 28 studies that compared the fitness of Ae. aegypti infected with wMel, wAlbB or wMelPop to an uninfected counterpart. The studies used are listed in table 1.2. (A) Wolbachia infections had a negative, positive or no effect on fitness. There was no effect when differences were not statistically significant (P > 0.05). Effects of Wolbachia infection on the magnitude of fitness changes were expressed in terms of (B) effect sizes and (C-E) relative fitness, where negative values below the dotted line indicate a fitness cost due to Wolbachia infection. Relative fitness was tested under (C) standard laboratory conditions or (D) stressful conditions, which includes rearing larvae with sub-optimal nutrition or under predation, supplying adults with non-human blood and experiments with quiescent eggs and old adults. (E) Relative fitness was compared between different trait types. wMelPop was separated into more categories due to a larger number of comparisons available. Error bars are medians and interquartile ranges.
Table 1.2 Effects of experimental *Wolbachia* infections on fitness traits of *Aedes aegypti* and *Aedes albopictus*.

<table>
<thead>
<tr>
<th>Host species</th>
<th>Wolbachia infection</th>
<th>Trait</th>
<th>Fitness effect of experimental infection</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Aedes aegypti</em></td>
<td>wAlbB, wMel (superinfection)</td>
<td>Fecundity</td>
<td>No effect (<a href="#">JOUBERT et al. 2016</a>)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Egg hatch rate</td>
<td>Reduced (<a href="#">JOUBERT et al. 2016</a>)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Adult longevity</td>
<td>Reduced (<a href="#">JOUBERT et al. 2016</a>)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Quiescent egg viability</td>
<td>Reduced (<a href="#">JOUBERT et al. 2016</a>)</td>
</tr>
<tr>
<td><em>Aedes aegypti</em></td>
<td>wAlbB</td>
<td>Fecundity</td>
<td>Reduced (<a href="#">ROSS et al. 2016</a>) or no effect (<a href="#">AXFORD et al. 2016; JOUBERT et al. 2016; ANT et al. 2018</a>)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Egg hatch rate</td>
<td>Reduced (<a href="#">XI et al. 2005; JOUBERT et al. 2016</a>)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Adult longevity</td>
<td>Reduced (<a href="#">AXFORD et al. 2016; ANT et al. 2018</a>) or no effect (<a href="#">BIAN et al. 2010</a>)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Quiescent egg viability</td>
<td>Reduced (<a href="#">AXFORD et al. 2016; JOUBERT et al. 2016</a>) or no effect (<a href="#">ANT et al. 2018</a>)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Larval development time</td>
<td>No effect (<a href="#">AXFORD et al. 2016</a>)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Adult size</td>
<td>No effect (<a href="#">AXFORD et al. 2016; ROSS et al. 2016; ROSS et al. 2017c</a>)</td>
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<tr>
<td></td>
<td></td>
<td>Male mating competitiveness</td>
<td>No effect (<a href="#">AXFORD et al. 2016</a>)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Larval survival under competitive conditions</td>
<td>Reduced (<a href="#">ROSS et al. 2016</a>)</td>
</tr>
<tr>
<td><em>Aedes aegypti</em></td>
<td>wMel</td>
<td>Adult longevity</td>
<td>Reduced (<a href="#">WALKER et al. 2011; AXFORD et al. 2016; JOUBERT et al. 2016</a>) or no effect (<a href="#">ANT et al. 2018</a>)</td>
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<tr>
<td></td>
<td></td>
<td>Fecundity</td>
<td>No effect (<a href="#">DUTRA et al. 2015; ANT et al. 2018</a>), reduced (<a href="#">TURLEY et al. 2013; HOFFMANN et al. 2014a; ROSS et al. 2016; DE OLIVEIRA et al. 2017b</a>) or increased (<a href="#">JOUBERT et al. 2016</a>)</td>
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<tr>
<td></td>
<td></td>
<td>Egg hatch rate</td>
<td>Reduced (<a href="#">DUTRA et al. 2015; JOUBERT et al. 2016</a>)</td>
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<td></td>
<td></td>
<td>Quiescent egg viability</td>
<td>Reduced (<a href="#">JOUBERT et al. 2016; FRASER et al. 2017</a>) or no effect (<a href="#">ANT et al. 2018</a>)</td>
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<tr>
<td></td>
<td></td>
<td>Larval development time</td>
<td>Reduced (<a href="#">WALKER et al. 2011; DUTRA et al. 2016a; KHO et al. 2016</a>), increased (<a href="#">ROSS et al. 2014</a>) or no effect (<a href="#">DE OLIVEIRA et al. 2017a</a>)</td>
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<tr>
<td></td>
<td></td>
<td>Body size</td>
<td>Reduced (<a href="#">DUTRA et al. 2016a</a>), increased (<a href="#">ROSS et al. 2014</a>) or no effect (<a href="#">KHO et al. 2016; ROSS et al. 2016; DE OLIVEIRA et al. 2017a; DE OLIVEIRA et al. 2017b; ROSS et al. 2017c</a>)</td>
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<tr>
<td></td>
<td></td>
<td>Glycogen levels</td>
<td>Increased (<a href="#">DUTRA et al. 2016a</a>)</td>
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<tr>
<td>Parameter</td>
<td>Results</td>
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<td>--------------------------------------------------------------------------</td>
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<tr>
<td>Larval survival under competitive conditions</td>
<td>Reduced (ROSS et al. 2016) or increased (ROSS et al. 2014; DE OLIVEIRA et al. 2017a)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male mating competitiveness</td>
<td>No effect (TURLEY et al. 2013; SEGOLI et al. 2014)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Attraction to human host</td>
<td>No effect (TURLEY et al. 2014)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Insecticide susceptibility</td>
<td>No effect (ENDERSBY AND HOFFMANN 2013)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Blood meal weight</td>
<td>No effect (DE OLIVEIRA et al. 2017b)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adult starvation survival after blood meal</td>
<td>No effect (DE OLIVEIRA et al. 2017b)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Time to blood meal excretion and oviposition</td>
<td>Increased (DE OLIVEIRA et al. 2017b)</td>
<td></td>
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</tr>
<tr>
<td><strong>Aedes aegypti wMelPop</strong></td>
<td></td>
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</tr>
<tr>
<td>Adult longevity</td>
<td>Reduced (MCMENIMAN et al. 2009; YEAP et al. 2011; AXFORD et al. 2016)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fecundity</td>
<td>Reduced (MCMENIMAN AND O'NEILL 2010; MCMENIMAN et al. 2011; WALKER et al. 2011; TURLEY et al. 2013; CARAGATA et al. 2014; ROSS et al. 2016)</td>
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<tr>
<td>Egg hatch rate</td>
<td>Reduced (MCMENIMAN et al. 2011; YEAP et al. 2011; TURLEY et al. 2013; CARAGATA et al. 2014)</td>
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<tr>
<td>Quiescent egg viability</td>
<td>Reduced (MCMENIMAN AND O'NEILL 2010; WALKER et al. 2011; YEAP et al. 2011; RITCHIE et al. 2015)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adult activity and metabolic rate</td>
<td>Increased (EVANS et al. 2009)</td>
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</tr>
<tr>
<td>Susceptibility of larvae to predation</td>
<td>No effect (HURST et al. 2012)</td>
<td></td>
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</tr>
<tr>
<td>Oviposition success</td>
<td>Reduced (MCMENIMAN AND O'NEILL 2010; YEAP et al. 2014)</td>
<td></td>
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</tr>
<tr>
<td>Blood feeding success</td>
<td>Reduced (MOREIRA et al. 2009b; TURLEY et al. 2009)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Larval development time</td>
<td>Increased (ROSS et al. 2014; SUH et al. 2017) or no effect (KHO et al. 2016)</td>
<td></td>
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</tr>
<tr>
<td>Adult size</td>
<td>Reduced (ROSS et al. 2014; ROSS et al. 2016) or no effect (KHO et al. 2016; ROSS et al. 2017c)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Larval survival under competitive conditions</td>
<td>Reduced (SUH AND DOBSON 2013; ROSS et al. 2014; ROSS et al. 2016; SUH et al. 2017)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male mating competitiveness</td>
<td>No effect (TURLEY et al. 2013; SEGOLI et al. 2014)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Response to light stimulation</td>
<td>Reduced (SUH AND DOBSON 2013)</td>
<td></td>
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</tr>
<tr>
<td>Attraction to human host</td>
<td>No effect (TURLEY et al. 2014)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Insecticide susceptibility</td>
<td>No effect (ENDERSBY AND HOFFMANN 2013)</td>
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<td><strong>Aedes aegypti wMelCS</strong></td>
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<td></td>
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</tr>
<tr>
<td>Fecundity</td>
<td>No effect (FRASER et al. 2017)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Egg hatch rate</td>
<td>Reduced (FRASER et al. 2017)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Quiescent egg viability</td>
<td>Reduced (FRASER et al. 2017)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adult longevity</td>
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<td></td>
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</tr>
<tr>
<td><em>Aedes aegypti</em></td>
<td>wRi</td>
<td>Fecundity</td>
<td>Increased (FRASER <em>et al.</em> 2017)</td>
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<td></td>
<td></td>
<td>Egg hatch rate</td>
<td>No effect (FRASER <em>et al.</em> 2017)</td>
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<td></td>
<td></td>
<td>Quiescent egg viability</td>
<td>Reduced (FRASER <em>et al.</em> 2017)</td>
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<tr>
<td></td>
<td></td>
<td>Adult longevity</td>
<td>No effect (FRASER <em>et al.</em> 2017)</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th><em>Aedes aegypti</em></th>
<th>wPip</th>
<th>Fecundity</th>
<th>Reduced (FRASER <em>et al.</em> 2017)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Egg hatch rate</td>
<td>Reduced (FRASER <em>et al.</em> 2017)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Quiescent egg viability</td>
<td>Reduced (FRASER <em>et al.</em> 2017)</td>
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<tr>
<td></td>
<td></td>
<td>Adult longevity</td>
<td>Reduced (FRASER <em>et al.</em> 2017)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th><em>Aedes aegypti</em></th>
<th>wAlbA</th>
<th>Female longevity</th>
<th>Reduced (ANT <em>et al.</em> 2018)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Fecundity</td>
<td>No effect (ANT <em>et al.</em> 2018)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Quiescent egg viability</td>
<td>Reduced (ANT <em>et al.</em> 2018)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th><em>Aedes aegypti</em></th>
<th>wAu</th>
<th>Female longevity</th>
<th>Reduced (ANT <em>et al.</em> 2018)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Fecundity</td>
<td>No effect (ANT <em>et al.</em> 2018)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Quiescent egg viability</td>
<td>Reduced (ANT <em>et al.</em> 2018)</td>
</tr>
</tbody>
</table>

| *Aedes albopictus* | wPip, wAlbA, wAlbB (superinfection) | Egg hatch rate | No effect (ZHANG *et al.* 2015b) |
|                   |                                  | Larval survivorship | No effect (ZHANG *et al.* 2015b) |
|                   |                                  | Larval development time | Reduced (ZHANG *et al.* 2015b) |

| *Aedes albopictus* | wMelPop, wAlbA, wAlbB (superinfection) | Fecundity | Reduced (SUH *et al.* 2016) |

| *Aedes albopictus* | wMel | Female longevity | No effect (BLAGROVE *et al.* 2013) |
|                   |      | Male longevity   | Increased (BLAGROVE *et al.* 2013) |
|                   |      | Male mating competitiveness | No effect (BLAGROVE *et al.* 2013) |
|                   |      | Egg hatch rate   | No effect (BLAGROVE *et al.* 2013) |

<p>| <em>Aedes albopictus</em> | wMelPop | Longevity | Reduced (SUH <em>et al.</em> 2009) |
|                   |         | Fecundity | Reduced (SUH <em>et al.</em> 2009) |</p>
<table>
<thead>
<tr>
<th>Species</th>
<th>Trait</th>
<th>Effect</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Aedes albopictus</em> wPip</td>
<td>Egg hatch rate</td>
<td>Reduced (SUH et al. 2009)</td>
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<td></td>
<td>Adult longevity</td>
<td>Reduced (CALVITTI et al. 2010)</td>
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<td></td>
<td>Male mating competitiveness</td>
<td>Reduced (ATYAME et al. 2016), increased (PUGGIOLI et al. 2016) or no effect (MORETTI AND CALVITTI 2013)</td>
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<td></td>
<td>Fecundity</td>
<td>Reduced (CALVITTI et al. 2010)</td>
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<td></td>
<td>Egg hatch rate</td>
<td>Reduced (CALVITTI et al. 2010)</td>
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<td></td>
<td>Development time</td>
<td>Reduced (PUGGIOLI et al. 2016)</td>
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In recent years, *Ae. aegypti* infected with wMel have been released in further trials in Asia, Oceania and South America to spread the infection into natural populations (http://www.eliminatedengue.com/project), though studies evaluating its success outside of Australia are yet to be published. Research since the initial releases in Australia suggests that several significant barriers may reduce the potential for the wMel infection to invade *Ae. aegypti* populations and interfere with viral transmission under some conditions. Though few deleterious effects of wMel on mosquito fitness were observed at the time of the first field releases (WALKER *et al.* 2011), later studies have demonstrated costs to fecundity (TURLEY *et al.* 2013; HOFFMANN *et al.* 2014a; ROSS *et al.* 2016), longevity (AXFORD *et al.* 2016), egg hatch rate (DUTRA *et al.* 2015; JOUBERT *et al.* 2016) and larval development and survival under competitive conditions (ROSS *et al.* 2014; ROSS *et al.* 2016). In contrast, other studies have observed no effect or some benefits under competitive conditions (DUTRA *et al.* 2016a; KHO *et al.* 2016; DE OLIVEIRA *et al.* 2017a). In areas where the wMel infection has successfully established, uninfected individuals have persisted at low frequencies (HOFFMANN *et al.* 2014a; SCHMIDT *et al.* 2017). The immigration of uninfected individuals could explain this persistence, at least in continuous populations (SCHMIDT *et al.* 2017), but there is now evidence for maternal transmission leakage which could also contribute to this observation. Incomplete transmission of wMel was reported under laboratory conditions in Brazil (DUTRA *et al.* 2015) and in an Australian field population (SCHMIDT *et al.* 2018). The wMel infection in *Ae. aegypti* is susceptible to cyclical heat stress (ULRICH *et al.* 2016; ROSS *et al.* 2017c), and high temperatures in larval habitats in the field could, therefore, contribute to maternal transmission leakage and incomplete cytoplasmic incompatibility.

Once *Wolbachia* invade a population of mosquitoes, their ability to interfere with arbovirus transmission under field conditions is critical for the success of these disease control programs. WALKER *et al.* (2011) demonstrated strong suppression of dengue by wMel in *Ae. aegypti*, with an observed 1500-fold reduction in viral titre compared to the wild type. However, they used a single DENV-2 isolate from 1992 which is not particularly infective (YE *et al.* 2014) and only tested viral titre in whole adults. Several studies since then have demonstrated substantially weaker effects when testing other dengue strains (FRENTIU *et al.* 2014; FERGUSON *et al.* 2015; YE *et al.* 2016), when looking at more relevant measures of transmission potential (YE *et al.* 2015) and by feeding mosquitoes on blood from dengue patients rather than laboratory cultured virus (FERGUSON *et al.* 2015; CARRINGTON *et al.* 2017). A mathematical model based on data from dengue patients estimates that the wMel infection is not sufficient to eliminate dengue transmission if established in a population (FERGUSON *et al.* 2015). Though the ability of wMel to interfere with dengue transmission has persisted in field mosquitoes returned to the laboratory (FRENTIU *et al.* 2014), it is unclear if arboviruses or *Wolbachia* infections will evolve to be more permissive in areas with higher levels of disease transmission, which could occur if wMel does not block arboviruses completely. The wMel
infection also does not prevent the vertical transmission of dengue (PACIDONIO et al. 2017), though vertical transmission of arboviruses tends to occur rarely (ADAMS AND BOOTS 2010).

Despite the limitations of the wMel strain, \textit{Wolbachia} infections demonstrate enormous potential to control arbovirus transmission through the population replacement approach. Alternative experimental \textit{Wolbachia} infections have been generated in \textit{Ae. aegypti} that could possess some advantages over wMel. For instance, a wMel-wAlbB superinfection provides greater dengue blockage than a single infection of wMel without imposing as large fitness costs as wMelPop (JOURBERT et al. 2016). The recently generated wMelCS strain also appears to induce greater viral blockage than wMel without additional fitness costs (FRASER et al. 2017). \textit{Wolbachia} infections generated in \textit{Aedes} mosquitoes differ considerably in their effects on host fitness (Table 1.2, Figure 1.1) and their ability to suppress arboviruses (FERGUSON et al. 2015; JOURBERT et al. 2016; FRASER et al. 2017). \textit{Wolbachia} infections that strongly inhibit viral transmission tend to exert greater fitness costs to the mosquito (MOREIRA et al. 2009a; JOURBERT et al. 2016). However, the diversity of \textit{Wolbachia} infections in nature suggests that some strains could possess desirable attributes without trade-offs (HOFMANN et al. 2015, ANT et al. 2018).

In contrast to population replacement strategies, the attributes required of \textit{Wolbachia} infections for population suppression are relatively simple. For this approach there is no requirement for the long-term stability of \textit{Wolbachia} infections in the field; its effectiveness relies primarily on the ability of infected males reared in the laboratory to inseminate wild females and cause cytoplasmic incompatibility (or other equivalent effects) under field conditions. \textit{Wolbachia}-driven suppression has been demonstrated in both semi-field cages (AYAME et al. 2015) and in the field, where persistent releases of \textit{Wolbachia}-infected males into a population substantially cut female fertility (O’CONNOR et al. 2012; MAINS et al. 2016) and even lead to extinction (LAVER 1967) through incompatible matings. These trials are now expanding in scope and location (WALTZ 2016). However, permanent eradication with this approach could be challenging due to the ability of \textit{Aedes} eggs to withstand long dry periods (FAULL AND WILLIAMS 2015), the potential for assortative mating to evolve in uninfected mosquitoes (CHAMPION DE CRESPIGNY et al. 2005; KOUKOU et al. 2006), reductions in density-dependent competition which regulate population size (HANCOCK et al. 2016a) and immigration of mosquitoes into release areas (BENEDICT AND ROBINSON 2003). The presence of any compatible (naturally \textit{Wolbachia}-infected) mosquitoes in the target population or any leakage in cytoplasmic incompatibility under field conditions could also be problematic for these approaches. Experimental \textit{Wolbachia} infections in \textit{Ae. aegypti} do not appear to affect mating competitiveness, a component which is critical to the success of incompatible male release programs (CHAMBERS et al. 2011; MORETTI AND CALVITTI 2013; TURLLEY et al. 2013; SEGOLI et al. 2014). However, the quality of released mosquitoes, independent of \textit{Wolbachia} infection, is also important. Inbred \textit{Ae. aegypti} exhibit greatly reduced mating competitiveness relative to wild populations (ROSS et al. 2017b) so it is
important that approaches such as outcrossing (YEAP et al. 2011) or the use of quality control devices (BALESTRINO et al. 2017) are used to maintain the fitness of colonized mosquito populations destined for release. It is also crucial that the release of infected females is avoided given that they will be able to produce viable offspring with released males (RITCHIE et al. 2018).

1.3 Environmental effects on *Wolbachia* infections

*Wolbachia* infections can vary considerably in their phenotypic effects under different environmental conditions. This is demonstrated clearly in comparisons between laboratory conditions and field conditions, where the latter is much more complex. *Wolbachia* density (the number of bacterial copies per insect cell) tends to be more variable in nature than in the laboratory (AHANTARIG et al. 2008; CORREA AND BALLARD 2012) where it can change seasonally (UNCKLESS et al. 2009; SUMI et al. 2017) and vary geographically (HOFFMANN et al. 2014b). This variation in density can translate to variation in the penetrance of *Wolbachia*’s effects. In laboratory populations of *Drosophila simulans*, the *Wolbachia* strain wRi exhibits perfect maternal transmission fidelity and complete cytoplasmic incompatibility, but in nature its fidelity is imperfect (HOFFMANN et al. 1990; TURELLI AND HOFFMANN 1995).

While the fitness, reproductive, and viral blocking effects of experimental *Wolbachia* infections in *Aedes* mosquitoes are well described (Figure 1.1, reviewed in HOFFMANN et al. 2015), studies have largely been conducted under controlled laboratory conditions. *Wolbachia* infections that impose complete cytoplasmic incompatibility, interfere strongly with viral transmission and are transmitted with perfect fidelity in the laboratory may not continue to do so under field conditions. Fitness costs of *Wolbachia* infections in *Ae. aegypti* tend to be minimal under standard laboratory conditions, but can be exacerbated under stress (Figure 1.1). On the other hand, the strength of dengue suppression by wMel is increased when mosquitoes are reared under natural conditions compared to laboratory-reared mosquitoes, indicating that environmental conditions will also influence arbovirus suppression phenotypes (CARRINGTON et al. 2017). Below I explore some of the key environmental conditions that affect *Wolbachia* infections in natural and novel hosts in terms of their effects on host fitness, their ability to block pathogens and their ability to manipulate the reproduction of their hosts. I also discuss the potential impact of these environmental conditions on the ability of experimental *Wolbachia* infections to invade mosquito populations and exert their effects.
1.3.1 Nutrition

Nutritional stress during development and adulthood can modulate the effects of *Wolbachia* infections on their host. Natural *Wolbachia* infections in *Ae. albopictus* larvae can shift from having a beneficial effect to a detrimental effect on host fitness under nutritional stress (GAVOTTE et al. 2010). Nutritional stress can also reduce the fidelity of cytoplasmic incompatibility in *D. simulans* (SINKINS et al. 1995; CLANCY AND HOFFMANN 1998) and *D. melanogaster* (YAMADA et al. 2007) and affect the density of *Wolbachia* in *D. simulans* (CORREA AND BALLARD 2014), *Ae. albopictus* (DUTTON AND SINKINS 2004; WIWATANARATANABUTR AND KITTAYAPONG 2009) and *Ae. aegypti* (ROSS et al. 2014). *Aedes* larvae commonly experience competition for nutrition during larval development (RUSSELL 1986; BARRERA 1996) and *Wolbachia*-infected mosquitoes will likely encounter competition during releases for disease control programs (HANCOCK et al. 2016a). Experimental *Wolbachia* infections in *Ae. aegypti* appear to remain stable in terms of their effects on cytoplasmic incompatibility, maternal transmission (ROSS et al. 2016) and dengue blockage (KHO et al. 2016) under nutritional stress. However, infected mosquitoes may suffer some fitness costs, including the survival of larvae under nutrient-poor and crowded conditions (ROSS et al. 2014; ROSS et al. 2016) and the fecundity of females that mate with *Wolbachia*-infected males reared under low nutrition conditions (TURLEY et al. 2013). Larval competition is also predicted to slow the speed of *Wolbachia* invasion considerably regardless of any fitness costs (HANCOCK et al. 2016a; HANCOCK et al. 2016b). Nutritional stress during adulthood could also affect pathogen blocking by *Wolbachia*; for instance, low levels of sucrose can reduce *Plasmodium* blocking by wMel in *Ae. aegypti*, though this is not the case for dengue (CARAGATA et al. 2016). In addition to the quantity of nutrition, the composition of a diet can also influence the effects of *Wolbachia* infections on their hosts. In *Drosophila*, sucrose-enriched diets can increase the density of *Wolbachia* in oocytes, while yeast-enriched diets can decrease it (SERBUS et al. 2015). Galactose, lactose, maltose and trehalose-enriched diets can also increase *Wolbachia* density (CAMACHO et al. 2017). Cholesterol-enriched diets reduce the density of some *Wolbachia* infections and their ability to protect their host against *Drosophila C* virus (CARAGATA et al. 2013). Different protein-carbohydrate ratios can modulate the effect on fecundity by wMel in *D. melanogaster* (PONTON et al. 2015), as can iron-restricted and iron-rich diets (BROWNLE et al. 2009). Because dietary composition affects competitive interactions between *Aedes* species (YEE et al. 2007a; YEE AND SKIFF 2014), and since *Wolbachia* require nutrients from their host (WU et al. 2004; CARAGATA et al. 2013), it is likely that dietary composition will also modulate *Wolbachia*’s effects in experimental infections of mosquitoes.

In nature, *Ae. aegypti* are highly anthropophilic; females will feed almost exclusively on human blood (SCOTT et al. 1993; SCOTT et al. 2000). In the laboratory, *Ae. aegypti* will readily feed on a variety of
birds and mammals (BENNETT 1970). *Ae. albopictus* also exhibit a preference for humans in nature, but tend to feed more often than *Ae. aegypti* on other animals (NIEBYLSKI et al. 1994; DELATTE et al. 2010). While their choice of blood meal does not usually have a substantial effect on their reproductive output, *Ae. aegypti* and *Ae. albopictus* fed on non-human blood have greatly reduced fertility when infected with *wMelPop* (MCLENIMAN et al. 2011; CARAGATA et al. 2014; SUH et al. 2016). Fertility can however be improved by supplementing their diet with amino acids (CARAGATA et al. 2014). *wMel-* and *wAlbB*-infected *Ae. aegypti* also suffer from fitness costs when fed on non-human blood, but the Wolbachia strains differ in their response (Cottingham et al. unpublished). Non-human blood sources can also affect the Wolbachia infections directly; in *Ae. albopictus*, the *wMelPop* infection is no longer transmitted with perfect fidelity when females are fed on mouse blood (SUH et al. 2016), while in *Ae. aegypti*, the density of *wAlbB* decreases over successive generations of feeding on pig and sheep blood (Cottingham et al. unpublished). Feeding *Aedes* mosquitoes on non-human blood in the laboratory could lead to loss of Wolbachia infections or reduced reproductive success, compromising the ability to produce large numbers of Wolbachia-infected mosquitoes for field release. Animal feeding in nature could also reduce the invasion prospects of Wolbachia infections due to associated fitness costs or Wolbachia transmission failure. The recent development of artificial blood meals could overcome some of the disadvantages associated with using animal blood for mass rearing Wolbachia-infected mosquitoes, but they also have their own shortcomings, with limitations related to storage time (DUTRA et al. 2017) and low egg hatch rates (BAUGHMAN et al. 2017).

1.3.2 Temperature

Wolbachia infections (TRPIS et al. 1981; STEVENS 1989; STOUTHAMER et al. 1990; JOHANOWICZ AND HOY 1996; JOHANOWICZ AND HOY 1998; VAN OPIJNEN AND BREEUWER 1999; JIA et al. 2009; ROSS et al. 2017c, reviewed in LI et al. 2014). These effects depend on both on the host species (PINTUREAU AND BOLLAND 2001; CORBIN et al. 2016) and Wolbachia strain (ROSS et al. 2017c; ANT et al. 2018). Temperature can also modulate the fitness costs of Wolbachia infections; for instance, the severity of life shortening by wMelPop in D. melanogaster is increased at higher temperatures (REYNOLDS et al. 2003; STRUNOV et al. 2013b; ROHRSCHIEB et al. 2016). Wolbachia strains can also affect the thermal tolerance of their host in different ways; in Drosophila, wMelCS infection increases host thermal tolerance while wMelPop decreases thermal tolerance (GRUNINENKO et al. 2017).

In Ae. aegypti mosquitoes, experimental Wolbachia infections are also susceptible to heat stress (ULRICH et al. 2016), though some strains are more resistant than others (ROSS et al. 2017c; ANT et al. 2018). The fitness costs of Wolbachia infections in Ae. aegypti are also modulated by temperature (MCMENIMAN et al. 2009; YEAP et al. 2011; Axford et al. unpublished). In a recent study, the ability of wMel to suppress dengue was unaffected by temperature (YE et al. 2016), though temperature affects pathogen protection by Wolbachia in other systems (CHROSTEK 2014; MURDOCK et al. 2014). Because Ae. aegypti can experience high temperatures in their natural habitats, the choice of Wolbachia strain for deployment in these areas will be an important consideration.

In addition to heat stress, Wolbachia infections are also affected by cold conditions. Wolbachia-infected D. melanogaster have reduced viability and fecundity compared to their uninfected counterparts when exposed to cold conditions for extended periods (KRIESNER et al. 2016). Cold treatment reduces the density of Wolbachia in Nasonia vitripennis (BORDENSTEIN AND BORDENSTEIN 2011) and the strength of thelytoky (a form of parthenogenesis where females are produced from unfertilised eggs) in Trichogramma evanescens (PINTUREAU et al. 2003). Temperature can also alter the composition of Wolbachia variants in Drosophila populations; in a cold environment certain clades of Wolbachia were lost while others increased in frequency over many generations (VERDACE et al. 2014). Cold temperatures during adulthood greatly reduce Wolbachia density in natural infections of Ae. albopictus (TSAI et al. 2017). The effects of low temperatures on Wolbachia in experimentally infected Ae. aegypti mosquitoes are unknown, though any effects might be concealed by the relatively high lower thermal limits of these mosquitoes (KEARNEY et al. 2009; RICHARDSON et al. 2011).
1.3.3 Diapause, quiescence and age

Wolbachia’s effects can change throughout the lifespan of their host insect, particularly in situations that extend the duration of certain life stages. For instance, in N. vitripennis, prolonged larval diapause can lead to the loss of Wolbachia infections (PERROT-MINNOT et al. 1996; BORDENSTEIN AND WERREN 2000). Many native Wolbachia infections can also weaken in their effects over time, for example, the fidelity of cytoplasmic incompatibility declines with male age in D. simulans (HOFFMANN et al. 1990), D. melanogaster (REYNOLDS AND HOFFMANN 2002; YAMADA et al. 2007), Culex pipiens (RASGON AND SCOTT 2003), Armigeres subalbatus (JAMNONGLUK et al. 2000) and the wAlbA infection in Ae. albopictus (KITTAYAPONG et al. 2002b). In contrast, experimental Wolbachia infections in Ae. aegypti continue to induce complete cytoplasmic incompatibility in older males (MCMENIMAN et al. 2009; Ross unpublished). However, the wMel infection in Ae. aegypti retains a relatively stable density (DUTRA 2014; AMUZU et al. 2015), Figure J.3) and level of dengue suppression (AMUZU et al. 2015) with adult age.

Several Wolbachia infections in Ae. aegypti impose fitness costs on eggs, adults or larvae that are not immediately apparent, but are exacerbated with age. Uninfected Ae. aegypti eggs can remain viable for up to a year under certain environmental conditions (RUSSELL et al. 2001; JULIANO et al. 2002), but Wolbachia-infected eggs often have reduced viability that deteriorates further with time (MCMENIMAN AND O’NEILL 2010; WALKER et al. 2011; YEAP et al. 2011; AXFORD et al. 2016; JOUBERT et al. 2016; FRASER et al. 2017). Wolbachia-infected eggs that do survive long periods of quiescence seem to retain their infection, at least for wMelPop (RITCHIE et al. 2015). Fitness costs of Wolbachia infections also extend to larval (ROSS et al. 2016) and adult (MCMENIMAN et al. 2009; TURLEY et al. 2009; YEAP et al. 2011; AXFORD et al. 2016) stages, where older individuals suffer from greater deleterious effects (Figure 1.1). Several environmental conditions such as dry periods or resource-limited larval habitats could, therefore, reduce the ability of experimental Wolbachia infections to invade natural populations of Ae. aegypti.

1.3.4 Environmental antibiotics

Antibiotics such as tetracycline and rifampicin are used widely in the laboratory to cure arthropods of their Wolbachia infections (LI et al. 2014). Antibiotics are also present in the environment, originating mainly from artificial sources such as wastewater treatment plants (WATKINSON et al. 2009) and the medical and agricultural industries (CHANG et al. 2015). Environmental antibiotics could have implications for biological control programs with Wolbachia-infected mosquitoes if they are present at high enough concentrations. Natural antibiotics can cure Wolbachia infections in Tribolium confusum; beetles fed on wheat flour inoculated with several species of Streptomyces bacteria or
Penicillium fungi were cured of Wolbachia due to the microbes producing antibiotics (STEVENS AND WICKLOW 1992). Antibiotics have been detected in many water sources in low concentrations (KUMAR et al. 2005; WATKINSON et al. 2009; CHANG et al. 2015) and could be present in septic tanks which are often highly productive containers for Ae. aegypti development (BARRERA et al. 2008). Wolbachia infections in Ae. aegypti are susceptible to low concentrations of antibiotics equivalent to levels in nature, but some strains are more resistant than others (Endersby-Harshman, Axford et al. unpublished). In a survey of water samples from Ae. aegypti larval habitats in São Paulo Brazil, concentrations of antibiotics were below the limit of quantification in all containers (CURTIS et al. 2015). It, therefore, seems unlikely that antibiotics will pose a threat for Wolbachia-infected mosquito releases in this location, but more work is needed in this area.

The presence of antibiotics in blood could also affect experimental Wolbachia-infections in Aedes mosquitoes. Antibiotics are widely used in agriculture (KUMAR et al. 2005) and mosquitoes feeding on livestock will likely be exposed to antibiotics, though it is unclear if concentrations will be high enough to have an effect. Antibiotics have been used in medicine for decades (KUNIN 1993); blood feeding on humans is another potential route for antibiotics to interfere with Wolbachia infections in mosquitoes, particularly in Ae. aegypti, but this also requires further investigation.

1.3.5 Genetic background

Wolbachia infections can have different effects in insect hosts with different genetic backgrounds. Examples include the wHa strain in D. simulans (DEAN 2006; WEEKS et al. 2007), wMel in D. melanogaster (OLSEN et al. 2001) and wMelPop in D. melanogaster (REYNOLDS et al. 2003), where fitness effects depend on the host origin. Host background can also affect reproductive manipulations induced by Wolbachia. The wRec infection in Drosophila subquinaria can express nearly complete male killing or have no effect depending on where the flies originated (JAENIKE 2007). Host genotype also affects cytoplasmic incompatibility levels in N. vitripennis (PERROT-MINNOT AND WERREN 1999) and Wolbachia density in C. pipiens (BERTICAT et al. 2002) and Callosobruchus chinensis (KONDO et al. 2005). Introgression of Wolbachia infections into closely related species can alter their reproductive effects, for example, by altering the fidelity of cytoplasmic incompatibility (BORDENSTEIN AND WERREN 1998) or even shifting from cytoplasmic incompatibility to male killing (JAENIKE 2007). Wolbachia strains introduced into distantly-related species through transinfection can exhibit drastically different phenotypes in their new hosts (reviewed in HUGHES AND RASGON 2014; HOFFMANN et al. 2015).

Experimental Wolbachia infections in Aedes mosquitoes could, therefore, have unexpected effects in populations with different genetic backgrounds. The wMel infection has similar fitness effects in both
Australian and Brazilian populations of *Ae. aegypti* (Dutra et al. 2015; Dutra et al. 2016a), but the density of infection can differ (Dutra 2014), and there is some evidence for maternal transmission leakage and incomplete cytoplasmic incompatibility in Brazilian laboratory populations (Dutra et al. 2015). *wAlbB*-infected *Ae. aegypti* mosquitoes suffer from substantial fitness costs in some combinations of nuclear and mitochondrial backgrounds from Australia and Malaysia (Cottingham et al. unpublished). These findings suggest that characterising infections in each background may be necessary before releases occur in new locations.

### 1.4 Stability of *Wolbachia* infections after field deployment

The long-term success of *Wolbachia* as a biological control agent for arboviruses may depend on the stability of *Wolbachia* infections after they have established in natural populations. One risk is that fitness costs of *Wolbachia* may attenuate, which could jeopardize approaches that rely on the deleterious effects of *Wolbachia* (Ritchie et al. 2015; Schutze et al. 2015). An attenuation of fitness costs induced by *Wolbachia* has been observed in both natural populations and in the laboratory. The *wRi* strain of *D. simulans* originally inflicted a fecundity cost, but evolved rapidly to provide a fecundity benefit over the span of 20 years (Weeks et al. 2007). In *D. melanogaster*, the life-shortening effect of *wMelPop* infection can be altered in response to selection (Carrington et al. 2009). Rapid evolution can also occur upon transfer of *Wolbachia* to novel hosts; infection of *D. simulans* with *wMelPop* from *D. melanogaster* initially caused severe deleterious effects (McGraw et al. 2002), but after many generations both the fertility (McGraw et al. 2002) and longevity (Carrington et al. 2010) costs had attenuated. In *Ae. aegypti* infected with *wMelPop*, the viability of quiescent eggs can be improved through selection, though this response is likely due to selection on the host genome and not *Wolbachia* (Ritchie et al. 2015). Theory predicts that vertically transmitted symbionts will evolve to be less costly to host fitness (Turelli 1994). The *wMelPop* infection is still highly virulent over seven years after being transferred to *Ae. aegypti* (Axford et al. 2016; Ross et al. 2016) and the *wMel* infection has also retained its fitness costs after field deployment (Hoffmann et al. 2014a), but it is unclear if these deleterious effects will persist in the long-term.

The potential for *Wolbachia*’s reproductive effects to attenuate after field establishment is also a risk. Populations of the butterfly *Hypolimnas bolina* recently evolved to eliminate the male-killing phenotype originally induced by its natural *Wolbachia* infection (Hornett et al. 2006). In *N. vitripennis*, laboratory selection can increase or decrease the strength of cytoplasmic incompatibility and even lead to the complete loss of *Wolbachia* (Perrot-Minnot and Werren 1999). Though *wMel* exhibits strong cytoplasmic incompatibility and maternal transmission fidelity in *Ae. aegypti* (Walker et al. 2011), this is not the case in its original *Drosophila* host where *wMel* is transmitted imperfectly and cytoplasmic incompatibility is weak (Hoffmann 1988; Hoffmann et al. 1998).
Theoretical studies suggest that selection should favour increased maternal transmission fidelity (TURELLI 1994). However, in populations polymorphic for *Wolbachia* infection, there are intense selective pressures on both infected males and uninfected females for increased compatibility with each other, as cytoplasmic incompatibility will sharply reduce their reproductive success (TURELLI 1994; HURST AND McVEAN 1996; KOEHNCKE et al. 2009).

Another potential concern is that uninfected females could evolve to discriminate against infected males to increase their reproductive success. Sympatric populations of *Drosophila recens* and *Drosophila subquinaria* exhibit asymmetrical mating preferences to avoid cytoplasmic incompatibility (JAENIKE et al. 2006) though this has not been observed in other cases, such as in *D. simulans* (CHAMPION DE CRESPIGNY AND WEDELL 2007). In theory, these preferences are more likely to evolve if maternal transmission is imperfect and infections have a fitness cost (CHAMPION DE CRESPIGNY et al. 2005).

Despite some examples of reproductive effects that have attenuated, there are also cases of these effects remaining stable in the face of intense selection. The male-killing *w*Inn infection of *D. innubila* has persisted for thousands of years and continues to mandate all-female progeny, with no sign of resistance evolving (JAENIKE AND Dyer 2008). Selection experiments on *D. simulans* for reduced levels of cytoplasmic incompatibility were unable to suppress the strong (but imperfect) expression of cytoplasmic incompatibility induced by *wRi* (CARRINGTON et al. 2011). *wMel*-infected *Ae. aegypti* have, so far, retained their ability to induce complete cytoplasmic incompatibility after field deployment (HOFFMANN et al. 2014a).

A crucial aspect of *Wolbachia*-based population replacement approaches is that the *Wolbachia* infections will continue to suppress arboviruses after establishment. *Wolbachia* infections tend to block arboviruses more effectively in novel hosts than in native hosts (BIAN et al. 2010; LU et al. 2012); pathogen blockage may attenuate due to strong selection for pathogens to circumvent *Wolbachia*-mediated protection, and a lack of selection for *Wolbachia* to maintain its protection (BULL AND TURELLI 2013). After field deployment in Cairns, Australia, the *wMel* infection in *Ae. aegypti* has so far remained stable in its ability to interfere with dengue transmission (FRENTIU et al. 2014). Experimental infections of *Wolbachia* have only recently been established in mosquito populations with persistent arboviral transmission, so it is unclear if changes will occur in the long-term.
1.5 Conclusion and research aims

This overview indicates that environmental conditions can have substantial impacts on *Wolbachia* infections, their effects on host fitness and their ability to invade populations of insects and interfere with arbovirus transmission. *Wolbachia* infections are extremely diverse in their effects, and different strains appear to have advantages and disadvantages under different environmental conditions. For my PhD research, I investigated the effects of different environmental conditions on *Ae. aegypti* mosquitoes and their *Wolbachia* infections.

Firstly, I evaluated the performance of *Wolbachia*-infected *Ae. aegypti* larvae under competitive conditions. I found that *Wolbachia* infections differ in their fitness costs under starvation conditions, but remain stable in their reproductive effects. I then investigated the effects of heat stress on *Wolbachia*-infected *Ae. aegypti* during larval development. I found that the wMel infection is susceptible to heat stress, which could limit its ability to invade wild mosquito populations. Based on these findings, I attempted to generate a heat-resistant strain of wMel in order to improve its utility as a biological control agent. Contrary to expectations, the selection regime had a negative effect, reducing *Wolbachia* density and the fidelity of cytoplasmic incompatibility in males. Finally, I investigated the effects of inbreeding and laboratory colonisation on mosquito performance. I found that *Ae. aegypti* mosquitoes are highly susceptible to inbreeding, but laboratory maintenance at large population sizes is not detrimental to performance. These findings will inform current and future disease control efforts using modified mosquitoes.
CHAPTER 2 - COSTS OF THREE WOLBACHIA INFECTIONS ON THE SURVIVAL OF AEDES AEGYPTI LARVAE UNDER STARVATION CONDITIONS

2.1 Abstract

The mosquito Aedes aegypti, the principal vector of dengue virus, has recently been infected experimentally with Wolbachia: intracellular bacteria that possess potential as dengue biological control agents. Wolbachia depend on their hosts for nutrients they are unable to synthesize themselves. Consequently, competition between Wolbachia and their host for resources could reduce host fitness under the competitive conditions commonly experienced by larvae of Ae. aegypti in the field, hampering the invasion of Wolbachia into natural mosquito populations. We assess the survival and development of Ae. aegypti larvae under starvation conditions when infected with each of three experimentally-generated Wolbachia strains: wMel, wMelPop and wAlbB, and compare their fitness to wild-type uninfected larvae. We find that all three Wolbachia infections reduce the survival of larvae relative to those that are uninfected, and the severity of the effect is concordant with previously characterized fitness costs to other life stages. We also investigate the ability of larvae to recover from extended food deprivation and find no effect of Wolbachia on this trait. Aedes aegypti larvae of all infection types were able to resume their development after one month of no food, pupate rapidly, emerge at a large size, and exhibit complete cytoplasmic incompatibility and maternal transmission. A lowered ability of Wolbachia-infected larvae to survive under starvation conditions will increase the threshold infection frequency required for Wolbachia to establish in highly competitive natural Ae. aegypti populations and will also reduce the speed of invasion. This study also provides insights into survival strategies of larvae when developing in stressful environments.

2.2 Author Summary

Dengue is currently the most important arboviral disease in the world. With no effective treatment or commercial vaccine available, strategies to control dengue focus on its mosquito vectors, primarily Aedes aegypti. A recent effort to reduce the burden of dengue aims to replace native Ae. aegypti with those refractory to the virus. This is achieved by infecting mosquitoes with Wolbachia, bacteria which can invade insect populations by exploiting host reproduction. Some strains of Wolbachia have harmful effects on the mosquito host which can inhibit its ability to spread. While these costs have
been characterized comprehensively in the laboratory, we must also consider any impacts when mosquitoes experience stresses that commonly occur in nature. For instance, *Ae. aegypti* larvae often develop in highly-occupied habitats where food is scarce. We investigated the effects of *Wolbachia* on mosquito larvae when they develop under extremely nutrient-limited conditions and found costs to survival for all strains. This will translate to a reduced ability of *Wolbachia*-infected mosquitoes to replace native populations in competitive habitats.

2.3 Introduction

Dengue fever is an increasing threat to global health. An estimated 50 to 390 million new cases of dengue occur annually, with 2.5 billion people living in areas at risk of infection (WHO 2009; BHATT *et al.* 2013). At present, dengue lacks an effective treatment or vaccine that protects against all serotypes of the virus. Thus, strategies to reduce infection incidence must rely on the control of its mosquito vector, principally *Aedes aegypti* (BROWN AND JAMES 2014; CAPEEDING *et al.* 2014). While permanent eradication is unlikely to be achieved, many emerging genetic and biological approaches aim to reduce mosquito vectorial capacity (FRANZ *et al.* 2015; LEFTWICH *et al.* 2015).

A promising new approach to dengue control utilizes the obligate intracellular bacterium, *Wolbachia*. *Wolbachia* are maternally inherited (HOFFMANN *et al.* 1986) and usually manipulate the reproduction of their hosts to enhance their own transmission (WERREN *et al.* 2008). The most common manipulation induced by *Wolbachia* is cytoplasmic incompatibility; a mechanism where embryonic lethality occurs when an infected male mates with a female that is not infected with *Wolbachia*, providing infected females with a relative reproductive advantage (YEN AND BARR 1973; TRAM AND SULLIVAN 2002). Many *Wolbachia* infections also provide protection to their host against pathogens, including RNA viruses (PANTELEEV *et al.* 2007; HEDGES *et al.* 2008; TEIXEIRA *et al.* 2008). These traits have enabled *Wolbachia* to be implemented in strategies to both suppress (LAVEN 1967; O'CONNOR *et al.* 2012) and replace (CURTIS 1976; DOBSON *et al.* 2002; HOFFMANN *et al.* 2011) insect populations.

While *Ae. aegypti* does not harbour a natural *Wolbachia* infection (KITTAYAPONG *et al.* 2000; POPOVICI *et al.* 2010), three infections have been stably introduced into the vector: the *wMelPop* and *wMel* strains originating from *Drosophila melanogaster* (MCMENIMAN *et al.* 2009; WALKER *et al.* 2011) and *wAlbB* from the mosquito *Aedes albopictus* (XI *et al.* 2005). All three infections are transmitted vertically at high rates and exhibit complete cytoplasmic incompatibility (XI *et al.* 2005; MCMENIMAN *et al.* 2009; WALKER *et al.* 2011), and these effects have remained stable after many years in the novel host (HOFFMANN *et al.* 2014a; YEAP *et al.* 2014; AXFORD *et al.* 2016). Crucially,
they also suppress the replication and transmission of dengue virus in *Ae. aegypti* (MOREIRA et al. 2009a; BIAN et al. 2010; WALKER et al. 2011), giving them potential to reduce dengue incidence in transformed populations. Establishment of *Wolbachia* in a field population is facilitated largely by maternal transmission and cytoplasmic incompatibility (CURTIS 1976; TURELLI AND HOFFMANN 1991; KRIESNER et al. 2013). However, because *Wolbachia*-infected mosquitoes must survive and reproduce in competition with the native inhabitants, lower relative fitness of infected mosquitoes can hamper the invisibility of *Wolbachia* (CASPARI AND WATSON 1959; BROWNSTEIN et al. 2003; CRAIN et al. 2011).

The experimental *Wolbachia* infections established in *Ae. aegypti* vary considerably in their effects on mosquito life-history traits. The wMel infection is relatively benign and has invaded both caged (WALKER et al. 2011) and field (HOFMANN et al. 2011) populations. wMel remains at a high frequency in mosquitoes collected from the release sites, three years after releases of wMel ceased in two suburbs of Cairns, Australia (HOFMANN et al. 2014a). Conversely, the wMelPop infection tends to overreplicate in host cells, leading to rapid tissue degeneration and early death (MIN AND BENZER 1997; McGRAW et al. 2002; McMENIMAN et al. 2008). It exacts a high fitness cost on *Ae. aegypti*; wMelPop shortens adult lifespan (McMENIMAN et al. 2009; YEAP et al. 2011), while fecundity (McMENIMAN AND O’NEILL 2010), blood feeding success (MOREIRA et al. 2009b; TURLEY et al. 2009) and quiescent egg viability (McMENIMAN AND O’NEILL 2010; YEAP et al. 2011; RITCHIE et al. 2015) deteriorate rapidly with age. wMelPop also modifies behaviour and metabolism (EVANS et al. 2009), reduces the response of larvae to light stimulation (SUH AND DOBSON 2013), delays larval development, and decreases viability and adult size when reared under crowded conditions (ROSS et al. 2014). The wAlbB infection has intermediate fitness costs, likely due to its moderate density in host tissues that lies between that of wMel and wMelPop (AXFORD et al. 2016).

While each of these infections can invade caged populations of *Ae. aegypti* (XI et al. 2005; WALKER et al. 2011; AXFORD et al. 2016), the mosquitoes were not exposed to many of the selective pressures that exist in the field (LEFTWICH et al. 2015). Suitable habitats for immature development in the field are limited; as a consequence, larvae are often subjected to competition for space and nutrition (SOUTHWOOD et al. 1972; SUBRA AND MOUCHET 1984; ARRIVILLAGA AND BARRERA 2004; BARRERA et al. 2006). Though Wolbachia infection has no clear effect on *Ae. aegypti* larval development in the absence of stress (McMENIMAN AND O’NEILL 2010; WALKER et al. 2011; YEAP et al. 2011; AXFORD et al. 2016), some costs emerge when larvae are crowded (ROSS et al. 2014). Many fitness costs of Wolbachia in *Ae. aegypti* also tend to become clearer with age in both adults and eggs (TURLEY et al. 2009; YEAP et al. 2011; AXFORD et al. 2016). As larval development times can reach several weeks, or even months in the field (COURET AND BENEDICT 2014) and often experience periods of food limitation (ARRIVILLAGA AND BARRERA 2004; BARRERA et al. 2006),
deleterious effects of *Wolbachia* on larvae undetected in laboratory studies could emerge when development times are prolonged, impacting *Wolbachia*’s invasive potential. This could explain a lack of invasion success by *wMelPop* in natural populations despite multiple attempts to establish the infection in the field (Nguyen et al. 2015).

*Aedes aegypti* larvae are adapted to nutrient poor-habitats as food limitation is a major regulator of their population size (Barrera and Medialdea 1996; Arrivillaga and Barrera 2004). Larvae decrease their rate of development in response to food scarcity, delaying metamorphosis until reaching a critical threshold of nutritional reserves (Chambers and Klowden 1990; Lan and Grier 2004; Nishiura et al. 2007; Telang et al. 2007), and larvae can resist starvation for several weeks at a time (Rasnitsyn and Yasyukevich 1989; Barrera 1996; Barrera and Medialdea 1996; Padmanabha et al. 2011). This is achieved largely by expending their accumulated reserves (Wigglesworth 1942; Gilpin and McClelland 1979; Perez and Noriega 2012), though larvae also scavenge on dead conspecifics (Daugherty et al. 2000; Bara et al. 2014) and may even prey on younger larvae (Edgerly et al. 1999) to increase their chance of survival. *Wolbachia* depend on their hosts for a wide range of resources they cannot synthesize themselves (Wu et al. 2004; Caragata et al. 2013; Caragata et al. 2014; Ponton et al. 2015). Since *Wolbachia* increase the activity and metabolic rate of *Ae. aegypti* in adults, at least for the *wMelPop* infection (Evans et al. 2009), we hypothesize that *Wolbachia* may also increase the rate at which energy reserves are depleted in larvae without food. *Aedes aegypti* breeding containers typically have low productivity and high food intermittency because leaf litter, animal detritus and the microorganisms that break them down are the primary source of nutrition (Daugherty et al. 2000; Kitching 2000; Yee et al. 2012). Thus, the ability to survive periods of limited food is a critical aspect of larval fitness (Barrera and Medialdea 1996; Arrivillaga and Barrera 2004). In the field, competition between *Wolbachia* and *Ae. aegypti* for resources could substantially reduce the survival of larvae, limiting the potential for *Wolbachia* to invade and persist in natural populations.

In this study we investigate the effects of *wMel*, *wAlbB* and *wMelPop* infection on the ability of *Ae. aegypti* larvae to survive and develop under extreme nutrient stress. We compare the survival and development of *Wolbachia*-infected and uninfected larvae under starvation conditions when held in groups, when infected and uninfected larvae are together in the same container, or when isolated, and test their ability to recover when an influx of resources is provided. We also examine the ability of *Wolbachia* to express their reproductive effects when *Ae. aegypti* larvae are held under starvation conditions for extended periods. We then consider the likely impact of any fitness costs imposed by *Wolbachia* on the potential for these infections to invade highly competitive populations.
2.4 Materials and methods

2.4.1 Colony maintenance and mosquito strains

*Aedes aegypti* mosquitoes were sourced from Cairns, Queensland and maintained under laboratory conditions for at least two generations before use in experiments. *Wolbachia*-infected lines were generated by crossing male uninfected Cairns mosquitoes to laboratory-reared female mosquitoes infected with *w*Mel (*WALKER et al. 2011), *w*AlbB (*XI et al. 2005) or *w*MelPop (*MCMENIMAN et al. 2009) to maintain a similar genetic background (>98%) between colonies. Mosquitoes were kept in the laboratory at 26°C ± 1°C and 80-90% relative humidity with a 12:12 light : dark photoperiod, and maintained according to methods described by AXFORD *et al.* (2016). Within one week of emerging, female adults were allowed to feed to repletion on the forearm of a single human volunteer. Blood feeding of female mosquitoes on human volunteers for this research has been approved by the University of Melbourne Human Ethics Committee (approval 0723847). All adult subjects provided informed written consent (no children were involved).

2.4.2 Rearing regime

Larvae were reared under a common regime before initiating the food-deprivation period for all experiments. At the beginning of each experiment, *w*Mel-infected, *w*MelPop-infected, *w*AlbB-infected and uninfected eggs were hatched synchronously in separate trays containing 3 L of RO (reverse osmosis) water, 2-3 grains of yeast and one crushed tablet of TetraMin tropical fish food (Tetra, Melle, Germany). Within three hours of hatching, cohorts of 200 1st instar larvae were transferred to plastic trays filled with 700 mL of RO water and fed TetraMin *ad libitum* for 72 hours. This rearing environment was chosen as development times do not differ significantly between *Wolbachia*-infected and uninfected larvae with abundant nutrition at this density. After the feeding period, larvae were pipetted into fresh trays of RO water. To remove any remaining food particles, larvae were rinsed by passing them through two additional trays of water before being added to experimental containers. All experiments used 72 hour old 3rd instar larvae of approximately the same size, and were conducted at 26°C ± 1°C and 80-90% relative humidity with a 12:12 light : dark photoperiod.
2.4.3 Survival of isolated larvae under starvation conditions

We tested the ability of *Wolbachia*-infected and uninfected larvae to survive starvation conditions in the absence of conspecific larvae, removing any effects of resource competition and also the ability to scavenge on dead larvae. Two independent experiments were conducted; in each, 96 larvae per infection type (see *rearing regime*) were added individually to wells of Costar 12-well cell culture plates (Corning, Corning, NY) filled with ~4 mL of RO water only. Plates were enclosed in stockings and held in a tray covered with a mesh lid to minimize external sources of food input, and RO water was topped up daily to counter evaporation. For both experiments, wells were monitored for mortality daily until all larvae had died. A larva was considered dead when no movement was observed after fifteen seconds of physical stimulation.

In the first experiment, plates were unmanipulated with the exception of maintaining a consistent volume of water in each well. In the second experiment, water was replaced completely twice per week to reduce the accumulation of microorganisms as a potential source of nutrition (e.g., bacteria, algae, protozoa, fungi) and waste in the water (YEE et al. 2007b). For this experiment, larvae were removed from wells and rinsed by pipetting through three trays of RO water, then returned to wells filled with a fresh change of water.

2.4.4 Survival and development of larvae held in groups under starvation conditions

Two independent experiments tested the ability of *Wolbachia*-infected and uninfected larvae to survive starvation conditions when held in the presence of conspecific larvae. Larvae (see *rearing regime*) were added to circular plastic containers (9.5-11.5 cm radius, 7 cm height) with mesh lids and filled with 200 mL of RO water only (no TetraMin was provided). Mortality was scored every second day by temporarily pipetting larvae into a separate container of RO water. Numbers of dead and live larvae were counted before all larvae (including dead larvae) were returned to the original container. Water was refreshed every four days by transferring all larvae to a new container of RO water. In the first experiment, larvae were added to containers in groups of 50. Each container was replicated eight times for the uninfected, *w*Mel, *w*AlbB and *w*MelPop strains. The experiment was terminated when all larvae had died or had reached adulthood.

During field releases, preferential mortality of *Wolbachia*-infected larvae in nutrient-deprived containers could release the remaining larvae from food stress, providing an advantage to uninfected larvae (AGUDELO-SILVA AND SPIELMAN 1984; WILSON et al. 1990). A second experiment was therefore conducted to determine whether there were differences in survival when *Wolbachia*-infected
and uninfected larvae were held together in mixed proportions within the same container. Cohorts of larvae were added to plastic containers filled with 200 mL of RO water in the following proportions (*Wolbachia*-infected to uninfected): 12:36, 24:24 and 36:12. Additional cohorts of 48 *Wolbachia*-infected and 48 uninfected larvae were set up as controls. Treatments (mixed proportions) were replicated eight times each, while the controls (pure cohorts) were replicated four times, and the experiment was repeated for the *w*Mel, *w*AlbB and *w*MelPop infections. Containers were monitored as per the previous experiment, with the exception that the five longest surviving larvae in each container were removed and screened for their *Wolbachia* infection status (see DNA extraction and *Wolbachia* detection). The proportion of individuals infected with *Wolbachia* in the longest surviving larvae was then compared with the initial proportion of larvae infected with *Wolbachia* in each container (see statistical analysis).

In both experiments, a few percent of larvae were able to reach the pupal and adult stages due to the availability of dead conspecific larvae as a food resource. All adults emerging throughout the two group experiments were stored in ethanol for wing length measurement and later tested for their *Wolbachia* infection status (see wing length measurements and DNA extraction and *Wolbachia* detection). Their development time and sex were also recorded.

### 2.4.5 Recovery from food deprivation

An experiment was carried out to test the ability of *Wolbachia*-infected and uninfected larvae to recover from starvation conditions after providing an influx of resources. Larvae (see rearing regime) were added to RO water in groups of 50 (see survival and development of larvae held in groups under starvation conditions). Containers were then divided into two treatments; larvae were re-fed TetraMin *ad libitum* after either 15 or 25 days of surviving starvation conditions. These two time points were chosen based on when substantial starvation-induced mortality had occurred; approximately 25% and 10% of larvae were remaining on Days 15 and 25 respectively (Figure A.1). For each infection type and treatment, the following observations were recorded: the number of surviving larvae upon the resumption of feeding, rates of pupation and survival to the pupal stage after re-feeding, rates of adult emergence and survival to adulthood, and the body size (see wing length measurements) and sex ratio of emerging adults. Containers were replicated between six and eight times for each infection type and treatment.
2.4.6 Cytoplasmic incompatibility when larvae are food-deprived then re-fed

We ran a series of experiments to determine if the reproductive effects caused by *Wolbachia* remain robust when larvae are held under starvation conditions for an extended period. To test the level of cytoplasmic incompatibility induced by *Wolbachia*-infected males, larvae (see rearing regime) were added to containers of RO water and their development was suspended for ~30 days by maintaining them in the absence of TetraMin. After this period larvae were again fed TetraMin *ad libitum* until pupation. Pupae were sexed (males are smaller than females), and male pupae pipetted into small cups of RO water and allowed to emerge in 1.5 L plastic containers with mesh sides and a stocking lid. Female pupae emerging from this treatment were set aside for an additional experiment on reproductive effects (see fecundity and maternal transmission). After confirming the sex of all males as adults, newly-emerged uninfected females that were reared under standard laboratory conditions (see colony maintenance and mosquito strains) were added to each cage and allowed to mate freely with *Wolbachia*-infected males. Seven *Wolbachia*-infected males and seven uninfected females were held in each experimental cage, and crosses were replicated eight times for the *w*Mel, *w*AlbB and *w*MelPop infections. Cages of adults were provided access to 10% sucrose solution and water throughout the experiment. Crosses between standard laboratory-reared *Wolbachia*-infected males and uninfected females were set up as controls, as these crosses are known to produce no viable offspring (Xi et al. 2005; McMeniman et al. 2009; Walker et al. 2011). Females were then blood fed and eggs were collected according to Axford et al. (2016) for three gonotrophic cycles.

2.4.7 Maternal transmission and fecundity when larvae are food-deprived then re-fed

This experiment assessed the rate at which *Wolbachia*-infected females transmit the infection to their offspring when their development time is greatly extended. Food-deprived and re-fed larvae from the *w*Mel, *w*AlbB and *w*MelPop lines (see cytoplasmic incompatibility) were sorted by sex, and 100 females per infection type were added to 12 L plastic cages and provided with 10% sucrose solution and a source of water. 100 uninfected males reared under standard laboratory conditions were then aspirated into each cage and allowed to mate freely. Females were then blood fed and isolated for oviposition according to Axford et al. (2016), and their progeny reared to adulthood and stored in absolute ethanol.

Ten progeny each from 30 isolated females per infection type were tested for the presence of *Wolbachia* using PCR to determine maternal transmission efficiency (see DNA extraction and *Wolbachia* detection). A set of control crosses was also completed for each infection type where both *Wolbachia*-infected females and uninfected males were reared under standard laboratory conditions.
Ten progeny from 15 Wolbachia-infected females were tested for each of the wMel, wAlbB and wMelPop infections. These crosses have expected maternal transmission rates of close to 100% (Xi et al. 2005; McMeniman et al. 2009; Walker et al. 2011). All female parents from the treatments and controls were scored for their fecundity, with a sample also measured for wing length (see wing length measurements). Data from uninfected females reared under standard laboratory conditions from a concurrent experiment were included as a point of comparison.

### 2.4.8 Wing length measurements

Linear measurements of wings were taken to give an indication of body size (Briegel 1990; Nasci 1990). The right wing was removed from each adult and fixed on a slide under a 10 mm circular coverslip (Menzel-Gläser, Braunschweig, Germany) using Hoyer’s solution (dH₂O : gum arabic : chloral hydrate : glycerin in the ratio 5 : 3 : 20 : 2) (Anderson 1954). Wings were observed under a dissecting microscope fitted with a camera and measured using NIS-Elements BR (Nikon Instruments, Japan). Wing length was determined by calculating the distance from the alular notch to the intersection of the radius 3 vein and outer margin, excluding the wing fringe scales (Huestis et al. 2011). Measurements in pixels were converted to millimetres by calibration with a graticule before the start of each set of measurements. Each measurement was repeated independently so that length represented the average of two measurements. Damaged or folded wings were excluded from the analysis.

### 2.4.9 DNA extraction and Wolbachia detection

To test for the presence of Wolbachia in adult and immature mosquitoes, we carried out DNA extraction and Wolbachia detection according to methods described previously (Lee et al. 2012; Hoffmann et al. 2014a; Oxford et al. 2016). DNA from whole adults or larvae was extracted using 150 µL of 5% Chelex 100 resin (Bio-Rad Laboratories, Hercules, CA). The PCR assay was conducted using a LightCycler 480 system (Roche Applied Science, Indianapolis, IN); mosquitoes were considered positive for Wolbachia when the mRpS6 (Aedes universal) and aRpS6 (Ae. aegypti-specific) primer sets were successfully amplified in addition to the appropriate Wolbachia-specific primer set (wMel, wAlbB or wMelPop). Wolbachia-free mosquitoes tested positive for mRpS6 and aRpS6 and negative for all Wolbachia-specific primer sets.
2.4.10 Statistical analysis

All data were analysed using SPSS statistics version 21.0 for Windows (SPSS Inc, Chicago, IL). Survival data were investigated using Kaplan-Meier analysis; log-rank tests compared rates of mortality between lines and treatments. Wolbachia infection frequency was calculated as the proportion of individuals that tested positive for Wolbachia. For containers where both Wolbachia-infected and uninfected larvae were present, deviations from expected infection frequencies in larvae and adults were analysed using Chi-squared tests. Maternal transmission rates of Wolbachia were expressed as the proportion of infected offspring produced by infected mothers, for which 95% binomial confidence intervals were calculated. All other data were tested for normality using Shapiro-Wilk tests. Data that were not normally distributed were arcsine square-root transformed (proportional data) or square-root transformed and tested again. Normally distributed data were then analysed with one-way ANOVA and Tukey’s honest significant difference tests, while data that failed Shapiro-Wilk tests were analysed with non-parametric Kruskal-Wallis and Mann-Whitney U tests. Associations between wing length and development time were assessed with Pearson’s correlation if data were normally distributed or Spearman’s rank-order correlation where data could not be transformed for normality.

2.5 Results

2.5.1 Survival of isolated larvae under starvation conditions

Kaplan-Meier (KM) analysis revealed a significant effect of Wolbachia infection type (KM: $\chi^2 = 123.273$, df = 3, $P < 0.0001$) and water-replacement regime (KM: $\chi^2 = 678.532$, df = 1, $P < 0.0001$) on the survival of larvae when isolated under starvation conditions. Whether water was refreshed in each well or left unmanipulated had a dramatic effect on survival, with the former (mean ± SE = 20.682 ± 0.221 days) reducing the mean survival time of larvae by half compared with unmanipulated experimental wells (40.286 ± 0.573 days, Figure A.2). An increased survival in the latter experiment is likely due to the build-up of microorganisms which are an important resource for mosquito larvae (MERRITT et al. 1992; YEE et al. 2007b; YEE et al. 2012).

When water was not replaced, all three Wolbachia infections reduced survival; the wMel, wAlbB and wMelPop infections decreased mean survival 15.8, 28.8 and 28.7% compared with uninfected larvae (Figure 2.1A). All pairwise comparisons between the infection types were highly significant (KM: all $\chi^2 > 24.087$, df = 1, all $P < 0.0001$), with the exception that wMelPop and wAlbB did not differ significantly in their survival patterns under starvation conditions ($\chi^2 = 0.717$, df = 1, $P = 0.397$).
Figure 2.1 Survival of *Ae. aegypti* larvae when isolated under starvation conditions. (A) Survival of larvae when there was no manipulation of the wells. (B) Survival of larvae when the water in each well was replaced every four days. Error bars are standard errors. Note that (A) and (B) differ in their x-axis values.
Although there was a significant effect of *Wolbachia* infection type in both experiments, survival differences between *Wolbachia*-infected and uninfected larvae were reduced markedly when water was replaced every four days (KM: $\chi^2 = 17.939$, df = 3, $P = 0.0005$) compared with wells that were unmanipulated ($\chi^2 = 150.024$, df = 3, $P < 0.0001$, Figure 2.1). When water was replaced, all pairwise comparisons between infection types were significant (KM: all $\chi^2 > 4.262$, df = 1, all $P \leq 0.039$) except for between uninfected and *w*Mel (KM: $\chi^2 = 1.707$, df = 1, $P = 0.191$), and *w*AlbB and *w*MelPop (KM: $\chi^2 = 0.630$, df = 1, $P = 0.427$) (Figure 2.1B). No pupae or adults emerged in either experiment where larvae were isolated.

### 2.5.2 Survival and development of larvae held in groups under starvation conditions

*Wolbachia* infection type also had a substantial effect on survival when larvae were held under starvation conditions in groups of 50 (KM: $\chi^2 = 225.821$, df = 3, $P < 0.0001$). Uninfected larvae had the greatest mean time of survival (mean ± SE = 28.289 ± 0.532 days), with the *w*Mel, *w*AlbB and *w*MelPop infections reducing survival times by 5.7, 15.7 and 29.5% respectively (Figure 2.2). All pairwise comparisons between lines were significant (KM: all $\chi^2 > 7.411$, df = 1, all $P \leq 0.006$). Note that emerging adults were excluded from Kaplan-Meier analyses rather than censored because the rate and number of adults emerging differed between infection types.
Figure 2.2 Survival of *Ae. aegypti* larvae under starvation conditions in groups of 50 per container. (A) Shows only larval mortality for each line and excludes those larvae that emerged as adults, while (B) is adjusted so that emerging adults are included in the survivors. Error bars are standard errors.
Larvae from both *Wolbachia*-infected and uninfected lines readily consumed dead conspecifics throughout the experiment. We inferred scavenging based on observations that the number of dead larvae in each container fluctuated with mortality rather than increasing proportionally (Figure A.3). Distributions of necrophagy closely matched larval mortality, with the mean time for larval consumption occurring less than one day after the mean time of death for both *Wolbachia*-infected and uninfected lines (Figure A.4). Necrophagy likely contributed to increased survival time; larvae lived for longer in groups compared with larvae kept in isolation under otherwise similar conditions. While survival began to decline earlier in the group experiment, rates of mortality became considerably slower when the majority of larvae had died (Figure A.2).

Less than five percent of larvae reached pupation or adulthood during this experiment (Table 2.1). *Wolbachia* infection type had a significant effect on the total number of larvae that survived to both the pupal (one-way ANOVA: $F_{3, 28} = 3.417$, $P = 0.031$) and adult ($F_{3, 28} = 5.647$, $P = 0.004$) stages, and also affected the development times of those pupae (Kruskal-Wallis: $\chi^2 = 31.499$, df = 3, $P < 0.0001$) and adults ($\chi^2 = 14.200$, df = 3, $P = 0.003$). Despite uninfected larvae having greater survival times under starvation conditions (Figure 2.2), they developed more slowly and pupated less often than *Wolbachia*-infected larvae, with the *w*MelPop infection displaying the greatest proportion of larvae reaching adulthood and the most rapid development on average (Table 2.1, Figure A.5). This observation is likely due to an earlier availability and greater abundance of conspecific carcasses as a source of nutrition in containers with *w*MelPop-infected larvae.
Table 2.1 Pupation and adult emergence from *Ae. aegypti* larvae held under starvation conditions in groups of 50.

<table>
<thead>
<tr>
<th>Infection type</th>
<th>Survival (%) ± SE</th>
<th>Development time (days) ± SE</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pupae*</td>
<td>Adults*</td>
</tr>
<tr>
<td>Uninfected</td>
<td>2.25 ± 0.35 a</td>
<td>1.25 ± 0.26 a</td>
</tr>
<tr>
<td><em>w</em>Mel</td>
<td>4.75 ± 0.80 ab</td>
<td>3.75 ± 0.61 ab</td>
</tr>
<tr>
<td><em>w</em>AlbB</td>
<td>3.00 ± 0.50 ab</td>
<td>1.25 ± 0.26 a</td>
</tr>
<tr>
<td><em>w</em>MelPop</td>
<td>7.50 ± 0.53 b</td>
<td>5.25 ± 0.32 b</td>
</tr>
</tbody>
</table>

* Within a column, values with the same letter in bold are not significantly different from each other ($P > 0.05$, by Tukey’s honest significant difference test)

† Within a column, values with the same letter in bold are not significantly different from each other ($P > 0.05$, by Mann-Whitney *U* tests on data pooled across replicates)

A second experiment was conducted where *Wolbachia*-infected and uninfected larvae were held together in the same container under starvation conditions. Control containers, where 48 larvae from each infection type were held separately, had a shorter starved survival period than in the previous experiment despite nearly identical methods, though the relative performance of each infection type was similar (Figure A.2). In each treatment container, the five longest-lived larvae were screened for their infection status to test for differential survival between infected and uninfected larvae when held together at different frequencies. The *w*AlbB and *w*MelPop infections were significantly underrepresented in the surviving larvae for all treatments, while for *w*Mel there were no significant deviations from any starting ratio (Table 2.2).
Table 2.2 *Wolbachia* infection frequencies in surviving *Ae. aegypti* larvae when held at different initial proportions under starvation conditions.

<table>
<thead>
<tr>
<th>Initial proportion <em>Wolbachia</em>-infected : uninfected</th>
<th>wMel : uninfected</th>
<th>wAlbB : uninfected</th>
<th>wMelPop : uninfected</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment*</td>
<td>Expected</td>
<td>Observed†</td>
<td>$\chi^2$‡</td>
</tr>
<tr>
<td>36:12</td>
<td>30:10</td>
<td>30:10</td>
<td>0 1</td>
</tr>
<tr>
<td>24:24</td>
<td>20:20</td>
<td>16:24</td>
<td>1.60 0.206</td>
</tr>
<tr>
<td>12:36</td>
<td>10:30</td>
<td>7:33</td>
<td>1.20 0.273</td>
</tr>
<tr>
<td>Total</td>
<td>60:60</td>
<td>53:67</td>
<td>1.63 0.201</td>
</tr>
</tbody>
</table>

* Cohorts of larvae were set up with initial ratios of 36:12, 24:24 and 12:36 (*Wolbachia*-infected : uninfected) and held under starvation conditions until five larvae per container were left alive.

† Observed proportion of *Wolbachia*-infected : uninfected in the longest five surviving larvae of each container

‡ Chi-squared tests assessed deviations from expected ratios which were based on the initial proportion of *Wolbachia*-infected larvae in each container.

Deviations from an expected 1:1 ratio were also tested when all treatments for each infection type were combined

§ *P*-values in bold denote significant deviations from expected ratios where all df = 1
Less than two percent of larvae from this experiment emerged as adults. Expected ratios of Wolbachia-infected and uninfected adults emerging were based on the initial proportion of larvae in each container. We found no significant deviations from expected proportions of adults for all treatments (Chi-squared test: all $\chi^2 < 3.267$, df = 1, all $P > 0.071$), except for the wMelPop infection which was significantly underrepresented when larvae were held in the ratio 36:12 (wMelPop : uninfected) (Chi-squared test: $\chi^2 = 24.2$, df = 1, $P < 0.0001$).

All adults that emerged from larvae held in groups were measured for wing length to test for effects on body size. Due to low numbers of adults, data were pooled across both experiments as they did not differ significantly (Student’s $t$ test: $P = 0.795$). Wing length was not associated with development time for either males (Spearman’s rank-order correlation: $\rho = 0.071$, $P = 0.455$, n = 56) or females ($\rho = -0.009$, $P = 0.924$, n = 58). As expected, there was a significant effect of sex on wing length (one-way ANOVA: $F_{1,106} = 285.910$, $P < 0.0001$), where males (mean ± SE = 1.659 ± 0.009 mm) were considerably smaller than females (1.973 ± 0.015 mm). However, we found no effect of Wolbachia infection type (one-way ANOVA: $F_{3,106} = 0.360$, $P = 0.782$); wings of mosquitoes with any infection type were approximately the same size (Table 2.3).

Table 2.3 Wing lengths of Ae. aegypti adults emerging from groups of larvae held under starvation conditions.

<table>
<thead>
<tr>
<th>Infection type</th>
<th>Males (mm) ± SE</th>
<th>Females (mm) ± SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uninfected</td>
<td>1.657 ± 0.013 (n = 21)</td>
<td>1.973 ± 0.024 (n = 24)</td>
</tr>
<tr>
<td>wMel</td>
<td>1.649 ± 0.019 (n = 11)</td>
<td>2.020 ± 0.024 (n = 8)</td>
</tr>
<tr>
<td>wAlbB</td>
<td>1.671 ± 0.021 (n = 7)</td>
<td>1.971 ± 0.042 (n = 10)</td>
</tr>
<tr>
<td>wMelPop</td>
<td>1.664 ± 0.020 (n = 17)</td>
<td>1.950 ± 0.027 (n = 16)</td>
</tr>
</tbody>
</table>

Data are pooled across experiments where infection types were held both separately and in mixed proportions. No values within a column differed significantly from each other by one-way ANOVA.
2.5.3 Recovery from food deprivation

25.5% and 12.3% of larvae across all infection types survived after 15 and 25 days of exposure to starvation conditions respectively. *Wolbachia* infection type had a significant effect on the number of larvae surviving after both 15 (one-way ANOVA: $F_{3,56} = 4.152, P = 0.010$) and 25 days ($F_{3,26} = 4.114, P = 0.016$). The $w$MelPop infection had the lowest survival at both time points (Figure A.1), consistent with other experiments (Figure 2.1B, Figure 2.2).

Recovery from food deprivation was assessed by scoring the proportion of surviving larvae that pupated and reached adulthood upon resuming feeding. The majority of surviving larvae were able to recover, though larval and pupal mortality occurred across both treatments for all infection types (Figure 2.3). We found a significant effect of treatment (day of re-feeding) (one-way ANOVA: $F_{1,52} = 5.576, P = 0.022$), but not *Wolbachia* infection type ($F_{3,52} = 1.461, P = 0.236$), on the proportion of surviving larvae that reached adulthood. Surviving larvae that were deprived of food for 25 days were less likely to reach adulthood than larvae deprived for 15 days, with the percentage surviving of larvae that died after re-feeding averaging 10.4% and 22.9% respectively. This is, in part, due to an increase in pupal mortality at the later time point (2.1% for Day 15, 9.0% for Day 25, Student’s *t* test: $P = 0.042$, Figure 2.3). The proportion of surviving larvae that reached adulthood was less for $w$MelPop than for other infection types, though this difference was not significant (Figure 2.3). Larvae that reached pupation before re-feeding (33.3% of $w$MelPop-infected larvae and 3.3% of $w$Mel-infected larvae) were counted as survivors. However, these individuals were excluded from development time and wing length analyses (see below) as they pupated before food was provided again *ad libitum*, and were similar in size to adults emerging from larvae held in groups under starvation conditions (Table 2.3).
Figure 2.3 Proportion of *Ae. aegypti* larvae developing when fed *ad libitum* after extended food deprivation. Larvae were provided with TetraMin *ad libitum* after (A) 15 and (B) 25 days of food deprivation. Light grey bars denote the proportion of surviving larvae that reached adulthood, while black and red bars correspond to the proportion of larval and pupal mortality respectively. Error bars are standard errors for the proportion of larvae that survived to adulthood. Within treatments, no proportions differed significantly from each other (*P* > 0.05, by Tukey’s honest significant difference test).

The number of days taken for larvae to reach pupation after re-feeding was significantly affected by infection type (one-way ANOVA: *F*₃,₄₈₈ = 5.377, *P* = 0.001) but not treatment (day of re-feeding) (*F*₁,₄₈₈ = 2.128, *P* = 0.145), though infection types within treatments did not differ significantly from each other (Table 2.4). Development times of both male and female adults were unaffected by infection type and treatment (one-way ANOVA: all *P* > 0.053). Female wing length was significantly affected by treatment (one-way ANOVA: *F*₁,₂₅₁ = 6.696, *P* = 0.010) but not infection type (*F*₃,₂₅₁ = 1.432, *P* = 0.234). Females re-fed after 25 days of food deprivation were smaller than those fed after 15 days for all infection types, though no pairwise comparisons were significant (Table 2.4). Conversely, male wing length was unaffected by both infection type (*F*₃,₁₉₄ = 0.844, *P* = 0.471) and treatment (*F*₁,₁₉₄ = 0.032, *P* = 0.859). We found no correlation between development time and wing length for both males and females for each treatment (Pearson correlation: all *P* > 0.175).
Table 2.4 Mean development time and wing length of *Ae. aegypti* when fed *ad libitum* after extended food deprivation.

<table>
<thead>
<tr>
<th>Infection type</th>
<th>Development time (days after re-feeding) ± SE</th>
<th>Wing length (mm) ± SE</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pupae</td>
<td>Males</td>
</tr>
<tr>
<td><strong>Re-fed on Day 15</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Uninfected</td>
<td>4.302 ± 0.042</td>
<td>6.075 ± 0.063</td>
</tr>
<tr>
<td>wMel</td>
<td>4.145 ± 0.022</td>
<td>5.877 ± 0.058</td>
</tr>
<tr>
<td>wAlbB</td>
<td>4.379 ± 0.026</td>
<td>5.953 ± 0.071</td>
</tr>
<tr>
<td>wMelPop</td>
<td>4.309 ± 0.055</td>
<td>6.129 ± 0.142</td>
</tr>
<tr>
<td><strong>Re-fed on Day 25</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Uninfected</td>
<td>4.478 ± 0.089</td>
<td>6.199 ± 0.256</td>
</tr>
<tr>
<td>wMel</td>
<td>4.264 ± 0.079</td>
<td>6.140 ± 0.127</td>
</tr>
<tr>
<td>wAlbB</td>
<td>4.404 ± 0.106</td>
<td>6.354 ± 0.171</td>
</tr>
<tr>
<td>wMelPop</td>
<td>4.250 ± 0.204</td>
<td>5.876 ± 0.281</td>
</tr>
</tbody>
</table>

Larvae were re-fed TetraMin *ad libitum* after either 15 (top) or 25 (bottom) days of food deprivation. Development time is defined as the number of days taken for larvae to reach pupation or adulthood after re-feeding. Within a column, values with the same letter in bold are not significantly different from each other (*P* > 0.05, by Tukey’s honest significant difference test).
2.5.4 Cytoplasmic incompatibility, maternal transmission and fecundity when larvae are food-deprived then re-fed

Males deprived of food for 30 days as larvae and then re-fed were tested for their ability to induce cytoplasmic incompatibility when crossed to uninfected females. All food-deprived and re-fed Wolbachia-infected males exhibited complete cytoplasmic incompatibility, with no viable offspring produced across three gonotrophic cycles (Table 2.5). Control crosses using standard laboratory-reared adults were also completely sterile, with the exception that a low proportion of eggs hatched in the wMelPop control cross due to contamination with uninfected males (Table 2.5).

Table 2.5 Percentage of hatching eggs from crosses between Wolbachia-infected males and uninfected female Ae. aegypti.

<table>
<thead>
<tr>
<th>Cross*</th>
<th>Gonotrophic cycle†</th>
<th>1</th>
<th>2</th>
<th>3</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Controls‡</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Uninfected ♀ × wMel ♂</td>
<td>0 (248.50 ± 25.67)</td>
<td>0 (247.38 ± 35.71)</td>
<td>0 (249.38 ± 41.28)</td>
<td></td>
</tr>
<tr>
<td>Uninfected ♀ × wAlbB ♂</td>
<td>0 (223.75 ± 24.56)</td>
<td>0 (188.62 ± 37.45)</td>
<td>0 (272.75 ± 18.25)</td>
<td></td>
</tr>
<tr>
<td>Uninfected ♀ × wMelPop ♂</td>
<td>0.30 (246.88 ± 22.96)</td>
<td>0.75 (234.63 ±14.30)</td>
<td>0.23 (221.75 ± 29.13)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Treatments§</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Uninfected ♀ × wMel ♂</td>
<td>0 (298.00 ± 53.84)</td>
<td>0 (258.50 ± 21.18)</td>
<td>0 (206.88 ± 39.91)</td>
<td></td>
</tr>
<tr>
<td>Uninfected ♀ × wAlbB ♂</td>
<td>0 (272.75 ± 39.88)</td>
<td>0 (211.00 ± 20.71)</td>
<td>0 (194.38 ± 37.70)</td>
<td></td>
</tr>
<tr>
<td>Uninfected ♀ × wMelPop ♂</td>
<td>0 (174.88 ± 39.79)</td>
<td>0 (219.25 ± 34.71)</td>
<td>0 (184.38 ± 41.13)</td>
<td></td>
</tr>
</tbody>
</table>

* Eight cages with each containing seven males and seven females were tested per cross. All females were reared under standard laboratory conditions
† Percentage hatch rates across three gonotrophic cycles are given, followed by the mean number of eggs laid per cross in parentheses, with standard errors
‡ Wolbachia-infected males were reared under standard laboratory conditions
§ Wolbachia-infected males were fed ad libitum as larvae for 72 hours, deprived of food for 30 days, then fed ad libitum until pupation
We also tested maternal transmission rates of *Wolbachia* when infected females were held under starvation conditions for 30 days as larvae and then re-fed. The *wMel*, *wAlbB* and *wMelPop* infections were transmitted with perfect fidelity by both standard laboratory-reared females (All infection types: maternal transmission rate = 1, lower 95% binomial confidence interval = 0.976), and females that were food-deprived then re-fed (All infection types: maternal transmission rate = 1, lower 95% binomial confidence interval = 0.988).

Female parents were also measured for their fecundity and wing length. Both *Wolbachia* infection type (one-way ANOVA: $F_{3, 227} = 33.011, P < 0.0001$) and treatment ($F_{1, 227} = 8.787, P = 0.003$) had significant effects on fecundity. The food-deprivation treatment reduced the mean fecundity of *wMel*, *wAlbB* and *wMelPop*-infected females by approximately 5-6 eggs relative to the controls, though no pairwise comparisons were significant (Table 2.6). All *Wolbachia*-infected females had considerably reduced fecundity compared with uninfected standard laboratory-reared females, regardless of the rearing treatment (Table 2.6). Female wing length was also significantly affected by both *Wolbachia* infection type (one-way ANOVA: $F_{3, 108} = 6.935, P = 0.0003$) and treatment ($F_{1, 108} = 8.852, P = 0.004$). For all infection types, females held under starvation conditions and then re-fed were smaller than standard laboratory-reared females, though only the *wAlbB* comparison was significant (Table 2.6).

Table 2.6. Average wing length and fecundity of isolated female *Ae. aegypti* tested for their maternal transmission fidelity.

<table>
<thead>
<tr>
<th>Infection type</th>
<th>Feeding regime</th>
<th>Wing length (mm) ± SE*</th>
<th>Fecundity ± SE*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uninfected</td>
<td>Control†</td>
<td>2.854 ± 0.018 a (n = 19)</td>
<td>68.21 ± 2.16 a (n = 39)</td>
</tr>
<tr>
<td><em>wMel</em></td>
<td>Control†</td>
<td>2.757 ± 0.020 abc (n = 16)</td>
<td>50.68 ± 3.73 b (n = 22)</td>
</tr>
<tr>
<td></td>
<td>Treatment‡</td>
<td>2.738 ± 0.027 bc (n = 16)</td>
<td>44.38 ± 2.34 bc (n = 42)</td>
</tr>
<tr>
<td><em>wAlbB</em></td>
<td>Control†</td>
<td>2.817 ± 0.019 ab (n = 16)</td>
<td>50.71 ± 2.46 b (n = 24)</td>
</tr>
<tr>
<td></td>
<td>Treatment‡</td>
<td>2.688 ± 0.022 c (n = 16)</td>
<td>44.65 ± 1.81 bc (n = 43)</td>
</tr>
<tr>
<td><em>wMelPop</em></td>
<td>Control†</td>
<td>2.703 ± 0.021 c (n = 16)</td>
<td>38.86 ± 1.86 cd (n = 22)</td>
</tr>
<tr>
<td></td>
<td>Treatment‡</td>
<td>2.665 ± 0.042 c (n = 16)</td>
<td>34.21 ± 1.36 d (n = 42)</td>
</tr>
</tbody>
</table>

* Within a column, values with the same letter in bold are not significantly different from each other ($P > 0.05$, by Tukey’s honest significant difference test)
† *Wolbachia*-infected females were reared under standard laboratory conditions
‡ *Wolbachia*-infected females were fed *ad libitum* as larvae for 72 hours, deprived of food for 30 days, then fed *ad libitum* until pupation
2.6 Discussion

We have demonstrated that Wolbachia infection reduces the tolerance of Ae. aegypti larvae to starvation conditions. Because Ae. aegypti larvae survive nutrient-poor conditions primarily by expending their own accumulated energy reserves (WIGGLESWORTH 1942; GILPIN AND MCCLELLAND 1979), we suspect that Wolbachia reduce survival by increasing the rate at which these reserves are depleted. Wolbachia do not appear to affect the rate at which larvae accumulate reserves because development times are unaffected by infection when larvae are well-fed (MCMeniman AND O'NEILL 2010; YEAP et al. 2011; AXFORD et al. 2016). However, when food is limited, Wolbachia may increase the drain on host reserves due to various nutritional requirements (WU et al. 2004; CARAGATA et al. 2013; CARAGATA et al. 2014; PONTON et al. 2015). Indeed, Wolbachia increase the metabolism of Ae. aegypti adults, at least for the wMelPop infection (EVANS et al. 2009), though this remains to be tested in larvae.

All three infections negatively affected the survival patterns of nutrient-deprived larvae but differed in their severity; wMelPop was highly costly to survival across all experiments, wMel either had a slightly deleterious or no significant effect relative to uninfected larvae, and wAlbB had an intermediate effect. These relative costs are consistent with their effects on mosquito adults and eggs; wMelPop drastically reduces adult lifespan and quiescent egg viability (MCMeniman et al. 2009; MCMENIMAN AND O'NEILL 2010; YEAP et al. 2011), wMel has relatively minor costs or no detectable effect (WALKER et al. 2011; HOFFMANN et al. 2014a), and wAlbB has an intermediate cost to these traits (AXFORD et al. 2016). Here, we demonstrate that infections with higher virulence in these life stages also have greater costs to the survival of larvae under starvation conditions. The differences between Wolbachia infections in terms of their deleterious effects are likely to be attributed to their density in mosquito tissues (HOFFMANN et al. 2015). High bacterial densities and broad tissue tropisms in host cells are often implicated in increasing fitness costs imposed by Wolbachia infection, both in Ae. aegypti (WALKER et al. 2011; AXFORD et al. 2016) and other insects (MOUTON et al. 2004; DURON et al. 2006; CHROSTEK AND TEIXEIRA 2015; MARTINEZ et al. 2015).

We found that as the survival period of larvae increased, the deleterious effects of Wolbachia became clearer. In adults and eggs of Ae. aegypti, the fitness costs of Wolbachia are also enhanced with age; wMelPop has relatively little cost to the reproductive success of young females, but fecundity (MCMeniman AND O'NEILL 2010) and rates of successful probing (MOREIRA et al. 2009b; TURLEY et al. 2009) decline severely with subsequent gonotrophic cycles. Additionally, the wAlbB and wMelPop infections impose increased costs on the viability of quiescent eggs over time (YEAP et al. 2011; AXFORD et al. 2016). If these age effects also occur in larvae as suggested by our results,
virulent *Wolbachia* infections could have difficulty invading populations where resources are scarce and thus development times are lengthened.

Adults emerging from starvation conditions were small in size, even in comparison with those produced through extreme crowding or nutrient limitation (e.g. BRIEGEL 1990; PONLAWAT AND HARRINGTON 2007; FARJANA AND TUNO 2012). Adult sizes were at the lowest end of natural variation found in Australian field populations of *Ae. aegypti*, from where these mosquitoes were sourced (TUN-LIN *et al.* 2000; YEAP *et al.* 2013). Adult body size reflects the feeding history of larvae after reaching a critical weight (TELANG *et al.* 2007); therefore adults emerging from starvation conditions likely obtained only the minimum nutritional reserves required for pupation. In contrast, larvae that were deprived of food for extended durations and then fed *ad libitum* emerged nearly as large as mosquitoes fed *ad libitum* throughout development, suggesting that they were able to attain a close approximation of their maximum weight despite the long interruption to feeding (CHAMBERS AND KLOWDEN 1990; LAN AND GRIER 2004; NISHIURA *et al.* 2007; PADMANABHA *et al.* 2011).

We found that *Ae. aegypti* larvae, regardless of *Wolbachia* infection type, recover well from long periods of nutrient deprivation. While the ability of larvae to resume their development has been reported previously (WIGGLESWORTH 1942; RASNITSYN AND YASYUKEVICH 1989; TELANG *et al.* 2007), we show that larvae exhibit low mortality, pupate rapidly and emerge at a large size when fed again after being deprived of food for as long as three weeks. In addition, infected males deprived of food as larvae for one month exhibited complete cytoplasmic incompatibility and females transmitted *Wolbachia* to their offspring with perfect fidelity despite a greatly extended development time. Maternal transmission rates of *Wolbachia* also remain high when eggs are held in a quiescent state for several weeks (RITCHIE *et al.* 2015). In insects, the maternal transmission efficiency of *Wolbachia* (KITTAYAPONG *et al.* 2002a; McGRAW *et al.* 2002; DUTTON AND SINKINS 2004; UNCKLESS *et al.* 2009) and the strength of cytoplasmic incompatibility (BOYLE *et al.* 1993; CLANCY AND HOFFMANN 1998; VENETI *et al.* 2003; MCWENIMAN *et al.* 2008; JAENIKE 2009) are known to be affected by bacterial density. Because environmental factors such as temperature (MOUTON *et al.* 2006; WIWATANARATANABUTR AND KITTAYAPONG 2006; MURDOCK *et al.* 2014) and nutrition (DUTTON AND SINKINS 2004; CORREA AND BALLARD 2014; PONTON *et al.* 2015; SERBUS *et al.* 2015) modulate *Wolbachia* density, extreme stress in the field could lead to changes in host effects derived from *Wolbachia*. However, the wMel infection of *Ae. aegypti* established in Australian field populations has so far remained stable in terms of its reproductive effects, fitness costs and dengue blockage (FRENTIU *et al.* 2014; HOFFMANN *et al.* 2014a).

We acknowledge some limitations of our laboratory study that should be addressed in future experiments. We were somewhat limited in our ability to discern any effects of *Wolbachia* on larval
development time and survival to adulthood when held under starvation conditions, due to low pupation rates. Future experiments testing these traits specifically should use larger cohorts with greater replication. Furthermore, we demonstrated the fitness costs of Wolbachia under rather arbitrary and specific scenarios. Nutrient input in the field is dynamic (Yee and Juliano 2012), but in this study larvae were fed for a single time period before either being deprived of food completely or re-fed at a later point. Breeding containers in the field are often populated by multiple cohorts (Southwood et al. 1972; Walsh et al. 2011; Walsh et al. 2013), and Suh and Dobson (2013) recently reported differential survival of Wolbachia-infected and uninfected 1st instar Ae. aegypti larvae in the presence of later instars. Because predatory behaviour is more likely to occur under nutrient-poor conditions (Edgerly et al. 1999), future experiments on survival under starvation conditions should also test interactions between larvae of mixed age classes. Our experiments also were conducted over multiple generations, and while all infection types were outcrossed to an uninfected colony, the number of generations spent in the laboratory varied between experiments. Laboratory adaptation can have substantial effects on fitness (Koenraadt et al. 2010; Leftwich et al. 2015), which could explain why larvae in some experiments had reduced survival under similar conditions (see Figure A.2).

Nevertheless, our study demonstrates consistent deleterious effects of Wolbachia on the survival of Ae. aegypti larvae under starvation conditions. To predict the impact on the invasion dynamics of Wolbachia in highly resource-limited habitats, we estimate changes to the unstable equilibrium frequency, denoted $\hat{p}$, when this cost to larval viability is considered. For Wolbachia to reach fixation in a population its frequency must reach or exceed $\hat{p}$; larger $\hat{p}$ values thus decrease the likelihood and speed of invasion, and will additionally reduce the potential for spatial spread once established in a population (Caspari and Watson 1959; Turelli and Hoffmann 1999; Barton and Turelli 2011).

Based on the mean survival time of larvae under starvation conditions (averaged across all experiments where larvae were held in groups), we estimate the relative fitness of the $w$Mel, $w$AlbB and $w$MelPop infections to be 92.3, 81.3 and 68.5% that of uninfected respectively. We detected no significant costs for other traits, thus only the cost to survival patterns under starvation conditions is considered. Following equation 17b of Turelli (2010), this produces a $\hat{p}$ of 0.08, 0.19 and 0.32 for $w$Mel, $w$AlbB and $w$MelPop respectively in the absence of any other fitness costs, assuming complete cytoplasmic incompatibility and no maternal transmission leakage as indicated by our results. Previous laboratory studies have estimated the fitness costs of the $w$Mel, $w$AlbB and $w$MelPop infections to be approximately ~24% (Walker et al. 2011), ~15% (Xi et al. 2005; Axford et al. 2016) and ~43% (Turelli 2010; Yeap et al. 2011) respectively. Using these estimates, $\hat{p}$ increases to
0.30, 0.31 and 0.61 for \( w\text{Mel} \), \( w\text{AlbB} \) and \( w\text{MelPop} \) respectively when both the costs to larval viability under starvation conditions and deleterious effects on other life stages are considered.

In a more extreme scenario, where larvae are deprived of food for 25 days before being provided access to food \textit{ad libitum}, the invasive potential of \textit{Wolbachia} decreases further. Assuming \textit{Wolbachia}-infected larvae are equally as capable of recovering from food deprivation as suggested by our results, the relative fitness of the \( w\text{Mel} \), \( w\text{AlbB} \) and \( w\text{MelPop} \) infections decrease to 90.6, 73.9 and 42.5\% that of uninfected respectively. This corresponds to increases of \( \hat{p} \) to 0.31, 0.37 and 0.75 when taking into account other fitness costs. The deleterious effects demonstrated here could in part explain why \( w\text{MelPop} \) was able to establish in semi-field cages (WALKER \textit{et al.} 2011; RITCHIE \textit{et al.} 2015) but has had great difficulty invading wild mosquito populations, both in Australia and Vietnam (NGUYEN \textit{et al.} 2015). In semi-field cages, any costs of \textit{Wolbachia} infection to larval viability under nutrient stress were likely to be masked by the fact that larvae were relatively well-fed. On the other hand, survival of larvae under starvation conditions was likely to be a critical fitness component in the field releases. The deleterious effects of \textit{Wolbachia} demonstrated here will, therefore, have an impact on the potential for these infections to invade natural mosquito populations where competition for resources is the major limiting factor of population size, particularly for \( w\text{MelPop} \).

\textbf{2.7 Acknowledgements}

The authors thank Jason Axford for experimental design and technical advice.
CHAPTER 3 – **Wolbachia** Infections in *Aedes aegypti* Differ Markedly in Their Response to Cyclical Heat Stress

3.1 Abstract

*Aedes aegypti* mosquitoes infected with *Wolbachia* bacteria are currently being released for arbovirus suppression around the world. Their potential to invade populations and persist will depend on interactions with environmental conditions, particularly as larvae are often exposed to fluctuating and extreme temperatures in the field. We reared *Ae. aegypti* larvae infected with different types of *Wolbachia* (*w*Mel, *w*AlbB and *w*MelPop-CLA) under diurnal cyclical temperatures. Rearing *w*Mel and *w*MelPop-CLA-infected larvae at 26-37°C reduced the expression of cytoplasmic incompatibility, a reproductive manipulation induced by *Wolbachia*. We also observed a sharp reduction in the density of *Wolbachia* in adults. Furthermore, the *w*Mel and *w*MelPop-CLA infections were not transmitted to the next generation when mosquitoes were exposed to 26-37°C across all life stages. In contrast, the *w*AlbB infection was maintained at a high density, exhibited complete cytoplasmic incompatibility, and was transmitted from mother to offspring with a high fidelity under this temperature cycle. These findings have implications for the potential success of *Wolbachia* interventions across different environments and highlight the importance of temperature control in rearing.

3.2 Author summary

There is currently great interest in using the bacterium *Wolbachia* to reduce the burden of dengue and Zika; viruses which infect millions of people globally each year. *Aedes aegypti* mosquitoes with *Wolbachia* infections can invade natural populations and interfere with the transmission of these viruses. However, we find that the *w*Mel strain of *Wolbachia* which is currently being used for dengue and Zika control in several countries may have reduced effectiveness at invading populations when mosquitoes experience heat stress. Since mosquito larvae experience extreme temperatures in their natural habitat, our results have implications for current and future releases of *Wolbachia*-infected mosquitoes and highlight the need for further investigation into alternative strains of *Wolbachia*. 

51
3.3 Introduction

*Aedes aegypti* mosquitoes transmit some of the most important arboviral diseases worldwide. They are widespread in tropical and subtropical regions (Bhatt et al. 2013), inhabiting urban environments where they have adapted to breed in artificial containers (Cheong 1967). Dengue and Zika are among the viruses they transmit and these are rapidly increasing their burden on global health. Dengue alone infects as many as 390 million people each year, and up to half of the world’s population is at risk of infection (Bhatt et al. 2013). Zika is an emerging threat that is experiencing an epidemic following an outbreak in Brazil in 2015 (Hennessy et al. 2016; Kindhauser et al. 2016). A vaccine for dengue has recently been licensed (Hadinegoro et al. 2015) but no vaccines for Zika are commercially available and there are risks associated with deployment of the licensed dengue vaccine (Ferguson et al. 2016). Efforts to reduce the spread of dengue and Zika therefore rely on the direct control of *Ae. aegypti* populations. Though permanent mosquito eradication is unlikely to be achieved, several genetic and biological approaches are being utilized to reduce the burden of arboviruses (McGraw and O'Neill 2013).

One such approach involves the release of *Ae. aegypti* infected with the bacterium *Wolbachia* into wild populations of mosquitoes in an effort to combat dengue and Zika (Hoffmann et al. 2011; Garcia Gde et al. 2016). *Wolbachia* are transmitted maternally and often manipulate the reproduction of their hosts to enhance their own transmission (Werren et al. 2008). These bacteria are of particular interest in the control of arboviral diseases as they are known to inhibit the replication of RNA viruses in insects (Hedges et al. 2008; Teixeira et al. 2008). Infections of *Wolbachia* from *Drosophila melanogaster* and *Ae. albopictus* were recently introduced experimentally into *Ae. aegypti* and were found to suppress the transmission of dengue (Moreira et al. 2009a; Ferguson et al. 2015), Zika (Aliota et al. 2016; Dutra et al. 2016b), chikungunya (Moreira et al. 2009a; Van Den Hurk et al. 2012), yellow fever (Van Den Hurk et al. 2012) and West Nile viruses (Hussain et al. 2013). This innate viral suppression makes *Wolbachia* a desirable alternative for arboviral control as it removes the need for mosquito eradication.

More than four *Wolbachia* infections have now been established in *Ae. aegypti* from interspecific transfers, including the wMelPop-CLA (McMeniman et al. 2009) and wMel (Walker et al. 2011) infections from *D. melanogaster*, the wAlbB infection from *Ae. albopictus* (Xi et al. 2005), and a wMel/wAlbB superinfection (Joubert et al. 2016). These *Wolbachia* infections induce cytoplasmic incompatibility in *Ae. aegypti*, a phenomenon that results in sterility when an infected male mates with an uninfected female. *Wolbachia*-infected females therefore possess a reproductive advantage because they can produce viable offspring with both infected and uninfected males as mates (Tram et al. 2003). These infections vary considerably in their effects on the mosquito host, from the minor deleterious fitness effects of wMel (Hoffmann et al. 2014a; Ross et al. 2014; Axford et al. 2016)
to the severe longevity and fertility costs of wMelPop-CLA (McMeniman and O'Neill 2010; Yeap et al. 2011; Ross et al. 2016). Variability also exists in the extent to which they suppress arboviruses; infections that reach a higher density in the host tend to block viruses more effectively (Walker et al. 2011; Ferguson et al. 2015; Joubert et al. 2016).

With its lack of severe fitness effects and its ability to cause cytoplasmic incompatibility, the wMel infection is suitable for invading naïve mosquito populations (Walker et al. 2011). This infection has become established in multiple wild populations of mosquitoes in Queensland, Australia (Hoffmann et al. 2011), and has persisted in these populations for at least two years after the associated releases ceased (Hoffmann et al. 2014a). wMel is currently the favoured infection for Wolbachia interventions on an international scale and is undergoing field release trials in Brazil, Indonesia, Vietnam and Colombia (Garcia Gde et al. 2016). Cage and field trials of the wMelPop-CLA infection demonstrate its difficulty in invading and persisting (Walker et al. 2011; Nguyen et al. 2015), though the infection could have utility in population suppression programs (Rašić et al. 2014a; Ritchie et al. 2015) due to its detrimental effect on quiescent egg viability (McMeniman and O'Neill 2010; Yeap et al. 2011). The wAlbB infection is yet to be released in the field but it has successfully invaded caged populations in the laboratory (Xi et al. 2005; Axford et al. 2016).

Since Wolbachia were introduced into Ae. aegypti, the four described infections have each displayed complete cytoplasmic incompatibility and maternal inheritance in the laboratory (Xi et al. 2005; McMeniman et al. 2009; Walker et al. 2011; Joubert et al. 2016). A high fidelity of these traits is necessary for the success of Wolbachia as a biological control; maternal transmission leakage and partial cytoplasmic incompatibility will increase the proportion of infected mosquitoes needed for the infection to spread, reduce the speed of invasion and prevent the infection from reaching fixation in a population (Turelli and Hoffmann 1999). Some natural Wolbachia infections in Drosophila exhibit perfect maternal inheritance and complete cytoplasmic incompatibility in the laboratory, but display incomplete fidelity under field conditions (Hoffmann et al. 1990; Turelli 1994).

The effects of Wolbachia on reproduction can depend on the density of Wolbachia in mosquito tissues. In insects other than Ae. aegypti, a decline in Wolbachia density can reduce the degree of male-killing (Dyer et al. 2005), feminization (Rigaud et al. 2001), parthenogenesis (Zchori-Fein et al. 2000), cytoplasmic incompatibility (Clancy and Hoffmann 1998; Ikeda et al. 2003) and maternal transmission of Wolbachia (Clancy and Hoffmann 1998; Unckless et al. 2009). Incomplete cytoplasmic incompatibility occurs when some sperm cysts in the testes are not infected with Wolbachia (Bourtzis et al. 1996; Veneti et al. 2003). Viral protection by Wolbachia is also density dependent, with higher densities in the host generally resulting in greater protection (Osborne et al. 2012; Martinez et al. 2014). However, environmental conditions such as temperature (Wiwatanaratatanabutr and Kittayapong 2009; Murdock et al. 2014), nutrition
(Dutton and Sinkins 2004; Correa and Ballard 2014) and pathogen infection (Tortosa et al. 2008; Mousson et al. 2010) are known to modulate Wolbachia densities in other insects. Given the importance of bacterial density in determining Wolbachia’s reproductive effects (cytoplasmic incompatibility and maternal transmission fidelity), fitness costs and viral blocking effects, work is needed to determine if environmental effects play a role in modulating densities in experimental infections of *Ae. aegypti*.

*Ae. aegypti* larvae often experience large diurnal fluctuations of temperature in nature, particularly in small containers of water and in habitats exposed to direct sunlight (Kearney et al. 2009; Richardson et al. 2013). While the thermal limits of *Ae. aegypti* are generally well understood (Mohammed and Chadee 2011; Richardson et al. 2011; Carrington et al. 2013), research has not assessed Wolbachia’s reproductive effects in *Ae. aegypti* at the high temperatures they can experience in the field. Ulrich et al. (2016) recently demonstrated that the density of wMel in *Ae. aegypti* decreased sharply when larvae experienced diurnally cycling temperatures of 28.5°C to 37.5°C during development. This suggests that the reproductive effects of Wolbachia could also be altered if infected larvae develop under similar conditions in the field.

We explored the hypothesis that the reproductive effects of Wolbachia infections could be diminished if *Ae. aegypti* experience stressful, high thermal maxima within a large diurnal cyclical temperature regime during development. We tested three Wolbachia infections: wMel, wMelPop-CLA and wAlbB, for their maternal transmission fidelity and ability to cause cytoplasmic incompatibility under temperature conditions that are representative of containers in the field (Richardson et al. 2013). We show for the first time that cyclical temperatures reaching a maximum of 37°C during development reduce the expression of cytoplasmic incompatibility in the wMel and wMelPop-CLA infections of *Ae. aegypti*. We also find a greatly diminished Wolbachia density under these conditions. wMel and wMelPop-CLA-infected mosquitoes exposed to this regime across their life cycle do not transmit the infection to their offspring. Conversely, the wAlbB infection is more stable in terms of its reproductive effects and density under cyclical temperatures. These findings suggest the need for multiple infection types suitable for different conditions when using Wolbachia infections in biological control strategies.
3.4 Results

3.4.1 Maximum daily temperatures of 37°C during development reduce the hatch rate of wMel-infected eggs

We compared the hatch rate of eggs from crosses between Wolbachia-infected Ae. aegypti females and Wolbachia-infected males reared under cyclical temperatures. Larvae of both sexes were reared in incubators set to cycle diurnally between a minimum of 26°C and a maximum of either 26°C, 32°C, 34.5°C or 37°C (Figure B.1), and crosses were then conducted at 26°C. We observed a sharp decrease in the hatch rate of eggs when wMel-infected mosquitoes were reared at 26-37°C compared to 26°C (Mann-Whitney U: Z = 2.802, P = 0.005), but found no effect of rearing temperature on hatch rate for the wAlbB (Kruskal-Wallis $\chi^2 = 2.587$, df = 3, P = 0.460) or wMelPop-CLA ($\chi^2 = 1.687$, df = 3, P = 0.640) infections (Figure 3.1). We hypothesized that reduced hatch rate in wMel-infected mosquitoes could reflect the loss of Wolbachia infection under heat stress, leading to partial cytoplasmic incompatibility.

Figure 3.1 Proportion of eggs hatched from Wolbachia-infected Ae. aegypti reared at cyclical temperatures. Wolbachia-infected females were crossed to Wolbachia-infected males reared at cyclical temperatures for the (A) wMel, (B) wAlbB and (C) wMelPop-CLA infections. Both sexes were reared under the same temperature regime and then crossed together at 26°C. Each data point shows the proportion of eggs hatched from a cage of 7 females and 7 males (n = 6 replicates per cross). Numbers for each bar denote the total number of eggs scored per cross. Error bars show 95% confidence intervals.
3.4.2 Wolbachia density is reduced in wMel and wMelPop-CLA, but not wAlbB-infected adults reared under cyclical temperatures of 26-37°C

We wanted to see if a reduction in Wolbachia density could explain the reduced hatch rate of wMel-infected eggs. We measured the density of Wolbachia in whole adults infected with wMel, wAlbB and wMelPop-CLA when reared at either 26°C, 26-32°C or 26-37°C using quantitative PCR. The density of wMel did not differ significantly between 26°C and 26-32°C for either males (Mann-Whitney U: Z = 1.190, \(P = 0.234\)) or females (\(Z = 1.112, P = 0.267\)), but sharply decreased at 26-37°C in both sexes (Figure 3.2). The density in females reared at 26°C (mean ± SD = 3.56 ± 1.87, \(n = 29\)) was 14.75-fold higher than those reared at 26-37°C (0.24 ± 1.04, \(n = 30\), \(Z = 6.239, P < 0.0001\)). For males the difference between 26°C (4.65 ± 2.71, \(n = 29\)) and 26-37°C (0.027 ± 0.025, \(n = 30\)) was 174.73-fold (\(Z = 6.688, P < 0.0001\)). For wMelPop-CLA, female Wolbachia density at 26°C (mean ± SD = 84.60 ± 89.19, \(n = 30\)) was 268.34-fold higher than those reared at 26-37°C (0.32 ± 0.66, \(n = 30\), \(Z = 6.631, P < 0.0001\)), while males reared at 26°C (45.62 ± 32.25, \(n = 30\)) had a 73.37-fold higher density than males reared at 26-37°C (0.62 ± 1.76, \(n = 30\), \(Z = 6.542, P < 0.0001\)). In contrast, there was no significant difference in wAlbB density between 26°C and 26-37°C for both females (\(Z = 0.47, P = 0.638\)) and males (\(Z = 1.678, P = 0.093\)). However, there was a significant effect of temperature overall due to an increased density at 26-32°C in both females (Kruskal-Wallis \(\chi^2 = 7.826, df = 2, P = 0.020\)) and males (\(\chi^2 = 16.311, df = 2, P = 0.0003\)).

Patterns of Wolbachia density were similar when larvae were reared under different levels of nutrition, but the effects of heat stress on wMel and wMelPop-CLA were more severe at lower levels of nutrition (Figure B.3). When larvae were provided 0.02 mg of food per larva per day, Wolbachia density was lower at 26-32°C than at 26°C for both wMel (Mann-Whitney U: \(Z = 7.964, P < 0.0001\)) and wMelPop-CLA (\(Z = 5.394, P < 0.0001\), Figure B.3), indicating that even moderate temperatures can reduce Wolbachia density when combined with nutritional stress.
Figure 3.2 Relative density of Wolbachia in Aedes aegypti reared at cyclical temperatures.

Relative Wolbachia density was measured in (A) female and (B) male adults reared at a constant 26°C, cycling 26-32°C or cycling 26-37°C. Each mosquito was tested with mosquito-specific and Wolbachia-specific markers to obtain crossing point values (see “Wolbachia quantification”). Differences in crossing point between the two markers were transformed by 2^n to obtain relative Wolbachia densities. 30 mosquitoes were tested per treatment. Each data point represents the average of three technical replicates.
3.4.3 Cytoplasmic incompatibility is partially lost in wMel and wMelPop-CLA, but not wAlbB-infected adults reared under cyclical temperatures of 26-37°C

Crosses between uninfected female and Wolbachia-infected male *Ae. aegypti* produce no viable offspring under standard laboratory conditions due to cytoplasmic incompatibility (Xi et al. 2005; McMeniman et al. 2009; Walker et al. 2011). We hypothesized that reduced *Wolbachia* densities in infected males reared at 26-37°C would coincide with reduced fidelity of cytoplasmic incompatibility. Incomplete cytoplasmic incompatibility leads to some viable progeny when infected males mate with uninfected females (Hoffmann et al. 1990). We crossed wMel, wAlbB and wMelPop-CLA males reared at 26°C and 26-37°C to uninfected females reared at 26°C, and scored the proportion of eggs that hatched (Figure 3.3A). 245 larvae hatched from 1747 eggs (14.02%) across all replicates when wMel-infected males were reared at 26-37°C. Conversely, we observed complete sterility when males were reared at 26°C (Mann-Whitney U: Z = 2.802, P = 0.005). We also observed incomplete cytoplasmic incompatibility in the wMelPop-CLA infection; 301 larvae hatched from 1846 eggs (16.31%) when males were reared at 26-37°C, but no larvae hatched when males were reared at 26°C (Z = 2.802, P = 0.005). In contrast to wMel and wMelPop-CLA, no eggs hatched from uninfected females that were mated to wAlbB-infected males reared under either regime (Z = 0.080, P = 0.936). The cytoplasmic incompatibility induced by wAlbB therefore appears to be stable under these conditions.
Figure 3.3 Effect of cyclical temperatures on cytoplasmic incompatibility in *Wolbachia*-infected *Ae. aegypti*. (A) Proportion of eggs hatched from uninfected females reared at 26°C and *Wolbachia*-infected males reared at either 26°C or a cycling 26-37°C. (B) Proportion of eggs hatched from *Wolbachia*-infected females reared at either 26°C or 26-37°C and *Wolbachia*-infected males of the same infection type reared at 26°C. For both sets of crosses, adults were mated at 26°C after a period of maturation. Each data point shows the proportion of eggs hatched from a cage of 7 females and 7 males (n = 6 replicates per cross). Numbers for each bar denote the total number of eggs scored per cross. Error bars show 95% confidence intervals.

We also scored the hatch rate of *Wolbachia*-infected females reared under a cycling 26-37°C when crossed to infected males reared at 26°C (Figure 3.3B). We hypothesized that reduced *Wolbachia* densities in the female could restore cytoplasmic incompatibility in this cross. For the wMel infection, mean hatch rates were drastically reduced to 22.7% in infected females reared at 26-37°C compared to 85.7% when reared at 26°C (Mann-Whitney U: Z = 2.802, P = 0.005). Conversely, we found no effect on the wMelPop-CLA (Z = 0.400, P = 0.689) and wAlbB (Z = 0.560, P = 0.575) infections; females possessed similar hatch rates regardless of the rearing temperature. Taken together, these results show that a cyclical rearing regime reaching a maximum of 37°C reduces both the ability of wMel-infected males to induce cytoplasmic incompatibility and the ability of wMel-infected females to retain compatibility. In contrast, this ability was unaffected in wMelPop-CLA-infected females (Figure 3.3B) despite the same regime causing incomplete cytoplasmic incompatibility in wMelPop-CLA-infected males (Fig 3.3A), while for wAlbB high rearing temperatures did not influence the level of cytoplasmic incompatibility induction through infected males or the ability to retain compatibility in infected females (Figure 3.3).
3.4.4 The wMel and wMelPop-CLA infections are not maternally transmitted, and wAlbB exhibits incomplete maternal transmission fidelity at 26-37°C

We tested the ability of wMel, wAlbB and wMelPop-CLA-infected females to transmit Wolbachia to their offspring when their entire lifecycle occurred at either a constant 26°C or a cycling 26-37°C. Females from each infection type were crossed to uninfected males which were reared at 26°C, and their progeny were reared to the 4th instar at the same temperature as the mother. wMel and wAlbB-infected females transmitted the infection to all of their offspring at 26°C. The wMelPop-CLA infection was also transmitted with a high fidelity at 26°C, though a single wMelPop-CLA-infected female produced two uninfected progeny (Table 3.1). In contrast, the wMel and wMelPop-CLA infections were lost completely when mothers and offspring were maintained at 26-37°C; all progeny were conclusively uninfected with Wolbachia. The wAlbB infection was transmitted to the majority of offspring at 26-37°C, but 11.5% lost the infection (Table 3.1). We also tested rates of maternal transmission when mothers were reared at 26-37°C and their progeny reared at 26°C; the wMel and wMelPop-CLA infections were still lost under these conditions (Table 3.1).

Table 3.1 Maternal transmission of Wolbachia under cyclical temperatures. Proportion of Wolbachia-infected offspring produced by wMel, wMelPop-CLA and wAlbB-infected mothers when mothers and progeny were maintained at a constant 26°C or a cycling 26-37°C. Ten progeny from five to eight mothers, for a total of 50-80 progeny, were tested per treatment.

<table>
<thead>
<tr>
<th>Wolbachia infection type</th>
<th>Maintenance temperature for mother</th>
<th>Maintenance temperature for offspring</th>
<th>Proportion of Wolbachia-infected offspring</th>
<th>Binomial confidence interval (lower 95%, upper 95%)</th>
</tr>
</thead>
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<tr>
<td>wMel</td>
<td>26°C</td>
<td>26°C</td>
<td>1</td>
<td>0.955, 1</td>
</tr>
<tr>
<td></td>
<td>26-37°C</td>
<td>26-37°C</td>
<td>0</td>
<td>0, 0.045</td>
</tr>
<tr>
<td></td>
<td>26-37°C</td>
<td>26°C</td>
<td>0</td>
<td>0, 0.076</td>
</tr>
<tr>
<td>wMelPop-CLA</td>
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<td>26°C</td>
<td>0.975</td>
<td>0.912, 0.997</td>
</tr>
<tr>
<td></td>
<td>26-37°C</td>
<td>26-37°C</td>
<td>0</td>
<td>0, 0.045</td>
</tr>
<tr>
<td></td>
<td>26-37°C</td>
<td>26°C</td>
<td>0</td>
<td>0, 0.074</td>
</tr>
<tr>
<td>wAlbB</td>
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<td>26°C</td>
<td>1</td>
<td>0.955, 1</td>
</tr>
<tr>
<td></td>
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<td>26-37°C</td>
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<tr>
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<td>26°C</td>
<td>0.917</td>
<td>0.800, 0.977</td>
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</tbody>
</table>
3.5 Discussion

We demonstrate for the first time that the wMel and wMelPop-CLA infections of *Ae. aegypti* exhibit incomplete cytoplasmic incompatibility when immature stages experience cyclical temperatures of 26-37°C during development. We also show that these infections are not transmitted to the next generation when infected mosquitoes experience these conditions over their entire lifecycle. wMel infected mosquitoes are currently being deployed in several countries for the control of arboviruses (http://www.eliminatedengue.com/project). Immature *Ae. aegypti* may experience extreme temperatures in the field that are similar to the conditions used in our study (RICHARDSON et al. 2013; RITCHIE et al. 2015); the thermal sensitivity of the wMel and wMelPop-CLA infections could therefore reduce their ability to establish and persist in natural populations. In contrast, the wAlbB infection retains its ability to induce complete cytoplasmic incompatibility under the same conditions, while maternal transmission fidelity remains relatively high. Densities of wAlbB are also stable, suggesting that it will also provide effective arboviral protection (BIAN et al. 2010; JOUBERT et al. 2016). The robustness of wAlbB when exposed to high maximum temperatures could make this infection more suited for field release in environments where temperatures in breeding sites fluctuate in comparison to wMel.

High temperatures have been known for some time to have a negative effect on *Wolbachia*. In other arthropods, high temperatures can reduce the density of *Wolbachia* in its host (WIWATANARATANABUTR AND KITTAYAPONG 2009; JEVAPRAKASH AND HOY 2010; BORDENSTEIN AND BORDENSTEIN 2011; SUGIMOTO et al. 2015), weaken the reproductive effects induced by *Wolbachia* (TRPIS et al. 1981; STOUTHAMER et al. 1990; CLANCY AND HOFFMANN 1998; JOHANOWICZ AND HOY 1998; FEDER et al. 1999; HURST et al. 2000) and even eliminate *Wolbachia* entirely (TRPIS et al. 1981; STEVENS 1989; STOUTHAMER et al. 1990; JOHANOWICZ AND HOY 1996; VAN OPIJNEN AND BREEUWER 1999). Only recently have the effects of temperature been characterised in experimental *Wolbachia* infections of *Ae. aegypti*. YE et al. (2016) reared wMel-infected larvae under diurnally cycling temperatures and assessed their vector competence and *Wolbachia* density. They concluded that the wMel infection should remain robust in terms of its ability to reduce dengue transmission under field conditions in Cairns, Australia. However, the authors only tested temperatures reaching a maximum of 32°C; we observed no effect on *Wolbachia* density or hatch rate under similar conditions. In nature, larvae and pupae are restricted to aquatic environments where average maximum temperatures can reach 37°C during the wet season in Cairns (RICHARDSON et al. 2013). Here we employed a larger temperature range to better reflect natural conditions in the field. Although we did not test vectorial capacity directly, we observed a greatly reduced density of wMel in adults when larvae experienced a maximum temperature of 37°C. These conditions will likely affect the viral suppression induced by wMel as the ability of *Wolbachia* to
interfere with transmission relies on high densities in relevant tissues (LU et al. 2012; OSBORNE et al. 2012).

In the majority of our experiments we exposed larvae to cyclical temperatures while maintaining adults and eggs at 26°C. However, we observed that when all life stages were maintained at 26-37°C the wMel and wMelPop-CLA infections were not transmitted to the next generation. The wAlbB infection also exhibited some maternal transmission leakage despite maintaining high densities and complete cytoplasmic incompatibility when only larvae were exposed. When we reared larvae under low nutrition conditions, the density of wMel and wMelPop-CLA was reduced even at 26-32°C. This suggests that both the duration of exposure and the maximum temperature reached will affect Wolbachia density, and the effects of heat stress could be exacerbated when combined with nutritional stress. ULRICH et al. (2016) provide additional evidence that the timing of heat stress is important; lowest wMel densities corresponded with the longest stress duration in immature Ae. aegypti, and densities varied considerably depending on their developmental stage at the time of exposure. More work is needed in these areas particularly as conditions and responses in the field are likely to be diverse.

We find that the wMel and wMelPop-CLA infections differ markedly from wAlbB in their response to heat stress; to our knowledge this is the first comparison of high temperature responses between multiple Wolbachia infections in the same host. The wAlbB infection also appears to have a higher optimum temperature than the other strains as its density at 26-32°C was higher than at 26°C. Differences in heat tolerance and preferred temperatures could result from different evolutionary histories; wMel and wMelPop-CLA are nearly genetically identical (SUN et al. 2003; WU et al. 2004; CHROSTEK et al. 2013) and originate from the same host, D. melanogaster (HOFFMANN 1988; MIN AND BENZER 1997). wAlbB occurs naturally in Ae. albopictus, a mosquito native to south-east Asia (BENEDICT et al. 2007; WIWATANARATANABUTR AND ZHANG 2016); this infection may have evolved a relatively higher heat tolerance and in response to the temperatures experienced by Ae. albopictus in its historical distribution. wAlbB density decreases only slightly when naturally infected Ae. albopictus are reared at a constant 37°C (WIWATANARATANABUTR AND KITTAYAPONG 2006; WIWATANARATANABUTR AND KITTAYAPONG 2009). The effects of high temperatures on the density of wMel and wMelPop-CLA in their natural host are however unknown. Whether there is an influence of the host on Wolbachia’s thermal tolerance requires further investigation.

While both wMel and wMelPop-CLA-infected males partly lost the ability to cause cytoplasmic incompatibility and exhibited a markedly reduced Wolbachia density when reared at 26-37°C, crosses between infected females and males reared at 26-37°C had different outcomes for the two infections. The reduced hatch rates in wMel may reflect the fact that infected males exposed to 26-37°C partly
maintain the ability to cause cytoplasmic incompatibility, whereas females reared under these temperatures have lost much of their ability to restore compatibility. For wMelPop-CLA which maintained a relatively high hatch rate regardless of temperature, the higher density of this infection compared to wMel (WALKER et al. 2011; AXFORD et al. 2016) may have allowed females to largely maintain compatibility even when reared under the 26-37°C regime. This requires further testing and other factors such as tissue tropism might also be involved.

The differential responses of Wolbachia infection types under heat stress may arise from factors other than their ability to tolerate high temperatures. Wolbachia densities can be influenced by interactions with WO, temperate bacteriophage which infect Wolbachia (GAVOTTE et al. 2007). Temperate phage undergo lysogenic and lytic cycles, the latter of which can be induced by heat shock (BORDENSTEIN AND BORDENSTEIN 2011; SHAN et al. 2014). During the lytic cycle, phage replicate and infect new Wolbachia cells, potentially reducing densities of Wolbachia through cell lysis (KENT AND BORDENSTEIN 2010). High densities of lytic phage reduce the density of Wolbachia and the strength of cytoplasmic incompatibility in the parasitoid wasp Nasonia vitripennis (BORDENSTEIN et al. 2006). Therefore, high temperatures may reduce Wolbachia densities in Ae. aegypti through the same mechanism. WO phage infect wMel (GAVOTTE et al. 2004) and wAlbB (CHAUVATCHARIN et al. 2006; TORTOSA et al. 2008) in their native hosts, but it is unknown if they persist following transfer to Ae. aegypti, though WO phage can be maintained upon interspecific transfer of Wolbachia in moths (FUJII et al. 2004). This too requires further investigation.

While the mechanism for the loss of Wolbachia at high temperatures is unknown, our results strongly suggest that the ability of wMel and wMelPop-CLA-infected Ae. aegypti to invade and persist in natural populations will be adversely affected by heat. We observed reduced cytoplasmic incompatibility and maternal transmission fidelity at cyclical temperatures approximating breeding containers in the field; constant high temperatures are therefore not needed to have adverse effects on Wolbachia. Incomplete cytoplasmic incompatibility and/or maternal transmission fidelity of Wolbachia will reduce the speed of invasion, increase the minimum infection threshold required for invasion to take place and decrease the maximum frequency that can be reached in a population (TURELLI AND HOFFMANN 1999). Maximum daily temperatures of larval mosquito habitats in nature can reach or exceed the maximum temperature tested in this study (PAAIJMANS et al. 2008; VEZZANI AND ALBICOCCO 2009; RICHARDSON et al. 2013) and this should be a careful consideration for additional research in this area. Though Wolbachia densities may partially recover if adults can avoid extreme temperatures (ULRICH et al. 2016), the loss of cytoplasmic incompatibility can still occur even when adults are returned to low temperatures for several days before mating, as we demonstrate here. These findings could in part contribute to the persistence of uninfected individuals in two populations near Cairns, Australia that were invaded by the wMel infection five years ago (HOFFMANN et al. 2014a). Mosquito suppression strategies which use Wolbachia-infected males as a
sterile insect may also be impacted by temperature, though results suggest males reared in the laboratory at lower temperatures are more likely to succeed in generating sterility.

As releases of *Ae. aegypti* infected with *wMel* are currently underway in several countries, researchers should assess the impact of heat stress on *Wolbachia* infections in the field. Surveys of temperature fluctuations and productivity in various container types should be conducted in planned release areas, as our current understanding of microclimate in breeding sites is limited. Our findings emphasize the need for further characterization of current *Wolbachia* infections under a range of temperature conditions, particularly in terms of the duration of exposure to extreme temperatures and the effects across generations. An enormous diversity of *Wolbachia* strains exist in nature (HOFFMANN et al. 2015); alternative strains, or current infections selected for increased thermal tolerance (PINTUREAU et al. 1999), should be considered. Our results also highlight the importance of temperature control in the laboratory rearing of *Wolbachia*-infected insects. Heat stress could be used to cure the *wMel* and *wMelPop-CLA* infections from mosquitoes in order to study their effects (VAN OPIJNEN AND BREEUWER 1999) as an alternative to tetracycline (DOBSON AND RATTANADECHAKUL 2001). A better understanding of the response of *Wolbachia* infections to varying environmental conditions is required particularly in the context of laboratory rearing and in their application as an arboviral biocontrol agent in the field.

### 3.6 Materials and methods

#### 3.6.1 Ethics statement

Blood feeding on human subjects was approved by the University of Melbourne Human Ethics Committee (approval 0723847). All volunteers provided informed written consent.

#### 3.6.2 Colony maintenance and *Wolbachia* infections

Uninfected *Aedes aegypti* mosquitoes were collected from Townsville, Queensland, in November 2015 and maintained in a temperature controlled insectary at 26°C ± 1°C according to methods described by AXFORD et al. (2016). *Aedes aegypti* with the *wMel*, *wMelPop-CLA* and *wAlbB* infections of *Wolbachia* were derived from lines transinfected previously (XI et al. 2005; MCMENIMAN et al. 2009; WALKER et al. 2011). Females from all *Wolbachia*-infected lines were crossed to males from the Townsville line for three generations in succession to control for genetic background. Female mosquitoes were blood fed on the forearms of human volunteers.
3.6.3 Rearing at cyclical temperatures

We chose diurnally fluctuating temperature regimes for our experiments based on water temperatures observed in breeding containers during the wet season in Cairns, Australia (RICHARDSON et al. 2013). Larvae for all experiments were reared in incubators (PG50 Plant Growth Chambers, Labec Laboratory Equipment, Marrickville, NSW, Australia) set to a constant 26°C or to one of the following cyclical temperatures: 26-32°C, 26-34.5°C and 26-37°C at a 12:12 light: dark photoperiod. Cycling incubators were set to maintain 26°C during the dark period and the maximum temperature during light, with 12 hours at each temperature. Water temperatures were monitored by placing data loggers (Thermochron; 1-Wire, iButton.com, Dallas Semiconductors, Sunnyvale, CA, USA) in sealed glass vials, which were submerged in plastic trays (11.5 × 16.5 × 5.5 cm) filled with 500 mL of water identical to larval rearing trays. Temperature was measured at 30 minute intervals. Representative daily temperature fluctuations that occurred in each incubator for the duration of the experiments are shown in figure B.1. Rearing at cyclical temperatures of 26-32°C or 26-37°C decreased the wing length of adults (Figure B.2), suggesting they were heat stressed (TUN-LIN et al. 2000).

For each experiment, eggs from the uninfected, wMel, wMelPop-CLA and wAlbB lines were hatched synchronously in 3 L trays of RO water at 26°C. Hatching trays were transferred to incubators within two hours of hatching. Larvae were provided TetraMin tropical fish food tablets (Tetra, Melle, Germany) ad libitum and maintained at a controlled density of 100 larvae in 500 mL water. Temperatures in each incubator deviated by up to ± 0.5°C from the set-point, depending on the location of data loggers. We randomised the location of rearing trays within incubators repeatedly during experiments to account for positional effects.

3.6.4 Hatch rate and cytoplasmic incompatibility

Crosses between Wolbachia infection types were conducted to determine the proportion of viable offspring from parents reared at different cyclical temperatures. Pupae were sexed according to size (females are larger than males) and added to 12 L cages held at 26°C ± 1°C within 24 hours of eclosion after confirming their sex. Sexes, infection types and adults reared at each temperature were maintained in separate cages. Adults were allowed to mature and acclimatise to 26°C for at least 48 hours; crosses were conducted only when all adults were at least 48 hours old as development times varied between sexes and rearing temperatures. After the period of maturation, 7 males and 7 females from their respective infection type were aspirated into 1.5 L cages and allowed to mate for 3 days. Each cross was comprised of 6 replicate cages; the combinations of sex, rearing temperature and Wolbachia infection status for each cross are described in the results section. Each cage was provided
with water for the duration of the experiment, and sugar until 24 h prior to blood feeding. Females were provided a blood meal through mesh on the side of each cage until all females had fed to repletion. Multiple human volunteers were used, with one volunteer per replicate cage. Pill cups were filled with 25 mL of water and lined with filter paper (Whatman 90mm qualitative circles, GE Healthcare Australia Pty. Ltd., Parramatta, New South Wales, Australia) and provided as an oviposition substrate. Eggs laid on filter papers were collected daily, dried on paper towel and photographed with a digital camera. The number of eggs laid was determined with a clicker counter. Eggs were hatched in containers of 200 mL of water four days after collection, and larvae were reared to the 3rd instar. Hatch proportions were defined as the number of larvae counted, including larvae that hatched precociously (visible on the filter papers).

3.6.5 Wolbachia quantification

The density of Wolbachia in adults reared at cyclical temperatures was determined for the wMel, wMelPop-CLA and wAlbB infections. We reared three trays of 100 larvae per infection type at 26°C, 26-32°C and 26-37°C (see “Rearing at cyclical temperatures”). Eclosing adults were collected daily at noon and stored in absolute ethanol for DNA extraction. We selected 10 males and 10 females at random per tray for Wolbachia quantification. DNA extraction and Wolbachia quantification were conducted according to methods described previously (LEE et al. 2012; HOFFMANN et al. 2014a; AXFORD et al. 2016). DNA from adults with both wings removed was extracted using 150 µL of 5% Chelex 100 resin (Bio-Rad Laboratories, Hercules, CA). We used a LightCycler 480 system (Roche Applied Science, Indianapolis, IN) to amplify mosquito-specific (mRpS6), Ae. aegypti-specific (aRpS6) and Wolbachia-specific (w1, wAlbB or wMelPop) genes (Table B.1). Mosquitoes used for Wolbachia quantification were considered positive for Wolbachia when there was robust amplification of mRpS6, aRpS6 and the appropriate Wolbachia-specific marker. Three technical replicates of the aRpS6 and Wolbachia-specific markers were completed for each mosquito; differences in crossing point between the two markers were averaged to obtain an estimate of Wolbachia density. These values were then transformed by $2^n$ to obtain relative Wolbachia densities.

3.6.6 Maternal transmission of Wolbachia

We tested the ability of wMel, wMelPop-CLA and wAlbB-infected females to transmit Wolbachia infections to their offspring. Wolbachia-infected females were reared from the egg stage in incubators set to a constant 26°C or a cycling 26-37°C (see “Rearing at cyclical temperatures”) and crossed to
uninfected males. Females were blood-fed en masse and isolated in 70 mL plastic cups filled with 20 mL of water and lined with a 2 × 12 cm strip of sandpaper (Norton Master Painters P80 sandpaper, Saint-Gobain Abrasives Pty. Ltd., Thomastown, Victoria, Australia). Females maintained at 26-37°C were split into two groups; eggs from one group of females stayed at 26-37°C while eggs from the other group were moved to the constant 26°C incubator. Eggs were hatched, progeny were reared to 3rd or 4th instar, stored in ethanol, then tested for the presence of Wolbachia (see “Wolbachia quantification”). We scored 10 offspring from 8 females per infection type at each temperature, except for progeny that were transferred from 26-37°C to 26°C where we scored 10 offspring from 5 females.

3.6.7 Statistical analyses

All analyses were conducted using SPSS statistics version 21.0 for Windows (SPSS Inc, Chicago, IL). Hatch proportions and Wolbachia densities were not normally distributed according to Shapiro-Wilk tests, therefore we analyzed all data with nonparametric Kruskal-Wallis and Mann-Whitney U tests.

3.7 Acknowledgements

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CHAPTER 4 – NEGATIVE RESPONSE OF WOLBACHIA STRAIN wMel TO SELECTION FOR INCREASED THERMAL TOLERANCE

4.1 Abstract

Aedes aegypti mosquitoes infected with the wMel strain of Wolbachia are being deployed by the World Mosquito Program to control the spread of arboviruses around the world. The success of this program relies on the stability of the wMel strain in natural mosquito populations in the tropics. Previous laboratory studies demonstrate that wMel is susceptible to cyclical heat stress during mosquito larval development. We attempted to generate a heat-resistant strain of wMel in Ae. aegypti through artificial laboratory selection. wMel-infected females reared under cyclical heat stress were crossed to wMel-infected males reared at 26°C. The low proportion of larvae that hatched founded the next generation, and this process was repeated for five generations. After two relaxed generations we tested the ability of the wMel heat-selected strain (wMel-HS) to induce cytoplasmic incompatibility in crosses with uninfected females and restore compatibility in crosses with wMel-infected males. Despite expectations that the wMel infection would improve its thermal tolerance, some negative effects were observed. wMel-HS was similar to wMel (unselected) in its ability to induce cytoplasmic incompatibility and restore compatibility when larvae were reared under cyclical heat stress. However, we observed incomplete cytoplasmic incompatibility by wMel-HS males at 26°C, and reduced Wolbachia density in males relative to wMel under all temperature conditions. Egg hatch proportions did not improve over successive generations and the infection was lost during the seventh generation of selection. Our results suggest that the capacity for the wMel infection in Ae. aegypti to adapt to high temperatures is limited and is unlikely to improve during deployment in the field, at least in the short-term. Though the impact of heat stress on the wMel infection in natural larval habitats remains to be tested, Wolbachia-based arbovirus control efforts should also consider deploying alternative strains that are more thermally robust.

4.2 Introduction

The open field release of Wolbachia-infected mosquitoes is a promising approach to control the spread of arboviruses and has gained considerable attention in recent years. Wolbachia are a diverse genus of intracellular bacteria that often alter the reproduction of their hosts to benefit their own transmission (Werren et al. 2008). Wolbachia infections can protect their hosts against pathogens, and this feature has been exploited to generate strains of mosquitoes with a reduced ability to transmit arboviruses (Moreira et al. 2009a; Bian et al. 2010). The principal vector of dengue, the Aedes aegypti mosquito, has now been infected experimentally with several strains of Wolbachia originating
from other insects (XI et al. 2005; RUANG-AREERATE AND KITTAYAPONG 2006; MOREIRA et al. 2009a; WALKER et al. 2011; JOUBERT et al. 2016; FRASER et al. 2017). These Wolbachia strains induce cytoplasmic incompatibility which greatly reduces or eliminates the production of viable offspring by uninfected females that mate with Wolbachia-infected males. This feature combined with Wolbachia-induced viral suppression has prompted the deployment of Ae. aegypti with artificial Wolbachia infections into the field in areas where dengue and other arboviruses are endemic. Efforts such as the World Mosquito Program (formerly Eliminate Dengue) (www.worldmosquitoprogram.org) and Wolbachia Malaysia (http://repository.imr.gov.my/wolbachia) aim to replace natural populations of Ae. aegypti mosquitoes with Wolbachia-infected mosquitoes that are less receptive to arbovirus transmission, while other groups aim to utilize cytoplasmic incompatibility to suppress mosquito populations through the release of only males (https://blog.debug.com).

The suitability of a given Wolbachia strain for population replacement programs will depend largely on its effects on mosquito fitness, the fidelity of maternal transmission and cytoplasmic incompatibility and its ability to block arboviruses in field populations (FERGUSON et al. 2015; TURELLI AND BARTON 2017). The wMel strain originating from Drosophila melanogaster is currently the only strain being deployed by the World Mosquito Program in their arbovirus control efforts. This strain was chosen for its relative lack of fitness costs and its ability to induce complete cytoplasmic incompatibility, inhibit dengue virus replication and be transmitted maternally with perfect fidelity in the laboratory (WALKER et al. 2011). wMel-infected Ae. aegypti have now established in multiple field populations in Queensland, Australia following field deployment (HOFFMANN et al. 2011; SCHMIDT et al. 2017). Infected mosquitoes persisted at high frequencies in release zones years after releases ceased (HOFFMANN et al. 2014a; SCHMIDT et al. 2017) and have retained their ability to inhibit dengue virus replication (FRENTIU et al. 2014) and induce cytoplasmic incompatibility (HOFFMANN et al. 2014a). In recent years, wMel-infected Ae. aegypti have been released in further trials in Asia, Oceania and South America (http://www.eliminatedengue.com/project), though the outcomes of releases outside of Australia are yet to be published.

The success of wMel demonstrates the utility of Wolbachia infections for arbovirus control, but some limitations of this strain could hamper its ability to invade natural populations and block viral transmission in certain environments. The wMel infection can reduce Ae. aegypti fecundity (TURLEY et al. 2013; HOFFMANN et al. 2014a; ROSS et al. 2016), longevity (AXFORD et al. 2016), egg hatch rate (DUTRA et al. 2015; JOUBERT et al. 2016) and larval development and survival under competitive conditions (ROSS et al. 2014; ROSS et al. 2016), though some studies observe fitness benefits or find no effect of wMel infection (DUTRA et al. 2016a; KHO et al. 2016; DE OLIVEIRA et al. 2017a). The wMel infection appears to be less costly than other strains (AXFORD et al. 2016; JOUBERT et al. 2016;
ROSS et al. 2016), but even minor fitness costs can impair the ability of infected mosquitoes to invade natural populations (CRAIN et al. 2011).

Environmental conditions such as temperature can have an enormous impact on Wolbachia infections and other insect symbionts (CORBIN et al. 2016) and therefore may have a potential impact on viral inhibition by Wolbachia. Heat stress can disrupt and deform the cellular membranes of Wolbachia, altering morphology and leading to degeneration (ZHUKOVA et al. 2008; STRUNOV et al. 2013a). In Ae. aegypti, cyclical heat stress reduces the ability of wMel to induce cytoplasmic incompatibility, lowers its density in the mosquito and can even eliminate the infection from subsequent generations (ULRICH et al. 2016; ROSS et al. 2017c). Incomplete maternal transmission of wMel was recently reported in an Australian field population (SCHMIDT et al. 2017), indicating that the wMel infection is less stable under field conditions than in the laboratory. This instability could result from heat stress experienced in mosquito larval habitats. wMel in its native D. melanogaster host is also temperature-sensitive, where its abundance relative to other microbes is greatly reduced when flies develop at 31°C (MOGHADAM et al. 2017). The thermal sensitivity of Wolbachia infections are variable and can differ considerably between host species (CORBIN et al. 2016). In Ae. aegypti, the wAlbB infection is more stable than wMel and wMelPop in its reproductive effects at high temperatures; Wolbachia strains in the same host can therefore also vary in their response to heat stress (ROSS et al. 2017c).

Wolbachia and other symbionts can exhibit a large amount of genetic diversity within a single insect host (SYMULA et al. 2013; RUSSELL AND CAVANAUGH 2017), and with this variation brings the potential for selection. There are now several demonstrations of Wolbachia infections rapidly adapting to changes in environment. Upon interspecific transfer, the Wolbachia strain wMelPop decreased its virulence dramatically in response to a novel host environment (MCGRRAW et al. 2002; MCMENIMAN et al. 2008; CARRINGTON et al. 2010). Wolbachia infections can also evolve rapidly in natural environments. The wRi strain originally inflicted a fecundity cost on its natural host D. simulans, but evolved in later years to provide a fecundity benefit (WEEKS et al. 2007). Experimental evolution studies also demonstrate rapid adaptation. D. melanogaster populations selected for increased survival after viral challenge led to the fixation of more protective Wolbachia variants (FARIA et al. 2016). Selection for Wolbachia variants with increased Octomom copy numbers increased the density and virulence of Wolbachia (CHROSTEK AND TEIXEIRA 2015) and selection can act within an individual host (CHROSTEK AND TEIXEIRA 2018). Experimental serial horizontal transfer of Wolbachia in Armadillidium vulgare resulted in a rapid increase of virulence and Wolbachia density (LE CLECH et al. 2017). Parthenogenic Trichogramma cordubensis exhibit variability in their response to high temperature conditions, and selection under a constant 30°C increased the strength of parthenogenesis under heat stress (PINTUREAU et al. 1999). In D. melanogaster, frequencies of different Wolbachia variants changed dramatically when flies were maintained at different
temperatures (VERSACE et al. 2014) and there is evidence of selection on the wMel strain for genes encoding heat shock proteins (BROWNLIE et al. 2007). These studies demonstrate that Wolbachia infections can evolve in response to a changing environment. Experimental evolution could therefore be used as a tool to select Wolbachia infections in Ae. aegypti mosquitoes for desirable attributes, improving their utility for arbovirus control applications.

In this study we subjected Ae. aegypti infected with wMel to cyclical heat stress over successive generations to improve the thermal tolerance of wMel. The generation of a heat-tolerant strain would demonstrate the potential for experimental Wolbachia infections in mosquitoes to adapt to heat stress in the field, and the strain could be deployed as an alternative to the current wMel strain. However, intense selection over five generations yielded no improvement in the response of the strain to heat stress. Contrary to expectations, the selected strain exhibited incomplete cytoplasmic incompatibility and the density of Wolbachia in males decreased. While there might be other ways to increase the tolerance of the wMel infection to heat stress, it seems likely that wMel in Ae. aegypti lacks sufficient variation in thermal tolerance to respond to direct selection.

4.3 Materials and methods

4.3.1 Ethics statement

Blood feeding on human subjects was approved by the University of Melbourne Human Ethics Committee (approval 0723847). All volunteers provided informed written consent.

4.3.2 Mosquito strains and colony maintenance

Ae. aegypti mosquitoes with the wMel infection were collected in 2013 from locations near Cairns, Australia where wMel had successfully established (HOFFMANN et al. 2011). Uninfected Ae. aegypti were collected in 2016 from locations where wMel-infected mosquitoes had not been released. The wAlbB infection was used for comparisons in cytoplasmic incompatibility experiments and is described in (XI et al. 2005) and (AXFORD et al. 2016). Wolbachia-infected mosquitoes were crossed to uninfected mosquitoes for at least three consecutive generations to ensure similar genetic backgrounds before use in experiments. Colonies were maintained in the laboratory at 26°C ± 1°C according to (ROSS et al. 2017a).
4.3.3 Selection regime

We wanted to see if the wMel infection could evolve increased thermal tolerance by subjecting infected *Ae. aegypti* to cyclical heat stress over successive generations. First instar wMel-infected larvae were placed in incubators (PG50 Plant Growth Chambers, Labec Laboratory Equipment, Marrickville, NSW, Australia) that were set to cycle diurnally between 26°C and 37°C, with 12 hours at each temperature, according to (ROSS et al. 2017c). Up to four trays with 100 larvae each in 500 mL of RO (reverse osmosis) water were reared under these conditions each generation. Data loggers (ThermoChron; 1-Wire, iButton.com, Dallas Semiconductors, Sunnyvale, CA, USA) in zip-lock bags were placed in each tray to monitor the temperature. Larvae were provided with TetraMin® tropical fish food tablets (Tetra, Melle, Germany) *ad libitum* until reaching pupation. Females were separated from males at the pupal stage and returned to 26°C for adult emergence, and males were discarded. Adult females were held at 26°C for three days before being crossed to wMel-infected males (unselected) reared at 26°C. Females were blood-fed on a single human volunteer, and all eggs were hatched in 3 L trays of RO water with a few grains of yeast. The resulting progeny founded the next generation and the selection regime was repeated. Subsequent gonotrophic cycles were initiated if more eggs were needed.

Crosses with wMel-infected males reared at 26°C were chosen to produce low hatch proportions, resulting from partial cytoplasmic incompatibility. This increased the intensity of selection as only females that restored compatibility when reared under heat stress would contribute to the next generation, eliminating any individuals that lost their *Wolbachia* infection. Hatch proportions also provided an indicator of the response to selection; high hatch proportions in subsequent generations would suggest an improvement in heat tolerance.

After each generation of selection, a subset of progeny was reared at 26°C as a backup in case the *Wolbachia* infection from the selected line was lost during the next selection event. After two generations of selection, all progeny were reared at 26°C to increase the population size as hatch proportions were low (Table 4.1). After five generations of selection, a subset of progeny was reared at 26°C for two generations, where females from the selected lines were crossed to wMel (unselected) males. The resulting progeny were then used for cytoplasmic incompatibility experiments and measured for *Wolbachia* density. Selection continued until the eighth generation where the experiment was terminated due to a lack of selection response and loss of *Wolbachia* infection from the selected line (Table 4.1). Egg hatch proportions were determined by observation under a dissecting microscope, and were recorded each generation from all cages. Hatched eggs had a clearly detached cap and no larva inside.
4.3.4 Cytoplasmic incompatibility and restoration of compatibility

After five generations of selection followed by two relaxed generations, we tested the ability of the selected wMel strain (from now on referred to as wMel-HS) to induce cytoplasmic incompatibility in crosses with uninfected females and restore compatibility in crosses with wMel-infected males. Larvae from the wMel, wMel-HS and wAlbB lines were reared at a constant 26°C or in incubators set to cycle diurnally between 26°C and 37°C, with 12 hours at each temperature. Uninfected larvae were reared at 26°C only for crosses with infected males. We generated two heat stress treatments which differed in the maximum daily temperature reached by 1°C by utilizing variation within the incubator. Trays were reared on different shelves of the same incubator, reaching average maximum daily temperatures of 36.5°C and 37.5°C respectively (Figure C.1). Variation between containers on the same shelf was negligible, where they were cycled in their position daily. After five days, larvae from the heat stressed treatments were returned to 26°C for pupation and adult emergence. Four trays with 100 larvae each were reared for each line and each temperature treatment.

Adults from each sex, temperature and infection status were maintained separately at 26°C for three days. We then set up nine crosses to test the ability of males from each infected line under each rearing condition to induce cytoplasmic incompatibility with uninfected females. We set up a further nine crosses with females from each infected line under each rearing condition to test their ability to restore compatibility with infected males. For crosses with females from the wMel and wMel-HS lines, males from the unselected wMel line were used. For crosses with wAlbB females, wAlbB males were used. We added 25 males and 25 females to 1.5 L cages for each cross and allowed two days to mate. After blood feeding, 20 females from each cross were isolated in 70 mL cups containing 20 mL of larval rearing water and lined with sandpaper for oviposition. Eggs were collected from each cup four days after blood feeding, partially dried, then hatched four days after collection. After one week, females were blood fed again and eggs were collected and hatched from a second gonotrophic cycle.

4.3.5 Wolbachia quantification

wMel and wMel-HS adults reared under the same conditions as the cytoplasmic incompatibility experiment were stored in absolute ethanol within 24 hr of emergence and measured for their Wolbachia density. DNA was extracted from twenty adults from each sex and infection, and Wolbachia density was estimated with qPCR using previously described methods (LEE et al. 2012; ROSS et al. 2017c).
4.3.6 Statistical analysis

All data were analysed in SPSS statistics version 24.0 for Windows (SPSS Inc, Chicago, IL). Egg hatch proportions and Wolbachia densities were compared with nonparametric Kruskal-Wallis and Mann-Whitney U tests, as data were not normally distributed according to Shapiro-Wilk tests. Fecundity data were normally distributed and were analysed with ANOVAs. We used a general linear model to test for an effect of female age on egg hatch proportions during the selection experiment.

4.4 Results

4.4.1 Egg hatch proportions during selection

We monitored egg hatch proportions each generation as an indication of the selection response. Overall hatch proportions during first generation of selection were intermediate and varied considerably between individual females, but from the second generation of selection onwards they fell consistently below 4% (Table 4.1). The wMel infection was lost in the selected line after the seventh generation of heat stress. No eggs hatched, and Wolbachia was not detected by qPCR in a sample of 30 individuals from the population.

| Table 4.1 Average maximum temperatures and egg hatch rates of wMel-infected Ae. aegypti females each generation during the selection experiment. | Females reared at the specified temperature were crossed to wMel-infected males reared at 26°C. Generation 3 (shaded in grey) is a relaxed generation. Only eggs and egg hatch proportions from cages that contributed to the next generation are shown. |
|---|---|---|---|---|---|---|---|---|
| Generation | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 |
| Mean maximum daily temperature | 37.2°C | 37.3°C | 26°C | 36.9°C | 36.9°C | 37.0°C | 36.8°C | 36.8°C |
| Egg hatch (wMel-HS ♀ × wMel 26°C ♂) | 30.02% | 2.64% | 69.88% | 2.12% | 2.87% | 2.90% | 3.67% | 0% |
| Eggs scored | 991 | 4040 | 934 | 2113 | 2115 | 8396 | 4741 | 4183 |
Egg hatch proportions of females from the first generation of heat stress increased dramatically in subsequent gonotrophic cycles (Kruskal-Wallis: $\chi^2 = 26.616$, df = 2, $P < 0.001$, Figure 4.1A). This restoration of compatibility over time indicates a recovery of *Wolbachia* within the female. However, we observed no such recovery in subsequent selection generations when initial hatch proportions were much lower (Figure 4.1B), or during the cytoplasmic incompatibility experiment, except for the $w$AlbB infection (Figure 4.3). Egg hatch proportions of females from selection generations 2-7 were consistently low and did not change with female age according to a general linear model ($F_{1,65} = 0.629$, $P = 0.841$, Figure 4.1B). We also tested to see if hatch proportions would increase when heat stressed females were aged before mating with *Wolbachia*-infected males, but found no improvement with female age (Figure C.2).

**Figure 4.1** Proportion of eggs hatching from crosses between $w$Mel-infected *Ae. aegypti* females reared under heat stress and $w$Mel-infected males reared at 26°C during the selection experiment. (A) Egg hatch proportions from single females during the first generation of selection were measured across three gonotrophic cycles. Black lines indicate the median hatch proportion for each gonotrophic cycle. (B) Egg hatch proportions across all cages maintained during selection generations 2-7. Each data point represents the proportion of eggs hatching from a single collection of eggs (median number 378, range: 53-986). Eggs were collected over a period of 15 days and over multiple gonotrophic cycles to obtain as many viable progeny as possible. The black line indicates the median hatch proportion across all cages for a given female age.
4.4.2 Cytoplasmic incompatibility in males reared under heat stress

After five generations of selection, we tested the ability of wMel-HS males reared under different temperature conditions to induce cytoplasmic incompatibility (Figure 4.2). When larvae were reared at 26°C, wMel and wAlbB males induced complete cytoplasmic incompatibility with uninfected females; no eggs hatched over two gonotrophic cycles. In contrast, some females that mated with wMel-HS males produced a low proportion of viable offspring, indicating incomplete cytoplasmic incompatibility induction by wMel-HS males. wMel and wMel-HS males induced partial cytoplasmic incompatibility when reared under heat stress, with weaker cytoplasmic incompatibility (and higher hatch proportions) under the more stressful temperature condition (Mann-Whitney U: Z = 5.545, P < 0.001). Egg hatch proportions from crosses with wMel and wMel-HS males did not differ from each other at 26-36.5°C (Z = 0.781, P = 0.435) or 26-37.5°C (Z = 1.419, P = 0.156), indicating a lack of response to selection. Hatch proportions also did not differ between gonotrophic cycles 1 and 2 for wMel (Z = 0.501, P = 0.617) or wMel-HS (Z = 0.123, P = 0.904) when males were reared under heat stress. In contrast to wMel and wMel-HS, wAlbB males induced complete cytoplasmic incompatibility under all rearing conditions tested, with no eggs hatching in either gonotrophic cycle.
Figure 4.2 Egg hatch proportions of uninfected female *Ae. aegypti* when crossed to males from the wMel, wMel-HS and wAlbB lines reared under different temperature conditions. Males from each infection type were reared at a constant 26°C, or held in incubators that cycled diurnally between 26°C and 36.5°C or 26°C and 37.5°C. Egg hatch proportions were measured over both the first (A) and second (B) gonotrophic cycles. Black horizontal lines indicate median hatch proportions.
4.4.3 Restoration of compatibility by females reared under heat stress

We tested the ability of \textit{wMel}-HS females reared under different temperature conditions to restore compatibility in crosses with infected males (Figure 4.3). Hatch proportions of infected females reared at 26°C were consistently high, and did not differ between the \textit{wMel}, \textit{wMel}-HS and \textit{wAlbB} lines (Kruskal-Wallis: \( \chi^2 = 5.533, \text{df} = 2, P = 0.063 \)). Egg hatch proportions declined when females were reared under heat stress conditions for the \textit{wMel} (Mann-Whitney U: \( Z = 7.416, P < 0.001 \)) and \textit{wMel}-HS lines (\( Z = 7.720, P < 0.001 \)) and to a lesser extent the \textit{wAlbB} line (\( Z = 5.004, P < 0.001 \)). The loss of compatibility was particularly severe at 26-37.5°C where median hatch rates were below 4% for the \textit{wMel} and \textit{wMel}-HS lines. Egg hatch proportions did not differ between the \textit{wMel} and \textit{wMel}-HS lines at 26-36.5°C (\( Z = 1.016, P = 0.308 \)) or 26-37.5°C (\( Z = 0.243, P = 0.810 \)), indicating a lack of selection response. Egg hatch proportions did not increase for heat-stressed \textit{wMel} (\( Z = 0.401, P = 0.689 \)) or \textit{wMel}-HS (\( Z = 0.422, P = 0.674 \)) females in the second gonotrophic cycle, but did increase for \textit{wAlbB} (\( Z = 2.131, P = 0.033 \)), suggesting that \textit{Wolbachia} strains can differ in their ability to recover from heat stress. The fecundity of females in this experiment did not differ between \textit{Wolbachia} infection types (one-way ANOVA: \( F_{2,154} = 0.284, P = 0.753 \)), but was greatly reduced in females reared under cyclical heat stress (\( F_{1,154} = 286.067, P < 0.001, \) Figure C.3).
Figure 4.3 Egg hatch proportions of female *Ae. aegypti* from the wMel, wMel-HS and wAlbB lines when reared under different temperature conditions and then crossed to infected males reared at 26°C. Females from each infection type were reared at a constant 26°C, or held in incubators that cycled diurnally between 26°C and 36.5°C or 26°C and 37.5°C. Egg hatch proportions were measured over both the first (A) and second (B) gonotrophic cycles. Black horizontal lines indicate median hatch proportions.
4.4.4 Wolbachia density

We wanted to see if Wolbachia density had changed in response to selection. We estimated Wolbachia density in whole wMel and wMel-HS adults when reared at a constant 26°C or under two cyclical temperature conditions. Wolbachia density was greatly reduced under heat stress in both wMel (Mann-Whitney U: Z = 8.899, P < 0.001) and wMel-HS (Z = 8.885, P < 0.001) lines (Figure 4.4). wMel-HS males had lower Wolbachia densities than wMel males at 26°C (Z = 3.544, P < 0.001), 26-36.5°C (Z = 5.396, P < 0.001) and 26-37.5°C (Z = 3.746, P < 0.00). For females, Wolbachia density did not differ between the wMel and wMel-HS lines (all P > 0.05) except for when they were reared at 26-36.5°C, where wMel had a higher density than wMel-HS (Z = 4.720, P < 0.001).

Figure 4.4 Relative Wolbachia density of (A) females and (B) male Ae. aegypti from the wMel and wMel-HS lines reared at either a constant 26°C or one of two cyclical temperature conditions: 26-36.5°C and 26-37.5°C. Black horizontal lines indicate medians.

4.5 Discussion

In this study we attempted to generate a heat-resistant strain of wMel that could serve as an alternative Wolbachia variant for mosquito control programs. In response to intense selection, wMel did not improve its thermal tolerance and instead exerted weaker effects in males compared to the original wMel strain. Our study indicates that wMel infections in Ae. aegypti that are already established in the field are unlikely to adapt rapidly to heat stress in larval habitats. wMel infections in Australian field
populations have remained stable in their effects (FRENTIU et al. 2014; HOFFMANN et al. 2014a) but our experiments indicate that long-term exposure to heat stress could reduce the stability of wMel. Field releases of the wMel strain are now occurring in Indonesia, Sri Lanka and Vietnam (http://www.eliminatedengue.com/project) where maximum daily temperatures during the hottest times of year are higher than in release zones in Australia. Unfortunately, weather reports are not necessarily good indicators of microclimates in mosquito larval habitats (MURDOCK et al. 2017), thus careful monitoring will be required to determine if heat stress will be a concern for wMel release programs in these areas.

After five generations of selection, the wMel-HS line induced incomplete cytoplasmic incompatibility at 26°C and had a reduced density in males relative to wMel (unselected). Conversely, wMel-HS and wMel females did not differ in their density and reproductive effects under most conditions. This sex-specific response could be due to a lack of selection for the maintenance of Wolbachia in males, which do not transmit the infection to their offspring. On the other hand, there was strong selection for females to maintain their Wolbachia infection under heat stress every generation. In Ae. albopictus, the wAlbA infection is maintained at a high density in females but is often lost during adulthood in males (TORTOSA et al. 2010). Sex-specific differences have not been observed for any experimental Wolbachia infection in Ae. aegypti, but could potentially arise under conditions where cytoplasmic incompatibility is incomplete (TURELLI 1994). It is possible that selection was too strong and did not sufficiently capture the most heat tolerant variants of wMel. During the first selection event and the cytoplasmic incompatibility experiment, we observed considerable variation in egg hatch proportions by heat-stressed females, but this variation was much lower in subsequent selection generations, and at higher temperatures during experiments. Selection experiments under less stressful conditions could perhaps capture more variation.

Though the density of Wolbachia was reduced in wMel-HS males at 26°C, it was only a minor (1.47-fold) reduction relative to the wMel infection. Incomplete cytoplasmic incompatibility would be expected to occur only at much lower densities (such as a 168-fold reduction in wMel density at 36.5°C); it was therefore surprising to observe incomplete cytoplasmic incompatibility induced by wMel-HS males at 26°C. Reproductive effects induced by Wolbachia infections are strongly associated with Wolbachia density (CLANCY AND HOFFMANN 1998; DYER et al. 2005), therefore the loss of complete cytoplasmic incompatibility could be due to something other than a reduction in whole-adult Wolbachia density, such as changing patterns of tissue tropism.

We provide further evidence that the wAlbB infection is more robust than wMel in its response to cyclical heat stress during larval development (ROSS et al. 2017c), but it is not completely resistant. wAlbB-infected males retained their ability to induce complete cytoplasmic incompatibility, but infected females partially lost their ability to restore compatibility with infected males. This finding
contrasts with the wMelPop infection where males exhibit incomplete cytoplasmic incompatibility, but females seem to retain their ability to restore compatibility under heat stress (ROSS et al. 2017c).

wAlbB-infected females reared under heat stress improved their ability to restore compatibility in their second gonotrophic cycle. This indicates that the strength of cytoplasmic incompatibility is not fixed at insemination, but can recover over time, likely due to an increase in Wolbachia density (ULRICH et al. 2016). Hatch rates of eggs from wMel and wMel-HS females reared under heat stress also increased with the age of the female, but only in some experiments. The reason for differences between experiments is unclear, but may indicate that subtle changes in temperature conditions could prevent the recovery of Wolbachia over time.
CHAPTER 5 – A COMPREHENSIVE ASSESSMENT OF INBREEDING AND LABORATORY ADAPTATION IN Aedes aegypti Mosquitoes

5.1 Abstract

Modified Aedes aegypti mosquitoes reared in laboratories are being released around the world to control wild mosquito populations and the diseases they transmit. Several efforts have failed due to poor competitiveness of the released mosquitoes. We hypothesized that colonized mosquito populations could suffer from inbreeding depression and adapt to laboratory conditions, reducing their performance in the field. We established replicate populations of Ae. aegypti mosquitoes collected from Queensland, Australia, and maintained them in the laboratory for twelve generations at different census sizes. Mosquito colonies maintained at small census sizes (≤100 individuals) suffered from inbreeding depression due to low effective population sizes which were only 25% of the census size as estimated by SNP markers. Populations that underwent full-sib mating for 9 consecutive generations had greatly reduced performance across all traits measured. We compared the established laboratory populations with their ancestral population resurrected from quiescent eggs for evidence of laboratory adaptation. The overall performance of laboratory populations maintained at a large census size (400 individuals) increased, potentially reflecting adaptation to artificial rearing conditions. However most individual traits were unaffected, and patterns of adaptation were not consistent across populations. Differences between replicate populations may indicate that founder effects and drift affect experimental outcomes. Though we find limited evidence of laboratory adaptation, mosquitoes maintained at low population sizes can clearly suffer fitness costs, compromising the success of “rear and release” strategies for arbovirus control.

5.2 Introduction

Aedes aegypti mosquitoes transmit some of the most important arboviruses in the world, including dengue, Zika and chikungunya viruses. These diseases are an enormous burden to global health and the elimination or disruption of their vectors is currently the leading approach to their control. Several of these strategies rely on rearing and releasing modified mosquitoes into the environment to reduce disease incidence. The sterile insect technique has been used for decades to suppress mosquito populations, though many programs using this technique have not succeeded in achieving substantial population suppression (BENEDICT AND ROBINSON 2003; BELLINI et al. 2007; BELLINI et al. 2013). In this approach, male mosquitoes are irradiated or chemically treated and then released into the field in large numbers to sterilize the wild females. Alternatives to this technique have recently emerged
which do not rely on traditional sterilization (reviewed in McGraw and O'Neill 2013; Ritchie and Johnson 2017). Transgenic Ae. aegypti males possessing a dominant lethal system have been released in multiple locations where they have reduced population sizes, at least in the short term (Harris et al. 2012; Lacroix et al. 2012; Carvalho et al. 2015; Garziera et al. 2017). When these males mate with wild females, most offspring die before reaching the late pupal stage, though a low proportion can emerge as functional adults (Curtis et al. 2015) and may persist for months after releases cease (Garziera et al. 2017). Aedes mosquitoes infected experimentally with the intracellular bacterium Wolbachia are also being released into the field for disease control programs. Certain strains of Wolbachia reduce the capacity for mosquitoes to transmit RNA viruses (Ferguson et al. 2015) and infected males can effectively sterilize wild, uninfected females through cytoplasmic incompatibility (Xi et al. 2005; Walker et al. 2011). Mosquitoes infected with Wolbachia are now being released into the field, both to suppress populations (O'Connor et al. 2012; Mains et al. 2016) and to replace populations with mosquitoes that are refractory to virus transmission (Hoffmann et al. 2011; Nguyen et al. 2015; Schmidt et al. 2017).

Rear and release approaches to arbovirus control require large quantities of mosquitoes to be reared in the laboratory for eventual release into the field. For sterile and incompatible male approaches, high ratios of modified to wild males are needed to achieve suppression, particularly if the modifications have deleterious effects on male fitness (Winskell et al. 2014). Laboratory environments are inherently artificial, and colonized mosquito populations experience an entirely different set of selective pressures compared to natural populations (Leftwich et al. 2015). Many laboratory mosquito populations are held at a controlled temperature, humidity and photoperiod, provided with abundant nutrition, and reared in discrete generations according to a schedule (Benedict 1997; Munstermann 1997; Carvalho et al. 2014). Rearing insects in discrete generations may select for an earlier, shorter and more productive reproductive period, as only individuals that adhere to the rearing schedule will contribute to the next generation (Sgro and Partridge 2000; Simoes et al. 2009). Laboratory populations of insects are often maintained at high adult densities due to space limitation which could lead to intense male-male competition and altered courtship behaviour (Reisen et al. 1985; Rull et al. 2005; Pereira et al. 2007). Laboratory environments can also lack selective pressures which could lead to declines in later life reproduction (Bryant and Reed 1999), a reduced ability to survive temperature extremes, dry conditions or starvation (Hoffmann et al. 2001) or a loss of insecticide resistance (Pimentel et al. 1953). Maintaining populations in the laboratory can also cause a reduction in genetic diversity resulting in low adaptive potential and inbreeding depression (Briscoe et al. 1992). Laboratory environments can therefore impose rapid genetic changes on insect populations, and laboratory-derived mosquitoes could be mal-adapted to the target population when eventually released into the field (Frankham 2008).
Competitive mosquitoes are critical for the success of rear and release programs. Past sterile insect interventions have failed due to the poor performance of released mosquitoes, possibly caused by laboratory adaptation (Reisen et al. 1982; Benedict and Robinson 2003; Helinski and Harrington 2013). For releases of sterile, dominant-lethal or incompatible males, the ability of modified males to seek and inseminate wild females is especially important (Chambers et al. 2011; Harris et al. 2011). For approaches where modified mosquitoes are intended to persist in the environment, it is often necessary for them to perform similarly to wild mosquitoes. Two attempts to establish the wMelPop Wolbachia infection in natural Ae. aegypti populations failed due to deleterious effects associated with the infection, including costs to fecundity, adult lifespan and egg viability (Nguyen et al. 2015). While trait variation related to fitness in mosquitoes has often been well-characterized, there are fewer attempts to compare laboratory strains intended for release against the wild mosquitoes against which they are intended to compete.

Across all mosquito species, there are numerous studies that compare life history, morphological and physiological traits between laboratory and field populations for evidence of laboratory adaptation (Table D.1). Substantial and rapid adaptation by mosquitoes to laboratory conditions is often observed (e.g. Watson et al. 2000; Oliva et al. 2011), but there are several instances of laboratory populations suffering reduced fitness (e.g. Huho et al. 2007; Ponlawat and Harrington 2007). Other studies find no clear differences between laboratory and field populations despite years of separation (e.g. Hassan et al. 2010; Faull and Williams 2015; Jong et al. 2017). Mosquitoes maintained in the laboratory can differ from wild populations for many traits, including blood-feeding duration (Chadee and Beier 1997; Chadee et al. 2002), wing shape (Yeap et al. 2013), oviposition behaviour (Allgood and Yee 2017), mating success (Haeger and O'Meara 1970; Knop et al. 1987; Lima et al. 2004), swarming behaviour (Reisen et al. 1985) and susceptibility to pathogens (Grimstad et al. 1977; Lorenz et al. 1984; Vazeille et al. 2003; Salazar et al. 2007). Researchers often compare a single wild population to a single long-established laboratory population (e.g. Haeger and O'Meara 1970; Lima et al. 2004), but these results could be confounded by inbreeding, drift and bottlenecks in the laboratory population rather than reflecting laboratory adaptation. Differences between populations could also be affected by rearing conditions, for example if the wild population is reared under field conditions (e.g. Huho et al. 2007; Oliva et al. 2011; Ng'Habi et al. 2015) or if measurements are conducted at different time points (e.g. Lorenz et al. 1984; Chadee et al. 2002). Other studies compare populations collected from different locations (e.g. Salazar et al. 2007; Allgood and Yee 2014; Allgood and Yee 2017) and any effects of laboratory maintenance could be confounded by local adaptation.

The extent of laboratory adaptation can vary between insect orders (Hoffmann and Ross 2018) and this could reflect differences in the range of conditions that can be tolerated relative to the conditions experienced in the laboratory (Ochieng'-Odero 1994). Laboratory environments that are suboptimal
will impose strong selective pressures on mosquito populations, leading to rapid adaptation (e.g. WATSON et al. 2000). Colonized mosquito species can require a specific set of conditions such as swarm markers (WATSON et al. 2000) artificial horizons (MARCHAND 1985), dusk periods (MARCHAND 1985) or exposure to stroboscopic light (LARDEUX et al. 2007) to improve their reproductive success in the laboratory. Other species will not freely reproduce in the laboratory at all, requiring induced copulation over successive generations before free-mating colonies can be established (MCDANIEL AND HORSFALL 1957; BRYAN AND SOUTHGATE 1978). In contrast, Ae. aegypti collected from the field perform well in the laboratory without any of these specific requirements (e.g. MUNSTERMANN 1997), and therefore less drastic differences in traits would be expected between laboratory and field populations due to a lack of selective pressures.

Rear and release programs with modified Ae. aegypti mosquitoes are now underway in several countries, and many of these programs rely on the use of mosquitoes that have been inbred or maintained in the laboratory for extended periods. We colonized replicate Ae. aegypti populations collected from Queensland, Australia to assess the effects of laboratory maintenance and inbreeding on life history traits in this species. We find that inbreeding is costly and is associated with a reduction in effective population size, but we find limited evidence of laboratory adaptation for most life history traits. Modified mosquitoes reared for disease control programs should therefore be maintained at large population sizes and/or crossed with field populations prior to field release. Our research highlights potential issues with maintaining colonized insects that are destined for field release, and informs protocols for the maintenance of Ae. aegypti in the laboratory.

5.3 Materials and methods

5.3.1 Replicate population establishment

Aedes eggs collected from ovitraps near Townsville, Australia, in September 2015 (RITCHIE 2001) were hatched and reared in the laboratory (see Colony maintenance). Ae. aegypti larvae were separated from other species based on an identification key (RUEDA 2004). A total of 327 Ae. aegypti adults (171 males and 156 females) were obtained and added to a single 19.7 L BugDorm colony cage (MegaView Science Co., Ltd., Taichung City, Xitun District, Taiwan). Females were blood-fed, and all eggs laid were pooled and hatched in a single tray containing 3 L of water. Larvae were selected at random and divided into groups to establish replicate populations (Figure 5.1). Five populations were maintained at a census size of 400 adults (large populations) and five populations were maintained at a census size of 100 adults (small populations). Twenty adult females were also isolated for oviposition. The offspring from five isolated females were used to establish five additional populations maintained at a census size of 100 adults (isofemale lines), while the offspring from ten females were maintained with a single male and female each (inbred lines). At least two mating pairs
were established for each inbred line (Table D.2), but only a single pair was used to found the next
generation. Their offspring underwent full-sib mating each generation for nine generations, then all
progeny were interbred during F12 to build up numbers for experiments. All replicate populations
were maintained until F13 when experimental comparisons were performed. All adults from the
ancestral Townsville population (F1) and the replicate populations at F13 were stored in absolute
ethanol at -20°C for pooled double-digest RADseq. Only two inbred lines had sufficient numbers for
RADseq due to the loss of most inbred lines over the course of full-sib mating (Table D.2).
_Ae. aegypti_ eggs can withstand desiccation and remain viable for up to one year (Faull and
Williams 2015). We utilised this ability to perform direct comparisons between the ancestral
population and the derived populations simultaneously. Eggs laid by F2 and F3 females were stored
under humid conditions for several months at 26°C and then hatched at the same time as eggs laid by
F11 females from the other populations. A colony derived from larvae that hatched was maintained
under standard conditions for one generation and their progeny (F4–5) were used for experiments
alongside the populations at F13. Colonies derived from eggs collected from Cairns and Innisfail,
Australia were also used for experimental comparisons. These colonies were maintained as single
caged populations with a census size of 400 individuals. Quiescent eggs from the Innisfail population
were also used to generate a colony that had experienced fewer generations of maintenance under
laboratory conditions. Eggs collected from Cairns at a later stage were used to establish a colony for
comparisons with the Cairns colony at F22.
Figure 5.1 Maintenance scheme for replicate *Ae. aegypti* laboratory populations. An ancestral population was established from eggs collected from Townsville, Australia that all other populations were derived from. Replicate populations were maintained separately beginning from F2 and were not interbred.

5.3.2 Colony maintenance

All populations were maintained in a controlled temperature laboratory environment (26 ± 0.5°C and 50-70% relative humidity, with a 12:12 h light:dark photoperiod) following the protocol described by ROSS et al. (2017a). This protocol is designed to reduce selection against individuals that are slow or quick to develop, mature, mate, blood feed, oviposit or hatch, and to minimize mortality at each life stage. To maintain each population, all eggs from the previous generation were pooled and a random subset of larvae was provided with food (TetraMin® tropical fish food tablets, Tetra, Melle, Germany) *ad libitum* and reared to adulthood. For the large populations, 400 adults were selected at random and added to 19.7 L cages, while for the small populations and isofemale lines, 100 adults were added to 12 L cages. For the inbred lines, a single male and female were added to a 1.5 L cage. Except for the inbred lines, sex ratios were maintained naturally, and equal numbers of males and females were not counted. All cages were provided with a source of water and 10% sucrose. Approximately three days after the last adult had emerged, females were blood fed on a single human volunteer. Two days after blood feeding, cups containing larval rearing water and lined with sandpaper strips were introduced into the cages. Eggs laid on the sandpaper strips were collected over the span of one week, and all
eggs were hatched three days after females had ceased oviposition. We followed this procedure until the Townsville populations were at F13, with each generation taking 28 days to complete. Blood feeding of mosquitoes on human subjects was approved by the University of Melbourne Human Ethics Committee (approval #: 0723847). All volunteers provided informed written consent.

5.3.3 Fitness comparisons between Townsville F13 populations

We compared all Townsville populations at F13 for their development time, survival to adulthood and wing length under two nutrition conditions, and the fecundity and egg hatch rate of females reared under high nutrition conditions. Not all inbred lines were included in the experiments as the majority were lost by F13 (Table D.2). Two of the four remaining inbred lines were only tested under high nutrition conditions due to low numbers, and these lines later became extinct (Table D.2). Cairns (F2 and F22), Innisfail (F4 and F10) and Townsville (F4/5) populations were included in all experiments.

One hundred larvae from each population were reared in containers with 500 mL of water and provided with TetraMin® ad libitum (high nutrition) or with 0.1 mg of TetraMin® per larva every 2 days (low nutrition). Four replicate containers were reared for each population, except for two inbred lines where less than 400 larvae were obtained. A random subset of females from each population that emerged from the high nutrition treatment were blood fed and then isolated for oviposition. Eggs collected from each female were counted and hatched three days post-collection. Egg hatch rates were determined by calculating the proportion of eggs that had a detached cap. Isolated females were blood fed again after one week and fecundity and egg hatch rate were measured for a second gonotrophic cycle. Wings from 10 males and 10 females selected at random from each population and each nutrition treatment were dissected and measured for their length according to methods described previously (ROSS et al. 2014).

Fitness data from the Townsville populations at F13 were used to estimate the performance of each population relative to the Townsville F4/5 ancestral population. We simplified an equation from LIVDAHL AND SUGIHARA (1984) to calculate performance from fecundity, egg hatch, survival and larval development time data. \( F \) is the mean fecundity of each population multiplied by egg hatch proportion, \( S \) is the mean proportion of larvae surviving to adulthood, and \( D \) is the mean larval development time in days. The performance index of each population at F13 was divided by the performance index of the ancestral population to determine their relative performance.

\[
\text{Performance index} = \frac{\ln(F \times S)}{D}
\]
5.3.4 Male mating competitiveness

We tested the male mating competitiveness of populations from Cairns that were at F2, F7 or F27 in the laboratory, and an inbred line from Townsville (Inbred A) at F18. Males from all populations competed against males infected with the wAlbB strain of Wolbachia for access to F2 females in a caged laboratory environment. Males infected with wAlbB induce complete sterility (eggs do not hatch) when crossed to uninfected females under standard laboratory conditions (Xi et al. 2005; Axford et al. 2016). Thus, the competitive ability of each population relative to wAlbB-infected males can be estimated by scoring egg hatch rate from crosses between uninfected females and a mix of Wolbachia-infected and uninfected males (Chambers et al. 2011; Segoli et al. 2014). We established 12 L cages containing 25 males from each population (F2, F7, F27 or inbred) and 25 males infected with wAlbB. Five replicate cages were established for each treatment. We then aspirated ten Cairns F2 females into each cage. This was repeated five times at 1 hr intervals, for a total for 50 females per cage. Staggered releases were chosen to increase the level of male-male competition; adding all females to a cage at once would not provide many males with an opportunity to inseminate multiple females. All individuals were reared under the same conditions for this experiment (see Colony maintenance), and males were at least 24 hr old, and females at least 48 hr old before the sexes were combined. Females were blood fed three days after mating and a single cup filled with larval rearing water and lined with a sandpaper strip was added to each cage. Sandpaper strips were collected daily and photographed, and the number of eggs on each strip was counted in ImageJ (Schneider et al. 2012) using the Cell Counter plugin (https://imagej.nih.gov/ij/plugins/cell-counter.html). Eggs were hatched three days post-collection and larvae were counted three days after hatching. Egg hatch rates were estimated by dividing the number of larvae counted by the number of eggs from each cage.

5.3.5 Pooled double-digest RADseq library preparation

We used pooled double-digest RADseq to determine the effective population size (Ne) of the seventeen replicate populations from Townsville at F13 relative to their ancestral population (F1). These included the five large populations, five small populations, five isofemale lines and two inbred lines. We prepared a library following methods described by Rasic et al. (2014b) and Schmidt et al. (2018), but modified the protocol for pooled mosquitoes. DNA was extracted from four pools of 20 adult mosquito heads from each population, with two pools for each sex, using a Roche DNA Isolation Kit for Cells and Tissues (Roche, Pleasanton, CA, USA). DNA from each pool was quantified using a Qubit dsDNA HS assay kit and a Qubit 2.0 fluorometer (Thermo Fisher Scientific, Life Technologies Holdings Pte Ltd, Singapore) and the four pools for each population were combined after a normalization step.
750 ng of DNA from each population was digested in a 50 µL reaction with 20 units each of Eco-RI-HF and SphI-HF restriction enzymes (New England Biolabs, Beverly, MA, USA), NEB CutSmart® buffer and water for three hours at 37°C with no heat kill step. Restriction enzymes that cut less frequently were chosen to produce fewer SNPs while providing more coverage. The digestion products were cleaned with 75 µL of Ampure XP™ paramagnetic beads (Beckman Coulter, Brea, CA) and then ligated with modified Illumina P1 and P2 adapters overnight at 16°C with 1000 units of T4 ligase and 1× T4 buffer (New England Biolabs, Beverly, MA, USA) in a 45 µL reaction volume, then heat deactivated for 10 minutes at 65°C. Ligations were cleaned using 75 µL of paramagnetic beads and adapter-ligated DNA fragments from all eighteen populations were pooled. We then used a Pippin-Prep 2% gel cassette (Sage Sciences, Beverly, MA, USA) to select fragments with a size range of 350-450 bp. The final library was generated by pooling 38 10 µL PCR reactions and run for 12 cycles; each reaction contained 5 µL of Phusion High Fidelity 2× Master mix (New England Biolabs, Beverly MA, USA), 2 µL each of 10 µM forward and reverse Illumina primers and 2 µL of size-selected DNA. The pooled PCR reactions were cleaned with 300 µL of paramagnetic beads, and a single library with DNA from 1440 Ae. aegypti from the eighteen populations was sequenced in a single Illumina HiSeq 2000 lane to obtain 100 bp paired-end reads.

5.3.6 Data processing and effective population size estimates

We checked the quality of the raw sequencing data with FastQC v0.11.5 (ANDREWS 2010) and then used the process_radtags component of Stacks v1.46 (CATCHEN et al. 2013) to demultiplex the populations, allowing for one mismatch. Reads were trimmed to 80 bp and then aligned to the Aedes aegypti reference genome AaegL4 (DUDCHENKO et al. 2017) using bowtie2 v2.3.0 (LANGMEAD AND SALZBERG 2012). Ambiguous alignments (minimum mapping quality below 20) were discarded and alignments were converted to SAM format and sorted using SAMtools v1.4 (LI et al. 2009). Sorted files were then converted to mpileup format, with each file containing the ancestral population and one of the seventeen derived populations. These files were converted to sync format using the mpileup2sync.jar tool from Popoolation2 (KOFLER et al. 2011). We then estimated effective population size (N_e) using the Nest R package v1.1.9 with three different methods (JONAS et al. 2016).

5.3.7 Statistics on life history traits

All data were analyzed using SPSS statistics version 24.0 for Windows (SPSS Inc, Chicago, IL). All data were checked for normality using Shapiro-Wilk tests. Data that were normally distributed were
analyzed using ANOVA and data that were not normal were analyzed with Kruskal-Wallis and Mann-Whitney U tests.

5.4 Results

5.4.1 Preliminary fitness comparisons

When the Townsville population had reached F2, we compared life history traits to an established laboratory population from Cairns (F11), however we observed no differences between populations for any trait (Appendix D.1). At F5, we compared life history traits between replicated large populations and inbred lines. After two generations of brother-sister mating, the inbred lines had reduced fitness relative to the large populations, with substantial costs to larval survival and development time (Appendix D.2). We also observed significant differences between replicate populations for some life history traits which likely arose due to founder effects or drift (Appendix D.2).

5.4.2 Larval development time

When the Townsville populations had reached F13, we performed fitness comparisons to test for inbreeding effects, laboratory adaptation, drift and founder effects. We measured larval development time for all populations under high nutrition and low nutrition conditions (Figure 5.2). Under high nutrition conditions, small populations were developmentally delayed compared to large populations (females: one-way ANOVA: $F_{1,38} = 48.080$, $P < 0.001$, males: $F_{1,38} = 27.895$, $P < 0.001$). There were significant differences in development time between replicate cages of the large populations (females: $F_{4,15} = 4.262$, $P = 0.017$, males: $F_{4,15} = 4.699$, $P = 0.012$), isofemale lines (females: $F_{4,15} = 81.888$, $P < 0.001$, males: $F_{4,15} = 18.956$, $P < 0.001$), inbred lines (females: $F_{3,9} = 10.413$, $P = 0.003$, males: $F_{3,9} = 8.575$, $P = 0.005$) and also females from the small populations ($F_{4,15} = 6.749$, $P = 0.003$) but not males ($F_{4,15} = 2.031$, $P = 0.141$). The isofemale and inbred lines were particularly diverse; some lines performed as well as (or better than) the large populations while others had greatly extended development times (Figure 5.2). Large populations (Townsville F13) were consistently faster to develop than the Townsville F4/5 ancestral population (females: $F_{1,22} = 33.462$, $P < 0.001$, males: $F_{1,22} = 15.434$, $P = 0.001$), suggesting a positive effect of laboratory maintenance on this trait. The Cairns F22 laboratory population was also faster to develop than the Cairns F2 field population (females: $F_{1,6} = 6.407$, $P = 0.045$, males: $F_{1,6} = 9.147$, $P = 0.023$), though the opposite was true for Innisfail, where the laboratory population was slower to develop. This difference was however only significant for females (females: $F_{1,6} = 6.653$, $P = 0.042$, males: $F_{1,6} = 5.938$, $P = 0.051$). Under low nutrition conditions, development times were greatly extended relative to high nutrition conditions (Mann-
Whitney U: $Z = 16.250$, $P < 0.001$, Figure 5.2). Differences between groups of populations maintained at different census sizes became less clear as there were significant differences between replicate populations at all census sizes (one-way ANOVA: all $P \leq 0.002$).

**Figure 5.2 Development time of *Aedes aegypti* F13 laboratory populations maintained at different census sizes.** Mean development time was measured for (A&C) female and (B&D) male larvae under (A&B) high nutrition (food provided *ad libitum*) and (C&D) low nutrition (0.1 mg of TetraMin® per larva every 2 days) conditions. Each data point represents the mean development time of individuals from a single container of 100 larvae. Replicates of the large populations (Townsville F13, census size 400) are in yellow, small populations (Townsville F13, census size 100) in orange, isofemale lines (Townsville F13) in red and inbred lines (Townsville F13) in purple. Other laboratory populations are shown in gray and ancestral / field populations in pale blue. Inbred lines B and C were not tested under low nutrition conditions. The dashed line represents the mean development time of the Townsville F4/5 ancestral population. Error bars are standard deviations.
5.4.3 Survival to adulthood and sex ratio

We compared the proportion of larvae that survived to adulthood between populations in the larval development experiment (Figure 5.3). Under high nutrition conditions, survival rates approached 100% in most populations that were maintained at a census size of 400. Small populations had reduced survival rates compared to large populations (Mann-Whitney U: Z = 3.273, P = 0.001), as did the isofemale (Z = 3.489, P < 0.001) and inbred (Z = 4.018, P < 0.001) lines. We observed significant variation between isofemale lines (Kruskal-Wallis: $\chi^2 = 14.872$, df = 4, P = 0.005), but not between large ($\chi^2 = 2.129$, df = 4, P = 0.712) or small ($\chi^2 = 7.507$, df = 4, P = 0.111) populations. Survival to adulthood was poorer under low nutrition conditions (Mann-Whitney U: Z = 6.343, P < 0.001), but populations maintained at a census size of 400 still performed consistently better than populations maintained at lower census sizes (Z = 7.084, P < 0.001). No differences between laboratory and field populations from Townsville, Innisfail or Cairns were evident (Mann-Whitney U: all P > 0.05), but the ability to detect any differences with this test was low due to low sample sizes for each population.

Sex ratios of adults emerging from the larval development experiment did not deviate significantly from 1:1 under high nutrition conditions for all populations (Chi-square: df = 3, all P > 0.05), except for the Cairns F2 population which was biased towards males (df = 3, P = 0.013). Sex ratios were skewed towards males under low nutrition conditions (df = 86, P < 0.001) which was likely the result of female larval mortality.
Figure 5.3 Survival to adulthood of *Aedes aegypti* F₁₃ laboratory populations maintained at different census sizes. The percentage of larvae surviving to adulthood was tested under (A) high nutrition (food provided *ad libitum*) and (B) low nutrition (0.1 mg of TetraMin® per larva every 2 days) conditions. Replicates of the large populations (Townsville F₁₃, census size 400) are in yellow, small populations (Townsville F₁₃, census size 100) in orange, isofemale lines (Townsville F₁₃) in red and inbred lines (Townsville F₁₃) in purple. Other laboratory populations are shown in gray and ancestral / field populations in pale blue. Solid black lines indicate the median survival of each population. The dashed line represents the median survival of the Townsville F₄₅ ancestral population.
5.4.4 Fecundity and egg hatch rate

A random subset of females emerging from the larval development experiment was scored for their fecundity (Figure 5.4) and egg hatch rate (Figure 5.5). Patterns of fecundity were similar between the first and second gonotrophic cycles, but fecundity was lower overall in the second gonotrophic cycle (one-way ANOVA: \( F_{1,624} = 17.660, P < 0.001 \)). We considered only the first gonotrophic cycle for the following analyses, as some females died before the second gonotrophic cycle. Inbred populations had greatly reduced fecundity compared to large populations (\( F_{1,100} = 130.395, P < 0.001 \)). Replicate populations differed significantly from each other for large populations (\( F_{4,69} = 3.573, P = 0.010 \)), isofemale lines (\( F_{4,68} = 10.300, P < 0.001 \)) and inbred lines (\( F_{3,24} = 12.087, P < 0.001 \)), but not small populations (\( F_{4,66} = 1.677, P = 0.166 \)), potentially reflecting drift or founder effects. The fecundity of large populations (Townsville F13) did not differ from that of Townsville F4/5 (\( F_{1,86} = 0.549, P = 0.461 \)), indicating that the effects of laboratory maintenance on this trait are minimal.

Egg hatch proportions did not differ between gonotrophic cycles (Mann-Whitney U: \( Z = 1.773, P = 0.077 \)), but were substantially affected by inbreeding, with both isofemale lines (\( Z = 6.895, P < 0.001 \)) and inbred lines (\( Z = 8.334, P < 0.001 \)) exhibiting reduced hatch proportions relative to large populations (Figure 5.5). There were differences between replicate populations for small populations (Kruskal-Wallis: \( \chi^2 = 10.405, df = 4, P = 0.034 \)), isofemale (\( \chi^2 = 19.639, df = 4, P = 0.001 \)) and inbred lines (\( \chi^2 = 11.222, df = 3, P = 0.011 \)), but not large populations (\( \chi^2 = 3.141, df = 4, P = 0.535 \)). Hatch proportions did not differ between the Townsville F4/5 population and the large populations at F13 (Mann-Whitney U: \( Z = 0.2137, P = 0.834 \)), but were improved in the Cairns F22 population relative to Cairns F2 (\( Z = 3.202, P = 0.001 \)), suggesting a positive effect of laboratory maintenance.
Figure 5.4 Fecundity of *Aedes aegypti* F₁₃ laboratory populations maintained at different census sizes. The number of eggs laid by females in each experimental population was scored during their first (A) and second (B) gonotrophic cycle. Fifteen females were tested per line, or as many as possible for inbred lines B and C. Replicates of the large populations (Townsville F₁₃, census size 400) are in yellow, small populations (Townsville F₁₃, census size 100) in orange, isofemale lines (Townsville F₁₃) in red and inbred lines (Townsville F₁₃) in purple. Other laboratory populations are shown in gray and ancestral / field populations in pale blue. The dashed line represents the mean fecundity of the Townsville F₄/₅ ancestral population. Error bars are standard deviations.
Figure 5.5 Egg hatch proportions of *Aedes aegypti* F13 laboratory populations maintained at different census sizes. Egg hatch proportions in each experimental population were scored in their first (A) and second (B) gonotrophic cycle. Fifteen females were tested per line, or as many as possible for inbred lines B and C. Replicates of the large populations (Townsville F13, census size 400) are in yellow, small populations (Townsville F13, census size 100) in orange, isofemale lines (Townsville F13) in red and inbred lines (Townsville F13) in purple. Other laboratory populations are shown in gray and ancestral / field populations in pale blue. Solid black lines indicate the median egg hatch proportion of each population. The dashed line represents the median egg hatch proportion of the Townsville F4/5 ancestral population.
5.4.5 Adult body size

We measured wing length from a random subset of adults emerging from the larval development experiment to estimate the body size of each population under different nutritional conditions (Figure 5.6). Wing lengths under high nutrition conditions were much larger than under low nutrition conditions (one-way ANOVA: $F_{1,918} = 221.749, P < 0.001$) and differences between populations were more distinct. Under high nutrition conditions there was a clear cost of inbreeding to wing length; adults from inbred lines were much smaller than adults from large populations (females: $F_{1,82} = 67.189, P < 0.001$, males: $F_{1,82} = 22.804, P < 0.001$). There were substantial differences in wing length between replicate isofemale lines (females: $F_{4,45} = 8.303, P < 0.001$, males: $F_{4,45} = 10.751, P < 0.001$) and inbred lines (females: $F_{3,30} = 20.703, P < 0.001$, males: $F_{3,30} = 9.729, P < 0.001$) and smaller, but still significant, differences between females of the large ($F_{4,45} = 3.102, P = 0.024$) and small ($F_{4,44} = 2.777, P = 0.038$) populations. Wing lengths of adults from the large populations at F13 were smaller than those from the ancestral population at F4/5 (females: $F_{1,58} = 10.472, P = 0.002$, males: $F_{1,58} = 10.519, P = 0.002$) which could reflect adaptation to artificial rearing conditions. However, there were no differences between the laboratory and field populations from Cairns and Innisfail (all $P > 0.05$).
Figure 5.6 Wing length of *Aedes aegypti* F\textsubscript{13} laboratory populations maintained at different census sizes. Wing lengths were measured from (A&C) female and (B&D) male adults when reared under (A&B) high nutrition (food provided *ad libitum*) and (C&D) low nutrition (0.1 mg of TetraMin\textsuperscript{®} per larva every 2 days) conditions. Replicates of the large populations (Townsville F\textsubscript{13}, census size 400) are in yellow, small populations (Townsville F\textsubscript{13}, census size 100) in orange, isofemale lines (Townsville F\textsubscript{13}) in red and inbred lines (Townsville F\textsubscript{13}) in purple. Other laboratory populations are shown in gray and ancestral / field populations in pale blue. Up to ten wings were measured for each group, though some inbred lines had less than 10 adults available, and several measurements were discarded due to damaged wings. Inbred lines B and C were not tested under low nutrition conditions. The dashed line represents the mean wing length of the Townsville F\textsubscript{4/5} ancestral population. Error bars are standard deviations.

5.4.6 Overall performance

We calculated an index of performance for each Townsville population at F\textsubscript{13} relative to the ancestral population (Townsville F\textsubscript{4/5}) using the data for fecundity, egg hatch proportion, larval development time and survival to adulthood (under high nutrition conditions) available for each population (Figure 5.7). The large populations (census size 400) consistently performed better than the ancestral population (one sample t-test, P = 0.008) which indicates a positive effect of artificial rearing.
conditions on performance in the laboratory. The increased performance of laboratory populations was largely due to shorter larval development time (Appendix D.3). The relative performance of populations declined substantially with increasing levels of inbreeding; isofemale lines and inbred lines had much poorer performance than the ancestral population. This fitness deficit could largely be restored by crossing inbred mosquitoes to an outbred population (Appendix D.4). The Cairns laboratory population (F_{22}) had increased performance over the field (F_{2}) population (relative performance index: 1.080) but the Innisfail laboratory population (F_{10}) had decreased performance over the field population (F_{4}) (relative performance index: 0.960). Laboratory populations therefore did not always exhibit increased performance over the populations that were colonized more recently.

![Figure 5.7](image)

**Figure 5.7 Relative performance of *Aedes aegypti* F_{13} laboratory populations maintained at different census sizes.** Each data point represents the performance index of a single replicate population relative to the ancestral population (Townsville F_{4,5}) which is represented by the black dotted line. Black bars indicate means and standard deviations.

### 5.4.7 Effective population size

We estimated the effective population size (N_{e}) of the replicate Townsville populations at F_{13} relative to the ancestral population (F_{1}) using pooled RADseq and the Nest R package v1.1.9 (JONAS et al.)
The $N_e(JR)$ and $N_e(P)$ methods provided similar estimates of $N_e$ but $N_e(W)$ provided estimates that were in many cases much larger than the census sizes. For estimates calculated using the $N_e(JR)$ and $N_e(P)$ methods, $N_e$ declined substantially with decreasing census size (Table 5.1). Ratios of $N_e$ to census size calculated using the $N_e(P)$ method were low, though the small populations (census size 100, mean $N_e/N = 0.250$) had higher ratios than large populations (census size 400, mean $N_e/N = 0.143$). The index of performance for each population increased dramatically with increasing $N_e$ but levelled off at higher $N_e$ (Figure D.5). These findings demonstrate a clear association between $N_e$ and fitness (Spearman’s rank-order correlation: $\rho = 0.973$, $P < 0.001$, $n = 17$) but suggests that an $N_e$ greater than used in the large populations will lead to only small fitness improvements.

Table 5.1 Effective population sizes ($N_e$) of *Aedes aegypti* F13 laboratory populations maintained at different census sizes, calculated using three temporal methods.

<table>
<thead>
<tr>
<th>Population</th>
<th>Replicate</th>
<th>$N_e$ estimate</th>
<th>$N_e(JR)$</th>
<th>$N_e(P)$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Large populations ($N = 400$)</td>
<td>A</td>
<td>661.073</td>
<td>71.566</td>
<td>55.827</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>903.292</td>
<td>55.903</td>
<td>53.776</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>568.557</td>
<td>74.208</td>
<td>60.054</td>
</tr>
<tr>
<td></td>
<td>D</td>
<td>846.441</td>
<td>73.280</td>
<td>60.850</td>
</tr>
<tr>
<td></td>
<td>E</td>
<td>1048.192</td>
<td>65.394</td>
<td>55.632</td>
</tr>
<tr>
<td>Small populations ($N = 100$)</td>
<td>A</td>
<td>526.797</td>
<td>24.428</td>
<td>24.435</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>190.602</td>
<td>20.666</td>
<td>17.814</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>761.790</td>
<td>28.871</td>
<td>34.351</td>
</tr>
<tr>
<td></td>
<td>D</td>
<td>183.127</td>
<td>22.279</td>
<td>20.863</td>
</tr>
<tr>
<td></td>
<td>E</td>
<td>162.276</td>
<td>24.785</td>
<td>22.982</td>
</tr>
<tr>
<td>Isofemale lines</td>
<td>A</td>
<td>191.258</td>
<td>10.015</td>
<td>11.437</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>182.663</td>
<td>10.366</td>
<td>9.782</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>154.080</td>
<td>9.541</td>
<td>9.038</td>
</tr>
<tr>
<td></td>
<td>D</td>
<td>188.197</td>
<td>11.173</td>
<td>11.507</td>
</tr>
<tr>
<td></td>
<td>E</td>
<td>148.858</td>
<td>10.139</td>
<td>9.694</td>
</tr>
<tr>
<td>Inbred lines</td>
<td>A</td>
<td>271.954</td>
<td>4.213</td>
<td>5.184</td>
</tr>
<tr>
<td></td>
<td>E</td>
<td>323.245</td>
<td>4.610</td>
<td>5.408</td>
</tr>
</tbody>
</table>
5.4.8 Mating competitiveness

The success of sterile and incompatible insect programs will depend on the ability of modified males to mate successfully in the wild. We tested to see if inbreeding and laboratory maintenance had any effect on male mating competitiveness. Males from the Cairns F2, F7 or F27 and inbred (Inbred A F18) populations competed for access to F2 females against a standard competitor infected with Wolbachia (wAlbB strain) (Figure 5.8). Hatch proportions did not differ significantly between the F2, F7 and F27 populations (one-way ANOVA: F2,12 = 0.829, P = 0.460), but were markedly reduced for inbred males relative to the other populations (one-way ANOVA: F1,18 = 39.784, P < 0.001). These results indicate that male mating success in laboratory cages is not affected by long-term laboratory maintenance, but can be decreased by inbreeding. The poor performance of inbred males was likely due to reduced mating success and not a paternal effect on female fertility, as crosses between inbred males and Cairns F2 females produced eggs with high hatch proportions (Appendix D.4).

Figure 5.8 Relative mating success of Aedes aegypti males maintained in the laboratory for different numbers of generations. We tested the relative mating success of males from Cairns F2, F7 and F27 populations when competing against Wolbachia-infected males for access to Cairns F2 females. An inbred colony (Inbred A F18) was included for comparison. Higher hatch proportions indicate increased mating success of the experimental males relative to Wolbachia-infected males. Each data point represents the mean egg hatch proportion from a cage of 50 females. Black bars indicate means and standard deviations.
5.5 Discussion

We performed a comprehensive assessment of inbreeding and laboratory adaptation in *Ae. aegypti* mosquitoes to inform rear and release programs for arbovirus control. Our study is the first to investigate the effects of inbreeding on *Ae. aegypti* fitness directly by comparing outbred and inbred lines derived from the same population, and the first that links fitness costs to reductions in effective population size as assessed through genomic markers. We look for evidence of adaptation by comparing laboratory populations to their direct ancestor concurrently and use replicate populations to separate fitness changes due to adaptation from drift and founder effects, two approaches which have not been previously applied in mosquitoes.

We find evidence of laboratory adaptation in colonized *Ae. aegypti* populations, but changes in trait means were small in magnitude and directions were often inconsistent between populations. All replicate laboratory populations from Townsville developed faster and were smaller than mosquitoes from the ancestral population. These changes could be a response to selection for abbreviated development in the laboratory, despite efforts to avoid selection in our laboratory rearing protocol (ROSS et al. 2017a). Shorter developmental periods are often observed in laboratory-adapted insects (ALLGOOD AND YEE 2014), particularly under mass-rearing conditions that favor the rapid production of insects (ECONOMOPOULOS 1992; MIYATAKE 1993). In contrast, development times can increase in colonized *Drosophila* maintained with overlapping generations, where there is less selection against slow developing individuals (SGRO AND PARTRIDGE 2000).

When fitness traits were combined into an overall index of performance, we found that laboratory populations maintained at a large census size usually had greater performance than field populations. This finding is consistent with other insects, where fitness under laboratory conditions tends to improve with laboratory maintenance (HOFFMANN AND ROSS 2018). The rate of adaptation in our laboratory colonies of *Ae. aegypti* was slower than other mosquito species and insects in general (HOFFMANN AND ROSS 2018). *Ae. aegypti* collected from the field performed well from the first generation in the laboratory; rates of adaptation are likely to be higher for species such as *Ae. notoscriptus* where the laboratory environment is suboptimal and only a small proportion of individuals are able to reproduce in the initial generations (WATSON et al. 2000). Populations tested at F2 did not tend to differ from laboratory populations in terms of trait means, but some traits exhibited greater variation at F2. This suggests that *Ae. aegypti* could lose variation with laboratory maintenance, though other traits for other populations at F2 had similar variances to laboratory populations. Our main comparisons were between populations at F4/5 and F13; if substantial laboratory adaptation occurs then we would be unable to detect it with these comparisons. Our population comparisons could also be confounded by selection on the ancestral population due to eggs experiencing quiescence (Townsville and Innisfail populations) or differences present in populations.
collected from the same location but at different times (Cairns populations). Other factors such as gut microbiota could also confound our comparisons between laboratory and field populations because the microbiome can greatly influence mosquito life history traits (COON et al. 2014; COON et al. 2016a). Gut microbiota are much less diverse in colonized mosquitoes (MWADONDO et al. 2017) and tend to be similar in laboratory populations regardless of geographic origin (DICKSON et al. 2017). This could be an issue when comparing field and laboratory populations.

Few studies on laboratory adaptation in insects attempt to separate the effects of laboratory adaptation from drift or founder effects (HOFFMANN et al. (2001) is one exception) which are likely to be substantial when establishing small laboratory colonies. We used replicate populations to avoid this issue; consistent divergence in colonized populations from the ancestral population indicates adaptation, variation between replicate populations immediately after establishment indicates founder effects, and divergence between replicate populations at the time indicates drift. We found that replicate populations at the same census size differ significantly from each other for several fitness traits, both at F5 and at F13, particularly for populations maintained at low census sizes. Fitness differences between replicate populations were not always consistent between F5 and F13, suggesting that both founder effects and drift occur. These findings are of concern for laboratory studies that compare traits between populations maintained separately. Researchers should consider using replicate populations when conducting experiments or outcross populations frequently to maintain similar genetic backgrounds (YEAP et al. 2011).

We demonstrate that inbreeding is extremely costly to *Ae. aegypti* fitness. Most inbred lines were lost across the experiment, and the remaining lines performed substantially worse than outbred populations. Relatively few studies have specifically addressed the effects of inbreeding on mosquito fitness. Powell and Evans (2016) observed that inbreeding *Ae. aegypti* through full-sib mating reduces heterozygosity by much less than expected based on theory, and deleterious recessive alleles must therefore be common. KOENRAADT et al. (2010) reported fitness costs of inbred *Ae. aegypti* larvae relative to a wild population, and inbreeding through full-sib mating reduces fitness in other *Aedes* species (ARMBRUSTER et al. 2000; O’DONNELL AND ARMBRUSTER 2010). We demonstrate that mosquito populations inbred intentionally, for instance, to generate homozygous transgenic strains (CATTERUCCIA et al. 2003; PHUC et al. 2007), will likely suffer from severe inbreeding depression. However, it may be possible to retain partial fitness if there is also selection for certain life history traits during inbreeding (SHETTY et al. 2016). We show that laboratory populations maintained at low census sizes (N = 100) also experience inbreeding depression, and the loss of fitness correlates strongly with decreased effective population size. Thus, laboratories should ensure that population sizes in colonized mosquitoes are sufficiently high to maintain their fitness. Our laboratory populations for these experiments were each established from only a few hundred individuals, and we would recommend that larger numbers be used to avoid bottlenecks.
Our laboratory populations at F13 had a substantially lower $N_e$ than field populations from Townsville (Endersby et al. 2011) and other locations around the world (Saarman et al. 2017). However, ratios of $N_e$ to census size ($N_e/N$) were similar to ratios reported in nature (Saarman et al. 2017). $N_e/N$ ratios were larger in the small laboratory populations ($N = 100$) than in the large ones ($N = 400$), consistent with a study of Drosophila populations maintained at different census sizes (Schou et al. 2017). Low $N_e/N$ ratios indicate that reproductive success varies greatly between individuals (Nunney 1995; Hedrick 2005) and this appears to be the case for large colonized populations of Ae. aegypti. Unequal contributions to the next generation occur because we sample only a few hundred individuals randomly from a pool of thousands of larvae, and we do not equalize offspring from each female to establish the next generation (Ross et al. 2017a).

We demonstrate that the consequences of laboratory maintenance in Ae. aegypti can be minimized by maintaining large population sizes, but there are several other ways to maintain the fitness of colonized mosquito populations. The simplest approach is to cross laboratory colonies to an outbred population (Yeap et al. 2011). Gene flow into inbred populations commonly leads to a fitness improvement (Frankham 2015) and we also show that the fitness of inbred Ae. aegypti can be improved through a single generation of outcrossing. Increased performance of hybrids has been demonstrated in Anopheles mosquitoes (Menge et al. 2005; Baeshen et al. 2014; Ekechukwu et al. 2015) and the Queensland fruit fly (Gilchrist and Meats 2014), with fitness improvements in the F1. Crosses between different laboratory lines can also be used to determine whether changes in fitness in laboratory populations are due to inbreeding or adaptation (Baeshen et al. 2014). Rates of laboratory adaptation can be slowed by using more natural rearing environments. Knop et al. (1987) compared two methods of rearing Culex tarsalis and found that colonies maintained in larger cages at a variable temperature and more complex environmental conditions had a slower rate of laboratory adaptation. Ng’habi et al. (2015) found that rearing Anopheles arabiensis under semi-field conditions preserved their similarity to the wild population and reduced the extent of inbreeding. Quality control methods such as screening mosquitoes for their flight capacity can also be used to increase fitness before their deployment for disease control programs (Balestrino et al. 2017).

In summary, we provide evidence for inbreeding depression effects and a small effective population size relative to census size in laboratory mosquito populations, along with some limited laboratory adaptation particularly in large populations. Our results have implications for the maintenance of insects in the laboratory, particularly for those destined for open field releases. While we find that life history traits of Ae. aegypti do not change consistently with laboratory maintenance, traits where selective pressures are absent in the laboratory, such as flight ability, feeding behaviour and thermal tolerance might still be compromised.
5.6 Acknowledgements

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CHAPTER 6 – CONCLUSIONS

In chapter 2, I demonstrate that *Wolbachia* infections reduce the ability of *Ae. aegypti* larvae to survive under starvation conditions. Some infections are costlier than others, likely making these strains less able to invade populations where competitive and resource-poor larval habitats are abundant. I tested the potential for *Wolbachia* infections to be transferred to uninfected larvae through the consumption of infected conspecifics. Uninfected larvae fed on a diet of exclusively *Wolbachia*-infected larvae did not acquire the infection; it is unlikely that the horizontal transmission of *Wolbachia* infections will affect invasion dynamics. I also show that *Wolbachia* infections do not affect the scavenging behaviour of larvae, or their ability to recover from periods without food. Finally, and most importantly, I show that *Wolbachia* infections in *Ae. aegypti* retain complete cytoplasmic incompatibility and maternal transmission fidelity under starvation conditions. The stability of *Wolbachia* infections under extreme nutritional stress will be critical to the success of *Wolbachia*-based disease control programs.

In chapter 3, I tested the response of *Wolbachia* infections in *Ae. aegypti* to heat stress under temperature conditions approximating larval habitats in Cairns, Queensland during the wet season. I found that *w*Mel- and *w*MelPop-infected mosquitoes had greatly reduced *Wolbachia* density and partially lost their ability to induce cytoplasmic incompatibility when reared under high temperature conditions. In contrast, I found the *w*AlbB infection to be more stable than the other strains, highlighting that *Wolbachia* infections in the same host can differ dramatically in their response to environmental conditions. These findings indicate that the *w*Mel infection, now being deployed by the World Mosquito Program in several tropical countries, could face difficulty invading some of these hot environments. The World Mosquito Program addresses this study in the frequently asked questions section of their website, stating that these laboratory results do not necessarily reflect the more complex situation in nature, and that the impact of heat stress on field trials appears to be minimal (http://www.eliminatedengue.com/faqs/index/index/type/wolbachia). The outcomes of field releases with *w*Mel outside of Australia have not yet been published and it is therefore unclear if these releases were successful. The impacts of heat stress on the *w*Mel infection are now being tested under field conditions; the findings should provide insight into whether heat stress will pose a legitimate threat to the success of *w*Mel release programs in tropical environments.

In chapter 4, I attempted to improve the tolerance of the *w*Mel infection to heat stress through experimental selection. After five generations of intense selection, *w*Mel showed no evidence of improvement in its response to heat stress, and instead exhibited reduced density in males at high temperatures and incomplete cytoplasmic incompatibility under standard laboratory conditions. This suggests that the *w*Mel strain has limited potential to adapt to higher temperature conditions in the
field, and any vulnerabilities to temperature will be retained following field release, at least in the short term.

The success of disease control efforts using *Wolbachia*-infected and other modified mosquitoes will depend on the quality of the released mosquitoes. Laboratory adaptation and inbreeding are likely to have compromised the success of disease and pest control efforts in the past, particularly when insects have been mass-reared for sterile insect programs. In chapter 5, I investigated this issue using colonised *Ae. aegypti* maintained at different census sizes. I found limited evidence that *Ae. aegypti* adapt to laboratory conditions, but found them to be highly susceptible to inbreeding effects. Mosquitoes maintained at low population sizes for 12 generations experienced consistent fitness costs, while consecutive generations of full-sib mating resulted in severe deleterious effects and eventually extinction. While these findings do not suggest that laboratory maintenance at large population sizes will compromise the fitness of released mosquitoes, laboratory adaptation will likely still be a problem in colonies where less care is taken to avoid selective pressures, such as in mass rearing programs.

In summary, my research demonstrates that environmental conditions can have major impacts on *Wolbachia* infections and their interactions with mosquitoes, and highlights potential issues with maintaining mosquitoes in the laboratory for disease control programs.
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Appendix A.1 Horizontal transfer of *Wolbachia* through necrophagy of conspecific larvae

*Wolbachia* infections can be transferred horizontally to naturally uninfected arthropods through the consumption of infected individuals, both intra- and inter-specifically (Heath et al. 1999; Wu and Hoy 2012; Le Clec'h et al. 2013; Brown and Lloyd 2015). While there is no evidence that *Wolbachia* from *Ae. aegypti* can be transferred to distantly related predators through consumption (Hurst et al. 2012), intra-specific transfer facilitated through the necrophagy of *Ae. aegypti* larvae is yet to be tested. *Ae. aegypti* larvae will readily consume dead conspecifics in the absence of higher quality nutrition (Daugherty et al. 2000; Bara et al. 2014) and may even cannibalise earlier instars (Edgerly et al. 1999), therefore the potential exists for intra-specific horizontal transfer to occur during field releases of *Wolbachia*-infected mosquitoes.

We tested whether *Wolbachia* infections could be transferred to uninfected larvae through direct consumption in two experiments. In the first, 24 hour old uninfected larvae were transferred to containers of RO water. Every two days, wMelPop-infected larvae of approximately the same size as uninfected larvae were fatally wounded and immediately added to the containers of uninfected larvae. Uninfected larvae were fed wMelPop-infected larvae *ad libitum* for 5-6 days and then stored in absolute ethanol. In the second experiment this method was repeated, but uninfected larvae were reared to adulthood and aged for one week before being stored in ethanol for *Wolbachia* detection. 20 4th instar larvae and 20 adults (whole bodies) reared exclusively on a diet of wMelPop-infected larvae were screened for their infection status (see DNA extraction and Wolbachia detection). To test if *Wolbachia* could be detected in the carcasses being consumed, we also screened dead wMelPop-infected larvae held in containers of water for 0, 36 and 72 hours (n = 16 per time point).

We found no evidence that *Wolbachia* infections could be transferred through the consumption of infected conspecifics. No traces of *Wolbachia* in uninfected larvae were detected when they were fed exclusively wMelPop-infected larvae. The ability to detect *Wolbachia* in carcasses declined rapidly; 100, 63 and 38% of wMelPop-infected larvae tested positive for *Wolbachia* when killed and left in water for 0, 36 and 72 hours respectively. While this could in part explain the inability to detect horizontal transmission, scavenging of carcasses in other experiments occurred rapidly (Figure A.4). These results suggest that horizontal transfer is unlikely to occur in the field and thus will not affect
invasion dynamics. The results of experiments in which infected and uninfected larvae are present in the same container are also unlikely to be confounded by any false positives for *Wolbachia* in screened larvae or adults.

Figure A.1 Survival of *Ae. aegypti* larvae under starvation conditions during the recovery from food deprivation experiment. Points A and B denote when larvae were re-fed TetraMin for the experiment. Survival curves are based on 12-16 replicates for each line until Day 15 and 6-8 replicates after Day 15. Error bars are standard errors.
Figure A.2 Comparison of larval survival under starvation conditions between experiments.
Larvae of *Ae. aegypti* were held under starvation conditions in isolation when water was replaced every four days (solid red line) or when water was left unmanipulated (dashed red line). Experiments where larvae were held in groups (grey lines) were conducted under similar conditions (water was replaced), but the mixed cohort (dashed grey line) and recovery (dotted grey line) experiments were
conducted at a later time on different generations. Data are averaged across all four infection types. Error bars are standard errors.

**Figure A.3** Larval mortality and dead larvae observed when *Ae. aegypti* larvae were held in groups under starvation conditions. Rates of larval mortality are shown by solid lines while the
numbers of dead larvae observed are shown by dotted lines. The dotted line being below the solid line suggests that mortality is occurring at a slower rate than the consumption of larvae.

Figure A.4 Larval mortality of *Ae. aegypti* and the number of larvae consumed when held in groups under starvation conditions. Rates of larval mortality are shown by solid lines while the numbers of dead larvae inferred to be consumed are shown by dashed lines. The delay between distributions of larval mortality and consumption provide an estimate of the rate of necrophagy in
group containers. Mean delays between mortality and consumption are as follows: Uninfected, 0.60 days; wMel, 0.32 days; wAlbB, 0.44 days; wMelPop, 0.81 days.
Figure A.5 Pupae and adults of *Ae. aegypti* observed when larvae were held under starvation conditions in groups of 50. Number of (A) pupae and (B) adults emerging in total from eight containers of 50 larvae for each infection type.

APPENDIX B – CHAPTER 3 SUPPLEMENTARY INFORMATION
Figure B.1 Diurnal temperature fluctuations in incubators. Incubators were set to a constant 26°C or a cycling 26-32°C, 26-34.5°C or 26-37°C. Temperature was measured by submerging data loggers in plastic trays filled with 500 mL water, identical to the trays used for rearing larvae. Data shown were averaged from seven days of measurements; error bars represent standard deviations.

Figure B.2 Wing length of (A) females and (B) males reared at a constant 26°C, cycling 26-32°C and cycling 26-37°C. Thirty adults were measured per treatment. Data were normally distributed according to Shapiro-Wilk tests. Analysis of variance finds a significant effect of temperature regime on wing length for both females (one-way ANOVA: F2,356 = 11.203, P < 0.0001) and males (F2,357 =
9.381, P = 0.0001) but no effect of infection type for either sex (females: F,3,355 = 0.313, P = 0.816, males: F,3,356 = 1.714, P = 0.164). Increasing maximum temperatures had a negative effect on wing length for all infection types and both sexes, with the 26-37°C regime being the most stressful.

Figure B.3 Relative density of *Wolbachia* in *Aedes aegypti* reared at cyclical temperatures and different levels of nutrition. Relative *Wolbachia* density was measured for wMel (A & B), wAlbB (C & D) and wMelPop-CLA (E & F) infections in both females (A, C, E) and males (B, D, F). Mosquito larvae were reared at a constant 26°C, cycling 26-32°C or cycling 26-37°C and provided different levels of nutrition: high food (food provided *ad libitum*), moderate food (0.1 mg per larva per day) and low food (0.02 mg per larva per day). Each mosquito was tested with mosquito-specific and
Wolbachia-specific markers to obtain crossing point values (see “Wolbachia quantification”). Up to 30 mosquitoes were tested per treatment, but fewer were tested under more stressful conditions where larval survival was lower. Each data point represents the average of three technical replicates.
Figure B.4 Wing lengths of Wolbachia-infected Aedes aegypti reared at cyclical temperatures and different levels of nutrition. Females (A, C, E) and males (B, D, F) reared at a constant 26°C, cycling 26-32°C or cycling 26-37°C were provided with (A & B) high food (food provided ad libitum), (C & D) moderate food (0.1 mg per larva per day) or (E & F) low food (0.02 mg per larva per day).

Table B.1 Primers used in qPCR for detection of Wolbachia in Ae. aegypti.

<table>
<thead>
<tr>
<th>Specificity</th>
<th>Name</th>
<th>Sequence (5’-3’)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aedes</td>
<td>mRpS6_F</td>
<td>AGTTGAACGTATCGTTTCCCCTTAC</td>
<td>LEE et al. (2012)</td>
</tr>
<tr>
<td></td>
<td>mRpS6_R</td>
<td>GAAGTGACGCGAGCTTTTGTCG</td>
<td></td>
</tr>
<tr>
<td>Aedes aegypti</td>
<td>aRpS6_F</td>
<td>ATCAAGAAGCGCGTGTGC</td>
<td>LEE et al. (2012)</td>
</tr>
<tr>
<td></td>
<td>aRpS6_R</td>
<td>CAGGTGAGGATCTTTATGTATTGC</td>
<td></td>
</tr>
<tr>
<td>wMel</td>
<td>w1_F</td>
<td>AAAATTTTGAGAGGATTCG</td>
<td>LEE et al. (2012)</td>
</tr>
<tr>
<td></td>
<td>w1_R</td>
<td>GCATGGAGAAGGAGGAAAGGG</td>
<td></td>
</tr>
<tr>
<td>wMelPop-CLA</td>
<td>wMelpop_F</td>
<td>CTCATTTATTCCGACTAAAAATTTC</td>
<td>RITCHIE et al. (2015)</td>
</tr>
<tr>
<td></td>
<td>wMelpop_R</td>
<td>TCTTTCTCATTAAAGAACCTCCTTTG</td>
<td></td>
</tr>
<tr>
<td>wAlbB</td>
<td>wAlbB_F</td>
<td>CCTACCTCCTGCACAACAA</td>
<td>AXFORD et al. (2016)</td>
</tr>
<tr>
<td></td>
<td>wAlbB_R</td>
<td>GGATGAGCCGTGGCCCTTA</td>
<td></td>
</tr>
</tbody>
</table>

qPCR = quantitative real-time polymerase chain reaction.
Figure C.1 Temperatures experienced in larval rearing trays during the cytoplasmic incompatibility experiment on *Ae. aegypti*. Each line represents the mean temperature collected from four data loggers placed in separate rearing trays. Dotted lines are standard deviations.
Figure C.2 Hatch proportions of eggs from wMel-infected female *Ae. aegypti* reared under cyclical heat stress and held at 26°C for 1, 4 or 7 days before being crossed to wMel-infected males reared at 26°C. Twenty females were blood-fed and isolated for oviposition from each cross, but some crosses exhibited high mortality after isolation. Each data point represents the hatch proportion of eggs from a single female. Egg hatch proportions did not differ between females that were mated at different ages (Kruskal-Wallis: $\chi^2 = 1.055$, df = 2, $P = 0.590$). Black horizontal lines indicate medians.
Figure C.3 Fecundity of wMel, wMel-HS and wAlbB female *Ae. aegypti* reared under different temperature conditions for the cytoplasmic incompatibility experiment. Error bars are means with standard deviations.
Table D.1 Studies of laboratory adaptation in mosquitoes. Studies compared mosquito populations that were maintained in the laboratory for different numbers of generations. Focal traits were either tested over successive generations in the laboratory, or field and laboratory populations were compared simultaneously.

<table>
<thead>
<tr>
<th>Species</th>
<th>Focal trait</th>
<th>Comparison</th>
<th>Effect of laboratory maintenance</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aedes aegypti</td>
<td>Blood feeding duration</td>
<td>Tracked changes over 4 generations</td>
<td>Reduced blood feeding duration</td>
<td>(CHADEE AND BEIER 1997; CHADEE et al. 2002)</td>
</tr>
<tr>
<td>Aedes aegypti</td>
<td>Susceptibility to yellow fever</td>
<td>Tracked changes over 11 generations</td>
<td>Significant differences between generations</td>
<td>(LORENZ et al. 1984)</td>
</tr>
<tr>
<td>Aedes aegypti</td>
<td>Susceptibility to dengue</td>
<td>Field population vs laboratory population</td>
<td>Reduced susceptibility to dengue</td>
<td>(SALAZAR et al. 2007)</td>
</tr>
<tr>
<td>Aedes aegypti</td>
<td>Quiescent egg viability</td>
<td>Field population (G1) vs laboratory population (G60)</td>
<td>No effect on egg viability under almost all conditions</td>
<td>(FAULL AND WILLIAMS 2015)</td>
</tr>
<tr>
<td>Aedes aegypti</td>
<td>Sperm quantity</td>
<td>Field population (G0) vs two laboratory populations</td>
<td>Reduced quantity of sperm in both laboratory populations</td>
<td>(PONLAWAT AND HARRINGTON 2007)</td>
</tr>
<tr>
<td><strong>Aedes aegypti</strong></td>
<td>Wing shape</td>
<td>Field population (G1) vs laboratory population (G8)</td>
<td>Altered wing shape</td>
<td>(YEAP et al. 2013)</td>
</tr>
<tr>
<td><strong>Aedes albopictus</strong></td>
<td>Development time and survival to adulthood, under different levels of nutrition</td>
<td>Field population (G2) vs laboratory population (G&gt;300)</td>
<td>No effect, except under low nutrition conditions where the field population has higher survival to adulthood</td>
<td>(JONG et al. 2017)</td>
</tr>
<tr>
<td><strong>Aedes albopictus</strong></td>
<td>Susceptibility to dengue virus</td>
<td>Field populations (G&lt;5) vs laboratory populations (G&gt;40)</td>
<td>Increased susceptibility</td>
<td>(VAZEILLE et al. 2003)</td>
</tr>
<tr>
<td><strong>Aedes notoscriptus</strong></td>
<td>Egg hatch rate</td>
<td>Tracked changes over 13 generations</td>
<td>Increased egg hatch rate (likely due to increased mating success)</td>
<td>(WATSON et al. 2000)</td>
</tr>
<tr>
<td><strong>Aedes triseriatus</strong></td>
<td>Susceptibility to La Crosse virus</td>
<td>Various field and laboratory populations</td>
<td>Varied effect on susceptibility</td>
<td>(GRIMSTAD et al. 1977)</td>
</tr>
<tr>
<td><strong>Culex tarsalis</strong></td>
<td>Swarming behavior and insemination rate (females)</td>
<td>Field populations (G0) vs laboratory population</td>
<td>Altered swarming behaviour and increased female insemination rate</td>
<td>(REISEN et al. 1985)</td>
</tr>
<tr>
<td><strong>Culex tarsalis</strong></td>
<td>Levels of essential fatty acids</td>
<td>Various field and laboratory populations</td>
<td>Reduced levels of triacylglycerol eicosapentaenoic acid and triacylglycerol arachidonic acid</td>
<td>(DADD et al. 1988)</td>
</tr>
<tr>
<td><strong>Culex tarsalis</strong></td>
<td>Insemination rate (females), mating competitiveness, blood</td>
<td>Tracked changes over 12 generations, compared various</td>
<td>Increased insemination success, mating competitiveness, blood</td>
<td>(KNOP et al. 1987)</td>
</tr>
<tr>
<td>Species</td>
<td>Characteristic</td>
<td>Population 1</td>
<td>Population 2</td>
<td>Result</td>
</tr>
<tr>
<td>------------------</td>
<td>---------------------------------</td>
<td>-----------------------</td>
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<td>--------------------------------------------------------------------------------------------------</td>
</tr>
<tr>
<td>Culex quinquefasciatus</td>
<td>Development time, survival to adulthood and body size</td>
<td>Field population (G2) vs laboratory population</td>
<td>Faster development time, increased survival to adulthood, larger size</td>
<td>(Allgood and Yee 2014)</td>
</tr>
<tr>
<td>Culex quinquefasciatus</td>
<td>Oviposition behaviour</td>
<td>Field population (G3) vs laboratory population</td>
<td>Altered oviposition behaviour</td>
<td>(Allgood and Yee 2017)</td>
</tr>
<tr>
<td>Culex nigripalpus</td>
<td>Insemination rate (females)</td>
<td>Field population (G1) vs laboratory population (G54)</td>
<td>Increased insemination success (independent of male strain)</td>
<td>(Haeger and O'Meara 1970)</td>
</tr>
<tr>
<td>Anopheles albitarsis</td>
<td>Insemination rate (males)</td>
<td>Field population (G1) vs laboratory population (G150)</td>
<td>Increased insemination success (independent of female strain)</td>
<td>(Lima et al. 2004)</td>
</tr>
<tr>
<td>Anopheles arabiensis</td>
<td>Insemination rate and recapture rate (males)</td>
<td>Field population (G0) vs laboratory population (G68)</td>
<td>No change in insemination rate or recapture rate</td>
<td>(Hassan et al. 2010)</td>
</tr>
<tr>
<td>Anopheles arabiensis</td>
<td>Rate of male sexual maturation</td>
<td>Field population (G0) vs laboratory population (G125)</td>
<td>Increased rate of sexual maturation</td>
<td>(Oliva et al. 2011)</td>
</tr>
<tr>
<td>Anopheles arabiensis</td>
<td>Abundance of energetic reserves (males), wing size (males)</td>
<td>Field population (G0) vs laboratory population (G10)</td>
<td>Increased glycogen and glucose abundance, reduced lipid abundance, reduced wing size</td>
<td>(Ng'Habi et al. 2015)</td>
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<tr>
<td><em>Anopheles</em> species</td>
<td>Trait descriptions</td>
<td>Study details</td>
<td>Reference</td>
<td></td>
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<tr>
<td>---------------------</td>
<td>--------------------</td>
<td>---------------</td>
<td>-----------</td>
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<tr>
<td><em>Anopheles coluzzii</em></td>
<td>Quantity of sperm and mating plug proteins, insemination rate, reproductive success, longevity</td>
<td>Field population (G1) vs two laboratory populations (8 y and &gt;35 y)</td>
<td>Substantial changes in several mating traits</td>
<td>(EKECHUKWU et al. 2015)</td>
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<td><em>Anopheles darlingi</em></td>
<td>Fecundity, egg hatch rate, adult emergence and susceptibility to <em>Plasmodium vivax</em></td>
<td>Tracked changes over 5 generations</td>
<td>No change in life history traits, no change in susceptibility to <em>Plasmodium vivax</em></td>
<td>(MORENO et al. 2014)</td>
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<td><em>Anopheles gambiae</em></td>
<td>Energetic reserves and body size of males</td>
<td>Field population (G0) vs laboratory population</td>
<td>Reduced body size and lipid reserves</td>
<td>(HUHO et al. 2007)</td>
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<tr>
<td><em>Anopheles gambiae</em></td>
<td>Testes size, accessory gland size</td>
<td>Field population (G1) vs laboratory populations</td>
<td>Increased testes size, decreased accessory gland size</td>
<td>(BAESHEN et al. 2014)</td>
</tr>
</tbody>
</table>
Table D.2 Maintenance and loss of inbred *Aedes aegypti* populations during successive generations of full-sib mating.

<table>
<thead>
<tr>
<th>Generation in the laboratory</th>
<th>Generations of full-sib mating</th>
<th>Inbred lines remaining</th>
<th>Replicate pairs maintained for each line*</th>
</tr>
</thead>
<tbody>
<tr>
<td>F3</td>
<td>0</td>
<td>10</td>
<td>2</td>
</tr>
<tr>
<td>F4</td>
<td>1</td>
<td>10</td>
<td>2</td>
</tr>
<tr>
<td>F5</td>
<td>2</td>
<td>10</td>
<td>2</td>
</tr>
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<td>F6</td>
<td>3</td>
<td>9</td>
<td>2</td>
</tr>
<tr>
<td>F7</td>
<td>4</td>
<td>7</td>
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<td>F8</td>
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<tr>
<td>F10</td>
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<td>8</td>
<td>4</td>
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</tr>
<tr>
<td>F12</td>
<td>9</td>
<td>4</td>
<td>10</td>
</tr>
<tr>
<td>F13</td>
<td>9</td>
<td>4</td>
<td>All offspring interbred for each line</td>
</tr>
<tr>
<td>F14</td>
<td>9</td>
<td>2</td>
<td>All offspring interbred for each line</td>
</tr>
</tbody>
</table>

* The number of replicate male and female pairs for each line increased as inbred lines were lost.

Appendix D.1 Pilot experiments comparing fitness differences between Townsville F2 and Cairns F11 *Aedes aegypti* populations.

*Materials and Methods*

We conducted a series of experiments on the Townsville population at F2 by comparing a range of fitness traits to an established laboratory population from Cairns (F11). Adult longevity was compared using methods described by Axford et al. (AXFORD et al. 2016). In short, eight replicate cages containing 25 females and 25 males for each population were checked three times per week for mortality until all adults had died. Adults were provided with a sucrose solution and a source of water, and females were blood-fed weekly. Larval development time was also assessed under two levels of...
nutrition. Eight replicate containers with 50 larvae each in 200 mL of water were provided with TetraMin *ad libitum* (high nutrition) or with 0.1 mg of TetraMin per larva every 2 days (low nutrition). Adult emergence was scored twice daily (high nutrition) or daily (low nutrition) until all individuals had completed development or died. The survival of larvae under starvation conditions was compared using methods described by Ross et al. (2016). Larvae were provided with food *ad libitum* for 72 hr and then transferred to reverse osmosis (RO) water with no food provided. Larvae were either held in containers of 200 mL of water in groups of 50 (starvation survival in groups) or individual larvae were held in wells of 12-well cell culture plates containing 4 mL of water (starvation survival in isolation). The number of larvae alive was then scored every two days until all larvae had died. For starvation survival in groups, two independent experiments were conducted with eight replicates each. For the individual experiment, 144 isolated larvae were tested per population.

Fecundity was also scored following the methods of Ross *et al.* (2016) over three gonotrophic cycles. Females were blood fed *en masse* and 30 females per population were isolated in 70 mL cups containing a moist sandpaper oviposition substrate. Sandpaper strips were collected five days after blood feeding and the number of eggs was counted using a clicker counter. Females were blood fed one and two weeks after the initial blood feeding to initiate further gonotrophic cycles. Survival data for adult longevity and larval starvation experiments were investigated using Kaplan-Meier analysis, with log-rank tests comparing rates of mortality between populations.

**Results**

We compared the Townsville F2 population to the Cairns F11 population. We observed no clear differences between the two populations for all traits tested (Figure D.1). Development time under two nutrition conditions (one-way ANOVA: $F_{1,50} = 0.150, P = 0.700$), fecundity across three gonotrophic cycles ($F_{1,118} = 2.568, P = 0.112$) and the longevity of females (Kaplan-Meier: $\chi^2 = 1.592$, df = 1, $P = 0.207$) and males ($\chi^2 = 3.725$, df = 1, $P = 0.054$) were not significantly different between the two populations. The two populations also did not differ in their ability to survive under starvation conditions, both when larvae were held in groups ($\chi^2 = 0.670$, df = 1, $P = 0.413$) and in isolation ($\chi^2 = 3.093$, df = 1, $P = 0.079$). While no clear differences were observed, the two populations were derived from locations 350 km apart, and differences between the original field populations could affect the results of experiments.
Figure D.1 Fitness comparisons between Townsville F2 (gray) and Cairns F11 (red) populations in the laboratory. We scored the longevity of (A) female and (B) male adults, (C) the fecundity of females over three gonotrophic cycles, the survival larvae under when held in (D) groups of 50 or (E) in isolation under starvation conditions and the development time of females and males under (F) high nutrition (1 mg per larva every 2 days) or (G) low nutrition (0.1 mg per larva every 2 days) conditions. Dotted lines on survival graphs represent 95% confidence intervals, while error bars on the fecundity and development time graphs are standard deviations.

Appendix D.2 Fitness comparisons between large Aedes aegypti populations (census size 400) and inbred lines from Townsville at F5.

Materials and Methods

We conducted a set of experiments on the Townsville populations at F5 to see if there were any differences between replicate populations after a few generations of being maintained separately. We compared the five large populations at F2 and five inbred populations that had experienced two consecutive generations of full-sib mating by F5 (Table D.2). We also included Cairns (F14) and Innisfail (F2) populations for comparison; isofemale lines and small populations were not tested at this stage. We measured larval development time, survival to adulthood and fecundity of each population under two levels of nutrition. Four replicate containers with 50 larvae each (except for Innisfail F2 and Cairns F14 which had 8 replicates) in 200 mL of water were provided with TetraMin ad libitum (high nutrition) or with 0.05 mg of TetraMin per larva every 2 days (low nutrition). For the high nutrition treatment, 20-30 females per population were isolated for fecundity measurements. For the low
nutrition treatment, all females that emerged from the containers were isolated for fecundity due to low survival rates.

Results

Fecundity under high nutrition conditions did not differ significantly between large and inbred populations (one-way ANOVA: $F_{1,181} = 0.126, P = 0.723$) but replicates of the large populations differed from each other ($F_{1,87} = 3.422, P = 0.012$, Figure D.2). Under low nutrition conditions fecundity was considerably more variable and differed between replicate inbred populations (Kruskal-Wallis: $\chi^2 = 10.519, df = 4, P = 0.033$) but not between large and inbred populations (Mann-Whitney U: $Z = 1.752, P = 0.080$). The survival of inbred larvae to adulthood was consistently lower than for the large populations under high nutrition conditions (Mann-Whitney U: $Z = 3.665, P < 0.001$). Differences were less clear under low nutrition conditions ($Z = 1.488, P = 0.136$) due to substantial variation between replicate inbred lines (Kruskal-Wallis: $\chi^2 = 13.273, df = 4, P = 0.010$). Both male (Mann-Whitney U: $Z = 3.963, P < 0.001$) and female ($Z = 4.693, P < 0.001$) development times were greatly extended for inbred lines under high nutrition conditions relative to the large populations. There were also significant differences between replicate inbred populations (Kruskal-Wallis: males: $\chi^2 = 13.986, df = 4, P = 0.007$, females: $\chi^2 = 13.700, df = 4, P = 0.008$) and large populations for males ($\chi^2 = 11.057, df = 4, P = 0.026$) but not females ($\chi^2 = 8.443, df = 4, P = 0.077$). Effects on development time were less clear under low nutrition conditions as the variation between replicate containers was much higher. Overall, survival to adulthood and female fecundity were drastically reduced, development times were greatly extended, and fitness differences between inbred and large populations became less clear under low nutrition conditions (Figure D.2). These results demonstrate that substantial fitness costs arise after only two generations of inbreeding, and differences between replicate populations appeared after only a few generations of being maintained separately in the laboratory.
**Figure D.2** Fitness comparisons between large populations and inbred lines at $F_5$. Fitness traits were compared between five large populations (gray) and five inbred lines (red) at $F_5$ under high nutrition (food provided *ad libitum*, upper panels) and low nutrition (0.05 mg of food per larva every 2 days, lower panels) conditions. Cairns $F_{14}$ and Innisfail $F_3$ populations (black) were also included for comparison. Populations were scored for their (A) fecundity, (B) survival to adulthood and development time for (C) females and (D) males. Error bars indicate means and standard deviations.

**Appendix D.3** The extent of laboratory adaptation in *Aedes aegypti* and comparisons with other insects.

**Methods**

We wanted to see if there was evidence for consistent directional changes in *Aedes aegypti* life history traits resulting from laboratory maintenance, and to assess the magnitude of these changes for comparisons with other studies. We took trait means from each large Townsville population at $F_{13}$ (census size 400) as these were the populations least affected by inbreeding, and compared them to the trait mean of the ancestral population (Townsville $F_{4/5}$) (Figure D.3). Traits were compared in terms of proportional changes in trait means per generation (Figure D.3A) and standard deviations from the trait mean per generation (Figure D.3B), a measure which considers the variation between measurements and not just differences in trait means. When the 95% confidence intervals of the proportional changes did not intersect with zero, we considered these changes to be significant. However, significant changes only indicate a trend in trait means relative to the ancestral population and do not consider the variation within populations.
Results

Most traits did not differ from the ancestral population at F_{4/5} and the laboratory populations at F_{13}, but other traits exhibited consistent positive or negative shifts in trait means (Figure D.3A). Under high nutrition conditions, laboratory populations developed around 0.3 days (3.5%) faster, and wing lengths were 0.08 mm (3%) smaller; though these changes were consistent they reflect only minor shifts. Survival to adulthood in the laboratory populations was also consistently lower under high nutrition conditions and consistently higher under low nutrition conditions, but these differences represent no greater than a few percentage points. When the laboratory and field populations from Cairns and Innisfail were included, fewer traits exhibited consistent changes in one direction (Figure D.4); patterns of trait changes with laboratory maintenance were therefore not always consistent.

We compared the extent of adaptation across all traits in this study to data from a review of laboratory adaptation in insects (HOFFMANN AND ROSS 2018). The median proportional change in trait means (unsigned) in our *Ae. aegypti* laboratory populations was 2.54% (n = 86 trait measurements), which was lower than the magnitude of adaptation across all mosquitoes (median = 5.70%, n = 43, Mann-Whitney U: Z = 2.665, P = 0.008) and substantially lower than magnitudes of adaptation across all insects (median = 12.05%, n = 369, Z = 8.841, P < 0.001). Median proportional changes per generation were 0.29% for *Ae. aegypti* in our study (n = 86) and 0.64% across insects in other studies (n = 296, Z = 4.795, P < 0.001). This indicates that mosquitoes, particularly *Ae. aegypti*, undergo less adaptation in response to laboratory rearing conditions than other insects. This is unsurprising given that *Ae. aegypti* are already somewhat adapted to artificial environments, where they preferentially feed on humans and breed in household-linked containers.
Figure D.3 Proportional changes in life history traits in the large populations at F13 relative to the ancestral population (Townsville F48). Proportional changes are expressed in terms of (A) changes in trait means per generation and (B) standard deviations of trait means per generation. Each data point represents the proportional change in a trait of a single replicate population relative to the ancestral population. The directions of trait changes were adjusted so that positive values indicate increased fitness with laboratory maintenance. Error bars are 95% confidence intervals; red asterisks indicate traits where the 95% confidence intervals do not include zero. Significant changes indicate a trend in trait means relative to the ancestral population but do not consider the variation within populations.
Figure D.4 Proportional changes in life history traits in laboratory populations from Townsville, Cairns and Innisfail relative to their respective ancestral or field populations. Proportional changes are expressed in terms of (A) changes in trait means per generation and (B) standard deviations of trait means per generation. Each data point represents the proportional change in a trait of a single replicate population relative to the ancestral population. The directions of trait changes were adjusted so that positive values indicate increased fitness with laboratory maintenance. Error bars are 95% confidence intervals; red asterisks indicate traits where the 95% confidence intervals do not include zero. Significant changes indicate a trend in trait means relative to the ancestral or field population but do not consider the variation within populations.
Figure D.5 The effective population size ($N_e$) of *Aedes aegypti* populations at F13 versus their index of performance. Large populations (Townsville F13, census size 400) are in yellow, small populations (Townsville F13, census size 100) in orange, isofemale lines (Townsville F13) in red and inbred lines (Townsville F13) in purple. The relative performance of the Townsville F4/5 population is represented by the black dotted line. Effective population size was calculated using the $N_e(P)$ method.

Appendix D.4 Outcrossing of an inbred *Aedes aegypti* population.

*Methods*

Egg hatch rates for the inbred line (Inbred A) in the mating competitiveness experiment were reduced markedly compared to other populations (Figure 7 main text). To confirm that this result was due to reduced mating competitiveness of inbred males and not reduced fertility of F2 females that mated with inbred males, we performed reciprocal crosses between inbred and Cairns F2 lines and scored their fecundity and the proportion of eggs that hatched. We then tested offspring from the F2 and inbred lines and reciprocal crosses for their development time, fecundity and egg hatch rate to determine the extent to which the fitness of the inbred line was restored when crossed to an outbred population. Eight replicate trays with 50 larvae in 500 mL of water were reared for each cross and
provided with TetraMin *ad libitum*. Twenty-five females from each cross were isolated for fecundity and egg hatch proportion measurements for experiments across both generations.

**Results**

We performed reciprocal crosses between Cairns F2 and inbred (Inbred A F18) populations to assess the effects of each male type on female fecundity (Figure D.6A) and egg hatch proportion (Figure D.6B). Cairns F2 females laid more eggs than inbred females (two-way ANOVA: $F_{1,97} = 15.921$, $P < 0.001$) and their fecundity was unaffected by male type ($F_{1,97} = 0.370$, $P = 0.544$). Similarly, inbred females had greatly reduced egg hatch proportions compared to Cairns F2 females (Mann-Whitney U: $Z = 3.182$, $P = 0.001$) regardless of male type ($Z = 0.109$, $P = 0.912$). We wanted to see if the fitness of the inbred line could be improved through reciprocal crosses to the Cairns F2 population. Larvae that hatched from each cross were scored for their development time (Figure D.6C-D). Inbred larvae developed substantially slower than Cairns F2 larvae for both females (one-way ANOVA: $F_{1,14} = 132.316$, $P < 0.001$) and males ($F_{1,14} = 246.030$, $P < 0.001$). Hybrids had greatly improved development times relative to inbred larvae (females: $F_{1,20} = 161.238$, $P < 0.001$, males: $F_{1,20} = 107.455$, $P < 0.001$) but differed in the extent to which they improved, with the progeny of Cairns F2 ♀ × Inbred ♂ developing faster than the progeny of Inbred ♀ × Cairns F2 ♂ for both females ($F_{1,20} = 9.366$, $P = 0.010$, Figure D.6C) and males ($F_{1,20} = 16.455$, $P = 0.002$, Figure D.6D). Conversely, egg hatch proportions improved to similar extents for the two hybrids, and did not differ significantly from Cairns F3 (Kruskal-Wallis: $\chi^2 = 2.416$, df = 2, $P = 0.299$, Figure D.6F). Fecundity also did not differ between the hybrids and Cairns F3 (one-way ANOVA: $F_{2,58} = 1.278$, $P = 0.286$) but the fecundity of the inbred population was lower than all other crosses ($F_{1,81} = 29.966$, $P < 0.001$, Figure D.6E). Overall, the fitness of an inbred line was greatly improved through one generation of outcrossing, though the extent of improvement, at least in terms of development time, depends on which sex is chosen from each group.
Figure D.6 Outcrossing inbred *Ae. aegypti* mosquitoes dramatically improves their fitness. (A) Fecundity and (B) egg hatch proportions in crosses between Cairns F₂ and inbred populations. The resulting progeny were then tested for the development time of (C) females and (D) males, (E) female fecundity and (F) egg hatch proportion. Reciprocal cross A refers to a cross between the male and female progeny of Cairns F₂ ♀ × Inbred ♂ and reciprocal cross B refers to a cross between the male and female progeny of Inbred ♀ × Cairns F₂ ♂. Error bars are standard deviations.
Appendix D.5 Larval competitiveness of *Aedes aegypti* laboratory populations.

**Materials and Methods**

The Cairns populations at F2, F7 or F27 and the inbred line (Inbred A) at F18 were tested for larval competitiveness. Fifty 1st instar larvae from each population and 50 wAlbB-infected larvae were added to containers with 500 mL of water, with eight replicate containers each. wAlbB-infected larvae were used as a standard competitor, as they can be distinguished from the other populations by qPCR (Lee et al. 2012; Axford et al. 2016). Containers were provided with 0.04 mg of TetraMin per larva every two days; this nutrition regime was chosen to reflect highly competitive conditions and result in low survival rates. All adults that emerged were stored in ethanol and screened for their *Wolbachia* infection status using previously described methods (Lee et al. 2012; Axford et al. 2016). The frequency of *Wolbachia* infection in the emerging adults was used to determine the relative competitiveness of each population; higher frequencies of adults without *Wolbachia* indicate increased competitiveness of the experimental population.

**Results**

Approximately 18.5% of larvae in the experiment survived to adulthood and development times were greatly extended relative to other experiments under high nutrition conditions (Figure D.7, c.f. Figures 5.2-5.4). All adults that emerged were screened for their *Wolbachia* infection status, with the proportion from each population that were uninfected providing an estimate of their larval competitiveness (Figure D.7A). No differences in larval competitiveness were found between the Cairns laboratory populations (Kruskal-Wallis: $\chi^2 = 1.915$, df = 2, $P = 0.384$). The proportion of uninfected adults for the inbred line was substantially reduced compared to the other populations, indicating greatly reduced larval competitiveness (Mann-Whitney U: $Z = 4.156$, $P < 0.001$). Development times of experimental larvae were slower than larvae from the standard competitor for females (Mann-Whitney U: $Z = 3.522$, $P < 0.001$, Figure D.7B) but not for males ($Z = 0.934$, $P = 0.352$, Figure D.7C).
Figure D.7 Relative larval competitive ability of the Cairns F2, F7, F27 and inbred F18 (Inbred A) populations versus Wolbachia-infected larvae when added in equal proportions at the start of the experiment. (A) The proportion of larvae surviving to adulthood that were not infected with Wolbachia. Higher frequencies indicate a greater competitive ability of the experimental population relative to the Wolbachia-infected standard competitor. Frequencies were calculated from all adults that emerged from each container, ranging from 10 to 29 adults per data point (males and females were combined). (B-C) Development times for individual (B) females and (C) males that survived to adulthood. Development time data were separated into groups for experimental individuals (blue) and Wolbachia-infected individuals (purple). Each data point for (B) and (C) represents a single individual rather than the mean development time for an entire container. Error bars are standard deviations.
APPENDIX E – Larval competition extends developmental time and decreases adult size of \textit{wMelPop} \textit{Wolbachia}-infected \textit{Aedes aegypti}
Larval Competition Extends Developmental Time and Decreases Adult Size of wMelPop Wolbachia-Infected Aedes aegypti

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Abstract. The intracellular endosymbiont Wolbachia has been artificially transinfected into the dengue vector Aedes aegypti, where it is being investigated as a potential dengue biological control agent. Invasion of Wolbachia in natural populations depends upon the fitness of Wolbachia-infected Ae. aegypti relative to uninfected competitors. Although Wolbachia infections impose fitness costs on the adult host, effects at the immature stages are less clear, particularly in competitive situations. We look for effects of two Wolbachia infections, wMel and wMelPop, on intra-strain and inter-strain larval competition in Ae. aegypti. Development of Wolbachia-infected larvae is delayed in mixed cohorts with uninfected larvae under crowded-rearing conditions. Slow developing wMelPop-infected larvae have reduced adult size compared with uninfected larvae, and larvae with the wMel infection are somewhat larger and have greater viability relative to uninfected larvae when in mixed cohorts. Implications for successful invasion by these Wolbachia infections under field conditions are considered.

INTRODUCTION

Wolbachia are intracellular endosymbiotic bacteria that infect as many as two-thirds of insect species.1,2 Wolbachia are transmitted maternally3 and commonly cause cytoplasmic incompatibility in their hosts; a mechanism resulting in embryonic lethality when an infected male mates with a female that is not infected. This incompatibility provides Wolbachia-infected females with a fitness advantage relative to uninfected females.4-5 The reproductive manipulation induced by Wolbachia enables the infection to proliferate rapidly, potentially leading to replacement of naturally uninfected populations.6-8

Although many mosquito species are infected with Wolbachia, the container-breeding Aedes aegypti, the principal dengue vector, does not harbor a natural Wolbachia infection.9 The wMelPop and wMel strains of Wolbachia, originally from Drosophila melanogaster, have been artificially transinfected into Ae. aegypti.10,11 These Wolbachia strains inhibit the replication of dengue virus in Ae. aegypti, preventing its transmission.11,12 Thus, Wolbachia may be used as an effective biological control for dengue that may eliminate the need for mosquito eradication.

The potential for Wolbachia-infected Ae. aegypti to replace natural populations and block dengue transmission depends on their ability to survive and reproduce in the field.8,15 The wMel infection is relatively benign; it primarily localizes to the mosquito salivary glands and gonads, and does not cause any substantial deleterious effects on fitness.11 The ability of wMel to invade natural mosquito populations has been demonstrated; in trial releases of wMel-infected Ae. aegypti at two locations near Cairns, Queensland, Australia, infection frequency approached 100% after two months of releases.14 Conversely, wMelPop is relatively virulent towards Ae. aegypti because it proliferates throughout the entire mosquito.11,12,15 The wMelPop infection reduces adult longevity by as much as 50% and decreases egg viability, fecundity,10,16,17 and blood feeding success of females.18

Although the costs of wMelPop infection on adult Ae. aegypti are well documented, studies that examine the effects of Wolbachia infection on immature development are limited, particularly those that evaluate direct competition with uninfected larvae under stress. Gavotte and others19 provided an example in Ae. albopictus. During field release Wolbachia-infected Ae. aegypti larvae will encounter competition with uninfected larvae for limited resources and space, as suitable habitats for oviposition are scarce.20 Larval crowding in Ae. aegypti causes delayed development rates,21 elevated mortality22 and reduced adult size.23 These deleterious effects on fitness may be explained by physical interference,24 increased levels of pollution caused by natural waste released into the environment,23,25 or through increased aggression leading to higher biting frequencies and increasing the incidence of disease transmission.26 Larval competition between species has been proposed as the primary mechanism for recently observed shifts in species distributions of Ae. aegypti and Ae. albopictus.27,28 Larval competition between infected and uninfected strains of Ae. aegypti may also play a critical role in the success of Wolbachia invasion.

For invasion to occur, the frequency of Wolbachia infection in a population must reach or exceed a certain threshold.29,30 This required frequency depends on variables, such as rates of maternal transmission and levels of cytoplasmic incompatibility induced by the Wolbachia infection. The fitness of infected mosquitoes relative to uninfected mosquitoes in the field is also an important factor; deleterious effects associated with the wMelPop infection mean a higher infection threshold must be reached for invasion to occur.13 An important component of this is relative larval viability.31 However, the competitive ability of Wolbachia-infected larvae relative to uninfected larvae under stress is poorly understood. Recent studies suggest Wolbachia infection may have deleterious effects on immature host survival and development,16,17,32 and studies on the naturally infected Ae. albopictus suggest that Wolbachia infection reduces fitness under high stress conditions.33 A minor cost to survival of Wolbachia-infected larvae relative to uninfected larvae could render invasion difficult.31 Therefore, it is important to assess the effect of

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wMelPop infection on competition for resources with uninfected larvae under stress.

In this study, we tested the effect of larval crowding on the fitness of Wolbachia-infected Ae. aegypti larvae relative to an uninfected strain of Ae. aegypti. We monitor wMelPop-infected and uninfected larvae competing directly for the same resources when present in different frequencies. Effects are examined in terms of survivorship, developmental time, sex ratio and wing length (a proxy for body size).17 We repeated this assay with the wMel strain. The results are discussed in terms of implications for facilitating invasions of Wolbachia strains in competitive environments.

MATERIALS AND METHODS

Mosquito strains and colony maintenance. Three lines of Ae. aegypti were used. Wild-type uninfected C14 Ae. aegypti were collected from Cairns, Queensland, Australia, in June 2012 and maintained under laboratory conditions for at least one generation. The wMelPop-CLA-infected and wMel-infected lines backcrossed to the Cairns background were generated before each experiment to maintain the same background as the uninfected strain. These infected lines were established from field-sourced mosquitoes from populations established near Cairns after releases. Lines were maintained in a controlled laboratory environment at 26°C ± 1°C and 80–90% relative humidity, with a 12:12 light: dark photoperiod. Mosquito colonies were maintained according to methods described by Yeap and others.17 Female mosquitoes were blood-fed on a single human volunteer, as approved by the University of Melbourne Human Ethics Committee (approval 0723847).

Larval competition. Preliminary experiments were conducted to determine a level of larval crowding that increased developmental time and reduced adult size. Larvae were provided with a fixed amount of crushed Tetramin fish food tablets (Tetra, Melle, Germany) daily (0.25 mg/larvae) and reared at the following densities: 50, 100, 200, 400, and 800 larvae in 200 mL of reverse osmosis (RO) water (0.25, 0.5, 1, 2 and 4 larvae/mL, respectively). Wolbachia-infected and uninfected larvae were tested in pure cohorts, in which each strain was reared in separate containers, and also in mixed cohorts where infected and uninfected larvae were present in the same container. Development rate, size, and survivorship decreased with greater larval densities, concordant with previous studies.22,33,35 A larval density of 2 larvae/mL affected these traits relative to the lowest larval density, increasing development time by approximately 1.5 days, decreasing wing length by nearly 10%, and decreasing survival by approximately 10%, and was chosen for the following experiments.

Experiments were conducted at 26°C ± 1°C and 80–90% relative humidity with a 12:12 light: dark photoperiod. Treatments, where Wolbachia-infected and uninfected larvae were present (mixed cohorts), comprised the following numbers of wMelPop or wMel to uninfected larvae: 300 to 100, 200 to 200, and 100 to 300. Pure cohorts of 400 infected or uninfected larvae were also included as controls. Eggs were hatched synchronously and first instar larvae were transferred to plastic containers with mesh lids (9.5–11.5 cm radius, 7 cm height) holding 200 mL of RO water. Containers were topped up with RO water daily to counter evaporation. A total of 100 mL of water from each experimental container was removed every second day, and replaced with 100 mL of RO water to reduce the build-up of toxic waste products. Adult eclosion was scored twice a day; adults were released into cages, chilled, and then stored in absolute ethanol. Experiments were terminated when all mosquitoes had eclosed or died.

Each treatment of the wMelPop versus uninfected experiment was replicated four times. Larvae were provided with TetraMin: 0.15 mg, 0.18 mg, 0.3 mg, 0.35 mg, and 0.35 mg per larva on days 1, 3, 4, and 5 of the experiment, respectively. In the wMel versus uninfected experiment, treatments were replicated five times. Larvae were provided with TetraMin: 0.08 mg, 0.08 mg, 0.32 mg, 0.37 mg, and 0.37 mg per larva on days 1, 2, 3, 4, and 5 of the experiment, respectively. A few containers were contaminated with bacteria (cloudy appearance) and were excluded from the experiments.

A subset of adults was collected on three occasions for wing measurement. Males were collected on days 8, 10, and 12, and females were collected on days 9, 11, and 13 to cover a range of development times. For each treatment, at least six adults per replicate were collected on each day; mosquitoes were screened for Wolbachia infection status (see below). All pure cohorts were also screened for Wolbachia to confirm infection status. Wing length measurements. Both wings were removed from each adult, placed under a 10-mm coverslip, and fixed with Hoyer’s solution (distilled water: gum arabic: chloral hydrate: glycerin in the ratio 5:3:20:2). Wing length was calculated as the distance from the alular notch to the wing tip, excluding the fringe scales.37 The lengths of both wings of a mosquito were averaged, and each measurement was repeated. Thus, length represented the average of four measurements. Damaged wings were excluded from the analysis.

DNA extraction and Wolbachia screening. Genomic DNA was extracted from adult mosquitoes by using a Chelex® 100 resin (Bio-Rad Laboratories, Hercules, CA) extraction method.38 Mosquitoes were ground in 3 μL of proteinase K (20 mg/mL) (Roche Diagnostics Australia Pty. Ltd., Castle Hill New South Wales, Australia) and 250 μL of 5% Chelex® solution, incubated at 65°C for 1 hour, followed by incubation for 10 minutes at 90°C and storage at −20°C. Wolbachia infection status was determined by using methods developed by Lee and others for a Roche LightCycler 480. Quantitative real-time polymerase chain reaction (PCR) was used to amplify three markers with three primer sets: a mosquito primer set to detect mosquitoes from the Aedes genus, Ae. aegypti specific primers to differentiate Ae. aegypti from other Aedes species, and Wolbachia specific primers to determine Wolbachia infection status, as well as density. Diagnosis was based on crossing-point values for the PCR and melting temperature from high-resolution melt analysis. More details of the screening method are provided by Yeap and others and Lee and others.

Statistical analyses. Chi-square tests were used to assess deviations from the expected numbers of infected and uninfected offspring surviving until adulthood, and to test departures from an expected 1:1 male to female ratio. Contingency analyses were performed to assess differences in developmental time between infections in mixed cohorts, accounting for differential survivorship. Analyses of variance (ANOVA) and linear regressions were conducted using PopTools version 3.2 for Microsoft (Redmond, WA) Excel to test for the effect of varying infection frequencies on size and development time.
Survival data were arcsine square-root transformed, and development time data were square-root transformed for normality as determined through Kolmogorov-Smirnov tests. Tukey's honest significant difference post-hoc tests were conducted for pairwise comparisons between strains and treatments using SPSS version 19 (SPSS, Inc., Chicago, IL) after finding significant effects with ANOVAs.

RESULTS

Survivorship. Survival to adulthood was scored to determine any differences in overall larval viability between treatments. No significant effect of initial Wolbachia proportion on overall survivorship was found between treatments in the wMel versus uninfected experiment (overall mean viability ± SE = 0.892 ± 0.013, by one-way ANOVA, \( F_{4,13} = 1.136, P = 0.382 \)) or the wMel versus uninfected experiment (0.916 ± 0.010, by one-way ANOVA, \( F_{4,17} = 0.691, P = 0.608 \)). Note that these comparisons address overall survivorship rather than the infection status of individual larvae. There were also no significant deviations from a 1:1 sex ratio in any of the treatments.

A subset of eclosing adults from each treatment was screened with PCR to differentiate between Wolbachia-infected and uninfected adults (Table 1). In the wMel versus uninfected experiment (Table 1A), no deviations from the expected number of Wolbachia-infected to uninfected adults were significant for any individual treatment (all \( P > 0.09 \)), or when all mosquitoes were pooled across treatments and sex (\( \chi^2 = 3.016, \text{degrees of freedom} = 1, P = 0.0824 \)). In the wMel versus uninfected experiment (Table 1B), a significantly greater number of adults were positive for Wolbachia than expected when sexes and treatments were pooled (\( \chi^2 = 14.341, \text{degrees of freedom} = 1, P = 0.0002 \)), suggesting higher relative survival of the infected strain when in a competitive environment.

Developmental time. Males (mean ± SE = 9.756 ± 0.051 days) developed around a day earlier on average than females (10.886 ± 0.051 days, \( P < 0.0001 \), by two-tailed \( t \)-test). The proportion of Wolbachia-infected larvae had no effect on mean developmental time for experiments with wMelPop (males: one-way ANOVA: \( F_{4,13} = 0.808, P = 0.542 \), females: one-way ANOVA: \( F_{4,13} = 0.331, P = 0.852 \)) or wMel (males: one-way ANOVA: \( F_{4,17} = 0.581, P = 0.681 \), females: one-way ANOVA: \( F_{4,17} = 0.361, P = 0.833 \)) when infection status of individual larvae was not considered.

Mosquitoes from the mixed cohorts, in which infected and uninfected larvae were present, were collected at three times for each sex and screened for Wolbachia to compare development rates of infected and uninfected larvae. If no differences in developmental time exist between strains, the infection frequency of adults emerging on each day would be expected to match expected numbers computed from the total number of infected and uninfected mosquitoes. Contingency analysis on numbers across days indicated significant differences in developmental time between Wolbachia-infected and uninfected larvae (Table 2). Relative to expectations, fewer mosquitoes that eclosed on day 8 (males) and day 9 (females) were infected with wMelPop (Table 2A). In contrast, on days 12 and 13, wMelPop-infected adults were overrepresented. This pattern is evident in males and females in all mixed cohorts (Table 2), and suggests that the development of infected larvae is delayed relative to uninfected larvae.

A similar but smaller delay in development was evident in the wMel experiment (Table 2B). The wMel-infected adults occurred less commonly than expected in the sample of early developers, and were more common than expected in late developers.

Wing length. Length differed significantly (\( P < 0.0001 \), by two-tailed \( t \)-test) between males (mean ± SE = 2.106 ± 0.003 mm, \( n = 828 \)) and females (2.743 ± 0.005 mm, \( n = 825 \)), and was also positively associated with developmental time for males (\( R^2 = 0.277, P < 0.0001 \)) and females (\( R^2 = 0.425, P < 0.0001 \)) (Figures 1 and 2).

For the wMelPop experiment, wing lengths of infected adults that emerged on days 12 and 13 were generally smaller relative to uninfected mosquitoes that eclosed on the same day. In contrast, they were similar for the earlier emerging mosquitoes (Figure 1). This wing length disparity in late developers was most pronounced in females when infected and uninfected strains were reared separately; infected females that eclosed on day 13 had a mean ± SE wing length of 2.762 ± 0.016 mm compared with the uninfected mean of 2.942 ± 0.017 mm (\( P < 0.0001 \), by two-tailed \( t \)-test). Differences tended to be reduced in mixed cohorts (Figure 1).

For the wMel experiment, wing lengths of infected adults from mixed cohorts (males: mean ± SE = 2.147 ± 0.006 mm, females = 2.791 ± 0.012 mm) were greater than that of uninfected adults from mixed cohorts (males = 2.089 ± 0.007 mm, \( P < 0.0001 \), by two-tailed \( t \)-test; females = 2.688 ± 0.009 mm, \( P < 0.0001 \), by two-tailed \( t \)-test). This observation can partially be explained by an overrepresentation of the wMel infection in slow developers, which generally have larger wings (Figures 1 and 2). However, wMel-infected mosquitoes also had significantly larger wings in some comparisons with uninfected adults on the same day of eclosion (Figure 2), suggesting an effect of wMel infection on wing length independent of developmental time.

Wolbachia density. Wolbachia density, an estimate of the number of copies of Wolbachia DNA relative to Ae. aegypti DNA, was higher on average in the wMelPop strain relative to the wMel strain (Figure 3), concordant with previous studies. Wolbachia density increased with developmental time in both infected strains, and for both sexes (Figure 3). This trend
was not observed in the 100 to 300 Wolbachia-infected to uninfected treatments, most likely because of low sample sizes of Wolbachia-infected mosquitoes (Table 1).

**DISCUSSION**

We examined the ability of Wolbachia-infected larvae to compete with uninfected larvae at different frequencies under crowded conditions. Our results indicate that survivorship of wMelPop-infected larvae was not significantly reduced relative to uninfected larvae in either mixed cohorts or pure cohorts. However, even a 5% relative viability cost of Wolbachia infection can make invasion difficult. The wMelPop-infected adults tended to be slightly underrepresented in mixed and pure cohorts, although whether this minor cost of infection to larval viability is sufficient to affect wMelPop invasion requires further investigation. In contrast, wMel was significantly overrepresented in screened adults; wMel infection appears to provide a viability benefit relative to uninfected larvae in mixed cohorts.

When strains were reared separately, neither Wolbachia infection had any significant effect on mean male or female developmental rate relative to the uninfected strain. However, development of wMelPop-infected and wMel-infected larvae was delayed relative to uninfected larvae when competing with them directly. This was observed in all treatments; Wolbachia delays development to a similar extent regardless of the initial infection frequency, although the delay was more severe in the wMelPop strain.

Wolbachia infection might therefore have several impacts on fitness under field conditions. Rapid development is particularly important for males because they can reach reproductive age faster and avoid competition for mates. Nutrition is limited in the field; slower developers risk food being depleted before they can complete development. Slow development also increases the duration of exposure to predation during immature stages and reduces the likelihood of survival in temporary habitats where water evaporates.

Although we were not able to determine the basis for this Wolbachia-induced developmental delay, recent studies suggest several potential explanations. Wolbachia is known to modify adult behavior, and Wolbachia might also reduce larval foraging capability, but this remains to be tested. In addition, immune up-regulation or increased metabolism.

### Table 2

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*Expected proportions are computed from the number emerging on a day and the number of infected and uninfected individuals overall (i.e., corrected for survivorship differences between strains).
†Days after hatching to eclosion. Males were sampled on days 8, 10, and 12 and females were sampled on days 9, 11, and 13.
‡Observed numbers of Wolbachia-infected and uninfected adults on each day of eclosion.
§Expected numbers of adults emerging on each day, adjusted for survivorship differences between strains. Expected values are equal for each day of eclosion.
¶P value for contingency table, χ² with degrees of freedom = 2, testing the deviation of observed ratio from expected due to chance (P < 0.05 in bold).
Figure 1. Mean wing length comparison between wMelPop-infected and uninfected *Aedes aegypti* for pure cohorts and mixed cohorts, and for females and males on three days of eclosion. Error bars indicate standard errors. Bars with the same letter (in bold) are not significantly different from each other (*P* > 0.05, by analysis of variance and Tukey’s honest significance difference test). *n* indicates number of samples per bar.

Figure 2. Mean wing length comparison between wMcl-infected and uninfected *Aedes aegypti* for pure cohorts and mixed cohorts, and for females and males on three days of eclosion. Error bars indicate standard errors. Bars with the same letter (in bold) are not significantly different from each other (*P* > 0.05, by analysis of variance and Tukey’s honest significance test). *n* indicates number of samples per bar.
observed in adults may also influence larval development rate. Our results are consistent with earlier observations; delayed development of wMelPop-infected larvae was shown by McMeniman and O’Neill16 and Yeap and others.17 However, neither of these studies conducted experiments with mixed cohorts, when Wolbachia-infected and uninfected larvae compete for the same resources. During release periods, direct competition will occur between infected and uninfected larvae. We predict that Wolbachia infections will disadvantage immature stages in the field relative to the wild-type population under competitive situations.

Although Wolbachia infection causes a developmental delay during inter-strain competition, the implications for adult fitness under field conditions are less clear. We observed a size trade-off in fast developers; when larvae were crowded, slow developers were larger than mosquitoes that were quicker to emerge, presumably because of increased feeding time and reduced competition. This finding is in contrast to a study of Ae. albopictus,19 in which delayed developers gained no size benefit when reared at a high density. Body size is an important indicator of female reproductive success; larger size is associated with greater fecundity,41,46 blood feeding success47 and mating success.48 The wMelPop infection significantly reduced body size (wing length) in slow developers relative to uninfected Ae. aegypti adults. However, no size difference between strains was observed in quicker developers. Slow-developing wMelPop-infected larvae therefore appear less able to take advantage of an increased development period to grow larger. Although wMelPop caused a deleterious effect regardless of the initial infection frequency, the cost to size appears amplified during intra-strain competition.

A body size reduction was not observed in the wMel infection; instead the infection was associated with a marginally increased size relative to uninfected mosquitoes, regardless of developmental time. We hypothesize that differential effects on size of wMel and wMelPop infections might be caused by differences in the level of replication between the Wolbachia strains.11 Wolbachia infection appears to be beneficial up until a certain density, but over-replication in host tissues becomes detrimental to size. We showed that Wolbachia density is highest in slow-developing wMelPop, in which we observed deleterious effects on size. However, wMelPop infection has no effect on the size of faster developers, in which Wolbachia density is lower. In the wMel infection in which Wolbachia densities are half that of wMelPop, size is increased over the uninfected strain.

Prior studies have noted differential effects of Wolbachia infection between sexes in response to larval competition.19,33 Sex-specific effects are expected in maternally transmitted endosymbionts because selection pressures acting on males are different than those acting on females. We found no clear sex-specific effects of either Wolbachia infection in our experiments. Wolbachia does not appear to cause differential survivorship between sexes because no deviations from a 1:1 male to female ratio in the surviving adults were significant. Wolbachia infection affected male and female developmental time in mixed cohorts equally, and wing length and Wolbachia density followed similar trends with respect to treatment and developmental time for both sexes.

Figure 3. Relative Wolbachia density of Wolbachia-positive males and females of Aedes aegypti from A, wMelPop versus uninfected treatments and B, wMel versus uninfected treatments. Mean values are given for each day of eclosion. Error bars indicate standard errors. Bars with the same letter (in bold) are not significantly different from each other (P > 0.05, by analysis of variance and Tukey’s honest significance test). n indicates number of samples per bar.
Despite a developmental delay in the wMel infection, this strain was able to invade two field populations, and continues to persist. For the wMelPop infection to become established in natural populations, strategies to counteract its deleterious effects must be used to maximize mosquito fitness. One potential strategy uses insecticides to crash target populations during release of insecticide-resistant Wolbachia-infected mosquitoes. This strategy will reduce the release numbers required for the Wolbachia infection to exceed its threshold frequency and reach fixation in a population. Although establishing the wMelPop infection in the field poses a challenge, there is continuing interest in the use of wMelPop for dengue control strategies; wMelPop provides a superior dengue blocking ability to wMel, and the life-shortening effect of wMelPop can further reduce potential dengue transmission (in the absence of its innate dengue protection) by killing the mosquito before the virus can be transmitted to a human host. We showed that wMelPop infection has deleterious effects on developmental time and adult size in competition with uninfected larvae; ideally competition should be avoided during releases to minimize these fitness costs. These results are important for assessing the invasion prospects of the wMelPop infection in areas to which dengue is endemic.

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REFERENCES


APPENDIX F – *Wolbachia* Strains for Disease Control: Ecological and Evolutionary Considerations
REVIEWS AND SYNTHESSES

Wolbachia strains for disease control: ecological and evolutionary considerations

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Keywords
Aedes, deployment issues, disease control, fitness, strain attributes, virus, Wolbachia.

Abstract

Wolbachia are endosymbionts found in many insects with the potential to suppress vectorborne diseases, particularly through interfering with pathogen transmission. Wolbachia strains are highly variable in their effects on hosts, raising the issue of which attributes should be selected to ensure that the best strains are developed for disease control. This depends on their ability to suppress viral transmission, invade host populations, persist without loss of viral suppression and not interfere with other control strategies. The potential to achieve these objectives is likely to involve evolutionary constraints; viral suppression may be limited by the ability of infections to spread due to deleterious host fitness effects. However, there are exceptions to these patterns in both natural infections and in novel associations generated following interspecific transfer, suggesting that pathogen blockage, deleterious fitness effects and changes to reproductive biology might be at least partly decoupled to achieve ideal infection attributes. The stability of introduced Wolbachia and its effects on viral transmission remain unclear, but rapid evolutionary changes seem unlikely. Although deliberate transfers of Wolbachia across species remain particularly challenging, the availability of strains with desirable attributes should be expanded, taking advantage of the diversity available across thousands of strains in natural populations.

Introduction

There is currently a high level of interest in using Wolbachia to indirectly suppress the incidence of vectorborne human diseases such as malaria, dengue fever or filariasis (McGraw and O’Neill 2013; Sinkins 2013; Bourtzis et al. 2014), or plant diseases caused by mosaic viruses and other disease agents (Box 1). Wolbachia are endosymbiotic bacteria living in all orders of insects and in other invertebrates. They are often promoted as a ‘natural’ way of manipulating insect hosts, in contrast to other approaches for manipulating vector biology, particularly through genetic modification, that are often regarded with suspicion because they involve novel constructs that are not present in the environment with the potential to spread to other nonvector species. While Wolbachia are already widespread in the environment, they are restricted to living exclusively within host cells and spread by changing the biology of their host species (O’Neill et al. 1997).

Wolbachia can be used in a variety of ways for disease suppression, by decreasing the size of a vector population through (i) the release of Wolbachia-infected males that are incompatible with females (O’Connor et al. 2012) or (ii) the invasion of a Wolbachia strain that produces deleterious fitness effects particularly under seasonally variable environments (Rašić et al. 2014a), and particularly by (iii) decreasing the ability of the vector population to transmit diseases through the invasion of a Wolbachia strain that interferes directly with transmission (Teixeira et al. 2008; Kambris et al. 2009; Moreira et al. 2009; Walker et al. 2011). The third option is considered particularly promising because it may not require ongoing management by health authorities; once a Wolbachia strain blocking disease transmission has invaded a target vector population by altering host reproduction, the Wolbachia strain should stay at a high frequency in that population without further releases being required (Hoffmann et al. 2011). It is also important to note that the three strategies are not mutually exclusive but rather complementary.
Despite the promise held by Wolbachia-based disease suppression programmes particularly for viral diseases spread by mosquito vectors (Box 1), there are still substantial challenges in their widespread deployment. In particular, strains of Wolbachia for release need to be carefully selected and evaluated to ensure long-term viability of the strategy in the face of ongoing evolutionary changes, and to meet any regulatory and community concerns. In this study, we focus on these issues, using information that has been collected on insects naturally infected with Wolbachia and on artificial introductions of Wolbachia into new hosts. We consider the development of strains and host backgrounds that combine desirable attributes for disease suppression with those required for rapid invasion into target vector populations, as well as the likely long-term evolutionary stability of effects generated by Wolbachia in these populations. Much of the information we discuss comes from research in Drosophila species where Wolbachia infections have been investigated within an evolutionary and ecological context since the early 80s, and where a large number of infections have been transferred across species to investigate the interaction and evolution of host and Wolbachia genomes.

**Diversity of Wolbachia and their effects**

There is an enormous diversity of Wolbachia strains in nature. DNA sequence data have been used to demonstrate the presence of multiple Wolbachia variants within the same individual host, the presence of variation among Wolbachia sequences collected from different conspecific individuals, and molecular changes in the same Wolbachia infection when it is transferred to different host species. Most molecular comparisons have focussed on describing variation in Wolbachia infections across related species to characterize patterns of horizontal and vertical transmission of infections across time (e.g. Bing et al. 2014; Morrow et al. 2014), using sets of conserved primers for a series of genes such as the MLST set (Baldo et al. 2006). Many studies have used primer sets to demonstrate variation in Wolbachia strains within the same host. An example of a species carrying a complex of infections is the tsetse fly, where 37 different Wolbachia variants have been identified (Symula et al. 2013). Numerous Wolbachia strains have also been identified in mosquitoes of the Culex pipiens species complex (Atyame et al. 2011; Morningstar et al. 2012) where they (rather than the nuclear background) largely control patterns of cytoplasmic incompatibility (Duron et al. 2006). Variability among Wolbachia within the same host could arise through recombination and mutation (Klasson et al. 2009; Atyame et al. 2011), and a new Wolbachia strain could spread if it has a selective advantage and/or generates patterns of cytoplasmic incompatibility or other changes to host reproduction that favour its spread. Horizontal transmission of Wolbachia across hosts such as mediated through a parasitoid could also result in the introduction of a new Wolbachia strain into a host population. Once new Wolbachia strains arise, they can displace existing strains at a rapid rate, as indicated by the replacement of wAu by wRi in Australian populations of Drosophila simulans (Kriesner et al. 2013), but these types of replacements are thought to occur very rarely (Richardson et al. 2012).
The majority of Wolbachia strains have undescribed effects, having been detected in organisms via molecular tools (Hilgenboecker et al. 2008; Ahmed et al. 2013) and not further studied experimentally. Many of these strains may well have little impact on their host, but nevertheless persist because of a high fidelity of vertical transmission from mothers to offspring. Such infections with no apparent phenotypic effects on hosts have been described in Drosophila species (e.g. Hoffmann et al. 1996). Other Wolbachia strains are likely to have dramatic effects on their host; the most widespread of these effects is cytoplasmic incompatibility, where Wolbachia presence leads to the death of embryos and sometimes immature offspring when infected fathers mate with uninfected mothers (or mothers carrying a different Wolbachia strain). There are also Wolbachia infections that cause the death of male offspring only (male killers) and others that lead to parthenogenetic reproduction in haplodiploid organisms or feminization of male offspring (reviewed in O’Neill et al. 1997). Even when Wolbachia appear to have no phenotypic effects on their host’s reproduction, they might nevertheless have other effects that only become evident once appropriate host challenges are provided; for instance, the viral blocking activity of Wolbachia strains only became apparent once infected Drosophila strains were challenged with RNA viruses (Teixeira et al. 2008; Hedges et al. 2008, Osborne et al. 2009).

Wolbachia effects exerted on hosts typically fall along a continuum; for instance, cytoplasmic incompatibility can range from complete (all offspring die) as in the case of many Wolbachia infections from mosquitoes (e.g. Rason and Scott 2003), to relatively weak (a small proportion of offspring die) as in the case of particular Drosophila infections (e.g. Reynolds et al. 2003). Moreover, the effects of Wolbachia on hosts can change markedly depending on environmental conditions and the age of the insect. Factors, such as the presence of natural antibiotics (Clancy and Hoffmann 1998; Lu et al. 2012), temperature extremes (Mouton et al. 2007; Bordenstein and Bordenstein 2011), the age of the male and female (Unckless et al. 2009; Tortosa et al. 2010) and interactions among these factors (Mouton et al. 2007; Bordenstein and Bordenstein 2011), can all influence the density of Wolbachia in host tissues and host effects such as cytoplasmic incompatibility.

Wolbachia density often varies substantially among individuals under field conditions (e.g. Ahantarig et al. 2008). This variation could potentially influence the transmission, fitness effects and expression of cytoplasmic incompatibility, which has been characterized in detail in Drosophila populations where variability in cytoplasmic incompatibility is high (e.g. Turelli and Hoffmann 1995) and in Culex populations where the variability is low (Rason and Scott 2003). However, it is not clear whether the variability reflects Wolbachia/host genomic variation or environment-induced variation that might only have a temporary effect on density and host phenotypes. For instance, when the wHa infection in D. simulans was tested in multiple host lines derived from the field, variation in the ovarian density of the Wolbachia infection among host lines was maintained for several generations, but was eventually lost (Correa and Ballard 2012). Therefore, while experimental studies might indicate a clear correlation between Wolbachia density and cytoplasmic incompatibility/deleterious effects (e.g. Clancy and Hoffmann 1998), it is not clear whether density variation is necessarily linked to variation in the Wolbachia genome. Recently, a group of Wolbachia genes associated with density variation (the Octomom region) has been identified in the wMelPop strain of D. melanogaster (Chrostek and Teixeira 2015) and might provide candidates for affecting density in field samples.

Unpredictable phenotypic effects in new hosts

A substantial number of Wolbachia strains have now been transferred through microinjection across species boundaries, particularly in the genus Drosophila, but also across genera within and among insect orders (Table 1, Appendix S1). Successful Wolbachia transfers can be challenging, although those involving Drosophila species have been undertaken for some time and have become fairly routine (e.g. Poinset et al. 1998; Charlat et al. 2002). Cross-infection experiments where Wolbachia are artificially transferred from one species to another have demonstrated (particularly in Drosophila) that host effects associated with a particular Wolbachia strain can persist or be modified after transfer to a new host (e.g. Ikeda et al. 2003; Osborne et al. 2012; Veneti et al. 2012).

The marked changes in cytoplasmic incompatibility and other reproductive effects, as well as host fitness effects, are typified by the wAu infection and lack of fitness effects in its native host but life shortening following transfer to D. melanogaster (Chrostek et al. 2014), and the absence of male killing when Wolbachia from Drosophila innubila are transferred to D. melanogaster and D. simulans (Veneti et al. 2012). As another example, wCaUA causes cytoplasmic incompatibility in its native host, Cadra cautella (Sasaki and Ishikawa 1999), but when transferred to Ephemia kuehniella, it causes male killing (Sasaki et al. 2002) (see Appendix S1). There are also several other instances where shifts in cytoplasmic incompatibility occur when Wolbachia from one host are transferred to a different species within the same genus (e.g. Boyle et al. 1993), and clearly, viral interference will also depend on host effects as reflected by the limited blockage provided by wAlB in its native Ae. albopictus host compared to strong blockage when this
### Table 1. Stable Wolbachia infections in native and transfected hosts, their reproductive effects (CI – cytoplasmic incompatibility, MT – maternal transmission), fitness effects, and viral blocking effectiveness where demonstrated (? – information unavailable). Effect size is denoted as: high (>90%), moderate/partial (20–90%), low (<20%) and none (no detectible effects). More details are found in Appendix S1.

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1Hoffmann (1988); 2Reynolds and Hoffmann (2002); 3Fry et al. (2004); 4Hoffmann et al. (1998); 5Harcombe and Hoffmann (2004); 6Montenegro et al. (2006); 7Fry and Rand (2002); 8Glaser and Meola (2010); 9Teixeira et al. (2008); 10Chrostek et al. (2013); 11Rances et al. (2012); 12Walker et al. (2011); 13Hoffmann et al. (2014a); 14Van den Hurk et al. (2012); 15Hussain et al. (2013); 16Blagrove et al. (2012); 17Blagrove et al. (2013); 18Poinso et al. (1998); 19Osborne et al. (2009); 20Martinez et al. (2014); 21Min and Benzer (1997); 22Reynolds et al. (2003); 23McGraw et al. (2001); 24Carrington et al. (2009); 25Hedges et al. (2008); 26McMeniman et al. (2009); 27Yeap et al. (2011); 28McMeniman and O’Neill (2010); 29Turley et al. (2009); 30Moreira et al. (2009); 31Suh et al. (2009); 32Carrington et al. (2010); 33Hoffmann et al. (1996); 34Yamada et al. (2011); 35Chrostek et al. (2014); 36Solignac et al. (1994); 37Holden et al. (1993); 38Serf et al. (2014); 39Dyer and Jainek (2004); 40Unckless and Jaenike (2011); 41Veneti et al. (2012); 42Hoffmann et al. (1986); 43Hoffmann et al. (1990); 44Turelli and Hoffmann (1995); 45Weeks et al. (2007); 46Xi et al. (2006); 47Fu et al. (2010); 48Boyle et al. (1993); 49Clancy and Hoffmann (1997); 50Zabel et al. (2004); 51Kang et al. (2003); 52Sinkins et al. (1995); 53Dobson et al. (2001); 54Dobson et al. (2004); 55Dobson et al. (2002); 56Kittayapong et al. (2002); 57Calvetti et al. (2009); 58Moens et al. (2012); 59Moens et al. (2010); 60Xie et al. (2005); 61Ruang-Areerate and Kittayapong (2006); 62Bian et al. (2010); 63Andrews et al. (2012); 64Bian et al. (2013a); 65Hertig and Wolbach (1924); 66Yen and Barr (1973); 67Rasgon and Scott (2003); 68de Almeida et al. (2011); 69Calvetti et al. (2010); 70Moretii and Calvetti (2013).

*High for one day old males, but decreases rapidly with increasing male age; +increases with higher Octomom copy numbers (Chrostek and Teixeira 2015); †unpublished work by J Axford and AA Hoffmann.
infection is transferred to *Ae. aegypti* (Bian et al. 2010) and other examples (Table 1, Appendix S1).

**Desirable attributes of *Wolbachia* strains for disease suppression**

With many thousands of *Wolbachia* strains existing in nature and interacting with host genomes and local environments in different ways, *Wolbachia* could be used in a variety of ways for disease control strategies aimed at suppressing vector populations and directly interfering with disease transmission. Some important transfers of *Wolbachia* to disease vectors have now been achieved, including transfers of *Wolbachia* from *Drosophila* to *Aedes* mosquitoes for the production of vectors that exhibit shortened lifespan (McMeniman et al. 2009) and suppression of RNA viruses and other disease agents (Kambris et al. 2009; Moreira et al. 2009; Walker et al. 2011). In addition, there have been successful transfers of *Wolbachia* from *Aedes albopictus* to *Aedes aegypti* to achieve virus suppression (Xi et al. 2005; Bian et al. 2010). These transfers capture a tiny fraction of the vast diversity of *Wolbachia* strains available in natural populations of insects related to mosquitoes. Yet, they are already raising questions about how *Wolbachia* strains and host backgrounds might be developed for disease suppression.

Different strain attributes are required by the three strategies that use *Wolbachia* to reduce disease transmission. The simplest requirement is for population suppression via male release where the main attribute is for released males to exhibit strong cytoplasmic incompatibility when they mate with field females. Released males also need to be competitive with males from natural populations. Competitive ability could be reduced if *Wolbachia* in the release strain directly reduces male competitive fitness and/or if the host nuclear background of the release strain has a detrimental effect on male field competitiveness. At least for *Ae. aegypti* carrying the wMel or wMelPop infection, there is no evidence that *Wolbachia* directly reduces male competitive fitness (Segoli et al. 2014), while *Ae. polynesiensis* carrying Wolbachia are also competitive in field releases (O’Connor et al. 2012). Detrimental host nuclear effects might develop if the release strain evolves and becomes adapted to conditions used for artificial rearing. This can be circumvented through backcrossing the release strain to field-sourced material prior to releases taking place, although it may then be more difficult to rear the strain under the artificial conditions if adaptation has taken place. Male competitiveness also needs to be high for successful *Wolbachia* strategies involving invasion (that utilize deleterious fitness effects and viral interference) because strong cytoplasmic incompatibility is required to drive the infection into a target population. In addition, several other attributes will be required for invasion-based strategies.

**Ease of invasion into field populations**

To produce disease suppression by interfering with pathogen transmission or expressing deleterious fitness effects, *Wolbachia* strains need to invade and reach high frequencies in focal populations. In *Wolbachia* strains that have so far been introduced into *Ae. aegypti* populations, cytoplasmic incompatibility has been complete or nearly complete with uninfected target populations (Xi et al. 2005; McMeniman et al. 2009; Walker et al. 2011; Yeap et al. 2011), facilitating invasions. As long as there are no substantial deleterious effects of the *Wolbachia* on the hosts and as long as the infection is transmitted with a relatively high fidelity, invasion should be possible under strong cytoplasmic incompatibility. However, if a focal population is already infected with a *Wolbachia* strain that shows bidirectional incompatibility with the release strain, invasion becomes more difficult. Under bidirectional incompatibility between two *Wolbachia* strains with equivalent deleterious effects on a host, the infection frequency of an introduced strain has to exceed 50% to achieve invasion (Hoffmann and Turelli 1997). This situation applies to the wMel infection introduced into *Ae. albopictus* (Table 1) which is bidirectionally incompatible with the naturally occurring *Wolbachia* of this species (Blagrove et al. 2012). Invasion will also depend on other fitness attributes such as the ability of females carrying the *Wolbachia* strain to feed and locate breeding sites and the ability of larvae with the *Wolbachia* strain to compete against other conspecific larvae and other species.

**Reduced pathogen transmission**

For effective suppression of vectorborne diseases (strategy (iii) from above), *Wolbachia* strains will need to directly interfere with pathogen transmission in vector species. In *Aedes* mosquitoes, this has often been assessed in laboratory-based assays where blood is mixed with virus cultures to mimic titres that might be found in infected humans (Moreira et al. 2009). However, it is ideally assessed by feeding mosquitoes directly on blood from infected humans and assessing pathogens in tissue through which transmission occurs, such as the salivary glands and saliva of mosquitoes (Ferguson et al. 2015).

The ability of *Wolbachia* to block viruses and other microbes will depend on the nature of the viruses and the *Wolbachia* strains. In *Drosophila*, it appears that some types of viruses (DNA viruses in particular) are not affected by the presence of *Wolbachia* in host cells,
whereas RNA viruses appear to be inhibited (Teixeira et al. 2008). The extent of inhibition varies dramatically between *Wolbachia* strains, such that some strains cause a dramatic reduction of the viral load in the host, whereas others have little impact (Table 1). In *Aedes* mosquitoes where stable *Wolbachia* infections have been established, the potential for *Wolbachia* to block different dengue virus serotypes and other RNA viruses seems to be high (Table 1, Appendix S1). The wMelPop infection appears to be highly efficacious in blocking different dengue serotypes as well as other arboviruses, at least in laboratory-based assays (Moreira et al. 2009; van den Hurk et al. 2012). For other *Wolbachia* infections, particularly wMel and wAlbB, blockage against dengue serotypes also appears robust (Bian et al. 2010; Frentiu et al. 2014), but somewhat weaker than provided by wMelPop (Walker et al. 2011). Recent data for wMel feeding on blood from infected human patients also point to strong blockage of dengue in saliva but show some differences among serotypes (Ferguson et al. 2015).

**Stable effects on hosts**

Once a high frequency of infection is reached through releases and subsequent invasion driven by cytoplasmic incompatibility and other effects, *Wolbachia* effects on hosts and on viral transmission need to be stable, even if there are evolutionary changes in the virus and/or changes in the host’s nuclear genome and *Wolbachia* genome. Data on the stability of *Wolbachia* effects following deliberate introductions are only just starting to emerge (Frentiu et al. 2014; Hoffmann et al. 2014a), but there is some relevant information from natural *Wolbachia* infections in other systems and particularly in *Drosophila* (Chrostek et al. 2013). Strategies that utilize the deleterious host effects associated with *Wolbachia* infections (strategy ii from above) also require that such effects remain stable even when there might be strong selection in the host genome to counter them.

**Evolutionary changes in the host genome**

Evolution of host genomes in response to *Wolbachia* is certainly possible and is dramatically illustrated by the changes that nullify male killing by a natural *Wolbachia* infection in the butterfly *Hypolimnas bolina* (Hornett et al. 2006). Other relevant sources of evidence for such changes include experimental populations and longitudinal studies of natural populations.

Phenotypic changes in the expression of *Wolbachia* effects due to changes in the host nuclear genome have been documented in experimental host populations maintained both with and without deliberate selection pressures. These include evidence for nuclear-based attenuation of wMelPop effects on longevity in *D. melanogaster* hosts (Carrington et al. 2009) and in the novel host *D. simulans* (Carrington et al. 2010). When the wMelPop infection was transferred from *D. melanogaster* to *D. simulans*, it initially caused large fitness effects such as reducing fecundity and decreasing longevity as in its native host (McGraw et al. 2002). However, these effects attenuated quickly (Reynolds et al. 2003), such that wMelPop-infected *D. simulans* eventually exhibited an increase in longevity in some genetic backgrounds (Carrington et al. 2010). In *Ae. aegypti* mosquitoes, host genome changes can cause a decrease in deleterious effects of the introduced wMelPop on egg viability (A. Callahan and A. A. Hoffmann, unpublished data). The impact of host nuclear genomic backgrounds on virus blocking by *Wolbachia* has not yet been systematically investigated within either *Drosophila* or mosquito species. However, because the upregulation of immune response genes seems to be restricted to recently transferred infections in mosquitoes rather than native infections, an eventual decrease in blockage might be expected, given the likely high cost of constitutive immune gene expression.

The deliberate release of *Wolbachia* infections into natural mosquito populations provides an opportunity to test for host nuclear responses in natural populations across a period of a few years. In particular, the release of wMel into uninfected *Ae. aegypti* populations in 2011 in two areas around Cairns, Australia (Hoffmann et al. 2011), provided an opportunity to monitor changes in both the viral interference effect and deleterious host effect across a three-year time span. These comparisons have indicated that dengue interference was not altered within this period (Frentiu et al. 2014) and neither were fitness effects of *Wolbachia* on its host (Hoffmann et al. 2014a). Because there is ongoing gene flow into these populations as inferred from infection frequencies and a lack of maternal leakage (Hoffmann 2014b), changes in the nuclear genome due to *Wolbachia* are only expected if selection is relatively strong.

Although the host genome can have a substantial effect on the expression of cytoplasmic incompatibility, deleterious effects and viral interference, it is not yet clear whether there will be rapid changes in the host genome that might affect the success of *Wolbachia* releases aimed at disease suppression. The most rapid host changes are expected in response to any deleterious effects induced by *Wolbachia*, whereas selection for altered effects of *Wolbachia* on viral interference should be weak unless the virus has a particularly large impact on host fitness (in which case selection would favour ongoing interference by *Wolbachia*). The host genome is therefore most likely to influence the success of a suppression strategy based

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on the expression of deleterious effects following invasion.

**Evolutionary changes in the Wolbachia genome**

Evidence for possible changes in the Wolbachia genome comes from analysis of changes in laboratory and natural populations. In addition, the phenotypic effects associated with particular Wolbachia strains that are maintained following interspecific transfers (as in the case of the wMelPop infection following transfer from *D. melanogaster* to *D. simulans* and *Ae. aegypti* – Table 1) also point to effects on hosts mediated by the Wolbachia genome rather than the host genome.

It is still difficult to predict whether genomic changes in Wolbachia will be rapid enough to be detectable in experimental populations. For the virulent wMelPop infection, there have only been minor genomic changes since its introduction from *D. melanogaster* into the new host *Ae. aegypti* (Woolfit et al. 2013). On the other hand, in laboratory *D. melanogaster* populations, Octomom copy number seems to be able to evolve rapidly to alter the density of wMelPop (Chrostek and Teixeira 2015). There is also evidence from comparisons of conspecific populations of *D. melanogaster* that interactions between wMel Wolbachia and host genomes can evolve fairly rapidly (Olsen et al. 2001; Fry et al. 2004). The wRi infection of *D. simulans* is another such example (Weeks et al. 2007). The deleterious effects of this infection on female reproduction were first characterized in the late 1980s (Hoffmann et al. 1990). Twenty years on, such effects were no longer evident, and some infected females even showed a fecundity advantage over uninfected hosts, largely attributable to changes in wRi or another maternally inherited factor (Weeks et al. 2007).

These findings suggest that while there is ample evidence for variation in the Wolbachia genome resulting in multiple strains of Wolbachia occurring in the same host and/or conspecific individuals carrying different Wolbachia strains, it is not clear whether there will be rapid changes in Wolbachia strains being released for disease suppression. As in the case of host genome changes, any changes will most likely be expected, particularly given that there are differences in the extent to which dengue serotypes are blocked by Wolbachia (Frentiu et al. 2014; Ferguson et al. 2015). However, only some types of interactions between Wolbachia and viruses (such as direct competition between viruses and Wolbachia) are expected to lead to evolutionary changes (Bull and Turelli 2013). Moreover, viral evolutionary dynamics are affected by a number of factors unconnected to Wolbachia that drive viral strain replacements (Vu et al. 2010; Lambrechts et al. 2012). Wolbachia and/or host genomes could also evolve in response to any changes in the virus, particularly if these affect the fitness of the vector host, although (at least in the case of dengue) viral effects on hosts remain unclear (Maciel-de-Freitas et al. 2011).

**Other effects of Wolbachia**

Even though Wolbachia can decrease transmission of many viral infections, its effects on others remain uncertain. A comparison of Wolbachia-infected and cured *D. melanogaster* strains and *Culex quinquefasciatus* strains suggested that Wolbachia might block West Nile virus (Glaser and Meola 2010). However, most *Culex quinquefasciatus* populations appear naturally infected with Wolbachia but are still capable of transmitting West Nile (Micieli and Glaser 2014). This may reflect the fact that Wolbachia densities in natural infections are too low to have much impact on transmission of this virus. On the other hand, in a recent study where Wolbachia from another mosquito were injected into *Culex dorsalis* females, the titre of West Nile virus increased (Dodson et al. 2014), although this may have been an effect of the infection process; the effect of Wolbachia on West Nile needs to be investigated in a host mosquito species carrying a stably introduced Wolbachia infection. In *Sporodoptera* moths, Wolbachia may also increase susceptibility to a virus (Graham et al. 2012); infection by nucleopolyhedrovirus was associated with moths carrying different strains of Wolbachia, and laboratory tests with one of the Wolbachia strains (likely a male killer) indicated much higher mortality levels following the viral infection. Because nucleopolyhedrovirus is being explored as a potential biopesticide, this result might point to a potentially novel application of Wolbachia releases for pest control.

It is not yet clear whether Wolbachia-based strategies will be effective against microbes other than viruses. Wolbachia introduced into the major malaria vector *Anopheles stephensi* protects against *Plasmodium* to some extent (Bian et al. 2013b), although perhaps insufficiently to provide much impact on disease transmission (Killeen et al. 2013). Moreover, it has been suggested that the presence of
Wolbachia may even enhance the incidence of malaria pathogens to some extent (Zélé et al. 2014) although this requires further validation. In Drosophila, Wolbachia infections appear to have few consistent effects on bacterial infections (Wong et al. 2011), while in mosquitoes, it has been suggested that any effects on bacteria will depend on whether the immune system is upregulated following Wolbachia transfer (Ye et al. 2013).

Another issue relevant to disease transmission is the potential interaction between Wolbachia and pesticide susceptibility. For Ae. aegypti mosquitoes that are artificially infected with Wolbachia, the infection does not affect susceptibility to commonly used insecticides (Endersby and Hoffmann 2013). However, in Culex pipiens naturally infected with Wolbachia, there was rapid evolutionary increase of Wolbachia density in an insecticide-resistant line (Echaubard et al. 2010), suggesting a dynamic interaction between the Wolbachia and/or host genomes evolving under insecticide exposure.

Because most Wolbachia-transfected lines originate from few or just one female (Xi et al. 2005; McMeniman et al. 2009), Wolbachia invasions can cause a dramatic reduction of mitochondrial haplotype diversity within and among populations (H. L. Yeap and A. A. Hoffmann, unpublished data; Armbruster et al. 2003). There is a growing body of evidence linking the mitochondrial polymorphisms with differences in metabolic rate and some fitness components in Drosophila (e.g. Ballard et al. 2007; Kurbalija Novićić et al. 2015), suggesting that mitochondrial diversity in natural populations is maintained by natural selection. Mitochondrial variation might play an important role in the epistatic interaction between the mitochondrial and nuclear genomes in determining insect metabolic rate under varying environmental conditions (Arnqvist et al. 2010). It is therefore possible that the loss of mitochondrial diversity following Wolbachia invasion could affect the performance of infected populations.

Finally, the various Wolbachia effects on host fitness could change the size and age distribution of the mosquito larval community in containers (Mains et al. 2013). These effects in turn might influence interspecific interactions, particularly under high-density larval conditions when fitness differences between Wolbachia-infected strains and uninfected strains can become accentuated (Ross et al. 2014). These ecological effects of Wolbachia need to be evaluated following invasions into natural communities and could have a substantial effect on disease transmission if vector populations become suppressed due to the detrimental effects of Wolbachia infection. The most dramatic example involves the wMelPop infection of Ae. aegypti, which reduces the viability of eggs when held in a dried state (Yeap et al. 2011). During a dry season, this effect could result in the complete collapse of an isolated population until there is a reinvasion from another source (Rašić et al. 2014a). Population cage experiments indicate that collapse is likely in populations that are completely Wolbachia-infected (S. Ritchie unpublished data).

**A pathogen interference/spread trade-off?**

It is possible that Wolbachia infections that provide the strongest blockage of pathogen transmission might not spread easily into populations (Fig. 1). This possibility arises because a high density of Wolbachia in hosts may increase viral blockage but decrease host fitness (Chrostek et al. 2013; Sinkins 2013; Martinez et al. 2014), and such a trade-off could have driven past cycles of Wolbachia strain replacements in natural populations. For instance, the wMel-CS strain in D. melanogaster which causes strong virus blockage (Table 1) may have been replaced with the wMel strain which causes weaker blockage but does not decrease longevity to the same extent in this host (Chrostek et al. 2013). Relevant information to explore the notion of such a trade-off comes from (i) comparisons of viral...
suppression, host fitness and *Wolbachia* density between infected hosts, (ii) inferences from natural populations and (iii) mechanistic understanding of the common basis of viral interference.

**Viral suppression vs host fitness and *Wolbachia* density**

Several authors have contrasted viral blockage (measured as survival/longevity following pathogen infection) in *Wolbachia* strains from *Drosophila* with effects on host fitness (mostly measured as longevity in the absence of the infection) and on cytoplasmic incompatibility (Table 1). These comparisons involve a relatively limited number of *Wolbachia* infections and a comparison of natural and introduced *Wolbachia* strains which may have different dynamics (Table 1, Fig. 1). In *D. simulans*, where the largest number of comparisons have been made involving 19 strains, survival following RNA viral infection is positively correlated with *Wolbachia* tissue density, although there are strains with relatively high *Wolbachia* densities that have a limited impact on survival (Martinez et al. 2014). Any association between deleterious *Wolbachia* effects and viral blockage may also not be particularly strong. In a comparison of five *Wolbachia* strains including one from a non-native host (*D. melanogaster*), the wAu infection caused the strongest blockage and had the highest density across tissues (Osborne et al. 2009, 2012), yet this strain does not cause detectable cytoplasmic incompatibility or have deleterious fitness effects, and is also transmitted at a high fidelity under field conditions (Hoffmann et al. 1996).

The wMelPop infection was transferred from *D. melanogaster* to *Ae. aegypti* to generate a strain that has a reduced longevity and thereby a reduced propensity to transmit diseases requiring a long incubation period through older females (McMeniman et al. 2009). In subsequent experiments, this strain was shown to have very strong blockage of viral replication and disease transmission in laboratory assays (Moreira et al. 2009). However, the wMelPop infection also causes substantial fitness costs, not just to longevity but also for egg viability, particularly when eggs are in a quiescent stage (McMeniman and O'Neill 2010; Yeap et al. 2011). The wMelPop infection also has deleterious effects on larval development under crowded conditions (Ross et al. 2014) and on some adult traits (e.g. Turley et al. 2009). In contrast, the wMel infection causes somewhat weaker blockage of dengue and other viruses than wMelPop, but has fewer deleterious effects as well as having a lower titre in adults (Walker et al. 2011; Hoffmann et al. 2014a).

The wMel infection was also transferred to *Ae. albopictus* where it causes strong blockage of chikungunya virus and dengue in laboratory assays, but has no apparent effects on longevity, hatch rates or other laboratory fitness parameters, despite generating strong cytoplasmic incompatibility (Blagrove et al. 2012, 2013). The wAlbB infection that blocks the transmission of dengue viruses in *Ae. aegypti* (Xi et al. 2005; Bian et al. 2010) has deleterious fitness effects on its host including a decrease in the viability of quiescent eggs and a reduction in longevity, although these deleterious effects are weaker compared to those exerted by wMelPop (J. Axford, unpublished data). When the native wPolA infection in *Ae. polynesiensis* was replaced with wAlbB from *Ae. albopictus*, there was an increase in *Wolbachia* density and evidence of dengue blocking in this species (Bian et al. 2013a), although it is not yet clear whether this transferred strain also produced deleterious fitness effects (Table 1).

*Wolbachia* density represents a complex phenotype, typically measured in three contexts: (i) whole body density, usually measured in newly eclosed adults; (ii) tissue specific density, focusing on heads, abdomens, ovaries, testes, salivary glands and so on; and (iii) age-specific (and life stage-specific) density, which can indicate whether *Wolbachia* continue to replicate when hosts have reached maturity or enter a quiescent phase. Changes in whole body density through exposure to low levels of antibiotics (usually tetracycline) typically reduce cytoplasmic incompatibility induced by *Wolbachia*, as demonstrated in the case of *D. simulans* (Clancy and Hoffmann 1998) and *Nasonia* wasps (Breeuwer and Werren 1993), and also reduce viral interference as shown for wAu in *D. simulans* (Osborne et al. 2012). These experimental data support the notion that differences in *Wolbachia* density can be linked to the expression of host effects and support the notion of a blocking/spread trade-off, particularly given that strain variation in *Wolbachia* density has a positive relationship to blockage in *D. simulans* as noted above (Martinez et al. 2014). However, the expression of strong cytoplasmic incompatibility in the *Drosophila paulistorum* species complex involves very low *Wolbachia* titres that can only be detected although nonconventional molecular methods (Miller et al. 2010), whereas high-density infections of other *Drosophila* species such as wAu (Osborne et al. 2012) have no detectable effects on cytoplasmic incompatibility or host fitness (Hoffmann et al. 1996). The effects of some infections can therefore be unconnected to their overall densities in hosts.

The tissue distribution of strains may influence pathogen blocking and host effects. For instance, the wRI and wHa infections in *D. simulans* are restricted mostly to gonadal tissues (Binnington and Hoffmann 1989; Correa and Ballard 2014), have mild deleterious effects (Hoffmann et al. 1990; Turelli and Hoffmann 1995) and cause mid- to low-level viral blockage (Osborne et al. 2009). On the other
hand, the wAu and wMelPop infections may block pathogens effectively because they are found in a variety of tissues (Min and Benzer 1997; Osborne et al. 2012). In mosquitoes, Wolbachia presence in a variety of tissues through which a virus needs to pass to be transmitted may be crucial for generating strong transmission blockage; for instance, wMelPop which causes strong blockage is found in many tissues including the salivary glands of Ae. aegypti (Moreira et al. 2009). This feature seems particularly important for dengue viruses, where a density-dependent cellular relationship between Wolbachia and viral load has been reported (Lu et al. 2012).

Some Wolbachia infections attain higher densities at eclosion and replicate at a higher rate than others when hosts reach adulthood (Chrostek and Teixeira 2015), resulting in very high densities throughout the body as hosts age. While this high density might result in strong pathogen blockage, it could also eventually kill the host and limit the potential of such infections to spread. The reduced longevity of D. melanogaster infected by the wMelPop strain is thought to be due to ongoing replication and increasing density of this virus (Min and Benzer 1997), as is the reduction in longevity and increased mortality of quiescent eggs in Ae. aegypti artificially infected by wMelPop (McMeniman and O’Neill 2010; Yeap et al. 2011). Continued Wolbachia replication may also contribute to hybrid sterility in crosses between D. paulistorum semi-species (Miller et al. 2010).

The distribution of Wolbachia within hosts is expected to be altered due to evolutionary changes in the host and Wolbachia. The distribution of Wolbachia densities across tissues in long-standing infections is expected to become more variable if there is no evolution towards obligate relationship with the host (Correa and Ballard 2014). Strong cytoplasmic incompatibility with infected sperm should favour accurate transmission of an infection across generations, resulting in strong tissue tropism. However, for old infections where cytoplasmic incompatibility is weak (e.g. wMa in D. simulans), Wolbachia density in tissues is expected to be variable because selection pressures for accurate transmission are weak (Correa and Ballard 2014). Such evolutionary changes are expected to weaken any blocking/spread trade-off.

These examples provide some support for a possible relationship between viral blockage, deleterious host effects and Wolbachia density, but too few strains have so far been examined. Moreover, the Drosophila data suggest that it is possible to identify infected lines demonstrating strong blockage, strong cytoplasmic incompatibility and no apparent fitness effects on the host. However, it is not yet clear whether such lines can be developed from novel combinations of hosts and infections generated through artificial transfers of Wolbachia.

Inferences from changes in natural populations

Although the potential benefits that hosts gain from pathogen blocking have so far only been demonstrated in laboratory tests (Chrostek et al. 2013), it seems likely that similar benefits will occur under field conditions. Recently, the wAu infection in D. simulans which causes strong viral blockage but no detectable cytoplasmic incompatibility (Hoffmann et al. 1996) has nevertheless been shown to increase rapidly in natural populations (Kriesner et al. 2013), suggesting that the infection provides a fitness advantage to its host which may include viral blocking. Another example is the wMel infection of D. melanogaster, which exhibits a stable cline in eastern Australia suggestive of selection (Hoffmann et al. 1994), but causes only partial cytoplasmic incompatibility in matings with young males (Reynolds et al. 2003). Given that this infection shows incomplete maternal transmission, it is hard to explain its persistence in D. melanogaster populations without assuming some sort of fitness benefit (Hoffmann et al. 1994). However, we still lack field data testing for a direct association between Wolbachia infection and natural viral load. If field strains exist that provide a fitness advantage under a high viral load but have fewer other effects on hosts, these would indicate that a blocking/spread trade-off can be avoided.

Mechanistic understanding of viral interference/host effects – immune priming and other effects

If the mechanisms involved in viral blockage, cytoplasmic incompatibility, and host fitness effects were understood, it might help in predicting likely interactions among Wolbachia effects. Viral blocking by Wolbachia seems to involve a number of subcomponents (Rances et al. 2013; Sinkins 2013). Part of the blockage may come from the upregulation of the immune system, as suggested by the increased expression of some immune response genes following recent Wolbachia transfers in mosquitoes (Kambris et al. 2009; Lu et al. 2012). However, cross-species transfers of Wolbachia do not necessarily lead to immune priming, as in the case of the experimental wAu infection of D. melanogaster (Chrostek et al. 2014). Other mechanisms have also been implicated, such as competition for resources such as cholesterol, interactions involving various metabolites, and the expression of microRNAs (Caragata et al. 2013; Zhang et al. 2013). Blockage mechanisms may be partly related to changes in the tissue distribution and density of Wolbachia particularly following transfer to a new host. For instance, native Wolbachia infections of Ae. albopictus have a relatively low density; the natural wAlbB infection of Ae. albopictus seems to cause some suppression of dengue and chikungunya viruses in its native host.
extended period of dry season will raise costs and the cost of disease control (Mousson et al. 2012). However, following transfer from Ae. albopictus into Ae. aegypti, the same infection develops a much higher density and blocking effect (Lu et al. 2012). Overall, these different lines of evidence point to a complicated pattern of interaction between pathogen blockage, deleterious fitness effects and cytoplasmic incompatibility. Host effects are not necessarily tightly linked mechanistically or through density, and a trade-off between blockage and spread might exist when host effects are predominantly related to density, but might in other cases be circumvented (Fig. 1). The Drosophila data indicate that strains such as wAu with strong blockage, no deleterious effects, high densities and no cytoplasmic incompatibility exist in populations alongside strains such as wHa that cause strong cytoplasmic incompatibility, but no blockage or large deleterious effects. A range of infections with different combinations of attributes occur in natural populations, including strains that might exhibit relatively strong blockage while also being able to easily spread in the absence of over replication after eclosion, and a high density in reproductive tissues to ensure strong cytoplasmic incompatibility and high maternal transmission. Unfortunately, the same combination of attributes might not be maintained after such a strain is transferred to a target vector host. For example, the wMel infection causes weak cytoplasmic incompatibility in its native Drosophila host but complete cytoplasmic incompatibility once transferred to Ae. aegypti, which has been essential for its successful spread (Hoffmann et al. 2011). Similarly, the wAu infection has no detectable fitness effect in its native host D. simulans, but causes a sharp reduction in lifespan and exhibits exponential growth when transferred to D. melanogaster (Chrostek et al. 2014). Therefore, intra- and intergeneric transfers across host species have unexpected consequences that may affect the suitability of strains for disease suppression.

Other deployment issues

Host population ecology

The successful invasion of Wolbachia infections will depend on the ecology of the host population. For example, if wMelPop is released into a host mosquito population where breeding sites lead to rapid egg hatch and where larvae develop under low densities, Wolbachia is more likely to invade. This is because the wMelPop infection does not strongly affect host viability and development time under low-density conditions and in the absence of dry conditions (McMeniman and O’Neill 2010; Yeap et al. 2011). On the other hand, there are development time and viability costs when wMelPop-infected mosquitoes are reared at a high density in competition with uninfected larvae (Ross et al. 2014). High-density conditions coupled with an extended period of dry season will raise costs and the threshold Wolbachia frequency required for a wMelPop invasion (Hancock et al. 2011; Yeap et al. 2014).

Areas of high mosquito density could be identified through factors such as housing characteristics, distribution of breeding containers and so on if this information is available from past surveys. Such information can be used to inform local invasion rates (Hoffmann et al. 2014b) and potential pockets where uninfected mosquitoes might persist and require additional treatment. Local knowledge of the ecology of mosquito populations should be used to inform release strategies; for instance, breeding containers that fill only occasionally after rain may need to be treated to remove sources of uninfected mosquitoes.

Release programmes also need to take into account expected movement patterns of mosquitoes and variation in host density across the region. Information on natural movement patterns from mark-release experiments or genetic analyses of local populations (e.g. Harrington et al. 2005; Olanratmanee et al. 2013) can provide a picture of likely movement patterns. By characterizing thousands of SNP markers, a much higher level of resolution of population structure can be obtained, and the movement of related individuals across a region can also be followed (Rašić et al. 2014b).

Wolbachia invasion into an isolated uninfected population of a target host only occurs if Wolbachia frequencies consistently exceed a particular frequency set by the size of the deleterious effects of Wolbachia on its host, levels of cytoplasmic incompatibility and to a lesser extent by the fidelity of maternal transmission (Hoffmann and Turelli 1997; Turelli 2010). If deleterious host effects associated with Wolbachia infections are too large, Wolbachia invasion into target host populations becomes difficult and high infection frequencies might not be sustained even if invasion succeeds. Invasion and persistence become increasingly unlikely if there is ongoing immigration of uninfected individuals into a relatively small release area (Barton and Turelli 2011) and if there are fitness effects of Wolbachia that decrease the size of the target population, making reinvasion by uninfected mosquitoes more likely (Rašić et al. 2014a).

A benefit of releasing infections with at least some deleterious fitness effects is that infections are expected to remain contained within an area rather than spreading rampantly (Barton and Turelli 2011; Hancock and Godfray 2012). This prediction is consistent with field experience from wMel releases around Cairns, Australia, where wMel did not spread outside areas where they were released even though Wolbachia were occasionally detected in other areas (Hoffmann et al. 2011, 2014b). Spread only occurs relatively slowly through a continuous residential area and is likely to be stopped by barriers to movement and high-density areas occupied by uninfected mosquitoes (Barton and
Although the likely benefits and costs of Wolbachia have been debated for many years (Barton and Turelli 2011; Hancock and Godfray 2012; Hoffmann et al. 2014b). Spread is much easier to achieve when a large area with a high host density has been invaded and the surrounding area has a low density; an increase in host density outside the invaded zone can stop Wolbachia spread, particularly if the invasion point is high (Barton and Turelli 2011), as in the case of wMelPop (Yeap et al. 2011). Moreover, invasions might then fail to persist with a moderate influx of migrants into a population (Hancock et al. 2011).

Although the host fitness costs associated with Wolbachia infections could be used to suppress and even eradicate mosquito hosts in some isolated areas (Rašić et al. 2014a), they provide challenges for the infection spreading in large and continuously distributed mosquito populations. So far, attempts to spread the high cost wMelPop infection into relatively isolated natural populations in Vietnam and northern Australia have failed, despite high release rates and some success in getting the infection to a high frequency (T. H. Nguyen, unpublished data). The wMelPop infection did successfully invade semi-field population cages, but only when release rates were high and sustained for many weeks (Walker et al. 2011). Several strategies could assist in spreading infections with high deleterious effects, such as through the suppression of host populations across all life stages just prior to release (Hoffmann 2014), through the release of male-biased sex ratios (Hancock et al. 2011) or through the use of pesticide resistance genes and application of pesticides during the release process (Hoffmann and Turelli 2013). These strategies should assist in introducing such infections into relatively isolated populations, but the infection is unlikely to spread further outside these areas (Barton and Turelli 2011).

Community acceptance

Although the likely benefits and costs of Wolbachia-based strategies for disease suppression can be identified to some extent, the final strategy and strain adopted will also depend on community acceptance and regulatory approval. A challenge for Wolbachia releases aimed at invasion and replacement is that there will be a period of time when mosquito numbers are increased above background levels to ensure that the Wolbachia infection exceeds an invasion threshold. As long as there are no fitness costs associated with the infection, Wolbachia is expected to spread from a very low starting frequency (close to 0%) depending on stochastic factors, with a slow rate of spread initially (Jansen et al. 2008). This type of spread has been observed in natural infections of D. simulans where resident populations number in the millions (Kriesner et al. 2013). However, with a threshold frequency of around 20–30%, the wMel invasion into uninfected Ae. aegypti required releases across 10 weeks, at which time adult numbers increased by a factor of 1.5–2 (Hoffmann et al. 2011; Ritchie et al. 2013). The period of time and relative increase in mosquito numbers required will be greater if infections are costly, and/or if the release material has a relatively low fitness.

While a 1.5–2 fold increase in mosquito numbers might seem trivial, particularly when only one mosquito species is being targeted in release areas where several species are likely to co-occur, implementation of such a strategy can be challenging. In most countries where dengue is endemic and attributable to Ae. aegypti mosquitoes which breed around houses, communities are encouraged to decrease the availability of breeding sites, removing containers that might hold standing water, treating containers with chemicals, and perhaps fogging an entire area with pesticides. Such combined programmes can be effective in reducing mosquito densities (Erlanger et al. 2008), but often there is little impact on mosquito populations due to factors such as cryptic breeding sites that cannot be easily targeted (Heintze et al. 2007; Eisen et al. 2009). These strategies can also generate additional problems such as the evolution of pesticide resistance in hosts (Maciel-de-Freitas et al. 2014). Furthermore, there is often a poor correlation between measures of mosquito numbers and disease incidence (Bowman et al. 2014), making it difficult to justify such campaigns. Nevertheless, while education and engagement campaigns can help increase acceptance of Wolbachia releases (McNaughton and Huong 2014), communities may be reluctant to participate in Wolbachia release programmes and regulatory authorities may be reluctant to approve strategies where there is a deliberate increase in mosquito numbers over a period of time.

This issue becomes particularly important where the Wolbachia strains being introduced have high invasion thresholds and therefore require high release numbers across an extended period of time. For instance, wMelPop failed to invade isolated field populations despite releases across several months where frequencies exceeded 70% (T. H. Nguyen, unpublished data). Even when this infection invaded semi-field cages, it required more than 80 days before the infection reached fixation in one cage, despite a starting infection frequency of 65% (Walker et al. 2011). In contrast, infections such as wMel seem to invade quite readily, at least based on experience in Australia.

One of the advantages of Wolbachia releases is that they are not necessarily incompatible with other control programmes. For instance, during the 2011 release of wMel around Cairns, Australia, pesticides were applied by the health authorities to a residential block within the release site where a dengue case had been reported, and this did not inadvertently affect the local rate of increase of Wolbachia (Hoffmann et al. 2011). In this case, both the resident uninfected population of Ae. aegypti and the released mosquitoes did not contain appreciable levels of insecticide.
resistance. In contrast, in many communities where there has been widespread application of pyrethroids and other insecticides over some time, resistance levels in uninfected *Ae. aegypti* are expected to be high (Ranson et al. 2009). In such cases, insecticide application during the release could lead to a preferential removal of the infected released mosquitoes. However, it should be possible to minimize this issue by backcrossing infected release stock to the local genetic background of a target population with high insecticide resistance.

Finally, when there are community concerns about release numbers increasing above background levels, suppression of mosquitoes prior to starting releases could help to alleviate community concerns, as well as speeding up *Wolbachia* invasions by increasing the frequency of *Wolbachia*, and by producing vacant breeding sites for infected released females. In addition, it may be possible to release large numbers of nonbiting infected male mosquitoes to facilitate invasions when these males generate cytoplasmic incompatibility with uninfected mosquitoes (Hancock et al. 2011). Pesticide applications could also assist invasions if the release material carries a higher level of resistance than the resident population (Hoffmann and Turelli 2013). Although there is little risk that resistance alleles will spread to the uninfected resident populations as long as cytoplasmic incompatibility is complete and maternal transmission is high, this strategy is unlikely to be approved by regulators except in limited circumstances, for instance, where relevant genes are already present in a target population.

**Conclusions**

Selecting a suitable strain of *Wolbachia* for release is not a straightforward process, and involves a balance between minimizing fitness costs while maximizing cytoplasmic incompatibility and blockage of disease agents, as well as considering community and regulatory issues. It is not yet clear to what extent desirable strain qualities can be combined or whether there are trade-offs that limit the options available. It seems essential to create and test a number of *Wolbachia* infections for releases, despite the challenges associated with this exercise that require thousands of microinjections to achieve success (McMeniman et al. 2009; Bian et al. 2013b). Nevertheless, there are many natural *Wolbachia* strains available within Diptera for potential introduction into disease vectors. Once a suitable strain has been identified, it will be necessary to monitor the long-term stability of the desirable effects because there may be further evolutionary changes in the host, *Wolbachia* and pathogen genomes that could modify *Wolbachia* effects, even though current data suggest they are relatively stable.

**Literature cited**


increase host resistance to West Nile virus infection. PLoS ONE 5: e11977.


Parameter estimates and infection dynamics in natural populations. Genetics 165:2029–2038.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

**Appendix S1.** Stable *Wolbachia* infections produced through microinjection, their effects on host reproduction and fitness, and potential blocking effectiveness where demonstrated.
Table F.1 Stable Wolbachia infections produced through microinjection, their effects on host reproduction and fitness, and potential blocking effectiveness where demonstrated. “?” denotes unavailable information. The level of pathogen blockage for each Wolbachia strain is defined as follows; High: the Wolbachia strain provides complete or nearly complete blockage of the pathogen. For example, the pathogen is absent or at low densities when Wolbachia-infected, or for pathogens that cause mortality, survival is unaffected by pathogen infection when also infected with Wolbachia. Low: statistically significant protection provided by Wolbachia, but pathogen densities remain high, or survival is only marginally improved when Wolbachia-infected. Moderate: intermediate protection that lies between the high and low categories. Yes: Wolbachia provides some level of protection, but the data are insufficient to determine the strength of protection. No: there is no significant difference between Wolbachia-infected and uninfected in terms of pathogen blockage. Reduced protection: Wolbachia infection decreases protection against the pathogen relative to uninfected. Abbreviations: CI, cytoplasmic incompatibility; MT, maternal transmission; DCV, Drosophila C Virus; FHV, Flock House Virus; DENV, Dengue Virus; WNV, West Nile Virus; YFV, Yellow Fever Virus; CHIKV, Chikungunya Virus; IIV-6, Insect Iridescent Virus 6; LACV, La Crosse Virus.

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<th>Strain</th>
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<th>Fitness effect</th>
<th>Pathogen</th>
<th>Protection</th>
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Native host

Transinfected Host
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**Notes:**
- CI: Cytoplasmic inclusion
- MT: Mitochondrial targeting
- Low: Low pathogen loads
- Moderate: Moderate pathogen loads
- High: High pathogen loads
- Minimal cost: Minimal cost
- No effect: No effect on female fitness
- Increased longevity: Increased longevity of blood-fed females
- Reduced fecundity: Reduced fecundity and hatch rate
- Increased hatch rate: Increased hatch rate
- Male mating competitiveness: Male mating competitiveness
- Male fitness: Male fitness
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* Level of CI declines with male age
* Level of pathogen protection determined by measurements of viral titer, bacterial load or pathogen density
* Level of pathogen protection determined by relative mortality of Wolbachia-infected and uninfected after exposure to pathogen
* Level of pathogen protection determined by semi-quantitative detection
* Protection against parasitoids measured by rates of egg encapsulation
* Unpublished work by JK Axford and AA Hoffmann
* Level of pathogen protection determined by estimating transmission rates (e.g. through detection in saliva or the head)
* wMel does not provide protection against pathogen-induced mortality, but it greatly reduces replication
* Depends on host genetic background
* The severity of life-shortening and the strength of pathogen blockage induced by wMelPop increase with higher Octomom copy numbers 158
* Rates of maternal transmission depend on the male infection status
* Cell line adapted wMelPop (wMelPop-CLA)
* Reduced severity of life-shortening and strength of CI in wMelPop-CLA compared to wMelPop
* Triple infection with wAlbA and wAlbB
* Also infected naturally with wStri
* Doubly infected with wAlbA and wAlbB. CI and fitness studies in Aedes albopictus test different combinations of infections (wAlbB only, wAlbB and wAlbA, or wAlbA only)
* Wolbachia in Aedes albopictus limits transmission 92, but does not affect dissemination 93, 94 or replication of DENV 92. Blagrove and others 23 report that Wolbachia infection increases rather than limits transmission, in contrast with Mousson and others 92
* Transinfected with wAlbB only
* Double transinfection with wAlbA and wAlbB
* Single transinfections with both wAlbA and wAlbB
* wRivB originated from Aedes riversi but was introgressed into Aedes polynesiensis,
Wolbachia protects against Plasmodium-induced mortality, but increases the susceptibility of the mosquito to infection with Plasmodium.

Unpublished work by Z Xi

Also infected with wCer1, but this infection is not present in artificial transfers. May also be infected with other wCer Wolbachia strains.

Stably transinfected with wCer2 only

Both wCer2 and wCer4 were transferred independently into Ceratitis capitata, with the same effect on CI, and these two stains are bidirectionally incompatible.

Supplementary References


APPENDIX G – FITNESS OF \textit{wAlbB} \textit{Wolbachia} INFECTION IN \textit{Aedes aegypti}: PARAMETER ESTIMATES IN AN OUTCROSSED BACKGROUND AND POTENTIAL FOR POPULATION INVASION
Fitness of wAlbB Wolbachia Infection in Aedes aegypti: Parameter Estimates in an Outcrossed Background and Potential for Population Invasion

Jason K. Axford,* Perran A. Ross, Heng Lin Yeap, Ashley G. Callahan, and Ary A. Hoffmann

Pest and Environmental Adaptation Research Group, Bio21 Institute and School of BioSciences, University of Melbourne, Parkville, Australia; Commonwealth Scientific and Industrial Research Organization, Acton, Australia

Abstract. Wolbachia endosymbionts are potentially useful tools for suppressing disease transmission by Aedes aegypti mosquitoes because Wolbachia can interfere with the transmission of dengue and other viruses as well as causing deleterious effects on their mosquito hosts. Most recent research has focused on the wMel infection, but other infections also influence viral transmission and may spread in natural populations. Here, we focus on the wAlbB infection in an Australian outbred background and show that this infection has many features that facilitate its invasion into natural populations including strong cytoplasmic incompatibility, a lack of effect on larval development, an equivalent mating success to uninfected males and perfect maternal transmission fidelity. On the other hand, the infection has deleterious effects when eggs are held in a dried state, falling between wMel and the more virulent wMelPop Wolbachia strains. The impact of this infection on lifespan also appears to be intermediate, consistent with the observation that this infection has a titer in adults between wMel and wMelPop. Population cage experiments indicate that the wAlbB infection establishes in cages when introduced at a frequency of 22%, suggesting that this strain could be successfully introduced into populations and subsequently persist and spread.

INTRODUCTION

Wolbachia bacteria are endosymbionts of insects that form the basis of novel approaches for suppressing disease transmission by mosquitoes. In particular, Wolbachia from Drosophila transferred to Aedes aegypti mosquitoes are being utilized in current releases aimed at suppressing dengue transmission.1,2 The main reason for this suppression comes from the fact that the presence of the bacteria reduces virus titer, particularly in tissues that the virus needs to invade for transmission between people.3,4 In addition, Wolbachia can influence disease transmission by causing deleterious effects on its host and triggering embryo mortality or cytoplasmic incompatibility (CI) when Wolbachia-infected males mate with uninfected females, potentially reducing vector population size.5,6 Effects on viral transmission interference and host fitness may last if the Wolbachia infection reaches a high and stable frequency in host populations after invasion, which has now been achieved in some field populations of Aedes aegypti.7

The ability of Wolbachia strains to generate viral blockage and influence host fitness depends on the nature of the Wolbachia strain and background host genome.4,8,9 This has become evident from research in Drosophila where multiple combinations of hosts and Wolbachia have now been generated and challenged with viruses.10,11 So far, in Aedes aegypti mosquitoes, three Wolbachia strains have been stably introduced and characterized: the wMel and wMelPop infections from Drosophila melanogaster12,13 and the wAlbB infection from Aedes albopictus.14 As in Drosophila, there are likely to be differences in fitness effects and blockage exhibited by these strains, which requires the strains to be compared in the same genetic background. The wMelPop strain generates very high viral blockage, whereas blockage by wMel is somewhat weaker.4,13 Blockage by wAlbB appears to be strong,15 but a direct comparison to the other strains has not yet been undertaken. The wMelPop infection generates large deleterious effects on adult longevity, egg viability particularly after quiescence, larval viability under high density, and other traits.12,16,17 These deleterious effects are either much weaker or absent in wMel.13 Less comprehensive data for wAlbB also suggest limited deleterious effects on a genetic background different from that used in experiments with wMel and wMelPop.14 The magnitude of deleterious effects as well as viral blockage may be partly related to bacterial titer and tissue distribution, with the wMelPop infection occurring at a higher titer and having a wider tissue distribution than wMel.13

Although strong viral blockage is clearly desirable from the perspective of curtailing disease transmission, large deleterious effects may be sufficient to prevent Wolbachia from invading into wild Aedes populations16 and subsequently spreading into surrounding areas.18 Invasion of uninfected populations depends on the process of CI, where infected males cause the death of embryos or immature stages when they mate with uninfected females (or females carrying an incompatible Wolbachia strain). Although CI is very strong for all three Wolbachia infections introduced into Aedes aegypti albeit with different genetic backgrounds,12–14 the presence of large deleterious effects can mean that invasion by wMelPop is difficult because of a high invasion threshold, particularly in the dry season when eggs have to persist in a quiescent phase.6 This is borne out by the difficulty of achieving invasion by this infection in semi-field cages13,19 and in field releases into relatively isolated areas.20 On the other hand, invasion by wMel has now been repeatedly achieved, and results in high and stable frequencies.7 The wAlbB infection has not yet been used for invasion in field releases although it has been shown to invade small laboratory population cages.14

In this article, we consider the three infections for fitness effects on a common genetic background with the aim of comparing their invasibility and also potential use in population suppression.2 We focus particularly on the wAlbB infection that has not been characterized in detail previously, and contrast its host effects and bacterial density with that of the wMel and wMelPop infections. We also provide estimates of
maternal transmission and CI to help assess the likely frequency of wAlbB in natural populations after invasion.

MATERIALS AND METHODS

Colonial maintenance. Aedes aegypti infected with wAlbB, wMel, or wMelPop were reared in a laboratory at 26 ± 1°C with a 12:12-hour (day:night) photoperiod, which included 1-hour dusk/dawn periods. Colonies of 450–500 adults (not differing significantly from a 1:1 sex ratio) were housed in 19.7-L BugDorm-1 cages (MegaView Science Co., Ltd., Taichung City, Xitun District, Taiwan), covered with plastic bags to maintain high humidity (~85%). Adults were provided with access to a 10% sucrose solution supplied by capillary action through a cotton wool–braided cord (7 × 0.5 cm) inserted through the lid of a 30-mL cup. Females were blood-fed by human volunteers for 15 minutes, 8 days after eclosion, to allow maturation and fertilization. Females oviposited on Norton® Master Painters P80 sandpaper (3.8 × 18 cm; Saint-Gobain Abrasives Pty. Ltd., Thomastown, Victoria, Australia) for routine maintenance or conical filter paper (Whatman® qualitative circles—15 cm Ø; GE Healthcare Australia Pty. Ltd., Parramatta, New South Wales, Australia) lining the inside of a plastic cup containing 150 mL reverse osmosis (RO) water. Eggs were conditioned by removing excess moisture with paper towel for 30 seconds on the second day post-oviposition, and then almost completely dried on the third day. Egg strips were then sealed in plastic zip-lock bags with a moist paper towel square (2 × 2 cm) to prevent desiccation. Egg hatching occurred in RO water (3 L for colony maintenance, but see specific methods for experimental volumes), deoxygenated with active dried yeast to stimulate hatching (~0.02 mg/L), and containing crushed TetraMin® fish food tablets (Tetra, Melle, Germany; hereafter referred to as hatching water). Immature stages were fed ad libitum with the fish food. Colonies were maintained by controlling the density of second instar larvae at 450–500 individuals per 4 L of RO water using a glass pipette and clicker counter. Colony pupae were collected 5 days later into 500 mL fresh RO water and placed in 19.7-L BugDorm-1 cages for eclosion. Colonies were routinely screened for Wolbachia to confirm their infection status (see section Wolbachia detection and primers) and maintained at a size of several hundred individuals. Aedes aegypti strains had been transinfected with the wAlbB, wMel, and wMelPop strains of Wolbachia by embryonic microinjection as described elsewhere. However, both the wMel and wMelPop cultures used in the experiments had been sourced from field material subsequent to releases. Colonies infected with each strain were maintained in our laboratory alongside an uninfected colony (CNS), sourced from eggs oviposited around Cairns. Host nuclear background effects were controlled by backcrossing females from all infected lines to CNS males for six generations before experimentation.

Fecundity. Reductions in female fecundity because of the wAlbB infection were tested relative to uninfected CNS females. Colonies were blood-fed by a single human volunteer. Twenty engorged females from infected and uninfected colonies were then isolated into 70-mL specimen cups with mesh lids using an aspirator. Access to a 10% sucrose solution was provided through a soaked cotton wool bud placed on the mesh. Cups were lined with a sand paper strip (2 × 12 cm, see section Colony maintenance) and filled with 20 mL water from larval rearing trays to promote oviposition. Eggs were collected daily and counted by eye under a dissecting microscope using a clicker counter.

Quiescent egg viability. The long-term viability of wAlbB-infected eggs in quiescence was assessed in comparison to eggs from the uninfected CNS colony. Eggs were collected daily en masse for 4 days from each line on a filter paper substrate (see section Colony maintenance). On the third day post-oviposition (day 0), filter papers were stored in a plastic environmental chamber, sealed with Blu-Tack (Bostik, Thomastown, Victoria, Australia). Relative humidity (RH) inside the chamber was maintained at 75% using a saturated solution of sodium chloride in a cup, which was monitored for 1 week with a hygrochron (1-wire; iButton.com) before the introduction of eggs. Ten replicate batches of at least 25 eggs from each line were hatched at days 0, 3, 10, 17, 24, 31, 61, 90, and 124. All batches were captured with a digital camera just before hatching and eggs were counted using the Cell Counter plugin in ImageJ. To avoid underestimating viability, eggs that hatched early (egg cap clearly detached) before immersion were removed from the analysis. Batches were immersed in plastic cups containing 140 mL hatching water (see section Colony maintenance). After 6 days, all individuals (dead or alive) were counted using a glass pipette and clicker counter.

Larval development time, survival, and adult body size. To test for any effects of Wolbachia on immature development, we reared cohorts of wAlbB-infected and uninfected CNS larvae under both high- and low-stress conditions. Eggs from both lines were submerged synchronously in RO water, and first instar larvae were added to treatments within 6 hours of hatching. Cohorts of 200 wAlbB-infected or 200 uninfected larvae were reared independently in either 4,000 mL (low density, one larva per 20 mL) or 200 mL (high density, one larva per 1 mL) of RO water. Containers were provided with either 0.25 mg per larva (high nutrition) or 0.05 mg per larva (low nutrition) of crushed TetraMin tablets daily. Each combination of density, nutrition, and Wolbachia infection status was replicated six times.

Cohorts were monitored to determine mean development time, survival, and adult body size. Pupae were transferred to separate containers of RO water as they appeared, and emerging adults were collected twice daily, in the morning and evening, and stored in absolute ethanol. Adults that emerged around the median development time for a particular level of nutrition and density were measured for their wing length to provide an estimate of body size. At least 25 males and 25 females sampled across all containers were measured for each treatment. The right wing was dissected from each adult and fixed under a 10-mm coverslip with Hoyer’s solution (distilled water: gum arabic:chloral hydrate: glycerin in the ratio 5:3:2:20). The distance between the alular notch and the intersection of the radius 3 vein and outer margin (excluding the fringe scales) provided a measure of wing length. Two independent measurements of each wing were averaged to give the final length. Damaged or folded wings were not measured.

Adult survival in groups. Adult survival of wAlbB, wMel, and CNS was assayed using groups of 50 individuals (1:1 sex ratio), replicated eight times. Pupae were sexed and added to 25 mL of RO water in plastic cups and allowed to eclose in 3-L plastic containers with stocking lids and mesh sides. A filter paper oviposition site and 10% sucrose solution were provided and refreshed twice a week (see section Colony maintenance).
To prevent desiccation, containers were maintained at high humidity (∼85% RH) in white plastic garbage bags. Females were blood-fed weekly for the duration of the experiment, and mortality was scored for males and females three times a week until at least 50% of females from the longest surviving line had died.

**Mating.** The mating success of wAlbB males competing with CNS males for mates was estimated. Infected and uninfected males were established in 19.7-L cages before the introduction of 80 virgin CNS females in the following groups: 1) 80 CNS males (negative control, 0% infected), 2) 40 wAlbB males and CNS males (treatment, 50% infected), and 3) 80 wAlbB males (positive control, 100% infected). Each group was replicated five times. Males and females were allowed to mature for 1 week before release. During the release, cages were tapped to ensure males were distributed throughout the cage. Mosquitoes were left to mate for 1 week before providing females with a blood meal. Eggs were collected en masse and blood fed by a single human volunteer within 1.5-L plastic containers with mesh sides and covered with a stocking. Adults were provided with access to a 10% sucrose solution (see section Colony maintenance). A minimum of 11 progeny each from 29 females were provided weekly and hatch rates determined for three generations. Eggs were collected daily over 4 days, photographed with a Canon LiDE 110 flatbed scanner (Canon USA), and later they were provided with a filter paper oviposition site. Eggs were collected separately within 1 week of oviposition in 3 L hatching water (see section Colony maintenance) and all individuals were counted 6 days later using a glass pipette and clicker counter.

**Cytoplasmic incompatibility.** The wAlbB line was reciprocally crossed with CNS to test for CI. Reciprocal crosses were performed between wAlbB males and CNS females (or wMel males mated to wAlbB females for the bidirectional test). Compatible crosses were performed between wAlbB males and CNS females and within each infected or uninfected parental line. All crosses were replicated eight times. For each cross, 14 pupae were sexed (1:1 sex ratio) and added to 20 mL of RO water in 30-mL cups. Pupae eclosed within 1.5-L plastic containers with mesh sides and covered with a stacking. Adults were provided with access to a 10% sucrose solution (see section Colony maintenance). Adults were left to mate for 1 week; they were then fed, and 3 days later they were provided with a filter paper oviposition site. Eggs were collected daily over 4 days, photographed with a digital camera and counted in ImageJ to determine the number of eggs. Within 1 week of embryonation, eggs were hatched in plastic containers with 500 mL hatching water (see section Colony maintenance). To test for age-related effects on CI, two further blood meals were provided weekly and hatch rates determined for three gonotrophic cycles in total.

**Maternal transmission.** The maternal transmission efficiency of the wAlbB infection was estimated by testing the proportion of infected offspring produced by an infected female. Females from the wAlbB line were crossed to CNS males en masse and blood fed by a single human volunteer within 1 week of emergence. Engorged females were isolated (see section Fecundity) and later stored in absolute ethanol at 4°C for at least 50% of females from the longest surviving line had died. Egg viability for the first month for wAlbB was 0.97 with no reduction in fecundity (μ = 0.03, see Fecundity and Quiescent egg viability results), giving rise to a UET lower than 0.15. The null hypothesis chosen for this experiment was therefore H0: ρ = 0.15 while the alternative was H1: ρ > 0.15. A power function was written in R 3.1.0.25 to determine the likelihood of invasion given a UET of 0.03 with an initial invasion frequency (p0) of 0.1.

** AU ETE To f**
detected based on the combinations of crossing point (Cp) and melting point (T_m) values of the PCR products as determined in a Light Cycler 480 (Roche Applied Science, Castle Hill, New South Wales, Australia). *Wolbachia* load was also determined in 25 females sampled from the *wAlbB*, *wMel*, and *wMelPop* colonies.

**Analysis.** All data were analyzed and graphed in R 3.1.0.\textsuperscript{20} Fecundity was scored as the number of eggs laid per female, and egg viability (or hatch rate) was defined as the proportion of eggs that hatched by counting the number of third or fourth instar larvae. Proportional data were arcsine square root transformed if they failed Shapiro–Wilk tests of normality and tested again. We checked for heteroscedasticity between groups being compared using *F* tests. Depending on whether we could assume equal variance and normality, we used the Student’s *t* test and Welch *t* test for comparing means. For data that could not be transformed to meet assumptions of parametric tests, we used nonparametric Mann–Whitney *U* tests.

Development time was calculated as the time in days from hatching to adult emergence while larval survival was defined as the proportion of larvae that reached adulthood. Larval development time, survival to adulthood, and wing length data were analyzed by analysis of variance (ANOVA) and Tukey’s honest significant difference post hoc tests or by nonparametric Kruskal–Wallis tests depending on normality. To identify deviations from a 1:1 sex ratio, *χ²* tests were run.

The equality of adult survival curves (separated by sex) was compared using the Cox regression procedure. Replicates were initially compared, and replicates detected as significant outliers were removed from the analysis. Strains were then compared for pooled data. *CNS* was set as the baseline, thus the hazard ratio (*e^β* ) for each infected line is the average relative mortality rate of infected to uninfected *CNS* for the entire monitoring period. We tested the proportional hazards assumption for each line. If nonproportionality was suspected, time was partitioned into blocks. Blocks were decided based on survival curves by visually identifying cutoff time points where hazard ratios were expected to change. The final blocks met the proportional hazard assumption test.

The degree of CI induced by *wAlbB* males in crosses to *CNS* or *wMel* females (and the reciprocal cross) was determined by computing the proportion of eggs that hatched (cages that laid ≤ 10 eggs were excluded). We also tested for the effects of cross and gonotrophic cycle (a proxy of female age), treated as factors, on hatch rates in an ANOVA if data were distributed normally or a Kruskal–Wallis test for non-normal data.

Assortative mating data were analyzed by applying a linear model on mean hatch rates resulting from cages possessing 0%, 50%, or 100% infection rates. Hatch rate was used as a proxy for the proportionate contribution of infected versus uninfected males.\textsuperscript{31} The mating competitiveness term, *β_am*, represents the deviation between observed and expected hatch rates given a particular rate of CI (see Supplemental Appendix 2). A Student’s *t* test was then performed against the null model (*β_am = 1*) to test for significant deviations, where *β_am > 1* or *β_am < 1* indicates an advantage or disadvantage, respectively, in *wAlbB* male–mating success relative to *CNS*.

The rate of *wAlbB* maternal transmission from parent to offspring was calculated as the mean proportion of infected progeny produced by infected mothers. Binomial 95% confidence intervals (95% CIs) were also computed.

**RESULTS**

**Fecundity.** No difference was found in the number of eggs laid (*P* > 0.9, *t* test) between *wAlbB* females (mean = 60.14, standard deviation [SD] = 7.63, *N* = 14) and *CNS* females (mean = 59.72, SD = 12.16, *N* = 18, relative fecundity = 1.007).

**Quiescent egg viability.** Egg batches ranged from 25 to 78 eggs. Student’s *t* tests were performed on hatch proportions (arcsine transformed). The average percentage of *wAlbB* eggs surviving at days 0, 3, 10, 17, 24, and 31 was not significantly different from equivalent time points for the *CNS* strain (Figure 1, *P* > 0.05). However, after day 31, the *wAlbB* infection caused a 27% reduction in mean egg hatch by day 61, 79% by day 90, and 99% by day 124, compared with *CNS*. The *wAlbB* egg hatch rates were significantly different from those for *CNS* (day 61: *t* = 6.85, *df* = 18, *P* < 0.001; day 90: *t* = 25.39, *df* = 18, *P* < 0.001; day 124: *t* = 28.86, *df* = 18, *P* < 0.001). In contrast, the *wMelPop* strain had a strong negative effect on quiescent egg viability in *Ae. aegypti* outbred to the *CNS* background (plotted in Figure 1 for comparison),\textsuperscript{16} while *wMel*-infected *Ae. aegypti* outbred to the same genetic background did not affect quiescent egg viability within a period of 1 month.\textsuperscript{13}

### Table 1

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qPCR = quantitative real-time polymerase chain reaction.

*Inaki Iturbe-Ormaetxe, personal communication.
The Ten replicates per time point. Egg batches ranged from 25 to 78 eggs.
and 124 days of quiescence. Error bars are standard error of means.

\[ P < 0.02, \] \[ P < 0.001, \] \[ P = 0.080 \]

Larval density (larval survival, development time, and wing length (mean ± SE) for
from a 1:1 sex ratio in both
survival to adulthood. There were no significant departures
adults for any treatment (\( P > 0.05, \chi^2 \) test).
We also found that nutrition (\( F_{1,40} = 14.38, P < 0.001 \)) and larval density
(\( F_{1,40} = 10.50, P = 0.002 \)) but not Wolbachia infection status
(\( F_{1,40} = 0.37, P = 0.548, \) relative wAlbB survival = 1.002) on
survival to adulthood. There were no significant departures from a 1:1 sex ratio in both wAlbB-infected and
infected adults for any treatment (\( P > 0.05, \chi^2 \) test).
We also found that nutrition (\( F_{1,180} = 3.082.52, P < 0.001 \)), larval density (\( F_{1,180} = 295.36, P < 0.001 \)), and sex (\( F_{1,180} =
144.95, P < 0.001 \)) but not Wolbachia infection status (\( F_{1,180} = 0.02, P = 0.891 \)) affected larval development time. Relative to
CNS, larval development time for wAlbB was 1.015 days for
males and 0.99 days for females (average across nutrition and
density). Low larval densities increased development time to
a greater extent when nutrition was also low (Table 2). Males
reached adulthood around 1.3 days earlier than females at
high nutrient levels (mean ± standard error (SE) for males =
9.002 ± 0.151 days, females = 10.302 ± 0.142 days), while
difference was extended to 4.4 days in the low-nutrition
treatments (males = 20.653 ± 0.759 days, females = 25.085 ±
0.800 days). No pairwise comparisons for development time
between wAlbB-infected and uninfected larvae were signifi-
cant (Table 2).

We estimated body size in the larval development experi-
ment by measuring wing length. As expected, female wings
(mean ± SE = 2.502 mm ± 0.011, N = 213) were considerably
larger than male wings (1.941 ± 0.006 mm, N = 222, \( F_{1,419} =
6.078.57, P < 0.001 \)). We also found a significant effect of
nutrition on wing length (\( F_{1,419} = 725.89, P < 0.001 \)), while
Wolbachia infection (\( F_{1,419} = 3.09, P = 0.080 \)) and larval den-
sity (\( F_{1,419} = 0.91, P = 0.340 \)) had no significant effects. Rela-
tive to CNS, wing length for wAlbB males was 0.99 and 1.00
for females. Larval density affected size differentially at each
level of nutrition. For the high nutrition level, the low-density
treatment resulted in larger wings, while for the low-nutrition
level, the high-density treatment resulted in larger wings
(Table 2). No pairwise comparisons between wAlbB-infected
and uninfected wings were significant, with the exception of
the low-nutrition, high-density containers where uninfected
females were significantly larger than wAlbB-infected females
(relative wAlbB wing length = 0.97, Table 2).

Adult survival in groups. Shortly after CNS had passed
50% survival, this experiment was terminated at day 86. Log-
rank tests on unpoole data identified CNS replicates 1 and 2
and wMel replicate 5 as significant outliers, which were excluded
before proceeding to analyze pooled data. Within infected
lines, wMel males survived the longest followed by wAlbB
and then wMelPop (Figure 2A). Hazard ratios for males
from each infected line remained proportional throughout
the monitoring period. The mortality rate of wAlbB males
differed significantly from CNS (Figure 2, \( z = 3.24, e^b = 1.43
\) [95% CI = 1.15–1.78, \( P = 0.001 \)], as did wMelPop (\( z = 5.77, \)
e^b = 1.98 [95% CI = 1.57–2.30, \( P < 0.001 \)], whereas wMel
and CNS did not differ significantly (\( z = -1.01, e^b = 0.89
\) [95% CI = 0.71–1.12, \( P = 0.312 \]). Within the infected lines,
wAlbB females performed the best up to around 40 days
before a sudden increase in mortality rate, which led to a

![Figure 1](image-url) Percentage hatch rate of wAlbB-infected (red, dashed
line), wMelPop-infected (green, dotted line), and uninfected CNS
(black, solid line) Aedes aegypti eggs after 0, 3, 10, 17, 24, 31, 61, 90,
and 124 days of quiescence. Error bars are standard error of means.
Ten replicates per time point. Egg batches ranged from 25 to 78 eggs.
The wMelPop curve, generated from eggs outbred to the Cairns
genetic background, is taken from the work of Yeap and others.16

Larval development time, survival, and adult body
size. Larval to adult survival was generally high (Table 2).
In an analysis of the overall data, we found significant effects
of nutrition (\( F_{1,40} = 14.38, P < 0.001 \)) and larval density
(\( F_{1,40} = 10.50, P = 0.002 \)) but not Wolbachia infection status
(\( F_{1,40} = 0.37, P = 0.548, \) relative wAlbB survival = 1.002) on
survival to adulthood. There were no significant departures from a 1:1 sex ratio in both wAlbB-infected and uninfected
adults for any treatment (\( P > 0.05, \chi^2 \) test).

<table>
<thead>
<tr>
<th>Nutrition*</th>
<th>Density†</th>
<th>Infection</th>
<th>Survival to adulthood (%)*</th>
<th>Development time (days)</th>
<th>Wing length (mm)†</th>
</tr>
</thead>
<tbody>
<tr>
<td>High</td>
<td>High</td>
<td>wAlbB</td>
<td>98.92 ± 0.154 a</td>
<td>8.32 ± 0.06 a</td>
<td>1.992 ± 0.012 c</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CNS</td>
<td>97.83 ± 0.792 ab</td>
<td>8.30 ± 0.08 a</td>
<td>2.000 ± 0.008 c</td>
</tr>
<tr>
<td>Low</td>
<td>wAlbB</td>
<td>98.17 ± 0.803 a</td>
<td>9.83 ± 0.09 ab</td>
<td>9.77 ± 0.10 a</td>
<td>2.027 ± 0.011 c</td>
</tr>
<tr>
<td></td>
<td>CNS</td>
<td>97.33 ± 0.703 abc</td>
<td>9.56 ± 0.09 ab</td>
<td>10.99 ± 0.08 b</td>
<td>2.017 ± 0.008 c</td>
</tr>
<tr>
<td>Low</td>
<td>wAlbB</td>
<td>96.50 ± 1.252 abc</td>
<td>17.23 ± 0.29 c</td>
<td>21.65 ± 0.44 d</td>
<td>1.869 ± 0.010 a</td>
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<tr>
<td></td>
<td>CNS</td>
<td>97.75 ± 0.793 ab</td>
<td>17.29 ± 0.31 c</td>
<td>21.63 ± 0.45 d</td>
<td>1.916 ± 0.012 b</td>
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<tr>
<td>Low</td>
<td>wAlbB</td>
<td>93.00 ± 1.494 bc</td>
<td>24.46 ± 0.56 e</td>
<td>28.21 ± 0.88 f</td>
<td>1.837 ± 0.011 a</td>
</tr>
<tr>
<td></td>
<td>CNS</td>
<td>93.00 ± 0.707 c</td>
<td>23.64 ± 0.91 de</td>
<td>28.85 ± 1.04 f</td>
<td>1.864 ± 0.017 ab</td>
</tr>
</tbody>
</table>

SE = standard error.
*High- and low-nutrition regimes consisted of 0.05 and 0.25 mg, respectively, of TetraMin per larva per day.
†High- and low-density treatments consisted of 200 larvae in 200 mL (one larva per 1 mL) and 200 larvae in 4,000 mL (1 larva per 20 mL), respectively.
‡For each trait, values with the same letter are not significantly different from each other (\( P > 0.05, \) Tukey's honest significant difference test).
significantly lower survival overall compared with \( w_{\text{Mel}} \), but this was still higher than for \( w_{\text{MelPop}} \) (Figure 2B, Supplemental Table 3). Hazard ratios were nonproportional for \( w_{\text{AlbB}} \) and \( w_{\text{MelPop}} \) females; however, \( w_{\text{Mel}} \) was proportional throughout the monitoring period. Separate Cox regressions were performed on days 0–20 (block 1), 21–40 (block 2), and > 40 days (block 3) as the hazards appeared to be different across blocks (Figure 2, see Supplemental Table 3). The mortality rate of \( w_{\text{AlbB}} \) females was significantly different from CNS in block 3 only (\( z = 13.27, \hat{e}^{\beta} = 8.54 \) [95% CI = 6.22–11.73], \( P < 0.001 \)), whereas \( w_{\text{Mel}} \) females became significantly different from CNS in blocks 2 (\( z = 3.34, \hat{e}^{\beta} = 3.77 \) [95% CI: 1.73–8.23], \( P < 0.001 \)) and 3 (\( z = 3.34, \hat{e}^{\beta} = 2.64 \) [95% CI = 1.95–3.56], \( P < 0.001 \)). Females infected with \( w_{\text{MelPop}} \) had the highest mortality rate, becoming significantly different from CNS in blocks 2 (\( z = 8.29, \hat{e}^{\beta} = 21.63 \) [95% CI = 10.45–44.75], \( P < 0.001 \)) and 3 (\( z = 12.85, \hat{e}^{\beta} = 21.87 \) [95% CI = 13.66–35.01], \( P < 0.001 \)) (see Supplemental Table 4).

Mating. The average number of eggs oviposited by CNS females in cages possessing 0%, 50%, and 100% infection frequencies was 2,364, 2,471, and 2,346, respectively, and their mean hatch rates were 0.01, 0.39, and 0.85, respectively (Figure 3). The number of eggs that hatched early and died on the paper (egg cap clearly detached) was negligible (< 40 per replicate). The relative mating success of \( w_{\text{AlbB}} \) males to CNS females (\( \hat{\beta}_{\text{am}} = 1.114 \), but this did not differ significantly from the null hypothesis (\( \hat{\beta}_{\text{am}} = 1 \)) in a \( t \) test (\( t = 0.89, df = 14, P = 0.195 \)). Therefore, there was no strong evidence for assortative mating in favor of males from either strain.

Cytoplasmic incompatibility. Complete CI was observed between \( w_{\text{AlbB}} \) males and CNS females resulting in sterility, regardless of gonotrophic cycle (Table 3). Similarly, reciprocal crosses between \( w_{\text{Mel}} \) and \( w_{\text{AlbB}} \) exhibited complete

![Figure 2](https://example.com/image2.png)

**Figure 2.** Survival of adult *Aedes aegypti* males (A) and females (B) infected with \( w_{\text{AlbB}} \) (red, dashed line), \( w_{\text{Mel}} \) (blue, dotted line), or \( w_{\text{MelPop}} \) (green, dot-dash line) outcrossed to the Cairns genetic background, represented by CNS (black, solid line). “+” represents right-censored data. Thin dotted lines are 95% confidence intervals.

![Figure 3](https://example.com/image3.png)

**Figure 3.** Mean hatch rates of *Aedes aegypti* eggs oviposited by 80 CNS females exposed to populations of males in the following groups: 0% infected (80 CNS males), 50% infected (40 \( w_{\text{AlbB}} \) and CNS males), and 100% infected (80 \( w_{\text{AlbB}} \) males). The solid line denotes the null model for the expected hatch rate (no difference in mating ability), whereas the dotted line represents the observed hatch rate and relative mating success (\( \hat{\beta} \)) of \( w_{\text{AlbB}} \) to CNS males and its probability (\( \rho \)).
bidirectional incompatibility (Table 3). One-way ANOVAs on arcsine transformed hatch rates of the compatible crosses indicated a nonsignificant effect of gonotrophic cycle ($F_{2,88} = 0.35$, $P = 0.708$), but a significant effect of cross ($F_{3,88} = 28.16$, $P < 0.001$). Fecundity did not differ between incompatible and compatible crosses in a $t$-test ($t = 0.28$, df = 141, $P = 0.782$), but showed a consistent decrease between gonotrophic cycles 1 and 3, most likely due to age effects (Table 3). Average hatch rates of $\geq 79\%$ were observed for all control crosses. Tests on Cairns outbred $w$Mel and $w$MelPop males crossed to Cairns wild-type females also indicated very strong CI.$^{7,13,16}$

Maternal transmission. Out of the 319 offspring produced by 29 $w$AlbB-infected females, 319 were positively infected with the $w$AlbB strain (maternal transmission rate = 1, lower 95% CI = 0.99).

Population cages. As outlined in the Materials and methods section, replicates were terminated from four to seven generations after it was clear that the $w$AlbB infection had successfully invaded in either the 10% or 22% groups (Figure 4). All remaining colonies were terminated at generation 7 when the trajectory of the populations was clear. By the seventh generation, one of the five cages in the 10% group had successfully invaded. Because we observed less than three invasions, the critical threshold to achieve $\alpha < 0.05$, we could not reject the null hypothesis that $\hat{p} = 0.15$ ($P = 0.482$; Figure 4, Supplementary Figure 1). The remaining four cages in the 10% group failed to invade, with infection frequencies ranging from 0% to 23% over seven generations. All of the 22% control cages successfully invaded uninfected populations in four to six generations (Figure 4).

Wolbachia load. The median difference in log concentration of template DNA between Wolbachia and its host genome ($\Delta C_P$) in order of highest to lowest was $w$MelPop (6.83), $w$AlbB (5.09), and $w$Mel (3.33) (Figure 5). Significant differences between groups were found in a Kruskal–Wallis

![Figure 4. Wolbachia infection frequency per generation after an initial release of $w$AlbB females and males (red, open markers: 10%; black, solid markers: 22%) into 10 cages possessing CNS populations.](image-url)
of infected eggs do not last longer than 1 month.\textsuperscript{19} The fitness effects of Wolbachia infections (but not their levels of CI) appear to be at least partly related to the density of the Wolbachia in tissues although only a modest number of infections have so far been tested.\textsuperscript{8,10} We confirmed the absence of any costs in terms of fecundity\textsuperscript{14} and also male mating success for wAlbB; wMelPop does exhibit fecundity costs,\textsuperscript{12,16} but neither this infection nor wMel show mating costs.\textsuperscript{31} We also confirmed the maternal transmission of wAlbB was 100\%\textsuperscript{14} and complete CI was shown in crosses with uninfected females from Cairns, which was also reported for wMel and wMelPop.\textsuperscript{13,16} Complete bidirectional incompatibility was found between wAlbB and wMel; given the relative fitness effects found here, wMel may outcompete wAlbB should these two infections be released in the same population at an equal frequency. However, a population that has been invaded by wAlbB is not expected to be invaded by wMel because of bidirectional incompatibility and frequency dependence; CI will be induced in crosses between wAlbB males and wMel females, and, when present at a low frequency, most wMel females will inevitably mate with wAlbB males. If two infected strains are present in a population, the outcome will depend mostly on the nature of incompatibility patterns among the strains.\textsuperscript{28} If strains are bidirectionally incompatible, both strains may persist when they have invaded different areas because common strains have an advantage in an area; females from the common strain will be more likely to engage in compatible matings with males from the same strain. If males from one strain show CI but males from the other strain do not, the CI-inducing strain is likely to invade as occurred in Australian populations of Drosophila simulans where the wAu strain that did not induce CI was replaced rapidly by the wRi strain that did induce it.\textsuperscript{38} A decade ago, the wAlbB infection was reported as generating strong CI and perfect maternal transmission while having minimal effects on host fitness in terms of fecundity and egg hatch.\textsuperscript{14} The wAlbB infection was shown to invade small population cages when introduced at a starting frequency of 20\%.\textsuperscript{14} We confirmed these results in a larger cage population with greater replication, suggesting relative fitness costs that are comparable to the frequency of Wolbachia required to obtain invasion,\textsuperscript{28} are most likely greater than 10\% but lower than 20\%. However, we found no significant fitness costs for male mating competitiveness, adult survival within 1 month, or larval development and survival to adulthood. Previous experiments on the natural host of the wAlbB infection, Ae. albopictus, suggest that the wAlbB infection in combination with the wAlbA infection can generate a fitness advantage for its host.\textsuperscript{39} Our results suggest that releasing wAlbB-infected adults so that they comprise 20\% of the target population may be sufficient to achieve rapid invasion, but this threshold may well be higher in natural populations, particularly given the costs we have found at the quiescent egg stage.

In summary, the wAlbB infection might be suitable for invasion into Ae. aegypti populations given that this infection appears to have moderate fitness costs that place it between wMel, which can successfully invade populations where it is stably maintained,\textsuperscript{17,35} and wMelPop, which can be invaded into semi-field populations\textsuperscript{13,19} but which has substantial fitness costs that make invasion into natural populations.
difficult. Given that wAlbB in Ae. aegypti can block arbovirus transmission, this strain may be suitable for release alongside wMel, although its ability to block different serotypes of dengue and other viruses remains to be established (cf.4). Because it influences the hatch rate of quiescent eggs, the wAlbB strain may also have utility in releases aimed at suppressing or eliminating populations of Ae. aegypti during the dry season.6,19

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Note: Supplemental appendices, tables, and figure appear at www.ajtmh.org.

REFERENCES


competitiveness of the dengue vector mosquito, *Aedes aegypti*. 


APPENDIX H – MAINTAINING *Aedes aegypti* MOSQUITOES INFECTED WITH *Wolbachia*
Introduction

Aedes aegypti mosquitoes are responsible for transmitting some of the most important arboviruses in the world, including dengue, Zika and chikungunya. These viruses are becoming an increasing threat to global health as the widespread distribution of Aedes aegypti in the tropics continues to expand. Female Aedes aegypti preferentially feed on human blood and thus tend to live in close proximity to humans, particularly in urban areas where populations are most dense. Through this close association with humans they have also adapted to breed in artificial habitats, including tires, pots, gutters and water tanks. Ae. aegypti also readily adapt to laboratory environments where they can be maintained without any special requirements after being collected directly from the field, unlike some other species in the Aedes genus. Their ease of maintenance has seen them studied widely in the laboratory in a broad range of fields, ultimately aiming to control the diseases mosquitoes may transmit.

Traditionally, arboviral control relies heavily on the use of insecticides to reduce mosquito populations. However, there is increasing interest in approaches where modified mosquitoes are reared in the laboratory and then released into natural populations. Released mosquitoes may be modified genetically, biologically, through irradiation, chemical treatment, or with combined techniques to either suppress populations of mosquitoes or replace them with mosquitoes that are refractory to arboviral transmission.

Wolbachia are bacteria that are currently being used as a biological control agent for arboviruses. Several strains of Wolbachia were recently introduced into Aedes aegypti experimentally using embryonic microinjection. These strains reduce the capacity of arboviruses to disseminate and replicate in the mosquito, diminishing their transmission potential. Wolbachia infections are transmitted from mother to offspring, however certain strains induce sterility when infected males mate with uninfected females. Wolbachia-infected males can therefore be released in large quantities to suppress natural mosquito populations, as recently demonstrated in other Aedes species. However, since Wolbachia also inhibit arboviral transmission in Aedes aegypti, mosquitoes can also be released to replace native populations with poorer vectors. Wolbachia-infected experimentally with Wolbachia are now being released into the field in several countries using this latter approach.

Wolbachia-based approaches for arboviral control rely on a sound understanding of the interactions between Wolbachia, the mosquito and the environment. Wolbachia occur naturally in a broad range of insects, and the strains introduced into mosquitoes are diverse in their effects. As new Wolbachia infection types are introduced into Aedes aegypti, it is necessary to characterize each strain for their effects on mosquito fitness, reproduction and arboviral interference under a range of conditions. Rigorous experimentation in the laboratory is therefore required to evaluate the potential for Wolbachia strains to succeed in the field.

Open field releases of Aedes aegypti with Wolbachia infections can often require thousands to tens of thousands of mosquitoes per release zone to be reared each week. The success of initial releases can be improved by releasing mosquitoes of a large size to maximize their fecundity.
and mating success\textsuperscript{34,35}. Mosquitoes should also be adapted to the conditions they will experience in the field, however long-term laboratory rearing may cause changes in behavior and physiology which could impact field performance\textsuperscript{36,37,38}.

We describe a simple protocol for rearing \textit{Ae. aegypti} in the laboratory using basic equipment. This protocol is suitable for both wild-type and \textit{Wolbachia}-infected mosquitoes, the latter of which can require special attention as some \textit{Wolbachia} strains have substantial effects on mosquito life-history traits\textsuperscript{39,40}. The rearing conditions avoid overcrowding and competition for food to produce mosquitoes of a consistent size, which is critical for vector competence and fitness experiments, and ensures that the mosquitoes are healthy for field release\textsuperscript{41}. We also take precautions to minimize laboratory adaptation and inbreeding by reducing selective pressures and ensuring that the next generation is sampled from a large, random pool. However, laboratory environments are distinctly different from field conditions, and long-term maintenance under relaxed conditions could reduce the fitness of mosquitoes upon release into the field\textsuperscript{42,43}. We therefore cross females from laboratory lines to field-collected males periodically, resulting in colonies that are genetically similar for experimental comparisons and that are adapted to the target field population\textsuperscript{42}. The methods do not require any specialized equipment and can be scaled up to rear tens of thousands of individuals per week for field releases. The protocol also prioritizes the fitness of mosquitoes within and across generations, an important consideration for insects destined for establishment in natural populations. The protocol is suitable for most laboratories that require maintenance of \textit{Ae. aegypti}, particularly for experimental comparisons where a consistent quality of mosquitoes and relatability to the field are important.

## Protocol

**Blood feeding of mosquitoes on human subjects was approved by the University of Melbourne Human Ethics Committee (approval #: 0723847). All volunteers provided informed written consent.**

### 1. Larval Rearing

**NOTE:** Mosquitoes are held at 26 ± 0.5 °C and 50-70% relative humidity, with a 12:12 h (light:dark) photoperiod for this colony maintenance protocol. These conditions are similar to the average climatic conditions in Cairns, Australia and within the optimal thermal range for \textit{Ae. aegypti} survival and development\textsuperscript{44,45,46}. High temperatures can result in the loss of \textit{Wolbachia} infections from mosquito colonies and should be avoided\textsuperscript{47}. We maintain at least 500 individuals per population to minimize inbreeding; maintaining colonies of a smaller size can have fitness consequences [Ross \textit{et al.} unpublished]. Under these conditions and assuming adequate nutrition, the average generation time is 28 days (see Table 1).

1. Submerge the eggs on substrate in trays (Figure 1A) containing 3 L of water (reverse osmosis water or aged tap water, generated by leaving tap water in trays for 24 h prior to use). ~300 mg of fish food (one crushed tablet, see Table of Materials) and a few grains of active dry yeast to induce hatching\textsuperscript{48}.

2. One day after hatching, use a glass pipette to transfer approximately 500 larvae to trays containing 4 L of water (Figure 1B), count using a clicker counter. Add two crushed fish food tablets to each tray. If needed, use containers of different sizes for rearing larvae (Figure 1A), but keep larval densities below 0.5 larvae/mL to avoid overcrowding.

3. Check the trays daily to ensure that the larvae have sufficient food; add approximately two food tablets to the trays every two days. Provide food \textit{ad libitum} but ensure that 0.5 mg/larva/day is available during this period to ensure development is synchronous and body size is consistent, otherwise the results of experiments may be confounded (see Representative Results).

4. Take care to avoid the overfeeding of larvae, particularly in smaller rearing containers with less water surface area and volume. If the water looks cloudy or if there is significant larval mortality, replace it with fresh water; mortality should be negligible if larvae are fed optimally.

### 2. Adult Emergence

**NOTE:** Larvae will begin to pupate from five days after hatching if well fed and the majority should pupate by seven days after hatching. Adults will begin emerging approximately two days after pupation if maintained optimally at 26 °C (see Representative Results). Larval development is typically unaffected by \textit{Wolbachia} infections when ample food is provided\textsuperscript{33,39,49}.

1. Seven days after hatching pour the entire contents of the tray through a fine mesh (pore size 0.4 mm). Keep the filtered larval water for later use in ovipups (see "Blood Feeding and Oviposition" section). Invert the mesh and dip it into a plastic container with 200 mL of water to transfer the pupae. Provide additional food if any larvae remain.

2. Prepare the adult emergence cages (Figure 1C) by providing two cups of 10% sucrose solution (Figure 1F) and two cups of moist cotton wool to prevent desiccation (Figure 1E).

3. If the pupae do not need to be sorted by sex, place the lidded containers of pupae into the cage and leave the lid slightly ajar to allow the adults to emerge into the cage. Alternatively, place an inverted funnel over the container to minimize drowning. Ensure all adults have emerged before removing the container from the cage to prevent selecting against slow developers.

### 3. Pupal Sexing for Outcrossing

1. If the pupae do need to be sorted by sex (e.g., for outcrossing), pipette the pupae from larval trays and separate the sexes (Figure 2) into plastic containers (Figure 1A) with 200 mL of water every 24 h until the desired number of each sex has been reached. Place lids on the containers and leave them closed.

2. Adults will emerge into the containers; confirm their sex before releasing into cages (Figure 2C). Remove any adults sexed incorrectly with an aspirator within 24 h of emergence before they reach sexual maturity. Once the sexes have been confirmed, release the adults into cages every 24 h.

3. To obtain \textit{Wolbachia}-infected colonies of a similar genetic background to a natural population, outcross by adding \textit{Wolbachia}-infected females from laboratory colonies to cages of uninfected males derived from eggs collected by ovitraps in the field\textsuperscript{36}, maintaining the prescribed density of 500 individuals per population.
1. Repeat the outcrossing for at least three consecutive generations to produce colonies that are at least 87.5% similar genetically to the field population. Critical: Ensure that the sexes are correct at this stage (see step 3.1).

4. Female *Ae. aegypti* are usually refractory to further insemination within hours of mating. When outcrossing colonies, allow the females and males to mature in separate cages for two days and then aspirate the females into the male cage to provide an equal opportunity to all males.

### 4. Blood Feeding and Oviposition

1. Wait for at least three days after the last female has emerged before blood feeding to allow ample time to mature. Blood feed the females within two weeks of emergence to prevent excessive mortality, particularly for mosquitoes with *Wolbachia* infections that adversely affect longevity. Remove the sugar cups the day prior to feeding to improve feeding rates.

   1. Ask a volunteer to insert their forearm into the cage to allow the female mosquitoes to feed. Most females should feed to repletion within 5 min, but to reduce selection against slow feeders, leave the forearm in the cage for 15 min, or until all females are visibly engorged; a latex glove to protect the hand from bites is optional but recommended.

   2. Two days after blood feeding, place two plastic cups containing larval rearing water and lined with a strip of sandpaper (Figure 1G) (or filter paper (Figure 1H)) into the cage for females to lay eggs. Partially submerge the sandpaper strip in the water to keep it moist. Remove other sources of water to prevent the females from laying their eggs outside the oviposition cup.

   NOTE: Tap water may be used in the cups, but larval-rearing water encourages oviposition and females will lay their eggs more synchronously.

### 5. Egg Collection and Conditioning

1. The females will lay eggs on the sandpaper just above the water line; collect and replace the strips of sandpaper daily until no more eggs are laid. Note that oviposition may continue for up to one week.

2. Partially dry the sandpaper strips by gently blotting them on a paper towel for 30 s, taking care not to dislodge the eggs. Then, wrap the strips in a sheet of dry paper towel and place it into a sealable plastic bag (Figure 1I).

3. Check the condition of the eggs under a dissecting microscope (Figure 3). If the sandpaper strips are too wet, eggs may hatch before being submerged in water (Figure 3B), but if dried too harshly, eggs may collapse (Figure 3C).

4. The eggs can be hatched at any time beyond three days post-collection; hatch all eggs from each colony, collected across all days, in the same container of water to ensure that the next generation is sampled from a large, random pool of individuals.

5. For long-term storage, keep the eggs in a sealed container at a high (> 80%) humidity at around 20 °C. Under these conditions, eggs without *Wolbachia* can be stored for several months while maintaining high hatch rates.

6. As some *Wolbachia* infections greatly reduce the viability of eggs with age, hatch the eggs from *Wolbachia*-infected lines within one week of collection to prevent excessive mortality for the relevant strains. Blood feed the females again after one week if more eggs are needed.

<table>
<thead>
<tr>
<th>Day</th>
<th>Step</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>Hatch eggs</td>
</tr>
<tr>
<td>1</td>
<td>Count larvae into trays</td>
</tr>
<tr>
<td>7</td>
<td>Transfer larvae and pupae to colony cages</td>
</tr>
<tr>
<td>17</td>
<td>Blood feed female adults</td>
</tr>
<tr>
<td>21</td>
<td>Begin collecting eggs</td>
</tr>
<tr>
<td>25</td>
<td>Finish collecting eggs</td>
</tr>
<tr>
<td>28</td>
<td>Hatch eggs</td>
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</tbody>
</table>

Table 1: Overview of the *Ae. aegypti* colony maintenance schedule at 26 °C. The timing of the female blood-feeding and the hatching of eggs is flexible, but long durations at these stages should be avoided, particularly for mosquitoes infected with *Wolbachia*, in order to minimize mortality. Following this schedule minimizes selection against mosquitoes that are fast or slow to develop or mature at all life stages, provided that larvae are fed optimally.
Figure 1: Equipment used for rearing *Ae. aegypti* in the laboratory. (A) Plastic containers used for hatching eggs or rearing larvae with volumes of 500, 750 and 5,000 mL (from left to right). (B) Trays used for rearing larvae at a controlled density, usually 500 larvae in 4 L of water. (C) 19.7 L and (D) 3 L cages used for housing adults. A density of 25 adults or less per liter should be maintained to provide sufficient space. (E) 35 mL cup with moist cotton wool provided as a source of water to adults. (F) 35 mL cup with sucrose solution provided through a cord or dental wick as a source of sugar. (G-H) Cups filled with larval rearing water and lined with an oviposition substrate of sandpaper or filter paper (G and H, respectively). (I) Zip-lock bag used for storage of sandpaper strips or filter paper. Black spots on sandpaper are mosquito eggs. Please click here to view a larger version of this figure.

Figure 2: Lateral (A) and dorsal (B) views of pupae and adult *Ae. aegypti* (C) demonstrating their sexual dimorphism. Males are positioned on the left and females on the right in each panel. When optimally fed, male and female pupae are distinguished by size; females are larger than males (A) and have a relatively bulbous cephalothorax compared to males which have flatter sides (B). Male adults are easily distinguished from females under all rearing conditions, primarily by their plumose antennae and long palps. Please click here to view a larger version of this figure.
Figure 3. Four day old *Ae. aegypti* eggs under different conditions. (A) Intact eggs on sandpaper strips maintained at a high (> 80%) humidity but without any visible moisture. Hatch rates should be above 90% for wild-type *Ae. aegypti* if maintained correctly. (B) Eggs that hatch before being submerged in water (precocious hatching) are distinguished by a detached egg cap and visible larva. This indicates that the sandpaper strip was kept too moist. (C) Eggs that are dried too harshly may collapse, and are clearly visible by their concave appearance. If the sandpaper becomes stiff this also indicates that the eggs may be too dry. Please click here to view a larger version of this figure.

Representative Results

Figure 4 demonstrates the effects of suboptimal nutrition on the development of *Ae. aegypti* larvae. When containers are provided with 0.25 mg of food per larva per day or less, the development time increases for both males and females, and is less synchronous than in containers provided with 0.5 mg of food. If adequate food is not provided throughout the duration of larval development, this could have an adverse impact on the maintenance schedule. Slow-developing individuals are at risk of being selected against, blood feeding may be delayed, and there is a higher risk of adult mortality before reproduction occurs.

Figure 5 shows the wing length (an estimate of body size) of *Ae. aegypti* adults reared under a variety of nutrition regimes. Wing lengths of both males and females decrease substantially and become more variable when nutrition is suboptimal. Uniform body sizes are important for experimental comparisons as body size is positively associated with fecundity, and large mosquitoes are expected to exhibit greater fitness under field conditions.

The effects of *Wolbachia* infections on the above traits are described in other studies but generally there is little to no effect.

Figure 4: Cumulative proportion of *Ae.aegypti* (A) females and (B) males developing to adulthood under different food regimes at 26 °C. 100 larvae were reared in containers of 500 mL water (a larval density of 0.2 larvae per mL), provided with different levels of food (see Table of Materials) and scored for their development time. Only larvae that survived to adulthood were included. Error bars are standard errors, with n = 4 replicates per treatment. Please click here to view a larger version of this figure.

Figure 5: Wing length of *Ae.aegypti* (A) females and (B) males developing under different food regimes at 26 °C. 100 larvae were reared in containers of 500 mL water (a larval density of 0.2 larvae per mL) and provided with different levels of food. A subset of adults was then measured for their wing length by using previously described methods. Error bars are standard deviations. Please click here to view a larger version of this figure.
Discussion

Following the protocol presented here for the maintenance of Wolbachia-infected Ae. aegypti should ensure that healthy mosquitoes of a consistent quality are produced for experiments and open field releases. In contrast to other protocols that prioritize the production of mass quantities of mosquitoes (see reference[57]), the methods are focused on maximizing their fitness, both within generations by implementing relaxed rearing conditions, and across generations by minimizing inbreeding, selection and laboratory adaptation. This protocol is also designed specifically for Ae. aegypti with Wolbachia infections but should be suitable for any type of Ae. aegypti. However, it is not suitable for rearing mass quantities (in the order of millions per week), which can be necessary for sterile or incompatible insect releases that require high numbers to achieve population suppression[57,58].

There are some critical steps that should be followed carefully. It is important to feed the larvae optimally and avoid overcrowding for the duration of their development. This will ensure that the mosquitoes develop synchronously and are of a consistent size. Special care should also be taken when conditioning the eggs; larvae can very easily hatch too soon or eggs can desiccate if the oviposition substrates are too wet or dry, respectively. At all steps in the protocol, we recommend allowing enough time for as many individuals in the colony as possible to complete each stage. Selection against individuals that are slow to develop, mature, blood feed, oviposit or hatch will likely lead to the loss of genetic variation.

We note some further considerations for the maintenance of Wolbachia-infected mosquitoes that are not described in the above protocol. It is possible for Wolbachia infections to be lost from laboratory colonies, and we therefore recommend that colonies be routinely monitored for their Wolbachia infection status. We use quantitative polymerase chain reaction (qPCR)[49,59] to test at least 30 individuals from each Wolbachia-infected line every generation. If any individuals test negative for the appropriate Wolbachia infection, colonies can be purified by isolating as many females as possible from the affected colony and then using progeny from infected mothers only, to find the next generation. The cause of the loss of some Wolbachia strains from laboratory colonies is largely unknown, but could be explained by the failure of temperature controls, as high temperatures can cause the loss of Wolbachia infections[57,60].

Colonies and experiments may also become contaminated with individuals from different lines if care is not taken when rearing. Contamination can result from careless pipetting of larvae, mixing up batches of eggs, mislabeling cages or incorrectly sexing pupae in crosses. Special care should therefore be taken when handling colonies with different Wolbachia infection types. Thoroughly clean any oviposition cups, rearing trays and cages before reusing them, clean out pipettes before handling each new tray of larvae and clean the mesh each time when transferring larvae and pupae to other containers. Furthermore, inspect fingers for eggs when handling sandpaper strips and use fresh paper towels when drying each strip, ensure that adults are the correct sex before releasing them into cages, and deal with any escapees quickly. Taking these precautions should avoid most contamination, but colonies should still be routinely monitored using diagnostic assays[59].

Female Ae. aegypti require a blood meal in order to lay eggs, and laboratories around the world provide them in a variety of ways, from membrane feeding systems[57], restrained animals[61] and, to a lesser extent, artificial blood[62]. However, mosquitoes with experimental Wolbachia infections often perform poorly on non-human blood, and may exhibit reduced fecundity and hatch rates, and incomplete transmission of Wolbachia to their offspring[63,64,65]. Maintaining the receptiveness of females to human odors is also important for mosquitoes to be released into the field, and feeding them through membranes or other animals may compromise this ability[57]. We therefore opt to use the forearm of human volunteers for this colony maintenance protocol, though other methods are required under some circumstances. Blood should be supplied through other means when working directly with arboviruses, and care should be taken when colonies originate from field populations with high viral loads as transovarial transmission of arboviruses may occur[66]. Human volunteers should also not blood feed if they have recently visited arboviral endemic countries as there may be a risk of transmission.

Our maintenance protocol aims to minimize laboratory adaptation and selective pressures, but there is room for improvement. Leftwich et al.[42] provide further recommendations for maintaining the fitness of mosquitoes for open field releases, including the use of more diverse and natural larval diets, reducing the density of adults in cages, and providing a complex environment. These considerations may enhance the fitness of Ae. aegypti to a greater level, though currently there is no evidence of fitness changes due to laboratory adaptation under the maintenance protocol described here [Ross et al. unpublished]. Some additional measures may not be feasible for laboratories with limited space and resources, but are nevertheless worth investigating. Maintaining large population sizes, avoiding major selective pressures through rearing protocols and implementing periodic outcrossing to field mosquitoes should help in ensuring high fitness of Ae. aegypti for open field releases. The principles involved in the approach can be applied to rearing other disease vector species for release to manipulate or suppress natural populations.

Disclosures

The authors declare that they have no competing financial interests.

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References


APPENDIX I – RATES AND PATTERNS OF LABORATORY ADAPTATION IN (MOSTLY) INSECTS
Rates and patterns of laboratory adaptation in (mostly) insects

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Abstract

Insects and other invertebrates can readily adapt to a range of environmental conditions and these include conditions used in artificial rearing. This can lead to problems when mass rearing insects and mites for release as biocontrol agents or in sterile insect control programs, and when using laboratory strains to understand field population dynamics. Laboratory adaptation experiments also help to understand potential rates of trait evolution and repeatability of evolutionary changes. Here we review evidence for laboratory adaptation across invertebrates, contrasting different taxonomic groups and providing estimates of the rate of evolutionary change across trait classes. These estimates highlight rapid changes in the order of 0.033 (median) haldanes and up to 2.4 haldanes, along with proportional changes in traits of more than 10% per generation in some cases. Traits tended to change in the direction of increased fitness for Coleoptera, Diptera and Hymenoptera, but changes in Lepidoptera were often in the opposite direction. Laboratory-adapted lines tend to be more sensitive to stress, likely reflecting relaxed selection for stress-related traits. Morphological traits show smaller changes under laboratory conditions than other types of traits. Estimates of evolutionary rates slowed as more generations were included in comparisons, perhaps reflecting non-linear dynamics although such patterns may also reflect variance differences among trait classes. The rapid rate of laboratory adaptation in some cultures reinforces the need to develop guidelines for maintaining quality during mass rearing, and highlights the need for caution when using laboratory lines to represent the performance of species in vulnerability assessments.

Key words: laboratory adaptation, domestication, insect, evolutionary rate
Introduction

Adaptation to artificial rearing conditions is an important issue in several situations. When biological control agents such as parasitoid wasps, predatory mites and lacewings are reared for release to provide pest control in agricultural settings, adaptation can decrease the effectiveness of release agents (Bertin et al. 2017). This also applies to the situation where insects are reared for sterile male releases that are used around the world for suppressing populations of pests and disease vectors (Schutze et al. 2015). Adaptation is an issue when invertebrates such as chironomid midges and *Daphnia* are reared as test organisms to assess the role of pollutants and other contaminants in the environment (Nowak et al. 2007), and when endangered animals are captively bred for later release in the wild (Frankham 2008).

Adaptation to artificial rearing conditions ("domestication") occurs when the artificial environments impose new selective pressures that are absent in nature, and/or where selection pressures that are strong in nature are weakened to allow populations to evolve in new directions. Invertebrates are often maintained in discrete generations in the laboratory, which selects for individuals that reproduce early and develop faster (e.g. Leppla et al. 1976, Matos et al. 2000, Diamantidis et al. 2011). Selection can favour behavioural changes that speed up courtship and mating under confined conditions (e.g. Raulston 1975, Kuriwada et al. 2014). Laboratory environments typically lack variation and complexity, and therefore selective pressures associated with variable conditions may be relaxed, reducing selection for tolerance to stresses associated with thermal extremes, desiccation and starvation (Hoffmann et al. 2001), or for the ability of animals to utilize a variety of food sources or oviposition substrates (Bravo and Zucoloto 1998).

Large numbers of insects and other invertebrates are typically reared under controlled and favourable environments. Yet the success of invertebrate release programs depends on the released individuals being able to survive, mate, predate and reproduce effectively in the wild. Adaptation to artificial environments could compromise these abilities and therefore the success of release programs. Released invertebrates could be maladapted due to loss of pesticide resistance, inability to tolerate stressful environmental conditions, and/or inability to use natural resources while being maintained in the laboratory (Kolliker-Ott et al. 2003). Laboratory ("lab") domestication could compromise the ability of laboratory colonies to predict the vulnerability of natural communities to pollution, climate change and other stresses (Nowak et al. 2007). In conservation, lab domestication in colonies of threatened species destined for future release could compromise the fitness and persistence of the species under field conditions (Frankham 2008).

Some researchers find that laboratory strains behave similarly to field strains in many ways, suggesting that the former can be representative of populations and species even after many years of
culture in artificial conditions (Kolliker-Ott et al. 2003, Jong et al. 2017). Others find that changes in the lab are extremely rapid and influences traits substantially, such that adapted strains no longer reflect field populations (Meats et al. 2004, Liedo et al. 2007). These differences may reflect the type of organism being investigated, and particularly its ability to thrive under artificial conditions. Some insects colonizing an artificial environment show a “production curve” (Boller 1972, Ochieng’-Odero 1994), where populations decline for several generations followed by a recovery (and substantial adaptation).

As well as lab adaptation, changes in traits can arise because of inbreeding and genetic drift. Inbreeding depression and drift can counter the effects of lab adaptation by changing traits in a direction that decreases fitness under lab conditions. In conservation, this process will further confound the recovery of such populations as they pass through a bottleneck. In practise, lab adaptation is difficult to separate from drift and inbreeding effects unless crosses are done to test specifically for inbreeding effects (Cacoyianni et al. 1995, Baeshen et al. 2014) or there are clear expectations around the direction of changes in traits (Sgro and Partridge 2001).

The rate of evolution of traits in invertebrates being held in artificial environments will depend on the intensity of directional selection acting on them and trait heritability; the response to selection in a trait, $R$, is given by $h^2S$, where $h^2$ is the additive genetic variance ($V_A$) divided by the phenotypic variation ($V_P$) and $S$ is the selection differential reflecting the intensity of selection. A low additive genetic variance will slow potential rates of lab adaptation, and the evolvability of a trait ($V_A/m^2$) provides a way of expressing this rate of change relative to the mean ($m$) of a trait (Houle 1992) although this measure is difficult to interpret for fitness traits and heritability may provide a similar measure (Hoffmann et al. 2016). Traits can also evolve under genetic drift (i.e. without selection being present) in which case trait means could shift in any direction and the rate of change will only depend on population size.

Rates of evolution across time periods are typically measured in terms of darwins and haldanes (Gingerich 2009). Darwins provide a measure of evolutionary change in trait mean relative to a particular time interval, whereas haldanes provide a measure of the difference between trait means relative to trait variability and the number of elapsed generations (Gingerich 1993). Surveys of rates of adaptation using these rates typically exclude lab adaptation and instead focus on changes in the wild (Hendry and Kinnison 1999).

Here we undertake a review of the literature to test whether particular trait classes and taxonomic groups change under laboratory conditions further than others, and we consider the direction and rate of adaptation. We test whether rates are linked to the length of time that has elapsed in comparisons, and we contrast rates of lab adaptation with those observed in the field. Implications of lab adaptation
are discussed within the context of issues of concern for researchers and managers dealing with invertebrate groups.

**Materials and Methods**

**Literature search**

To identify papers on lab adaptation, we searched Web of Science for the term “laboratory adaptation”. We searched for related terms by combining the words “adaptation” and “artificial” and “rearing”, as well as “adaptation” and “laboratory rearing”. We also considered the term “domestication” although this yielded few additional studies. We undertook wider searches for some genera where there was a substantial literature that appeared to be relevant by including searches for genera (*Aedes, Drosophila, Ceratitis, Anopheles, Bactrocera*) and other terms such as “artificial” and “colony”. We also located some references by backtracking to papers through identified studies.

In total we started with 96 papers which compared laboratory and field populations for evidence of laboratory adaptation. These were then filtered to identify relevant studies. We excluded papers where comparisons between populations or generations involved individuals that were not reared under the same conditions, such as when traits were scored on samples taken directly from the field (e.g. Rull et al. 2005, Pereira et al. 2007) or when there were changes in culturing conditions (Blatt et al. 2012). We excluded studies where populations were deliberately inbred. We also excluded studies where strains representing laboratory and field populations came from geographically separated regions (e.g. Carpenter and Bloem 2002) or where there were different genetic strains involved in the comparisons (e.g. Edwards and Hoy 1995, Tayeh et al. 2012). This resulted in 52 remaining studies covering 35 organisms. We included studies that raised individuals on different food sources, which might include more “natural” sources as well as artificial diets. While the natural resources will more closely reflect those encountered under field conditions, the organism are still being reared under artificial conditions, and we did not want to make a subjective assessment of “naturalness”. The issue of diet was particularly stark where pest organisms from the field were reared on artificial diets as opposed to resources that they attacked under field conditions (e.g. Ekesi et al. 2007).

The studies fell into two main classes: those that compared old and newly established populations originating from the same location (the “comparative” approach), and those studies which looked for changes across time in the same populations (the “temporal” approach) (Matos et al. 2004). Some studies (e.g. Krebs et al. 2001) combined both approaches, while others used tools such as cryopreservation to compare the laboratory population to its ancestor concurrently (Wang and Grewal 2002). Note that the two classes of studies are similar to the “synchronic” (comparison of different
populations with common ancestry at the same time) and “allochronic” (comparison of the same population at different times) designations in Hendry and Kinnison (1999).

The temporal and comparative approaches have strengths and weaknesses in measuring rates of lab adaptation (Matos et al. 2004). When comparing populations from the same area that has been repeatedly sampled, there is the potential for that population’s genetic constitution to change over time due to gene flow and/or selection in that area, potentially invalidating population comparisons. On the other hand, when the same population is tracked and not adequately replicated, there is potential for genetic drift as well as adaptation to contribute to changes within the populations. Drift effects are particularly likely when populations undergo bottlenecks during laboratory colonization, as seen commonly in Lepidoptera (Leplla et al. 1980). When populations are repeatedly measured, it is also possible that small but undetected changes in experimental conditions might influence traits. This issue can be avoided to some extent by running a control population (perhaps an inbred line, or a well-adapted laboratory line) alongside the populations being monitored. An inbred line might become an integral part of an assay to measure fitness changes, as in the case where competition experiments are run against it (Woodworth et al. 2002). In *Drosophila* studies, the comparative and temporal approaches have provided similar results for some traits but not others (Matos et al. 2004).

When using the comparative approach, studies differ in the elapsed generation number between collecting individuals from the field and starting measurements. These are often taken when populations have only been held for 1-2 generations under the laboratory conditions, but this can also extend to 10 or more generations (e.g. Rössler 1975a, b). Any effects of initial rapid adaptation following colony establishment might then go undetected even though these can be substantial (Raulston 1975). The level of replication in studies varies markedly depending on the material available to the investigator and difficulty of culturing the organism as well as other factors including practical limitations such as the ease of measuring the trait under consideration. In many cases, studies might compare one established laboratory stock with a recently collected population (Jong et al. 2017), or evolutionary trajectories that involve a few time points (Krebs et al. 2001). In other studies, comparisons involve multiple populations (Sgro and Partridge 2001) and/or trajectories with several sampling points (Griffiths et al. 2005). The former are essential in tracking whether lab adaptation is consistent across genetic backgrounds (Matos et al. 2002, Griffiths et al. 2005), while the latter help in tracking non-linear patterns across time which have been detected in studies with *Drosophila* in particular (Matos et al. 2000, Johnstone et al. 2017).

**Data extraction**

From each study, we extracted information on traits where there were data for two populations that had experienced different numbers of generations in the laboratory. For each trait we determined the
mean of each population, and when available, its standard deviation (SD) (extracted directly, or computed from its standard error (SE) and sample size). We also included the number of generations between the two trait measurements and the generation time of the insect in days when available. We grouped traits into five classes: life history, behaviour / reproduction, morphology / size, physiology and stress response. We considered stress response traits separately from physiology and life history as we were interested in the response to laboratory populations to stressful conditions. Traits were considered stress response traits when the testing environment was not under standard laboratory conditions (e.g. under desiccation, starvation or thermal stress).

When extracting information, we assumed that separate estimates of lab adaptation were provided in cases where different populations, treatments (Kolliker-Ott et al. 2003) or approaches (Krebs et al. 2001) were used for particular traits. Because we often had limited data on evolutionary trajectories, where multiple points were available we only included the first time point nearest the field population (i.e. the first time controlled conditions were used for culture and traits were measured) and the final time point in the experiment, unless there was clear evidence of non-linear dynamics. The first suitable time point was sometimes unclear (where a parental generation was specified, this might indicate adults collected from the field, or adults emerging from immatures that had been reared under controlled conditions from the eggs of field-collected adults).

To extract data from papers, we often had to use graphs to identify trait means and SDs / SEs). These were obtained with the web-based webplotdigitizer (http://arohatgi.info/WebPlotDigitizer/app/) program. In many cases SDs (or SEs and sample sizes) were not provided, or could not be readily estimated because traits were measured on groups. In some cases there was confusion about whether error estimates represented SEs or SDs such as where both are mentioned (e.g. Jong et al. 2017, Table 1). Some traits only make sense within the context of a group, such as the time taken for a group to reach 50% mortality under stress, or the percent survival of a life cycle stage. For these traits, we did not compute SDs even when relevant data were presented. For percentage data, 95% confidence intervals based on SDs or SEs often exceed biologically meaningful boundaries (such as when mortality levels fall below 0!) and these data were not considered for estimates of evolutionary rates reliant on SDs (see below). In a few cases, it was not possible to extract data from papers even for mean changes. This occurred when only adjusted values were presented in papers (such as changes in trait values across generations adjusted by values for long term laboratory lines) and/or when regression lines were provided only as line slopes without intercepts (Simoes et al. 2009).

When extracting SDs of trait estimates from papers, there is a danger that SDs reflected variation across group means rather than among individuals within groups. If traits are measured on groups, SDs refer to average values rather than individuals. While these might be used to estimate individual-based SDs if sample sizes are known, these were often not specified in papers, or else were specified
but not all individuals were measured for experimental reasons (e.g. Lewis and Thomas 2001). Moreover, individuals in groups often share a common testing environment, such as when insect larvae develop in the same vial; estimates of individual-based SDs for traits like development time would be too low if extrapolated from averages obtained from vials. We therefore tried to ensure that SDs extracted from papers represent variation among individuals, but this was not always possible. It is likely that evolutionary trajectories will often be non-linear and that rates of adaptation to lab conditions will decline over time (Gilligan and Frankham 2003) but this pattern could not be tested in most studies and we therefore had to assume that responses were linear. Traits may also show sudden shifts directly after laboratory culture that may be unrelated to lab adaptation (Krebs et al. 2001) and instead reflect maternal factors or other types of carry over effects (Schiffer et al. 2013) but these were rarely measured. We were therefore unable to control for these sources of variation when making comparisons across traits and taxa.

**Comparing the extent and rate of laboratory adaptation**

To measure the degree to which traits changed under lab adaptation, we computed the change in trait mean across time \((m_2 - m_1)\), where \(m_1\) is the trait mean of the (near) field population, and \(m_2\) is the trait mean of the laboratory population (Appendix 1), i.e:

\[
\frac{m_2 - m_1}{m_1}
\]

This approach is appropriate for metrical traits such as wing length but not for traits measured as percentages (which typically involved measurements on groups of individuals). For traits measured as percentages, we considered proportional changes between timepoints or populations rather than relative changes. We added direction to the changes to reflect fitness and assumed that increased fitness was associated with increased reproduction, size, mating success, resistance to stress and so on (Appendix 1). For traits where larger values reflect lower fitness such as developmental time and pre-mating period, we reversed the direction of the relative change to indicate increased fitness. To capture the size of changes as well as their direction, we consider the absolute value of the changes as a consequence of lab adaptation as well as (fitness-related) signed changes.

When the extent to which traits change or the speed at which traits change are compared across groups, it is important to control for noise that can lead to erroneous conclusions about patterns of evolutionary changes (Morrissey 2016). In comparing the extent to which traits changed across life history classes or taxonomic groupings, we therefore focussed initially on the consistency of the direction of changes, before (more cautiously) considering the size of the changes which are difficult to compare meaningfully when underlying variances and distributions might vary among the groups.
being compared. For the quantitative comparisons, we considered proportional changes in traits, and 
also changes in terms of darwins and haldanes, two commonly-used evolutionary rate metrics 
(Gingerich 2009). We calculated darwins using the formula 
\[
rate (d) = \frac{\ln(m_2) - \ln(m_1)}{\Delta t}
\]
where \(\Delta t\) is the difference in time between populations in millions of years. Haldanes were calculated 
from untransformed values using the formula 
\[
rate (h) = \frac{(m_2/S_p) - (m_1/S_p)}{\Delta g}
\]
where \(S_p\) is the pooled trait standard deviation and \(\Delta g\) is the number generations in the laboratory that 
separate the populations. In order to calculate darwins, a measurement of generation time is required, 
though some studies provided only an approximate estimate (e.g. Bravo and Zucoloto 1998) and 
many did not provide any estimate. Where generation times were not indicated, we estimated 
generation times from life history data presented in the paper (e.g. Kuriwada et al. 2010). If these 
were not available, we estimated generation times based on other laboratory studies of the organism. 
Haldanes were only calculated when standard deviations for traits were provided. As standard 
deviations are expected to increase with the mean, raw data are usually log transformed (Hendry and 
Kinnison 1999), however as we did not have access to the raw data in many cases, we calculated 
haldanes from untransformed data. This resulted in 291 trait estimates expressed in darwins and 138 
in haldanes.

All statistics were performed in SPSS 24.0 for Windows (SPSS Inc, Chicago, IL). In the absence of 
directional selection, we expected traits to either increase or decrease (or show no change) under 
laboratory adaptation, while in the presence of selection we expected them to change in the direction 
of increased fitness in the lab environment. The relative number of changes in an upward or 
downward direction were therefore compared with a Sign test, while differences in the direction of the 
changes among groups were compared with a contingency test. These analyses were undertaken 
irrespective of whether differences between trait means were significant. The relative magnitude of 
changes in different traits or taxa were also compared but care is required in their interpretation 
because of different levels of noise. Morphological traits typically have lower measurement noise than 
life history traits given that they can be measured with greater accuracy (Biro and Stamps 2015). This 
could result in higher apparent evolutionary rates for life history traits than morphological traits given 
that noise (as well as small sample sizes) can then inflate directional changes in means or medians.
To measure changes in evolutionary rates between trait classes and taxa relative to trait variation (including noise) or elapsed time, we also compared taxa and trait groups for estimates of evolutionary rates based on haldanes and darwins. We tested if these measures were linked negatively to elapsed time as previously shown for evolutionary changes in natural populations (Hendry and Kinnison 1999). A negative relationship between elapsed time and evolutionary rate might arise as an artefact if there is no change in a trait under lab adaptation or genetic drift regardless of generation time. Because of this issue, we also divide comparisons into those where statistical significance has been demonstrated, or where there were >2 SE/SD separating means (reflecting substantial divergence in estimates of means). This allowed us to test for the same association but only considering differences in means that are unlikely to represent noise. We use general linear models to compare groups and traits for evolutionary rates.

Results

We extracted 369 estimates of lab adaptation across all traits from the 52 studies. The studies used either comparative (n = 233 trait measurements), temporal (n = 132) or occasionally both of these approaches (n = 4) to test for adaptation to artificial rearing conditions. We found no clear difference between the comparative (median = 0.108, n = 233) and temporal (median = 0.143, n = 132) approaches (Mann-Whitney U: Z = 1.638, P = 0.101) in terms of absolute proportional changes in traits, however when we adjusted the data to reflect absolute proportional changes per generation, the difference between the two approaches was much larger (comparative approach: median = 0.003, n = 160, temporal approach: median = 0.017, n = 132, Mann-Whitney U: Z = 8.254, P <0.0001). The number of generations between trait measurements averaged 61.19 generations for the comparative approach and 10.95 generations for the temporal approach.

For many trait estimates we were not able to determine if differences between laboratory and near-field populations were significant. Many studies did not compare the two populations directly with statistical tests or did not include any statistics. When statistics were unavailable, we considered differences to be substantial when traits changed by more than two SDs or two SEs. For the 238 trait estimates where we could determine significance or where SEs/SDs were available, 144 were assessed as differing between near-field and laboratory populations in this manner, and 94 did not differ in this way.

Patterns of laboratory adaptation

Under lab adaptation, we anticipated trait changes in the direction of increased fitness but also anticipated negative changes due to relaxed selection (such as for stress resistance) and any deterioration of strains due to inbreeding depression. It is also possible that the accumulation of
disease or other factors having a negative effect on fitness might accumulate and decrease fitness under laboratory culture, although in practice these effects would be difficult to separate. We looked at the frequency of trait estimates that responded positively or negatively to artificial rearing conditions. For 221 of the 369 trait estimates (59.9%), the laboratory population outperformed its near-field counterpart; most responses to artificial rearing were therefore positive but there were many exceptions (Figure 1). Patterns were similar when only substantial changes were included, with 94 out of 144 (65.3%) traits responding positively (Figure S1). The direction of responses to artificial rearing differed depending on the trait class (Figure 1). The majority of life history (Sign test: $P < 0.0001$, $n = 193$), behavioural ($P = 0.0002$, $n = 45$), morphological ($P = 0.072$, $n = 61$) and physiological ($P = 0.0129$, $n = 14$) traits exhibited positive changes with artificial rearing. Stress response traits did not show a strong tendency in either direction ($P = 0.471$, $n = 48$) but changes were overwhelmingly negative when only substantial changes were considered ($P = 0.0007$, $n = 18$, Figure S1). Differences among trait classes in the direction of change (ignoring the few cases where equal values were recorded) were highly significant by a contingency test ($\chi^2 = 15.15$, df = 4, $P = 0.004$). This likely reflects a lack of selection for stress resistance in laboratory environments. The largest declines in laboratory populations were observed in *Heterorhabditis bacteriophora*, with reductions in heat, UV and desiccation tolerance (Wang and Grewal 2002), in the survival of *Aedes albopictus* larvae under low nutrition conditions (Jong et al. 2017), and in *Drosophila* desiccation and starvation resistance (Hoffmann et al. 2001, Simoes et al. 2009). However, a few laboratory populations had higher resistance relative to field populations.
Figure 1. Frequencies of trait measurements in laboratory populations that fell into positive, negative, and “no change” categories for each trait class, across all taxonomic groups. The direction of changes assumed to increase fitness (larger size, faster development, more mating etc.) are given in Appendix 1.

For the four insect orders where substantial data were available (Figure 2, S2), changes were mostly positive for Diptera (Sign test: $P < 0.0001$, $n = 252$), and they also showed (non-significant) changes in this direction for the Coleoptera ($P = 0.119$, $n = 15$) and Hymenoptera ($P = 0.267$, $n = 13$) with far smaller numbers of relevant studies. There were no strong directional trends for the Lepidoptera ($P = 0.908$, $n = 74$). Order differences were not significant by a contingency test ($\chi^2 = 7.46$, df = 3, $P = 0.059$). Among the Diptera, it was possible to consider *Drosophila* vinegar flies, tephritid fruit flies and mosquitoes separately because of the substantial amount of data available for these three groups. All three groups of Diptera showed a similar pattern for life history traits (Figure 3, S3), with a contingency analysis indicating no difference between them for the number of positive or negative responses ($\chi^2 = 0.97$, df = 2, $P = 0.616$). The other trait groups could not be compared across the three taxa, although patterns tended to be consistent, with behavioural/reproductive traits tending to show positive changes as was the case for size and physiology, in contrast to the stress response traits.
**Figure 2.** Frequencies of trait measurements in laboratory populations that fell into positive, negative, and “no change” categories for each insect order (and nematodes), across all studies. The direction of changes assumed to increase fitness (larger size, faster development, more mating etc.) are given in Appendix 1.
Figure 3. Frequencies of trait measurements in laboratory populations that fell into positive, negative, and “no change” categories in laboratory Dipteran populations. The direction of changes assumed to increase fitness (larger size, faster development, more mating etc) are given in Appendix 1.

Extent and rates of laboratory adaptation

We looked at the extent of adaptation for traits in terms of their proportional changes (Table 1, Figure S2), irrespective of the number of generations that had elapsed. Proportional changes tended to be variable across groups and studies, with large changes of 50% or more recorded in many groups. Median changes were substantial in some groups, particularly for Lepidoptera (behaviour/reproduction) and Diptera (physiology). Relative changes (unsigned) in behavioural and reproductive (median absolute proportional change = 0.237, n = 47), physiological (0.202, n = 16) and life history (0.160, n = 195) traits were larger than those for morphological (0.039, n = 63) and stress response (0.053, n = 48) traits. On a per generation scale, changes of more than 10% were evident for several trait/order combinations (Table 1), including behaviour in Lepidoptera and physiology/life history in Diptera.
Table 1. Proportional changes under lab adaptation for different trait classes measured with and without adjusting for the number of elapsed generations. The median, maximum and minimum values are presented for changes that are signed relative to fitness. The number of values involved is also given.

<table>
<thead>
<tr>
<th>Trait type</th>
<th>Order</th>
<th>Signed proportional change</th>
<th>Per generation proportional change</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>median</td>
<td>min</td>
</tr>
<tr>
<td>behaviour/reproduction</td>
<td>Coleoptera</td>
<td>0.337</td>
<td>-0.470</td>
</tr>
<tr>
<td></td>
<td>Diptera</td>
<td>-0.033</td>
<td>-0.636</td>
</tr>
<tr>
<td></td>
<td>Hymenoptera</td>
<td>0.025</td>
<td>0.025</td>
</tr>
<tr>
<td></td>
<td>Lepidoptera</td>
<td>0.530</td>
<td>-0.636</td>
</tr>
<tr>
<td></td>
<td>nematode</td>
<td>-0.140</td>
<td>-0.140</td>
</tr>
<tr>
<td>life history</td>
<td>Coleoptera</td>
<td>0.429</td>
<td>-0.064</td>
</tr>
<tr>
<td></td>
<td>Diptera</td>
<td>0.019</td>
<td>-0.505</td>
</tr>
<tr>
<td></td>
<td>Hymenoptera</td>
<td>-0.030</td>
<td>-0.333</td>
</tr>
<tr>
<td></td>
<td>Lepidoptera</td>
<td>0.018</td>
<td>-0.488</td>
</tr>
<tr>
<td></td>
<td>nematode</td>
<td>-0.150</td>
<td>-0.767</td>
</tr>
<tr>
<td>morphology/size</td>
<td>Coleoptera</td>
<td>0.006</td>
<td>-0.031</td>
</tr>
<tr>
<td></td>
<td>Diptera</td>
<td>0.023</td>
<td>-0.141</td>
</tr>
<tr>
<td></td>
<td>Lepidoptera</td>
<td>-0.010</td>
<td>-0.124</td>
</tr>
<tr>
<td>physiology</td>
<td>Diptera</td>
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<td>-0.721</td>
</tr>
<tr>
<td></td>
<td>nematode</td>
<td>0.300</td>
<td>-0.186</td>
</tr>
<tr>
<td>stress response</td>
<td>Coleoptera</td>
<td>0.047</td>
<td>0.042</td>
</tr>
<tr>
<td></td>
<td>Diptera</td>
<td>-0.001</td>
<td>-0.510</td>
</tr>
<tr>
<td></td>
<td>nematode</td>
<td>-0.818</td>
<td>-0.909</td>
</tr>
</tbody>
</table>
Absolute proportional changes were converted to darwins and haldanes to enable direct comparisons with other studies that measure rates of evolution. We observed median rates of change in the order of $8.789 \times 10^4$ darwins (range: $0-4.777 \times 10^6$) and $0.033$ haldanes (range = $0.0006-2.4$). These evolutionary rates are similar to those observed in a review of selection experiments (c.f. Gingerich 1983), and larger than those observed in a review of studies on vertebrate microevolution in natural populations (c.f. Table 1 (Hendry and Kinnison 1999), median darwins: 1425, range: $0-3.959 \times 10^5$, n = 57). We observed a negative association between the extent of adaptation and its measurement interval for both darwins and haldanes, concordant with other studies (Gingerich 1993, Hendry and Kinnison 1999, Gingerich 2009) (Figure 4). This makes comparisons between studies that had different measurement intervals difficult and reinforces the notion that evolutionary rates fall off with increasing measurement intervals. The non-linear response suggests that there are evolutionary constraints to lab adaptation, even across periods of tens of generations.
**Figure 4.** Associations between measurement interval (years or generations between the near-field and laboratory populations) and the estimated rate of evolutionary change in (A&C) darwins and (B&D) haldanes for all trait measurements (A&B) or when only significant changes (or changes >2 SE/SD) were considered (C&D). The direction of changes were not considered thus trait measurements were transformed to absolute values. Trait classes are plotted in different colors as classes significantly affected evolutionary rates (when measured as darwins).
We then investigated differences between trait classes and orders for studies that provided estimates of (log) darwins and (log) haldanes. A GLM indicated a highly significant effect of (log) measurement interval on the rate of evolution as measured by darwins ($F_{1,281} = 58.094, P < 0.001$) and haldanes ($F_{1,136} = 60.048, P < 0.001$) as expected. Negative associations between (log) measurement interval and (log) evolutionary rate were observed regardless of whether differences in trait measurements were significant (GLM: darwins: $F_{1,80} = 9.519, P < 0.001$, haldanes: $F_{1,53} = 51.838, P < 0.001$, Figure 4) or non-significant (darwins: $F_{1,45} = 6.438, P < 0.001$, haldanes: $F_{1,41} = 33.463, P < 0.001$), but evolutionary rates were much larger when differences were significant (darwins: $F_{1,167} = 34.274, P < 0.001$, haldanes: $F_{1,96} = 30.420, P < 0.001$). For darwins, where a substantial number of studies were available, there was also a significant effect of trait class ($F_{4,281} = 7.773, P < 0.001$); life history, behavioural and physiological traits tended to have higher evolutionary rates than stress response and morphological traits. An effect of invertebrate order on evolutionary rate ($F_{4,281} = 3.152, P = 0.015$) was no longer significant when we excluded nematodes which had a limited number of trait measurements ($F_{3,273} = 1.230, P = 0.299$) or when we only considered life history ($F_{4,158} = 1.123, P = 0.348$), the trait class with the most available data. Evolutionary rates within Diptera were also affected significantly by trait class ($F_{4,184} = 6.959, P < 0.001$) and (log) measurement interval ($F_{1,184} = 30.636, P < 0.001$). However, for the more limited data set where haldanes could be computed, there were no significant differences among trait classes ($F_{4,133} = 1.317, P = 0.267$), suggesting that changes may not differ between trait classes once expressed relative to trait variation.

**Discussion**

We found that lab adaptation can result in a very rapid (and often significant) evolutionary changes in traits, with the range of estimates encompassing values at the high end of changes seen in natural populations (c.f. Gingerich 1983, Hendry and Kinnison 1999). Where changes are detected across laboratory generations, these are likely to reflect genetic changes occurring because of selection, inbreeding depression or genetic drift rather than plasticity because in population comparisons the environment is controlled and in temporal studies the same environmental conditions are repeatedly used. Genetic drift and inbreeding effects result from a small effective population size decreasing genetic variation, which is expected if a field population performs poorly after having been brought into the laboratory. Unfortunately, there are few attempts to estimate effective population size along with lab adaptation with some exceptions (Gilligan and Frankham 2003). A decline in genetic variation has been documented in several laboratory populations (as in the case of the fruitfly *Anastrepha fraterculus* (Parreno et al. 2014) and olive fly *Bactrocera oleae* (Zygouridis et al. 2014)) and is expected to lead to a loss of adaptive responses when novel environmental conditions are subsequently encountered (Woodworth et al. 2002, Reed et al. 2003) as well as heterosis in population
crosses once these have diverged (Rössler 1975a) and genetic divergence among replicate lines as documented in *D. subobscura* (Santos et al. 2013).

In most empirical studies where the effective population size might be small, the effects of adaptation to lab conditions and inbreeding are not clearly separated. In a few cases crosses are undertaken to generate F1s which (if independently derived) should mask inbreeding effects due to recessive deleterious genes. Baeshen et al. (2014) crossed established laboratory stocks to outbred field stock to address inbreeding in *Anopheles gambiae* for sperm length, testes size and accessory gland size. They reasoned that inbreeding might account for genetic changes if means in crosses recovered to similar levels as seen in the field stock, whereas intermediate means were unlikely to reflect inbreeding effects. For gland and testes size, means were intermediate, whereas for sperm length they returned to field levels, suggesting trait-specific effects of inbreeding/adaptation. Additional crosses could be carried out to ensure that a return to field levels reflects inbreeding rather than a genetic architecture involving directional dominance (which tends to be expressed in the direction of increased fitness (Mather and Jinks 1982)). For instance, independently generated laboratory lines could be crossed to determine if F1s show trait changes in the direction of increased fitness, as expected under inbreeding depression because lines should randomly be fixed for different deleterious genes. It would be worthwhile to test if lines showing negative effects of laboratory culture are generally associated with a sharper reduction in effective population size given that adaptive rates and extinction probabilities (Willi et al. 2006) are often directly linked to this parameter or levels of genetic variation.

Founder effects and drift can also confound studies of laboratory adaptation and it is difficult to separate these effects from directional selection without a suitable study design. Variation between replicate laboratory populations after establishment would indicate founder effects; if differences during laboratory maintenance appear it could suggest drift. However, few studies use replication (Hoffmann et al. 2001) is one exception) and therefore the effects we observe may not be due to laboratory adaptation alone.

Although some cases of apparent lab adaptation may reflect inbreeding effects which is expected to decrease fitness, the most common pattern we observed across different orders and trait classes was towards an increase in fitness likely reflecting adaptive genetic changes, which will depend on levels of genetic variation in the colonizing population and the intensity of selection in the laboratory environment. Our observations of relatively low proportional changes in morphological traits and relatively high changes for other traits contrast with patterns for trait narrow-sense heritability (ratio of additive genetic variance (*V₁*) over phenotypic variance (*V₂*)), which tends to be relatively higher for morphological traits (Mousseau and Roff 1987, Hoffmann 2000). However, this measure of genetic variability does not consider levels of genetic variation relative to trait means, which is more accurately reflected by trait evolvability estimates (such as *V₁* over m²). Morphological traits do not
necessarily have a relatively higher evolvability than other trait classes (Hansen et al. 2011) although it is difficult to estimate the evolvability of life history traits which are typically not normally distributed (Hoffmann et al. 2016). We found that when lab adaptation was expressed in terms of haldanes, which reflect changes in means relative to trait variability, there was no difference in evolutionary rates between trait classes, unlike for the proportional changes and evolutionary rates measured in terms of darwins. Morphological changes therefore evolve more slowly than other types of traits under artificial conditions when rates are expressed relative to trait means but not trait variances.

The level of selection on traits during lab adaptation can clearly be strong to produce the changes in trait means recorded in some of the studies. Given the potential for lab adaptation to rapidly and substantially change traits, we suspect that invertebrate colonies destined for use in field release need to be reared under conditions that reduce the likelihood of directional changes that lowers field fitness, or else that steps are taken to ensure that colonies for field releases are renewed regularly from field material.

The feasibility of these approaches will depend on the specific context and taxon being considered. For instance, Aedes mosquitoes reared for field release in sterile release or Wolbachia release programs typically develop as larvae under favourable conditions that produce large adults (Yeap et al. 2013), whereas under natural conditions immature stages will often experience nutritional stress that slows development and reduces adult size, resulting in different population dynamics (Hancock et al. 2016). Nutrition and density could be altered in maintenance colonies to produce mosquitoes of variable size and a wide distribution of developmental rates. However, this means a reduction in the number of mosquitoes that can be produced and released particularly because development time is extended under nutritional stress (Ross et al. 2016). It may instead be possible to renew colonies for release regularly by continuously introducing field material into them.

For threatened species being reared in captivity but destined for reintroduction, it may be possible to use rearing conditions that are relevant to conditions likely to be experienced in the field. To date, the success of captive breeding efforts has mostly focussed on the number of founders used to establish colonies (Schultz et al. 2008) but the importance of adaptation in successful re-introductions is at least recognized (Aardema et al. 2011). Minor changes in rearing procedures such as the type of container used to maintain stock populations could have a marked effect on patterns and rates of lab adaption, as noted for life history traits in replicate populations of Drosophila melanogaster by Sgro and Partridge (2001).

In summary, this overview has indicated that lab adaptation rates in invertebrate populations can be extremely high, matching or exceeding rates seen in natural populations. The changes include a mix of increased and decreased fitness under laboratory conditions. The direction of changes seems to
vary between insect orders and Lepidoptera appear particularly prone to negative effects although there are also some cases within this order of very rapid adaptive changes. There is relatively little information on the implications of these changes for fitness under natural conditions although the experience with mass release strains where field fitness of released individuals can be low (Sørensen et al. 2012) suggests that this issue needs to be carefully examined. The speed of lab adaptation in many species (and frequent reduction in stress resistance) also raises the issue of whether environmental vulnerability assessments based on laboratory cultures are always useful.

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References


Figure S1. Frequencies of trait measurements in laboratory populations that fell into positive, negative, and “no change” categories for (A) each insect order (and nematodes) and (B) each trait class. Only traits that differed significantly between (near) field and laboratory populations, or traits where there were >2 SE/SD separating means were included. The direction of changes assumed to increase fitness (larger size, faster development, more mating etc.) are given in Appendix 1.
Figure S2. Histograms of the distribution of proportional changes under lab adaptation for each insect order/nematode and trait class across all studies, without adjusting for the number of elapsed generations.
**Figure S3.** Histograms of the distribution of proportional changes under lab adaptation for each trait class among Diptera, without adjusting for the number of elapsed generations.
APPENDIX J – SUPPLEMENTARY DATA

The following appendix includes experimental results that do not fit into any other chapter but may nevertheless be of interest.

Table J.1 Survivorship and development time of wAlbB-infected and uninfected Ae. aegypti larvae when reared under competitive conditions in different proportions. Larvae were provided with food *ad libitum*, but conditions were crowded, with 400 larvae in 200 mL of water. Six replicate containers were reared for each treatment.

<table>
<thead>
<tr>
<th>wAlbB : Uninfected proportion</th>
<th>Mean % survivorship ± SE</th>
<th>Mean development time (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Males ± SE</td>
</tr>
<tr>
<td>400:0</td>
<td>95.208 0.699</td>
<td>9.548 0.047</td>
</tr>
<tr>
<td>300:100</td>
<td>94.550 0.310</td>
<td>9.583 0.079</td>
</tr>
<tr>
<td>200:200</td>
<td>95.071 1.360</td>
<td>9.433 0.049</td>
</tr>
<tr>
<td>100:300</td>
<td>95.250 0.816</td>
<td>9.654 0.096</td>
</tr>
<tr>
<td>0:400</td>
<td>95.417 1.083</td>
<td>9.436 0.102</td>
</tr>
</tbody>
</table>
Figure J.1 Cumulative adult eclosion of males and females from the wAlbB-infected and uninfected pure cohorts. Data were averaged from six replicate containers each and are controlled for differential survivorship between cohorts.
Figure J.2 Longevity of \( w \)Mel (green), \( w \)AlbB (blue) and uninfected (black) adults when reared under different conditions. (A&C) Males and (B&D) females were reared at a low larval density of 70 larvae in 700 mL of water (0.1 larvae / mL) (A&B) or at a high larval density of 400 larvae in 200 mL (2 larvae / mL) (C&D). Survival curves were averaged from 4 (low larval density) or 8 (high larval density) replicate cages with 25 males and 25 females each.
Testing for multiple mating in *Aedes aegypti* using *Wolbachia*-induced cytoplasmic incompatibility

In this experiment, I used cytoplasmic incompatibility to assess the frequency of multiple mating in caged *Aedes aegypti* populations. Uninfected females were crossed to either uninfected males or *w*AlbB-infected males. Immediately after mating was observed, females were aspirated into cages with males of the opposite infection status and allowed to mate freely for 2 days before blood feeding and isolation for oviposition.

Normally, crosses between two uninfected mosquitoes produce eggs with high hatch rates, while crosses between uninfected females and *Wolbachia*-infected females produce no viable eggs (Figure J.4). When uninfected females were mated to a *w*AlbB-infected male and then immediately introduced into a cage of uninfected males, almost no eggs hatched, but a single female produced a low number of viable offspring, suggesting that it was successfully inseminated by an uninfected male (Figure J.4). When females were crossed to an uninfected male and then *w*AlbB-infected males, a substantial proportion (10/32 females) had intermediate (<80%) hatch rates and 4/32 were completely incompatible, suggesting high rates of multiple insemination. The frequency of hatch rates that indicate multiple insemination differed substantially between the two experimental crosses (4.5% versus 43.8%). Partial or complete cytoplasmic incompatibility therefore seems to occur frequently if an uninfected female mates with an infected male after mating with an uninfected male, but compatibility is often not restored in the reverse situation. These findings indicate that multiple
mating is frequent in densely populated laboratory cages, and that a second mating with an incompatible male seems to override the compatible mating to some extent. These findings could have implications for mating success experiments using *Wolbachia* and for releases of *Wolbachia*-infected males for mosquito population suppression, but more work is needed to determine the reason for this asymmetry in compatibility.

**Figure J.4 Egg hatch proportions of uninfected *Aedes aegypti* females in sequential mating experiments.** In control crosses, uninfected females mated with *wAlbB*-infected males (purple) or uninfected males (orange) only. For experimental crosses, uninfected females mated with a single *wAlbB*-infected male before being aspirated into a cage of uninfected males (blue) or a single uninfected male before being aspirated into a cage of *wAlbB*-infected males (pink).
Does a disparity in *Wolbachia* density between males and females result in partial cytoplasmic incompatibility?

In this experiment, I tested the hypothesis that a cross between two *Wolbachia*-infected mosquitoes could result in partial cytoplasmic incompatibility if males had a relatively high *Wolbachia* density compared to females. Egg hatch rates were obtained from each cross and both sexes had their *Wolbachia* density estimated. The results provide no evidence for any correlation between *Wolbachia* density and hatch rate (Figure J.5), and I suspect that this is because *Wolbachia* densities in laboratory-reared females are well above the threshold to completely restore compatibility. When densities are reduced through heat stress there is a substantial loss of compatibility (Chapter 3); positive correlations between (female - male) *Wolbachia* density and egg hatch rate would therefore be expected under conditions where *Wolbachia* densities are more variable, such as in the field.

![Figure J.5](image)

**Figure J.5 Plots of *Wolbachia* density versus egg hatch proportion in crosses between *wMel*-infected males and females.** Panel (A) plots the difference in standardised *Wolbachia* density between the female and male in each pair on the x-axis, while (B) and (C) compare hatch proportions against male and female density respectively. *Wolbachia* density values were averaged from two independent measurements. No correlations were significant.
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