Ecological and Policy Studies to Forecast the Future of Mosquito-borne Disease Control

Jill Ulrich
University of Miami, j.ulrich1@umiami.edu

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ECOLOGICAL AND POLICY STUDIES TO FORECAST THE FUTURE OF MOSQUITO-BORNE DISEASE CONTROL

By
Jill Nicole Ulrich

A DISSERTATION

Submitted to the Faculty of the University of Miami in partial fulfillment of the requirements for the degree of Doctor of Philosophy

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ECOLOGICAL AND POLICY STUDIES TO FORECAST THE FUTURE OF
MOSQUITO-BORNE DISEASE CONTROL

Jill Nicole Ulrich

Approved:

John C. Beier, Sc.D.
Professor of Public Health Sciences

Gina L. Maranto, M.A.
Senior Lecturer of Ecosystem
Science and Policy

Imelda K. Moise, Ph.D., M.P.H.
Assistant Professor of Geography and
Regional Studies

Guillermo J. Prado, Ph.D.
Dean of Graduate School

Leon E. Hugo, Ph.D.
Senior Research Officer
QIMR Berghofer Medical Research Institute
Now is a critical time for mosquito-borne disease (MBD) control. Although the reductions in the burden of malaria over the past decade and a half have saved more than a million lives, during the same period many mosquito-borne arboviruses have expanded in incidence and geographical range, causing large epidemics. With traditional mosquito control tools losing the battle against insecticide resistance and new biological control tools still in field trials, it is critical that MBD experts take a systematic approach to prepare for the future. The new biocontrol strategy of combating dengue fever with Wolbachia-infected *Aedes aegypti* is being tested at multiple field sites around the world, but the ecology of this group of bacteria in natural mosquito hosts remains largely unexplored. Hence, the evolutionary path of *Wolbachia* in the new host *Ae. aegypti* is uncertain. In some Australian mosquito species with natural *Wolbachia* infections such as the container-breeder *Aedes notoscriptus* and saltmarsh inhabitant *Culex sitiens*, *Wolbachia* infection frequencies range from 25-85% and 50-100%, respectively. To investigate the ecology of *Wolbachia* in natural mosquito hosts, I colonized *Cx. sitiens* and *Ae. notoscriptus* from the field populations and monitored their *Wolbachia* infection frequencies for ten generations after establishment. I found that after colonization of these species, *Wolbachia* infection frequencies remained relatively constant over subsequent generations, with a portion of each population remaining uninfected. This persistent polymorphism in regards to
Wolbachia infection could be due to environmental conditions during larval development or to mosquito host factors, such as other ovarian microbiota. I explored these possibilities in wild and caged Ae. aegypti, Ae. notoscriptus, Cx. sitiens populations. I found that Wolbachia infection levels were drastically reduced in Wolbachia-infected Ae. aegypti adult females after exposure to high temperatures during larval development, although the levels subsequently recovered to some extent once the heat was removed. I also found no association between Wolbachia infection and the abundance of other bacterial species inhabiting the ovaries of Ae. aegypti and Cx. sitiens, suggesting that factors other than bacterial competition are likely responsible for regulating Wolbachia infection levels within mosquito ovaries. These findings shed light on potential futures for Wolbachia in MBD control. At the same time, I addressed critical policy issues relevant to the future by applying the methods of horizon scanning to the field of MBD control. Content analysis of twenty-five interviews with MBD experts and editorial-type scholarly articles was coupled with expert-formulated scenarios to form a forecast of the next twenty years of MBD control, including potential opportunities and threats. In addition to characterizing expert opinions on the current state of MBD, I identified eight drivers of change that could be influential in shaping the future of MBD control. I also described four sets of future scenarios for MBD control based on the input of experts. Overall, my work contributes key findings relating to Wolbachia ecology and MBD control policy that can be used to forecast and prepare for the future of MBDS.
ACKNOWLEDGEMENTS

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LIST OF ABBREVIATIONS

ACT – artemisinin-based combination therapy
BMGF – Bill and Melinda Gates Foundation
CDC – Centers for Disease Control and Prevention
CHIKV – chikungunya virus
CI – cytoplasmic incompatibility
DDT – dichlorodiphenyltrichloroethane
DENV – dengue virus
EIR – entomological inoculation rate
GM – genetically modified
GMEP – Global Malaria Eradication Program
IIT – incompatible insect technique
IRS – indoor residual spraying
IVCC – Innovative Vector Control Consortium
LLIN – long-lasting insecticide-treated net
MBD – mosquito-borne disease
MIM – Multilateral Initiative on Malaria
NIH – National Institutes of Health
NMCP – National Malaria Control Program
OTU – operational taxonomic unit
PAHO – Pan American Health Organization
PCR – polymerase chain reaction
qPCR – quantitative polymerase chain reaction
RBM – Roll Back Malaria
RIDL – Release of Insects carrying a Dominant Lethal
SIT – sterile insect technique
TDR – Special Program for Research and Training in Tropical Diseases
ULV – ultralow volume
UN – United Nations
UNICEF – United Nations Children's Fund
USAID – United States Agency for International Development
VCAG – Vector Control Advisory Group
WHA – World Health Assembly
WHO – World Health Organization
ZIKV – Zika virus
LIST OF PUBLICATIONS

Contributions and status of Dissertation Chapters

Chapter 1: The changing face of mosquito-borne disease control

Synthesis and review were conducted by Jill N. Ulrich. Text written by Jill N. Ulrich.

Chapter 2: Ecology of Wolbachia infections in natural and novel mosquito hosts

Mosquito colonies were established and maintained by Jill N. Ulrich, Chen Wu, and Elise Kho. *Culex sitiens* field samples were collected in collaboration with Brisbane City Council. *Aedes aegypti* samples were provided by Brian Johnson at James Cook University. All experimental work was carried out by Jill N. Ulrich. Bioinformatics for microbiome data was carried out by Martha Zakrzewski. Text written by Jill N. Ulrich, Martha Zakrzewski, Leon E. Hugo, and John C. Beier. Manuscript in preparation.

Chapter 3: Heat sensitivity of wMel Wolbachia during *Aedes aegypti* development

Experimental work and data analysis were carried out by Jill N. Ulrich. Text written by Jill N. Ulrich, John C. Beier, Greg J. Devine, and Leon E. Hugo. Published in *PLoS Neglected Tropical Diseases* 2016.

Chapter 4: Horizon scanning to forecast the future of mosquito-borne disease control

Journal mining and directed interviews were carried out by Jill N. Ulrich. Content analysis was performed by Jill N. Ulrich. Text written by Jill N. Ulrich, Imelda K. Moise, Gina L. Maranto, and John C. Beier. Manuscript submitted to *PLoS Neglected Tropical Diseases*.

Chapter 5: Summary and conclusions

Text written by Jill N. Ulrich
Chapter 1: The changing face of mosquito-borne disease control

1.1. The current state of mosquito-borne diseases

Now is a critical period for mosquito-borne diseases (MBDs). The reductions in malaria over the past decade and a half have been one of the biggest success stories in global health, with more than a million lives saved [1]. The malaria death rate per 100,000 at risk has plummeted by 60% worldwide and by 66% in Africa [2], and some countries in sub-Saharan Africa have cut their malaria burden in half [1, 2]. However, during the same period, many mosquito-borne pathogens have been introduced to new regions or have re-emerged, causing explosive epidemics [3, 4]. Lack of effective mosquito control in urban centers is driving the increasing abundance and geographic expansion of the arbovirus vector *Aedes aegypti* [5]. As urban centers multiply, insufficient infrastructure leads to storage of water for drinking and washing as well as the accumulation of non-biodegradable trash such as bottles, cans, and automobile tires, all which support the development of the immature stages of *Ae. aegypti* [6]. As a result, the annual number of dengue virus (DENV) cases has more than doubled since 2000, and now over 100 countries are endemic [7]. The Indian Ocean region has seen large outbreaks of *Aedes*-transmitted chikungunya virus (CHIKV) affecting millions of people [8]. In late 2013, a chikungunya outbreak that began in Saint Martin in the Caribbean quickly spread to 31 other countries or territories in the Americas, including the United States [8-10]. The alarming incidence of Zika virus (ZIKV), also transmitted by *Ae. aegypti*, and the associated microcephaly cases in newborns in the Americas [11] have raised global awareness of the potential for pandemic expansion of previously geographically limited arboviruses due to the dire state of mosquito-borne disease control around the globe [12].
There is bolstered resolution from the World Health Organization (WHO) and international funding bodies to step up MBD control efforts in order to achieve widespread disease control and elimination [2, 7]. But MBD control is currently restricted by several challenges. Despite the achievements made against malaria in the past decade and a half, the reality is that all MBD control is threatened by the steady loss of effective insecticides. Sixty countries report mosquito resistance to at least one insecticide, and forty-nine countries face resistance to two or more insecticide classes [2]. The dearth of control options for the urban mosquito *Ae. aegypti* is particularly alarming considering that it transmits viruses of pandemic potential – dengue, chikungunya, Zika, and yellow fever [13].

The traditional malaria vector control tools of long-lasting insecticide-treated nets (LLINs) and indoor residual spraying (IRS) are still effective in reducing the burden of malaria [14, 15]. However, only 53% of the people at risk in sub-Saharan Africa sleep under an insecticide-treated net or live in a household that has received IRS [2]. In addition, LLINs and IRS do not control the proportion of mosquitoes biting outdoors [16], so alone these tools cannot eliminate malaria transmission.

On top of these ecological hurdles, MBD control is limited by social, economic, and political factors that are difficult to measure and forecast. In rapidly growing megacities, vector control faces competition with other public health priorities for scarce funding, so often resources for mosquito control are only committed during epidemics [6, 17]. Failure to appropriately engage the public in mosquito control has led to rebounding *Ae. aegypti* populations in domestic environments and increased public contention over the use of new technologies involving the genetic modification of mosquitoes. Global economic restriction has led to instability in the research environment, especially in the area of basic research.
Governments in most developing countries have insufficient resources to sustain qualified entomologists and to establish adequate monitoring and surveillance programs [2]. In addition, they lack the specific policy guidance they need to make evidence-based decisions for MBD control in their countries. In light of this dynamic landscape, it is critical that the MBD community take steps to systematically forecast and prepare for the future.

1.2. The legacy of global eradication campaigns

The idea that MBDs can be eradicated through concerted global efforts originated at the dawn of the 20th century following the mathematical modeling of malaria eradication by Ronald Ross and the declaration by William C. Gorgas in 1909 that yellow fever could be eradicated [18]. Ross and Gorgas were in support of large-scale vector control campaigns and mass drug administration to quickly eradicate MBDs [19]. Other experts opposing these blitz strategies, including the so-called Italian and Dutch schools of malariology, advocated for environmental sanitation, strengthening of health systems, and economic development on a local level to slowly but steadily diminish malaria morbidity and mortality [19, 20]. However, as described in this section, the approach advocated by Ross and Gorgas was the course of action adopted. Many lessons pertinent to the future can be gleaned from these past attempts to eradicate MBDs.

The first example of a strategic eradication effort on a regional scale was the yellow fever vector eradication in the Americas during the first half of the 20th century. In 1901, after receiving confirmation of the role of the mosquito as the sole vector of yellow fever, Gorgas applied mechanical control and patient screening to stop yellow fever transmission in Havana, Cuba [21]. These first efforts against the vector *Ae. aegypti* were made using environmental or source management, which consists of eliminating mosquito breeding
sites or making them uninhabitable for mosquito larvae [21, 22]. Shortly after, Gorgas met equal success in eliminating *Ae. aegypti* from the Panama Canal [23]. In response to these initial successes, in 1915 the newly created Rockefeller Foundation catalyzed efforts to eradicate yellow fever from the globe [18]. Despite initial progress against yellow fever in several countries, tables turned in 1928 when a severe yellow fever epidemic broke out in Rio de Janeiro and quickly spread to other cities in Brazil, Colombia, Venezuela, and Bolivia [24]. In response to the epidemic, the Rockefeller Foundation was widely criticized for imposing its eradication agenda on the health systems of countries, and the idea of single-disease eradication campaigns temporarily fell out of favor [18].

Meanwhile, local efforts to combat yellow fever in Brazil continued under the leadership of American epidemiologist Dr. Fred L. Soper, who was the administrative head of Rockefeller's regional office in Rio de Janeiro [25]. With Soper’s discovery of jungle yellow fever in 1932, it was understood that mosquito species other than *Ae. aegypti* perpetuated yellow fever virus transmission in South American forests, and unless *Ae. aegypti* were eliminated from Brazil entirely, resurgence of yellow fever in cities would always remain a threat [24, 26]. Consequently, Soper turned his attention from controlling mosquito populations in urban centers to eliminating *Ae. aegypti* from all of Brazil [24, 27]. Using comprehensive application of larvicidal mineral oils, cresols and potassium permanganate to larval development sites, fumigation with sulfur fumes and volatile substances, and destruction of water containers, *Ae. aegypti* elimination from all of Brazil was achieved under Soper in 1933 [18, 23, 24]. This achievement, along with the eradication of the malaria vector *Anopheles gambiae* from Brazil in 1940 under Soper’s guidance, re-established public faith in MBD eradication efforts.
Efforts to eradicate *Ae. aegypti* were accelerated by the discovery of the potent insecticide dichlorodiphenyltrichloroethane (DDT) during WWII. The U.S. military first applied DDT against a typhus outbreak in Naples in 1943, and then it was applied extensively in the Pacific to fight malaria [28]. Following WWII, the Pan American Health Organization (PAHO) used the newly discovered DDT along with earlier methods to attempt *Ae. aegypti* elimination from the Americas [27]. The notable exception was the United States, which refused to participate in the eradication efforts [18]. The first application of DDT to combat *Ae. aegypti* was in 1945 in Bolivia, and it was quickly adopted by other countries with absolute success [24]. The long residual action of DDT, which can kill adult *Ae. aegypti* emerging from hidden breeding sites, made much of the eradication from countries in the Americas in the late 1940’s and 1950’s possible [24]. By 1962, eighteen countries in the Americas and many Caribbean islands had eradicated the species, and yellow fever disappeared from all the major ports in South America “as if by magic” [24, 27].

Importantly, the United States, Cuba, Venezuela, and several Caribbean countries had not eliminated *Ae. aegypti*, and the regional achievements of the previous decades quickly dissolved through loosened surveillance, rapid urbanization, expansion of travel, and the development of mosquito resistance to DDT [27]. Following the widespread adoption of DDT for use against agricultural pests, resistance to DDT was found in *An. sacharovi* in Greece in 1951 [29], becoming more widespread in vector species in the following years [30]. Two dengue fever epidemics struck the Caribbean in the mid- and late 1960’s, and in the 1970’s and 1980’s dengue epidemics in the Caribbean, Central America, and South America became commonplace [23].
Due to the overwhelming success achieved against *Ae. aegypti* following WWII, the newly formed World Health Assembly (WHA) and the United Nations Children's Fund (UNICEF) launched the Global Malaria Eradication Program (GMEP) in 1955 with optimism that the application of DDT would also eradicate malaria from the globe. In light of emerging mosquito resistance to DDT, several advocates argued that the GMEP be launched before the valuable tool was lost [19]. Optimism for the GMEP was based on the extrapolation of a handful of successful local elimination experiences, such as the *An. gambiae* eradication from Brazil achieved by Soper [18, 19]. Critics of the GMEP cited the many operational hurdles that would need to be overcome in tropical Africa [19]. However, the belief in a rigid, central eradication plan without exceptions for local circumstances was maintained by the United Nations (UN) and the WHO Expert Committee on Malaria, and the malaria eradication agenda pressed forward [19].

While success of the GMEP was seen in many European countries [31], by the 1960’s it became apparent that this approach would not eliminate malaria from the heart of high malaria transmission in sub-Saharan Africa and the tropics due to technical challenges, and the momentum of malaria eradication efforts subsequently dwindled [32-35]. As a result, in 1969 the global eradication campaign was abandoned [35]. On top of the growing mosquito resistance to DDT, the toxicity, environmental persistence, and bioaccumulation of DDT caused a global trend away from its use [36]. DDT is still used for malaria vector control in some areas [37], but most countries use newer insecticides such as pyrethroids, which are safer for the environment and for human health [38]. In the years following the dissolution of the GMEP, interrupting transmission would no longer be the main concern, the new focus being curtailing malaria morbidity and mortality [39].
One lesson that can be taken from early MBD eradication attempts is that success can be achieved through vertical programs supported by national buy-in and the resolution of global institutions. Although the Rockefeller Foundation was influential in early stages of yellow fever control, the PAHO, which had been influential in the Americas even before WWII, was instrumental in unifying the countries of the Americas to approach *Ae. aegypti* eradication simultaneously. In doing so, the PAHO was able to achieve substantial buy-in from national governments. For instance, the Brazilian government financed the National Yellow Fever Service to take command of national yellow fever efforts, as opposed to leaving the task to the divided authority of the National Health Service, and consequently, yellow fever was eliminated from Brazil [18]. Similarly, the GMEP was launched after WWII through the World Health Assembly with backing from the newly created UN. The WHO was given the task of guiding the technical aspects of implementing the GMEP [19], and being a branch of the UN, was able to gain the support of many malaria-endemic countries. The influence of global public health institutions in gaining the participation of individual countries is apparent from these early eradication attempts.

A second lesson from the yellow fever and malaria eradication campaigns is that the overreliance on “magic bullet” solutions while neglecting key MBD control infrastructure is unsustainable. In 1939, tropical medicine specialist Mark F. Boyd conveyed the sentiments of much of the public health community regarding the need for a development approach to eradication:

*Malaria control should not be a campaign, it should be a policy, a long-term program. It cannot be accomplished or maintained by spasmodic effort. It requires the adoption of a practicable program, the reasonable continuity of which will be sustained for a long term of years* [19, 40].
Present-day programs have clearly not heeded the early warnings. The Roll Back Malaria (RBM) campaign was initially proposed by the WHO in 1998 with an emphasis on the need to strengthen health systems [41]. However, despite growing recognition that socioeconomic development is a potentially effective long-term intervention against malaria, current elimination approaches still rely heavily on the tools of LLINs and IRS while ignoring health systems and socioeconomic development [42]. The continued overreliance on insecticide-based approaches leaves modern MBD elimination campaigns just as vulnerable to the effects of insecticide resistance as it did the eradication campaigns in the DDT era. Before diminishing progress as a result of insecticide resistance dissuades donors against eradication for a second time in history, the local development approach to MBD control should be re-evaluated as a more sustainable alternative.

A final lesson is that without regional coordination, any reductions in MBDs achieved by single countries are temporary. The PAHO was able to achieve astounding regional cooperation during the early years of the yellow fever eradication campaign in the Americas, which was fundamental to preventing re-introductions of the disease. However, the regional achievements were later undone by the few countries that still harbored *Ae. aegypti* populations [27]. As a result, *Ae. aegypti* was re-introduced throughout the region, eventually leading to its current range extending from the southern United States to northern Argentina [43, 44]. With the need for regional coordination in mind, the Multilateral Initiative on Malaria (MIM) was formed in 1997 as a collaboration among 37 African countries, three non-governmental organizations, and three inter-governmental agencies to reduce the burden of malaria in Africa [41]. In addition, the WHO established a multilateral funding mechanism through the Special Program for Research and Training in Tropical
Diseases (TDR) to assist MIM members in building research capacity in Africa [45]. However, the collaborative nature present among African governments for malaria control is not echoed in other parts of the world or in other fields of MBD control. Therefore, the importance of regional alliances to MBD control that was established during the yellow fever eradication campaign must be re-emphasized.

1.3. New faces, new direction

Due to the success of long-lasting insecticide-treated nets (LLINs) and indoor residual spraying (IRS) in reducing the malaria burden on a global scale in the years following the dissolution of the GMEP [46], the policy initiative of eliminating malaria from endemic countries has regained attention in the past fifteen years. The Abuja Declaration in 2000 proposed that malaria mortality in Africa be halved by 2010 through increasing intervention coverage targets and by strengthening local health systems in African countries [47]. Between 2000 and 2010, the number of deaths from malaria in Africa decreased by 17%. During the same time period, countries in the Western Pacific and the Americas saw even greater reductions in malaria mortality, 37% and 48%, respectively [48]. Based on substantial progress towards global targets, in 2007 the idea that malaria eradication was achievable and should be a global goal was put back on the table [32, 35].

Unlike during the previous GMEP, today there are multiple organizations and individuals involved in MBD control, all with distinct agendas. The Bill and Melinda Gates Foundation (BMGF) was instrumental in advocating for a global campaign of malaria elimination, with the Gates Malaria Forum in October 2007 serving as a key setting for experts to debate the pros and cons of the endeavor [35]. Other philanthropists to join in on
malaria elimination include the UK-based Wellcome Trust and more recently Sean Parker (the founder of Napster). Public funders including the Global Fund to Fight AIDS, Tuberculosis and Malaria, the World Bank’s Booster Program, the US President’s Malaria Initiative, the UK Department for International Development, the United States Agency for International Development (USAID), and several others have also joined the cause of malaria elimination [35].

With the arrival of many new funders to the scene of MBD control and the dwindling resources allocated to the WHO by the UN, the WHO has gradually lost the authority over global MBD control that it once held during the GMEP. Although the WHO still publishes technical advice on the best practices in MBD control through its Malaria Policy Advisory Committee and the Strategic and Technical Advisory Group of the Department of Control of Neglected Tropical Diseases [49, 50], the choice of tools and the logistics of how control is carried out within each country are largely determined by the specific agendas of the funders providing support. As a result, the major role of the WHO within MBD control today is to review new vector control paradigms in light of available evidence using the technical expertise of the Vector Control Advisory Group (VCAG). Many National Malaria Control Programs (NMCPs) and Ministries of Health still look to the WHO for specific policy guidance, but with minimized WHO personnel due to funding cuts and the influence of donors on national budgets, the hands of national governments and the WHO are tied to some extent by their funders.

Facing the growing threats to MBD control, including wide-spread insecticide resistance and the emergence and expansion of arboviruses, will require the global coordination of fragmented donor agendas. To achieve this, a central coordinating body,
whether it be a public institution like the WHO or a consortium of philanthropic organizations, must be nominated to strategically plan for the future of MBD control and to lead global efforts. Multiple donor agendas must be pointed in the same direction and consolidated in order to achieve the maximum benefit. During this process it is important that MBD experts be consulted to determine how to best tackle disease transmission within distinct environments and which of the new tools on the horizon will be the most relevant for the future. Given that MBDs now threaten most of the world, a global strategic plan for the future based a substantial body of MBD expertise is the only way that success can be achieved against MBDs in coming decades.

1.4. New tools on the horizon

Sadly, little has changed in our approach to mosquito control since the major global eradication campaigns of the 20th century—current mosquito control still relies heavily on the use of insecticides. Currently synthetic pyrethroids, such as permethrin and deltamethrin, are the only recommended insecticides for treatment of bed nets because they are safe for close human contact and they have a rapid, persistent effect on mosquitoes at low doses [38]. In areas of high transmission, LLINs can reduce malaria incidence by 50%, and IRS is capable of reducing prevalence by 62% [14, 51]. However, pyrethroid resistance in major African malaria vectors has increased dramatically over the past decade [52]. Aircraft-delivered and truck-mounted ultralow volume (ULV) formulations of pyrethroids represent the standard policy of killing adult Ae. aegypti even though they neglect cryptic indoor mosquito resting sites [17, 53-55]. Peridomestic space spraying with ULV insecticides can be used to reduce adult Ae. aegypti populations quickly, but mosquito populations may recover rapidly after spraying [55, 56], so multiple treatments would be
needed in the event of an epidemic [57]. In addition, *Aedes* resistance to all four classes of insecticides has developed and is already limiting the success of some control interventions in the Caribbean [58]. As a result of these limitations, efforts are currently being made to develop insecticides with new modes of action and greater residual activity [59, 60]. However, more promising technologies on the horizon are tools that do not rely on insecticides at all.

Field trials using a genetically modified strain of *Ae. aegypti* carrying a sterilizing dominant lethal gene are planned or are in progress in several countries around the world [61]. The concept of releasing insects to introduce sterility in wild pest populations, today known as sterile insect technique (SIT), was conceived in the 1930’s and 1940’s. A. S. Serebrovskii at Moscow State University laid the conceptual framework for SIT in 1940 using Mendelian principles [62]. In 1947, F. L. Vanderplank successfully controlled a tsetse population in rural Tanganyika through sterility from species crosses [62, 63]. The fundamental principle behind SIT is that if high frequencies of sterile matings occur and if each female only mates once, then the sterile males increasingly outnumber the fertile males, and the target population eventually collapses [64].

SIT has a long history of success, starting with its first major application in the late 1950’s by the United States Department of Agriculture against the New World screwworm, *Cochliomyia hominivorax* [62, 65-67]. The New World screwworm is an abuser of livestock and humans that was once present in a range extending from Argentina to the southern United States. The first trial release of sterilized screwworms on Sanibel Island off the coast of Tampa, Florida, nearly achieved elimination, with only a single fertile egg mass appearing in the twelfth week [68]. Further trials conducted by Knipling in Curacao
in the Netherlands Antilles were able to eradicate the species from the island [69, 70]. After news of the success in Curacao, the governor of Florida, whose economy was losing USD 20-40 million per year due to the screwworm, pushed for an elimination program. The program received ample funding from the state and federal levels, and releases began in 1957. By the end of 1959, eradication from the southeastern USA was declared [71]. Optimism following the southeastern eradication led to many other successful SIT screwworm programs in the US Southwest (1960-1966), the US Virgin Islands and the British Virgin Islands (1971-1972), Mexico (1972-1991), Puerto Rico (1975), and Central America (1988-2000) [72]. SIT has since been applied to other pests, such as fruit flies, weevils, tsetse flies, moths, and mosquitoes.

A modern approach to SIT involves releasing male insects carrying a dominant lethal gene or genetic system, which is called Release of Insects carrying a Dominant Lethal (RIDL). The lethal element is a gene that causes the death of mosquito offspring. Most RIDL systems operate with a dependence on tetracycline or other chemical dietary additives to repress the lethal element in the laboratory, making reproduction possible. The absence of these dietary supplements in the wild cause activation of the sterility gene [73, 74]. When males carrying the dominant lethal gene are released in large numbers into wild populations, they mate with wild females, but the offspring are not viable. In this way RIDL can be used to crash insect populations. Early demonstrations of the feasibility of RIDL in *Drosophila melanogaster* induced expression of a female-specific lethal gene controlled by tetracycline. Lab-reared strains could be released into the wild at any life stage since the suppression mechanism is genetic [75].
In 2010, a RIDL strain of *Ae. aegypti* called OX513A (expressing a single transgenic sequence encoding a red fluorescent marker and tetracycline-repressible late-acting dominant lethal gene produced by Oxitec Ltd.) was tested in a 10-hectare area in Grand Cayman. Approximately 3.3 million engineered OX513A males were released in a 23-week period. Preliminary results showed that the released males could mate with wild females, as demonstrated by 88% of field-collected eggs carrying the fluorescent marker in the fifth month of releases and an 80% reduction in the ovitrap index (bucket traps with mosquito eggs present) relative to untreated areas by the end of the trial [76]. The finding that released males were competitive in mating in the field offered encouragement for further trials [77]. Field trials using OX513A *Ae. aegypti* are planned or are in progress in several countries around the world [61]. This approach has several advantages over the classical approaches to SIT involving irradiation or chemosterilization to induce sterility. Finding a dose of radiation that will sterilize without having too many impacts on fitness was a major challenge overcome by the use of dominant lethal genes. Also, the fitness of sperm is not compromised by embryo-specific dominant lethals, in contrast to irradiation [74]. Dominant lethals also have the advantage of being able to suppress only females, which are of greater public health concern [74].

A newer development, referred to as incompatible insect technique (IIT), involves releasing insects that have not been genetically modified but rather have been infected with the bacterium *Wolbachia* [78]. *Wolbachia* is a common endosymbiotic bacterium infecting the reproductive system of many insects [79, 80]. Inherited maternally, *Wolbachia* can cause mortality of offspring if infected males mate with uninfected females or if crosses between individuals infected with different *Wolbachia* strains take place [81, 82]. This
phenomenon is known as cytoplasmic incompatibility (CI). Because of CI, infected females have a selective advantage, causing the frequency of *Wolbachia* in a population to steadily increase [83]. This mechanism of altering insect reproduction makes *Wolbachia* capable of spreading within host insect populations, potentially reaching complete infection of the population [84].

The first uses of *Wolbachia* by scientists were as a form of insect population control. It was first used as a measure against filariasis vectors in 1967. Large numbers of *Wolbachia*-infected male *Culex quinquefasciatus* mosquitoes were released into wild populations in Burma, eliminating the local mosquito populations [85]. *Wolbachia*-infected *Aedes polynesiensis* were released on isolated islands near Tahiti. Even with relatively small release numbers, decreases in the number of females able to produce viable embryos were seen at the treatment sites, from 100% successful hatches before the trial releases to 76% during the trial phase [86]. Cage experiments in 2011 with the *Wolbachia* wPip strain in *Cx. quinquefasciatus* showed promise for the implementation of field tests for a control strategy on four islands in the south-western Indian Ocean [87]. An experimentally infected line of *Aedes albopictus* was also developed in Italy for population suppression [88, 89].

Although population suppression with *Wolbachia* has been effective in some cases, a more promising approach for disease control involves replacing a native *Wolbachia*-free vector population with *Wolbachia*-infected individuals that are incapable of transmitting disease. While *Wolbachia* are not normally found in *Ae. aegypti*, one strain from *Drosophila*, wMelPop, was introduced to *Ae. aegypti*, and it was found to shorten *Ae. aegypti* lifespans and reduce dengue virus transmission [90, 91]. However, the fitness costs
associated with the wMelPop strain have limited its ability of the strain to spread throughout the population, making it a poor candidate for large-scale dengue control [92-95]. The wMel strain has fewer fitness effects than wMelPop, facilitating its spread through wild populations while maintaining pathogen interference [96-99]. Promising reductions in dengue and Zika transmission induced by wMel in Ae. aegypti raise the possibility that Wolbachia could one day be used to disrupt dengue and Zika transmission cycles [91, 97, 99-102].

Due to the promising effects of Wolbachia against mosquito-borne pathogens observed in the lab, a consortium of researchers formed an initiative to test Wolbachia for mosquito-borne disease control on a larger scale. This initiative, called Eliminate Dengue, has encompassed field trial releases of the Wolbachia-infected Ae. aegypti in Australia, Vietnam, Brazil, Indonesia, and Colombia [103]. It is essential that the reproduction-altering mechanisms drive the Wolbachia infection into the wild population and the infections become self-sustaining for the resulting mosquito population to have a substantially reduced capacity to transmit disease [84, 104]. There has been some success of the wMel Wolbachia strain in invading wild populations [97, 105]. According to an Eliminate Dengue media release, in recent trials in Brazil, 65% of the Ae. aegypti trapped in the release community of Tubiacanga were infected with Wolbachia at the end of the 20-week mosquito release period, but the infection frequency was said to have dropped considerably since then [103, 106]. The drop has been attributed to the higher susceptibility to insecticides of the released mosquitoes compared with the local mosquito population [103]. One model shows that if the released infected females were also to carry insecticide resistance alleles and the wild population were suppressed with weekly fogging, only half
as many females would be needed to achieve a similar level of invasion [107]. The ecological mechanisms controlling *Wolbachia* infection frequencies are still not understood. Epidemiological evidence of a reduction in dengue incidence as a result of the *Wolbachia*-infected *Ae. aegypti* releases is still lacking, but multiple, simultaneous field trials are meant to address issues of broad-scale deployment and show efficacy of the intervention in reducing arbovirus transmission [108].

Insecticide-free strategies to combat mosquito vectors that are less environmentally damaging, better adapted to the biology and behavior of particular vector species, and more efficiently applied are being developed every day, but most still lack the epidemiological evidence necessary to implement them on a global scale. As a result, many new vector control tools have not progressed from small-scale field studies. Given that vector control is the only effective strategy against most MBDs, it is surprising that more resources are not channeled towards its improvement. While drug and vaccine initiatives against malaria have attracted more funding since 2000, improvements to vector control technology have received little attention [60]. The lack of resources for developing new vector control tools limits the extent to which researchers can conduct the large-scale, rigorous studies that are needed to show efficacy of new interventions against epidemiological MBD outcomes [50]. As a result, national MBD control programs are forced to “pick winners” for application within their countries and to predict future scenarios of MBD transmission based on limited evidence. A robust framework to assist decision makers in forecasting the coming decades of MBD control has not existed until now, leaving much of MBD control open to chance.
1.5 Strategic planning for the future of mosquito-borne disease control

The overreliance on insecticides and the failure to understand vector populations, which are biologically complex and are often able to evade interventions, continues to impede well-intentioned efforts to control and eliminate MBDs [109, 110]. Still, future policies incorporating a new arsenal of biological control tools are rarely discussed. Horizon scanning is a foresight tool used to think about, debate, and shape the future in a systematic way [111]. In the last decade it has been used by many businesses, national governments, and non-governmental organizations to predict societal and environmental needs as well as to forecast emerging science and technology [112-114]. Scanning for what might happen in the future, including threats and opportunities, is a critical component of science policy. Looking for early warnings and finding blind spots in the field of MBD control could assist in the strategic planning of MBD control and could reduce the likelihood of negative outcomes in the future [115].

The future utility of *Wolbachia* as a MBD control tool is also uncertain. The sustainability of the transmission-blocking benefits of *Wolbachia* in endemic areas is unknown because at present there is no ecological forecast for *Wolbachia* in mosquito host populations. An investigation of ecological mechanisms that could affect *Wolbachia* in mosquito populations, including reduction of *Wolbachia* density in response to environmental stress and the effects of other ovarian microbiota, could provide insight into the predicted course of evolution for *Wolbachia* in the novel host *Ae. aegypti*.

1.6 Specific aims and hypotheses

**Specific Aim 1.** Characterize *Wolbachia* infections in natural mosquito host populations and within the microbial communities of mosquito ovaries. In polymorphic caged
populations of the saltmarsh mosquito *Culex sitiens* and the container breeder *Aedes notoscriptus* and in field populations of *Cx. sitiens* and *Ae. aegypti*, I tested four hypotheses: 1.1) *Cx. sitiens* and *Ae. notoscriptus* can be colonized in the laboratory from field populations. 1.2) Following colonization, the *Wolbachia* infection frequency will reach fixation over several generations. 1.3) Cytoplasmic incompatibility is imperfect in these *Cx. sitiens* and *Ae. notoscriptus*. 1.4) Interactions among bacterial species within mosquito ovaries account for the different *Wolbachia* infection patterns in *Cx. sitiens* and *Ae. aegypti*.

**Specific Aim 2.** *Investigate environmental variables accounting for the low *Wolbachia* levels in individual mosquitoes.* In caged populations of *Ae. aegypti*, I tested four hypotheses: 2.1) Heat exposure during larval development produces decreased *Wolbachia* levels in adult females. 2.2) Eggs and early instar larvae are more susceptible to heat clearing of *Wolbachia* than later instar larvae. 2.3) There is an inverse relationship between the duration of heat exposure during larval development and the *Wolbachia* levels in adult females. 2.4) If adult females are removed from the heat treatment, *Wolbachia* levels will subsequently recover.

**Specific Aim 3.** *Forecast what mosquito-borne disease control will look like in twenty years’ time.* An ethnographic approach with components of grounded theory was used to address this question. For this reason, no *a priori* hypotheses derived from existing theories or models were used. Using content analysis of transcripts of directed interviews with MBD experts and of editorial literature, I achieved three objectives: 3.1) Describe the current state of MBD control. 3.2) Identify potential drivers of change in MBD control
over the next twenty years. 3.3) Build scenarios of what MBD control will look like in twenty years’ time.

1.7 Dissertation outline

The structure of the dissertation is as follows: Chapter One gives background information on MBD control policy and on the development of Wolbachia as a new biocontrol tool. In this chapter the problem statement, specific aims, and hypotheses are presented. Chapter Two explains cytoplasmic incompatibility and polymorphia of Wolbachia infections in arthropod host populations and presents studies I conducted in Cx. sitiens and Ae. notoscriptus populations infected with Wolbachia as well as a microbiome survey I conducted on Cx. sitiens and Ae. aegypti ovaries. Chapter Three provides evidence that environmental conditions such as heat exposure during larval development may limit Wolbachia levels in mosquito hosts. Chapter Four presents a qualitative horizon scanning study I carried out using expert interviews and editorial literature analysis, which provides a forecast of the next twenty years of MBD control. Chapter Five gives an overview of my thesis, discussing policy implications as well as limitations, and provides future research directions.
Chapter 2: Ecology of *Wolbachia* infections in natural and novel mosquito hosts

2.1. Background

The success or failure of *Wolbachia* in controlling vector-borne diseases is intimately tied to mosquito ecology. Although *Wolbachia* occurs naturally in many arthropod populations, little is understood about the bacteria-host interactions in mosquitoes. Hilgenboecker et al. (2008) described a “most-or-few” infection pattern of *Wolbachia*: either very few or most individuals of a species are infected. However, even when infection rates are high, it is rare to find 100% infection rates in large samples (>100) [116]. For instance, field *Wolbachia* infection frequencies in two Australian mosquito species, the container-breeder *Aedes notoscriptus* and the saltmarsh inhabitant *Culex sitiens*, range from 25-85% and 50-100%, respectively (Hugo et al., manuscript in preparation). In wild populations of arthropods that naturally carry *Wolbachia*, there is strong evidence that ecological factors play a role in limiting the spread of *Wolbachia* [117, 118]. It is still unclear whether ecological factors such as environmental stress or mosquito microbiota could also limit infection frequencies of *Wolbachia* in populations of the novel, artificial host *Ae. aegypti*, as there has been little exploration of the ecology of *Wolbachia*-infected mosquitoes until now.

The ability of *Wolbachia* to spread through arthropod populations arises from the reproductive manipulations it causes in the host. The most common reproductive manipulation is cytoplasmic incompatibility (CI), by which the mating between uninfected females and *Wolbachia*-infected males results in unviable offspring, while all of the other three crosses are compatible and produce viable offspring. This bias in favor of infected females, combined with maternal transmission of *Wolbachia*, causes the proportion of
infected individuals in the population to gradually increase to fixation [84]. The frequency dynamics of symbionts that cause cytoplasmic incompatibility were first modelled by Caspari and Watson (1959), and they concluded that if an infection produces no fitness costs to the host, its frequency in the population should increase [119]. However, if the symbiont imposes fitness costs such as reduced fecundity of the host, or if maternal transmission of the symbiont is imperfect, an unstable equilibrium exists. In such an equilibrium, the reproductive advantage of CI must exceed the disadvantages of the fitness costs in order for the infection frequency to increase. A deterministic age-structured model proposed by Rasgon et al. (2003) [120] requires knowledge of age-specific survival, fecundity, and mating effects in infected and uninfected individuals, which is proving laborious to demonstrate in caged populations and impossible in the field [121]. The model proposed by Turelli (2010), which assumes age-independent reproduction and survival, random mating between all reproductive adults, and equal survival rates and development times for males and females, can be easily validated in caged populations [121]. Like the Caspari and Watson (1959) model, it assumes that if the advantages of cytoplasmic incompatibility to the spread of *Wolbachia* outweigh the disadvantages that *Wolbachia* poses to its hosts, then the infection frequency should increase in the host population.

On the other hand, a large body of literature on the evolution that occurs in heritable symbioses shows that the dynamics in symbiont-infected populations will rarely follow these models. Host populations that acquire a heritable symbiont like *Wolbachia* can undergo unique patterns of genome evolution [122]. Wilson and Duncan propose that host/symbiont genomes can evolve in three ways: collaboration, in which there is metabolic codependency; acquisition, in which the symbiont genome degrades because of reduced
selective pressure and the host population evolves new mechanisms or acquires new symbionts to compensate for the lack of functionality; and constraint, in which symbiont genome evolution is constrained by the gene content of symbiont-hosting tissues [123]. A clear example of metabolic codependency is in bedbugs, where a *Wolbachia* strain has evolved to supplement its hosts with the B vitamins lacking in their blood meals [124]. As a result of this co-evolution, symbiont hosts are often referred to as holobionts, organisms whose phenotypes are determined not only by their own genomes but also by the genome(s) of all the symbionts they carry [125]. In some cases the host species undergoes ecological expansion, as the symbionts supply nutrients that are lacking in new ecological niches. The classic example is the macroevolution of symbiont-dependent sap-feeding insects, which evolved to feed on xylem or phloem sap having harbored obligate symbionts for millions of years [122]. As symbiotic partners evolve together, often an irreversible codependence forms and vulnerabilities in the system may arise, such as suppressed or modified immune responses in the host or decreased environmental tolerance of the symbiont [125, 126]. Because the symbiont evolves to lose functionality and to depend more and more on the host, the host population is selected to compensate. In this way, symbiont-infected host populations may evolve rapidly [122].

The existence of polymorphic populations of arthropods, in which some members are infected with *Wolbachia* and others are not, could be evidence that novel ecological mechanisms are emerging from *Wolbachia*-host co-evolution. In natural populations of *Drosophila simulans* in Eastern Australia, *Wolbachia* was found to occur at a low frequency and to not display cytoplasmic incompatibility despite perfect maternal transmission [127]. In natural populations of *Drosophila melanogaster*, stable *Wolbachia*
frequencies were found in some populations, but marked fluctuations were found in others [128]. Similarly, in East African and West African populations of *D. simulans*, *Wolbachia* infection frequencies were 18.4% and 32%, respectively, and no CI was induced [129]. Recently polymorphia of *Wolbachia* infection has been discovered in two mosquito species in Australia, container-breeder *Aedes notoscriptus* and saltmarsh inhabitant *Culex sitiens*, with infection frequencies in the field ranging from 25-85% and 50-100%, respectively (Hugo *et al.*, manuscript in preparation). Nothing about the *Wolbachia*-host interactions, such as CI or maternal transmission, has been characterized in these species, so the cause of their low infection frequencies remains unknown.

The microbiome of mosquito hosts may also influence the distribution of *Wolbachia* in populations. The bacterium *Asaia* was shown to inhibit the maternal transmission of *Wolbachia* in *Anopheles gambiae* and *An. stephensi* [130]. *Asaia* is a symbiont of many mosquito species, localizing in the gut, salivary glands, and reproductive organs. Like *Wolbachia*, *Asaia* is an α-proteobacterium that is transmitted maternally, but it is also horizontally transmitted through an oral route and through a venereal route during mating [131, 132]. Co-localization studies in *Ae. aegypti* have found that *Asaia* density in reproductive organs is lower in the presence of *Wolbachia* than in its absence, supporting the hypothesis that competition between the two bacteria occurs within the reproductive organs, particularly in the ovaries [133]. It is unknown whether other bacteria localize in the ovaries that could represent competitors for *Wolbachia* for this tissue niche. Despite their potential effects on cytoplasmic incompatibility and maternal transmission, and consequently their importance to *Wolbachia*-based strategies for disease control, the microbiota of mosquito ovaries have not been fully characterized.
Recent high-throughput sequencing of the 16S ribosomal RNA gene has provided a more accurate look at the vast bacterial diversity in mosquitoes and has assessed the impact of host and environmental factors on the microbiome composition [134]. Studies on the midgut have predominated, as this is where parasites and viruses first come into contact with mosquito epithelial tissues, and the composition of the bacterial community likely affects the probability of disease transmission [135, 136]. These studies have revealed that the bacterial diversity of the midgut is greater in field-collected mosquitoes than in their lab-reared counterparts [137-139], and it is also affected by sex [140], stage of development [137], seasonality [141], and locality [141, 142]. Bacterial competition within the mosquito midgut has also been described. When *Proteobacteria* isolates from *Ae. aegypti* midguts were cultured *ex vivo*, *Serratia marcescens* and *Klebsiella pneumoniae* inhibited the growth of *Sphingomonas* spp. and members of the *Burkholderiaceae* family [143]. Competition between two bacteria species or a bacterium and a pathogen could occur within the mosquito directly by the formation of a physical barrier or by the production of enzymes and toxins by the bacteria, or it could occur indirectly through bacteria-induced changes to host metabolism or activation of the mosquito immune system [144]. Overall, there is a growing understanding that mosquito endosymbiont interactions are complex and must be taken into account in microbial disease control strategies.

Understanding the microbiome of mosquito ovaries could have important implications for the success of *Wolbachia* in invading *Ae. aegypti* populations. In the 2011 releases of *Wolbachia*-infected *Ae. aegypti* near Cairns, Australia, the *Wolbachia* infection frequency approached 100% in the suburb Yorkeys Knob (area of 614 houses) and reached
90% in the suburb Gordonvale (area of 668 houses) after just five weeks of weekly releases [105]. Collections from the same communities in 2012 and 2013 showed long-term average Wolbachia infection frequencies of >95%, although the frequencies briefly fell as low as 80.3% and 82.6% in Yorkeys Knob and Gordonvale, respectively [145]. Following the release of Wolbachia-infected Ae. aegypti in Brazil, maternal transmission of Wolbachia was found to be lower in the Brazilian genetic background than what had previously been described in the Australian background (96% as opposed to 100%) [90, 97, 106]. Whether differences in microbiome composition in the two backgrounds can account for the differences in maternal transmission is unknown. Investigation into the microbiota of other mosquito populations that are known to be incompletely infected with Wolbachia could shed light on potential mechanisms of bacterial competition. The saltmarsh mosquito and Japanese encephalitis vector, Cx. sitiens, was recently found to harbor Wolbachia, with infection frequencies varying between 20-80%, depending on the trap location (Hugo et al., manuscript in preparation). Identifying bacterial species that could either facilitate or inhibit Wolbachia invasions in mosquito populations could have implications for the success of vector-borne disease control efforts using Wolbachia.

Wolbachia-host interactions can shed light on potential ecological mechanisms limiting Wolbachia prevalence in mosquito populations. In the following studies I investigated the dynamics of Wolbachia-host interactions, including cytoplasmic incompatibility and maternal transmission, in two natural mosquito host populations, Aedes notoscriptus and Culex sitiens. Furthermore, I described the ovary microbiomes of important disease vectors Ae. aegypti and Cx. sitiens using high-throughput bacterial diversity sequencing to search for any differences that may explain their different
population patterns of Wolbachia infection. Findings have implications for the future use of Wolbachia in MBD control.

2.2. Methods

2.2.1. Mosquito collections

The Ae. notoscriptus laboratory colony was established from eggs collected in ovitrap in the back yard of a residence in Kelvin Grove, QLD (27°26'60"S; 153°0'37"E) from September 2014 through February 2015. The Cx. sitiens laboratory colony was established from larvae collected at Boondall Wetlands Park in Boondall, QLD (27°21'35"S; 153°6'6"E) in May 2015 (Fig. 2.1). Laboratory Ae. aegypti negative controls were obtained from a colony established from eggs collected in Cairns in 2005. The outcrossed wMelPop-infected PGYP1 Ae. aegypti line obtained from University of Queensland [90] was used as a positive control for Wolbachia screening and for the Cx. sitiens ovary sequencing.

For the ovary microbiome study, thirty virgin, 4-day-old, female F1 Ae. aegypti were contributed by collaborators at James Cook University, and three field-collected female F0 Ae. aegypti were contributed by collaborators at the University of Queensland. Fifteen of F1 females were offspring of Wolbachia-infected females collected in Parramatta Park (W+ F1), and the other fifteen were offspring of Wolbachia-free females collected in Innisfail (W− F1) (Fig. 2.2). Parramatta Park and Innisfail are similar in climate and humidity, and sampling was conducted at the same time of year at both sites. The F1 Ae. aegypti were transported from Cairns to Brisbane on dry ice and were stored at −80°C. The three F0 Ae. aegypti females (W− F0) were collected as fourth instar larvae in Innisfail and were reared to emergence in the lab, when they were aspirated and stored at −80°C until
dissection. These three samples were included to account for any differences in microbiota between laboratory F₁ mosquitoes and field collected F₀ mosquitoes. Thirty female Cx. sitiens were collected in CO₂-and-octenol-baited light traps by Brisbane City Council (BCC) near a salt marsh in Hemmant, Queensland. The mosquitoes were morphologically identified with the aid of taxonomic keys and were stored at −80°C until dissection.

2.2.2. Colony maintenance

*Ae. notoscriptus* eggs were kept moist for at least 48 hours before they were hatched in aged (≥48 hours) tap water. Larvae were maintained at densities of approximately 300 larvae/L for 1st- and 2nd-instar and 100 larvae/L for subsequent instars in aged tap water and were fed daily with ground Tetramin Tropical Fish Food (Tetra Melle, Germany). Adult mosquitoes were kept in a 1-mm mesh screened cage (50 × 50 × 50 cm), the interior of which was darkened by a red towel over the top of the cage [146]. The cage contained two black ovitraps lined with brown packing paper and filled with larval rearing water for oviposition and emergence. A bromeliad plant was included in the cage to serve as a mating marker for the initial generations as described previously [146] (Fig. 2.3). Adult mosquitoes received 10% sucrose solution *ad libitum*, and females were blood-fed weekly on a human volunteer as per QIMR Berghofer Human Ethics Approval P361. All life stages were maintained in a climate controlled insectary at 27 ± 1°C and 70 ± 10% relative humidity with a 12:12 hour light:dark cycle and crepuscular periods. Both *Ae. aegypti* Cairns and wMelPop-infected *Ae. aegypti* colonies were maintained under the same conditions as *Ae. notoscriptus*.

Cx. sitiens larvae were reared in 5% seawater in aged tap water at densities of approximately 300 larvae/L for 1st- and 2nd-instar and 100 larvae/L for subsequent instars.
in aged tap water and were fed daily with ground Tetramin Tropical Fish Food (Tetra Melle, Germany). Adult mosquitoes were kept in a tall 1-mm mesh screened cage (50 × 50 × 250 cm) (Fig. 2.3), which was constructed after several attempts to colonize the species in a standard sized cage had failed. Adult mosquitoes received 10% sucrose solution ad libitum, and females were blood-fed weekly on an anesthetized guinea pig after initial colony establishment feeding on a human volunteer. Females oviposited in a black ovitrap inside the cage filled with water collected from a larval site at Boondall Wetlands Park, and egg rafts were collected five days after blood-feeding. Pupae were transferred to larval water in plastic containers 18 × 12 × 6 cm placed inside a cage for adult emergence. All life stages were maintained in a climate controlled insectary as described above.

All Ae. notoscriptus and Ae. aegypti used for experiments were fed as larvae using the “medium” diet developed by Hugo et al. (2010), and Cx. sitiens larvae received three times this amount [147].

2.2.3. Species confirmation

Given that the markings of Cx. sitiens closely resemble those of its sister species Cx. annulirostris, incorrect morphological identification is thought to occur 4.5% of the time [148, 149]. For this reason, a PCR-based species confirmation test was performed on all Culex field samples and was performed periodically on the colony following the methodology of Beebe et al. (2002) [150] with minor modifications. Each 25 µL sample contained 20 pM of the ITS1A forward primer, the flanking segment of the 18S gene (5´-CCTTTGTACACACCGCCCGTCG-3´), 20 pM of ITS1B, the reverse complement of the ITS2A primer on the 5.8S gene (5´-ATGTGTCTCTGAGGTCCACA-3´) [151], 1-10 ng of template DNA, 1.25 mM MgCl₂, 1.5 mM of each dNTP, 1× Taq reaction buffer and 1 U
of Taq DNA polymerase. Thermal cycling was performed in a C1000 Touch™ Thermal Cycler (Bio-Rad) or a MJ Mini™ Personal Thermal Cycler (Bio-Rad) with an initial denaturation at 94°C for 4 min, followed by 30 cycles of 94°C for 30 s, 51°C for 40 s, and 72°C for 30 s. Next, restriction endonuclease digestion was carried out, in which a mixture of 3 µL of PCR product and 3 µL of 2 × Rsal buffer (premade stock) containing 1 U Rsal enzyme/reaction (NEB II, New England Biolabs, Beverly, MA) was incubated at 37°C for 1 h. The digest was run on a 3.0% agarose gel containing 0.0013% SYBR®Safe DNA gel stain (Life Technologies Corporation) at 100 V for 30 min. Cx. sitiens was identified from the presence of a cluster of three bands at ~210, 180, and 170 bp and a fourth band at ~130 bp [150]. For each species confirmation test, a Cx. annulirostris adult female from the laboratory colony was used as a negative control, producing two clear bands at 300 and 180 bp, with less defined bands under 100 bp.

2.2.4. Wolbachia screening

Wolbachia infection status of both Ae. notoscriptus and Cx. sitiens colonies was tested for nine to ten generations after colony establishment using a polymerase chain reaction (PCR) assay. Adults (10 individuals for the first screening and 20 individuals for subsequent screenings) were aspirated from the colony cage, their heads removed, and each placed in a microtube with two sterile 2 mm glass beads and 180 µL of digest solution. Adult bodies were homogenized in a Mini-BeadBeater™ (Biospec Products) for 1 min 30 s. For Cx. sitiens adults, genomic DNA was extracted using the DNAEasy Blood and Tissue kit (Qiagen) as per the manufacturer's instructions. For Ae. notoscriptus adults, genomic DNA was extracted using QuickExtract™ DNA Extraction Solution (Epicentre Technologies Corporation) as per the manufacturer’s instructions and was diluted 1:10 in
purified water. PCR was carried out in a C1000 Touch™ Thermal Cycler (Bio-Rad) or a MJ Mini™ Personal Thermal Cycler (Bio-Rad) with primers amplifying a fragment of the wsp gene of Wolbachia (590-632 base pairs) [152]. PCR was performed in 20 mL reaction using 1× Phusion HF Buffer, wsp 81F (5'-TGGTCCAATAAGTGATGAAGAAAC-3') and wsp 691R (5'-AAAAATTAAACGCTACTCCA-3') primers [152] (0.5 µM each), 0.4 U of Phusion® High-Fidelity DNA Polymerase (New England Biolabs, Inc.) and at least 50 ng of DNA template. Thermal cycling consisted of 98 °C for 30 s, 35 cycles of 98 °C for 10 s, 59 °C for 30 s and 72 °C for 30 s, with a final extension at 72 °C for 10 min. The PCR products were electrophoresed on a 2% agarose gel with a 100 bp ladder. Samples were scored as positive for Wolbachia infection if a 590 to 632 bp band was present. If the wsp gene did not amplify for the positive control, or if the negative control showed wsp amplification, the PCR and gel electrophoresis was repeated.

2.2.5. Cytoplasmic incompatibility and maternal transmission

To determine the reproductive effects of Wolbachia on Ae. notoscriptus and Cx. sitiens, experimental mating crosses were performed for individual pairs of both species to assess the cytoplasmic incompatibility and maternal transmission of Wolbachia.

Two-day-old eggs collected from the Ae. notoscriptus colony were hatched in aged (≥48 hours) tap water, and larvae were maintained as described previously. Pupae were sexed by size, and each sex was placed into a separate cage to ensure virginity of the adults prior to the crosses. Two days after emergence from pupae (three days for females), pairs were combined in small 300 mL containers with darkened bottoms (first pilot study) (Fig. 2.4) or in 50 mL falcon tubes with a tongue depressor (second pilot study) and were given access to sucrose ad libitum. In the third pilot study, one male and four females were placed
in 500 mL containers to increase the prevalence of mated females. Three to five days after combination, the females in each container were blood-fed on a volunteer’s arm (first and second pilot studies) or on an artificial membrane with defibrinated sheep’s blood (third pilot study) (Fig. 2.4). Four days after blood-feeding, the females were removed from their containers and were individually placed for oviposition into black ovitraps containing aged larval rearing water and lined with brown packing paper. The next day, the total number of eggs laid per female was counted using a stereomicroscope. For those females which laid no eggs, the spermathecae were checked for the presence of spermatozoa to confirm the occurrence of mating.

Egg rafts collected from the *Cx. sitiens* colony were hatched in 5% seawater, and larvae were maintained as described previously. Pupae were placed in a plastic container in a cage and were allowed to emerge for four days. Each day, males were aspirated out and were moved to a separate cage to ensure virginity of the adults prior to the crosses. Two days after male emergence from pupae and three days for females, one male and four females were placed in each 500 mL container for mating. Adults were provided with sucrose *ad libitum* but were starved in the 24 h prior to blood-feeding. Three to five days later, females were blood-fed on defibrinated sheep’s blood using an artificial membrane feeding apparatus with Baudruch bovine caecum membrane. Four days after blood-feeding, the females were removed from their containers and were individually placed into black ovitraps containing water collected from a breeding site at Boondall Wetlands Park to stimulate oviposition. The next day, the total number of eggs laid per female (fecundity) was counted using a stereomicroscope. For those females which laid no eggs, the
spermathecae were checked for the presence of spermatozoa to confirm the occurrence of mating.

2.2.6. Sterile ovary dissections

All mosquitoes were thawed and were surface sterilized prior to dissection with five second washes in 100% ethanol, sterile water, and sterile PBS, as described previously [153, 154]. The ovaries were extracted on a sterilized microscope slide under a stereo dissecting microscope in sterile PBS using sterilized forceps and dissection needles. Ovaries were washed in sterile PBS to minimize contamination from other organs prior to their transfer to DNA extraction buffer in a 1.5 mL microcentrifuge tube.

2.2.7. Ovary DNA isolation and purification

Genomic DNA from mosquito ovaries was extracted using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) as per the manufacturer's instructions and was eluted into 30 µL purified water. I included a blank Buffer ATL sample from the QIAamp DNA Mini Kit (Qiagen) as an extraction control, which was processed in the same way as the ovary samples. Genomic DNA in each sample was quantified using the PicoGreen dsDNA Quantitation Reagent (Molecular Probes, Inc.) as per the manufacturer’s instructions. The Wolbachia density was quantified by quantitative PCR prior to applying high-throughput sequencing. All DNA samples were stored at −80 °C.

2.2.8. Wolbachia density in ovaries

Wolbachia densities in individual ovary pairs were determined by quantitative PCR. Multiplex qPCR was performed, amplifying the target Wolbachia-specific wsp gene and the somatic Actin5c gene, which acted as a reference gene to standardize for mosquito body size.
(wsp F: 5′-CATTGGTGTTGGTGTTGGTG-3′, R: 5′-ACACCAGCTTTTACTTGACCAG-3′, Actin5c F: 5′-GACGAAGAAGTTGCTGCTCTGGTTG-3′, R: 5′-TGAGGATACCACGCTTGCTCTGC-3′) [155-157].

The qPCR reactions were performed in 10 µl total volume containing 5.0 µl Platinum® SYBR® Green qPCR SuperMix-UDG (Invitrogen™), 1 µM of each primer, and 2 µL of DNA template. Cycling was performed using a RotorGene 6000 system (Corbett Research) with the following program: 95°C for 2 min, 50°C for 2 min, and 50 cyclic repeats of 95°C for 10 s, 52°C for 10 s, and 72°C for 20 s. This was followed by a standard melt analysis to confirm that only the expected product had been amplified. Quantification cycles (C_q) were calculated using the Comparative Quantification algorithm in the RotorGene 6000 software (Corbett Research). Quantification cycles (C_q) were normalized by taking into consideration the different amplification efficiencies of the wsp and Actin5c genes, and Wolbachia to host genome ratios were calculated using Q-Gene [158].

2.2.9. 16S rDNA amplicon generation, sequencing and analysis

16S rRNA gene amplicons were generated and sequenced for Ae. aegypti ovaries as described previously [159], applying high-throughput paired-end sequencing on the MiSeq Illumina next-generation sequencing (NGS) platform (Illumina, Inc., San Diego, USA) at the Australian Genome Research Facility (Brisbane, Australia). First, PCR was performed to amplify a region of 16S: 27F–519R (covering V1–V3 regions) using universal 16S rDNA primers. Next, gel electrophoresis and gel extraction were applied to obtain only amplicons with the correct length. The PCR was repeated in order to attach
barcode tags as well as adaptors to the amplicons. Finally, the amplicons were sequenced on a MiSeq Illumina genome sequencer (Illumina, Inc.).

Sequence filtering criteria were applied through Illumina's quality control procedure, and then 16 rRNA amplicon paired end sequences were joined using the software PEAR [160]. Next, the Quantitative Insights into Microbial Ecology (QIIME) [161] pipeline was used to remove adaptors, PCR primers, and low quality reads using default settings. The UPARSE [162] pipeline was applied to cluster the sequences into operational taxonomic units (OTUs) at the 97% similarity level by reference-based picking using the software package USEARCH version 8.0 [163, 164]. Next, representative sequences were selected for each cluster and aligned to core set of 16S rRNA Greengenes gene sequences using the Infernal software package v1.1 [165]. All reads not matching the reference set of sequences were excluded. The representative sequences were assigned to taxonomic groups using the USEARCH [163, 164]. All singleton OTUs and OTUs identified in the extraction control sample were removed from the analysis. Welch's t-test was applied to test for significant differences between the Parramatta Park (W+) and Innisfail (W−) samples in the mean number of reads per sample. Statistical analysis was performed using the software Calypso (http://cgenome.net:8080/html/wiki/index.php/Calypso). Briefly, Shannon diversity and richness were calculated and rarefaction analysis was performed. The relatedness between samples was analysed using Principal coordinates analysis (PCoA) based on Jaccard distance.
2.3. Results

2.3.1. Wolbachia infection frequencies following establishment

The *Wolbachia* infection frequency in both colonies was monitored for nine to ten generations following their establishment from the field. Infection frequencies for both colonies remained relatively stable over time (Fig. 2.5). There were large dips in infection frequencies in the *Ae. notoscriptus* and *Cx. sitiens* colonies at the 4th and 6th generations, respectively.

2.3.2. Cytoplasmic incompatibility in *Ae. notoscriptus* and *Cx. sitiens*

The first three pilot studies on the cytoplasmic incompatibility of the *Ae. notoscriptus* caged population were unsuccessful due to an apparent inability of *Ae. notoscriptus* to mate in small containers. This was confirmed by absence of sperm in spermathecae of females (Fig. 2.6). It is postulated that *Ae. notoscriptus* may need more space or larger numbers of males to mate. Similarly, a cytoplasmic incompatibility study in *Cx. sitiens* and maternal transmission studies in caged populations of both species were conducted, but neither species oviposited in the individual containers provided due to lack of insemination (confirmed by spermathecae dissections), which precluded further analysis.

2.3.3. Wolbachia density in mosquito ovaries

Fifteen ovary samples collected from Parramatta Park *Ae. aegypti* offspring (*W*+ *F*1), fifteen ovary samples from Innisfail *Ae. aegypti* offspring (*W*− *F*1), three ovary samples from field-collected Innisfail *Ae. aegypti* (*W*− *F*0), and thirty ovary samples from female *Cx. sitiens* collected in Hemmant were assessed for *Wolbachia* density using qPCR. *Wolbachia* densities in *Ae. aegypti* ovaries were uniformly high in the positive group (*W*+...
F₁), and the *Wolbachia* densities in the negative group (*W⁻* F₀ and *W⁻* F₁) were below the detection limit of qPCR (Fig 2.7). These results confirmed my expectations based on the exposure of the Parramatta Park *Ae. aegypti* population to *Wolbachia* and the lack of exposure to *Wolbachia* in Innisfail. There was a wide distribution of *Wolbachia* densities among *Cx. sitiens* ovaries, with over half of the samples having *Wolbachia* densities below the detection limit. The remaining *Cx. sitiens* ovaries had *Wolbachia* densities ranging from very low to very high, but no samples had densities as high as the *wMel*-infected *Ae. aegypti* from Parramatta Park.

### 2.3.4. Sample quality

After quality control and excluding contaminant sequences identified in the extraction control sample, we obtained a total of 2,057,952 high-quality reads for the *Ae. aegypti* ovaries and 2,542,203 high-quality reads for the *Cx. sitiens* ovaries. The mean number of high-quality reads generated for each *Ae. aegypti* sample was 62,362 (SD = 81,477), and the mean number of reads generated for each *Cx. sitiens* sample was 84,740 (SD = 104,747). The Parramatta Park *Ae. aegypti* samples produced significantly more reads than the Innisfail samples (*t* = 7.735, df = 14.02, *p* < 0.0001). The extraction control contained 183 reads resulting from bacteria in the genera *Wolbachia*, *Propionibacterium*, *Ralstonia*, *Corynebacterium*, *Moraxella*, *Bradyrhizobium*, *Streptococcus*, *Alloiococcus*, and *Planktothrix*. The detection of *Wolbachia* in the extraction control implies that there was some contamination of *Wolbachia* during the sample preparation, PCR, or sequencing [166]; however, the low number of reads suggest the contamination was minimal. As *Wolbachia* has not been described as a contamination in 16S rRNA gene samples so far [167] and is known to be a commensal microbe of arthropods species [116], we have
excluded taxa occurring in the extraction control (with the exception of Wolbachia) from further analysis. Here I present results of analyses including and excluding Wolbachia from the microbial profile.

2.3.5. Diversity of bacterial symbionts in Culex sitiens ovaries

Bacterial diversity was high for most of the Cx. sitiens ovaries, although Wolbachia predominated in ten of the ovaries (Fig. 2.8A). The greatest abundance of bacteria in Cx. sitiens ovary samples was in the phyla Proteobacteria and Bacteroidetes. Prominent species included Wolbachia, Acinetobacter rhizosphaerae, Sphingomonas yabuuchiae, Ralstonia spp., Pseudomonas spp., and Methylobacteriaceae spp. After Wolbachia was excluded from the analysis, there was a similar abundance of these species among all Cx. sitiens samples (Fig. 2.8B).

2.3.6. The ovary microbiome is predominated by Wolbachia in positive F1 group

On the phylum level, Proteobacteria highly dominated the ovary microbiome of Ae. aegypti in the Wolbachia-positive W+ F1 group. The family Rickettsiaceae was highly abundant in the W+ F1 group with Wolbachia being the dominating genus within this family. Finally, an OTU assigned to Wolbachia predominated by contributing 99% of all reads of the ovary microbiota in all samples of the W+ F1 group (Fig. 2.9). Wolbachia sequences were excluded from the microbial profile to examine the microbial composition of the remaining phylotypes in the W+ F1 group (Fig. 2.10). Proteobacteria was the most abundant phylum in the ovary microbiota of the W+ F1 group followed by Firmicutes. Among the phylum Proteobacteria, OTUs of the families Erytrobacteraceaea (order Sphingomonadales), Comamonadaeae (order Burkholderiales) and of the order PYR10d3 were identified in some samples of the W+ F1 group.
The ovary microbiome of Wolbachia-negative $W^-$ F$_0$ and $W^-$ F$_1$ groups has a high intragroup variability. In the $W^-$ F$_0$ and $W^-$ F$_1$ groups, Proteobacteria was the most abundant phylum followed by Firmicutes and Actinobacteria (Fig. 2.10). A negative correlation was observed between the relative abundance of Firmicutes and Proteobacteria in samples in the $W^-$ F$_1$ group (Pearson correlation, $p = 0.03$) (Fig. 2.11). Besides Rickettsiaceae, the families Bacillaceae and Pseudomonadaceae were also identified in the $W^-$ F$_0$ and $W^-$ F$_1$ groups as the most abundant families. OTUs belonging to Wolbachia, Pseudomonas, Bacillus and Acetobacteraceae were most abundant phylotypes in $W^-$ F$_0$, while OTUs assigned to Wolbachia, Bacillaceae, Comamonadaceae and Pseudomonas are the most abundant phylotypes in $W^-$ F$_1$ (Fig. 2.9). The high dominance of Wolbachia sequences in the positive group and also identification of the same genus in the negative groups, despite previous qPCR analysis failed to detect it, indicates potential cross-over contaminations between the samples. Apart from Wolbachia, no other predominantly described endosymbionts such as Asaia or Thorsellia were identified on genus level.

Overall, the ovary microbiota was very variable between samples within $W^-$ F$_0$ and $W^-$ F$_1$ groups, which has been also described in the ovaries of the mosquito Anopheles gambiae and An. coluzzii [168] or which may be also the result of limited power as a consequence of the low sequence counts and low sample size of the $W^-$ F$_0$ group. However, a few bacteria were identified which were also already characterized in ovaries of the Anopheles mosquito. For example, the OTUs assigned to Acinetobacter, Staphylococcaceae, Enterobacteriaceae, Comamonadaceae and Micrococcus, previously described to be part of the “core” OTUs of two Anopheles species, were present in some of the $W^-$ F$_1$ samples. Notable is an OTU assigned to Bacillaceae, which was present in
10/15 samples in the $W^-$ F1 but only in 2/15 samples in the $W^+$ F1 group. The *Bacillaceae* OTU was absent in $W^-$ F0. Similarly the family Comamonadaceae was not identified in $W^-$ F0 but in 4 samples in the $W^-$ F1 group. OTUs of the family Acetobacteraceae were only predicted in samples of the F0 generation (2/3 samples), but in none of the offspring $W^-$ F1 samples and also not in any samples from the $W^+$ F1 group. Acetobacteraceae includes genera such as *Asaia*, another common endosymbiont of mosquitos. Diversity analysis indicates a significant change in Shannon diversity and richness between $W^-$ F0, $W^-$ F1, and $W^+$ F1 groups, with both negative groups having an increase in diversity and richness (Fig. 2.12).

### 2.4. Discussion

Monitoring of *Wolbachia* infection frequencies in *Ae. notoscriptus* and *Cx. sitiens* for several generations after their colonization revealed the persistence of polymorphia with respect to *Wolbachia*, a phenomenon which has also been described in field populations (Hugo et al., manuscript in preparation). Unlike the exponential increase in *Wolbachia* infection frequencies seen during generations after the establishment of *Wolbachia* in *Ae. aegypti* populations [97], *Wolbachia* infection frequencies for *Ae. notoscriptus* and *Cx. sitiens* remained relatively stable over time. Incomplete maternal transmission or weak cytoplasmic incompatibility may explain the maintenance of stable equilibria of *Wolbachia* infections in these species, but unfortunately I was unable to provide results to this end due to challenges with paired mating experiments. Other researchers characterizing natural *Wolbachia* infections in *Anopheles* spp. met similar challenges and resorted to single pairings by forced mating [169], an option which I determined was not within the time frame of this study. Still, the presence of faint bands
on gels at the position where the wsp gene would appear during the PCR-based *Wolbachia*
screens of the colonies and the variety of *Wolbachia* densities I found in *Cx. sitiens* lead
me to believe that *Wolbachia* could be present at higher frequencies than I described in
these species but at densities that are below the detection limit of standard PCR. If this is
the case, then any variations in *Wolbachia* infection frequency I observed over the
generations may be the result of the natural variance of *Wolbachia* densities among
individuals in these populations.

The distinct patterns of *Wolbachia* distribution between the two host species *Cx. sitiens* and *Ae. aegypti* are notable. Whereas *Ae. aegypti* follow a clear “all-or-none” pattern of infection, *Cx. sitiens* show a variety of densities at which *Wolbachia* can persist. This could be due to genetic differences in the strains of *Wolbachia*, as *Wolbachia* density is known to be affected by strain [170], or it could be due the other microbiota present or differences in the host immune systems. Given that *Wolbachia* density has been found to explain between-strain differences in antiviral protection [170], we might expect lower general pathogen protection in *Cx. sitiens* that in wMel-infected *Ae. aegypti*. The finding that *Wolbachia* infections persist in *Cx. sitiens* ovaries at a range of intermediate densities contrasts with the theory proposed by Hilgenboecker *et al.* (2008) that *Wolbachia* infections follow a “most-or-few” pattern in arthropods [116]. As a result of this finding, it is important that the possibility of intermediate *Wolbachia* densities be considered when modeling mosquito-endosymbiont population dynamics and predicting impacts on mosquito fitness and *Wolbachia*-pathogen interactions.

My results show that within *Wolbachia*-infected *Ae. aegypti*, *Wolbachia* is the
dominant feature in the ovary microbiome. In my *W+ F₁* samples, *Wolbachia* comprised
99% of all reads of the ovary microbiota. As expected, *Wolbachia* was absent from the samples from Innisfail using qPCR, which is geographically isolated from the original wMel *Ae. aegypti* release sites. I also show that apart from *Wolbachia*, Bacillaceae-related bacteria and *Pseudomonas* spp. dominate the ovary microbiome of *Wolbachia*-infected *Ae. aegypti*. Excluding experimental contamination from *Wolbachia*, I found that *Pseudomonas*, *Bacillus* and *Acetobacteraceae* are the most common phylotypes in uninfected F₀ *Ae. aegypti*, while in uninfected F₁ *Ae. aegypti*, *Bacillaceae*, *Comamonadaceae* and *Pseudomonas* are the most abundant phylotypes. It is possible that the observed differences in ovary microbiome composition between *Wolbachia*-infected and uninfected *Ae. aegypti* could be due to the sampling locations [141, 142, 154] and not to presence or absence of *Wolbachia*. Therefore, future studies should aim to collect both *Wolbachia*-infected and uninfected *Ae. aegypti* from the same locations and to sample at least 2–3 different sites. Such a design would likely only be possible shortly after the introduction of *Wolbachia* to an area when the *Ae. aegypti* population is still polymorphic for *Wolbachia* infection.

In addition to detailing the differences between *Wolbachia*-infected and uninfected *Ae. aegypti*, my results show the different microbiome compositions of field-caught and lab-reared samples, which has been described by previously [137-139, 168]. OTUs of the family Acetobacteraceae, which includes genera such as *Asaia*, were only found in the ovaries field-caught F₀ mosquitoes. On the other hand, the *Bacillaceae* OTU was only found in lab-reared F₁ mosquitoes, suggesting this species could have been introduced during colonization. The small sample size and the low sequence counts of the W⁻ F₀ group
in this pilot study limited my ability to detect other possible differences between field-caught and lab-reared *Ae. aegypti*.

The limited number of reads in the \( W^- F_0 \) and \( W^- F_1 \) groups is evidence that the analysis of single ovary pairs approaches the sensitivity limits of Illumina sequencing. The number of reads in the \( W^+ F_1 \) group was also low after the exclusion of Wolbachia. To avoid any domination effects of *Wolbachia* during sequencing, future studies could incorporate a third primer in sequencing to suppress *Wolbachia*. This analysis of single ovary pairs allowed for assessment of variation at the individual mosquito level, but for some research questions pooling ovary samples would be appropriate and would allow for a greater number of reads. The inclusion of only thirty-three ovary pairs in this small pilot study also limited my ability to detect associations among bacterial species. My study also suffered from cross-contamination of samples, as evidenced by presence of *Wolbachia* reads in the \( W^- F_0 \) and \( W^- F_1 \) samples. This could have occurred during the sample preparation, PCR, or sequencing [166]. Commonly used DNA extraction kits are a known source of contamination in sequencing studies [167]. The problem of contamination is of greater concern when working samples with low numbers of reads, as in this study.

My findings in *Ae. aegypti* ovaries are similar to findings in the ovaries and lower reproductive tracts of swarming *Anopheles gambiae* and *An. coluzzii* in Burkina Faso, which are comprised of a core microbiome including *Acinetobacter, Pseudomonas, Staphylococcus, Enterobacteriaceae*, and *Corynebacterium* [168]. These similarities suggest that the primers used in my study successfully amplify many common mosquito ovary endosymbionts. The absence of *Spiroplasma* spp. from my samples is one notable difference between my study and midgut microbiome characterizations in *Ae. sollicitans*.
Ae. stricticus and Ae. vexans [172], Cx. annulus [173], and Cx. tritaeniorhynchus [174]. This could be due to my limited sample size, as Segata et al. (2016) isolated Spiroplasma from only one out of twenty female Anopheles lower reproductive tracts [168]. Much like Wolbachia, Spiroplasma spp. can provide an indirect fitness advantage to female hosts by inducing male killing [175, 176] and may provide the host with natural pathogen protection [177, 178], making them ideal candidates for disease control. Although I did not find Spiroplasma spp. in Ae. aegypti ovaries, it is possible that they may occur in other Ae. aegypti tissues, as these species have been found primarily in the hemolymph and gut of insects [179].

There is still a great deal to be learned about the microbial interactions within Ae. aegypti ovaries. In this study I observed a negative correlation between the relative abundance of Firmicutes and Proteobacteria in the $W^{-} F_{1}$ samples (Pearson correlation, $p = 0.03$), but the limited number of reads for ovaries in this group makes it difficult to determine whether there is a true association. Competition between other bacterial species and Wolbachia in Ae. aegypti ovaries could influence the spread of Wolbachia through Ae. aegypti populations and the maintenance of high Wolbachia infection frequencies in the field. Greater understanding of how other bacteria affect Wolbachia densities is needed. In addition, future studies should elucidate the influence of mosquito immune systems and virus-bacteria interactions on Wolbachia. Enhanced understanding of the ovarian environment is needed to predict the spread and pathogen interference effects of Wolbachia when introduced to new mosquito populations for disease control. I provided the first characterization of bacterial species that inhabit Ae. aegypti ovaries. The species I identified in the phyla Proteobacteria, Firmicutes, and Actinobacteria may serve as
candidates for future paratransgenesis strategies. Understanding the microbiome of mosquito ovaries is essential for identifying new candidates for genetic modification and for predicting bacterial interactions within mosquito ovaries, which may determine the success of microbial disease control strategies.
Fig. 2.1. Egg and larval collection sites for *Aedes notoscriptus* and *Culex sitiens* colonies. *Ae. notoscriptus* eggs were collected in ovitraps in the back yard of an urban residence, and *Cx. sitiens* were collected as fourth instar larvae from a saltmarsh reserve with low salinity (<5% salinity).
Fig. 2.2. Collection sites for *Aedes aegypti*. Parramatta Park, in central Cairns, is near the original wMel *Ae. aegypti* release sites. Innisfail, being isolated from the original release sites, has uninfected *Ae. aegypti*. Ovaries of fifteen offspring of *Wolbachia*-infected *Ae. aegypti* from Parramatta Park (*W*+ *F*1) and fifteen offspring of uninfected *Ae. aegypti* collected in Innisfail (*W*− *F*1) were sequenced. Ovaries of three female *Ae. aegypti* collected as larvae from Innisfail (*W*− *F*0) were also included in the sequencing as field controls.
Fig. 2.3. Adult colony cages. The cages constructed for the establishment of A. *Aedes notoscriptus* and B. *Culex sitiens* colonies from field populations are shown.
Fig. 2.4. Cytoplasmic incompatibility experiment. Mosquito pairs were placed in individual containers with darkened bases for mating and oviposition (top). The artificial membrane system used for blood-feeding during the latter CI experiments is also shown (bottom).
Fig. 2.5. Colony *Wolbachia* infection frequencies in *Culex sitiens* and *Aedes notoscriptus*. Frequency of *Wolbachia* infection in caged populations of *Culex sitiens* (blue) and *Aedes notoscriptus* (red) for nine to ten generations after establishment. Ten to twenty adults from each generation were assayed for each species.
Fig. 2.6. Spermathecae of female *Aedes notoscriptus*. The three spherical organs in each image are the spermathecae, where female mosquitoes store sperm after insemination. Translucence of the spermathecae indicate that no sperm are present.
**Fig. 2.7. Wolbachia densities in Culex sitiens and Aedes aegypti ovaries.** Wolbachia densities in the ovaries of field-caught Cx. sitiens, Wolbachia-infected F1 generation (W+ F1), uninfected F1 generation (W− F1), and uninfected F0 generation (W− F0) Ae. aegypti are shown. Wolbachia density was measured by qPCR of the Wolbachia-specific wsp gene and the somatic insect gene Actin5c. Displayed values are relative concentrations of wsp and Actin5c calculated in Q-Gene. The horizontal line at $y=10^{-2.244}$ represents the detection limit of Wolbachia by qPCR, which was established by the Cq values for negative control. Bars denote means bounded by their 95% confidence intervals. The lower 95% confidence limits for the Cx. sitiens and the W− F0 groups cannot be represented on the log scale. Each point represents an ovary pair.
Fig. 2.8. Relative bacterial species abundance in *Culex sitiens* ovaries. Shown are the relative species abundance plots in thirty *Cx. sitiens* ovaries A. including *Wolbachia* sequences and B. excluding *Wolbachia*. Samples along the x axis labeled with “S” are *Cx. sitiens* ovaries. “C” samples are the *Aedes aegypti* uninfected negative controls, and “P” samples are *wMelPop*-infected *Ae. aegypti* positive controls. On the y axis are the OTUs to which the sequences were assigned. The size of the square represents the relative abundance (expressed as a percentage) of the OTU within each sample, with a key on the bottom left of each plot.
Fig. 2.9. Bubbleplot of (a) family level and (b) Operational Taxonomic Unit (OTU) level of *Aedes aegypti* ovary microbiota including *Wolbachia*. Relative abundances of families and OTUs in *Wolbachia*-infected F₁ generation (*W⁺ F₁*), the uninfected F₁ generation (*W⁻ F₁*) and the uninfected F₀ generation (*W⁻ F₀*) of *Ae. aegypti* mosquitoes are indicated by the size of the squares. OTUs assigned to *Wolbachia* are included in the taxonomic profile. Only the top 20 taxa are shown for each level.
Fig 2.10. Bubbleplot of (a) family level and (b) Operational Taxonomic Unit (OTU) level of *Aedes aegypti* ovary microbiota excluding *Wolbachia*. Relative abundances of families and OTUs in *Wolbachia*-infected F$_1$ generation (*W+ F$_1$), the uninfected F$_1$ generation (*W− F$_1$) and the uninfected F$_0$ generation (*W− F$_0$) of *Ae. aegypti* mosquitoes are indicated by the size of the squares. OTUs assigned to *Wolbachia* are excluded from the taxonomic profile. Only the top 20 taxa are shown for each level.
Fig 2.11. Scatterplot of the relative abundance of Proteobacteria and Firmicutes based on the taxonomic profile after OTUs assigned to *Wolbachia* were removed. Each circle represents a sample from the negative F1 group. The size of the circle represents the relative abundance of the phylum Actinobacteria. The relative abundance of Proteobacteria and Firmicutes correlate significantly. In some samples, also Actinobacteria tend to decrease with Proteobacteria increasing.
Fig 2.12. Shannon diversity and richness in *Aedes aegypti* ovaries. Based on ANOVA test, Shannon diversity (a) and richness (b) differs significantly between three groups of *Ae. aegypti* ovaries: the Wolbachia-infected F1 generation ($W^+ F_1$), the uninfected F1 generation ($W^- F_1$) and the uninfected F0 generation ($W^- F_0$). A pairwise comparison was performed between $W^+ F_1$ and $W^- F_1$ groups using a $t$-test. Diversity analysis is based on all OTUs including Wolbachia. Notation: *** $p<0.001$
Chapter 3: Heat sensitivity of \textit{wMel} \textit{Wolbachia} during \textit{Aedes aegypti} development

3.1. Background

The possibility of using \textit{Wolbachia} to disrupt dengue transmission cycles is currently being tested by releasing \textit{Ae. aegypti} infected with the \textit{wMel} strain of \textit{Wolbachia} at sites in Australia, Vietnam, Brazil, Indonesia, and Colombia [103]. The \textit{wMel} strain was originally transinfected from \textit{Drosophila melanogaster} into \textit{Ae. aegypti}, and it blocks dengue virus (DENV) infection in the mosquito [91, 96, 97]. The success of each \textit{Wolbachia} strain in invading insect populations is determined by the net fitness effect of the strain coupled with the extent to which it manipulates host reproduction [84]. One key mechanism of reproductive manipulation is cytoplasmic incompatibility (CI). When a strain causes complete CI, \textit{Wolbachia}-infected females can mate successfully with \textit{Wolbachia}-infected males, while uninfected females cannot [84]. The \textit{wMel} \textit{Wolbachia} strain causes complete CI and has had some success in invading wild \textit{Ae. aegypti} populations [97, 105]. However, the prevalence of \textit{wMel} \textit{Wolbachia} must remain high in the \textit{Ae. aegypti} population in order for \textit{wMel} to reliably and substantially reduce the capacity of the mosquito population to transmit pathogens [84, 104]. Protection against DENV in field-collected \textit{wMel} \textit{Ae. aegypti} is similar to that observed in the original transinfected \textit{wMel} line [99], indicating that this strategy might be used to reduce dengue transmission in endemic areas [91, 97, 101]. Recently the WHO recommended the use of \textit{Wolbachia} for dengue and Zika control [180], although there is currently insufficient epidemiological evidence to know if the approach is effective. It is also unknown whether the prevalence of \textit{wMel}-infected \textit{Ae. aegypti} and the \textit{wMel Wolbachia} levels within
individual mosquitoes will remain high enough to prevent DENV and ZIKV transmission in all environments.

The levels of wMel Wolbachia load throughout the various stages of the Ae. aegypti lifespan have not been described, as most studies have focused on population dynamics and fitness effects of wMel Wolbachia after adult emergence [97-99, 105, 181-183]. The early stages of development comprise a sensitive period during the Ae. aegypti lifespan; immature forms are confined to their aquatic habitats, whereas adults can seek out favorable microclimates to increase their chances of survival [53, 184, 185]. Immature Ae. aegypti develop in containers in the domestic environment that hold water, including flower pots, tanks, and drums as well as bottles, cans, and automobile tires [6, 186]. These containers sometimes hold as little as 5 mL of water [187]. Female Ae. aegypti preferentially lay their eggs in shaded containers, but it is not uncommon to find immatures in containers fully exposed to the sun [188, 189]. Although comprehensive temperature measurements in sun-exposed containers have not been carried out, lab-reared Ae. aegypti larvae can tolerate aquatic temperatures as high as 43°C if they are pre-exposed to high but sublethal temperatures [190]. The ability of wMel Wolbachia to tolerate the same elevated temperatures as immature Ae. aegypti has not been investigated.

The heat sensitivity of Wolbachia with respect to its hosts has been characterized in other arthropods. Exposure to high temperatures during development cured the Wolbachia infections of two-spotted spider mites Tetranychus urticae [191], Tribolium flour beetles [192], and Drosophila spp. [126, 193-195]. In the mosquito Aedes scutellaris, the reproductive effect of CI caused by Wolbachia was lost when larvae were reared at 32.5°C, but it was unknown whether the loss of Wolbachia or host expression of
heat-shock proteins was responsible [196-198]. In *Ae. albopictus* all life stages maintained at 37°C had a lower levels of *Wolbachia* than those reared at 25°C, indicating that high temperatures may reduce *Wolbachia* levels in mosquito hosts [199].

Reduced *Wolbachia* levels in response to high temperatures during larval development could represent a barrier to the spread of wMel *Wolbachia* in *Ae. aegypti* populations if fundamental drive mechanisms such as maternal transmission and CI are affected. Because only *Wolbachia*-infected females produce viable offspring with *Wolbachia*-infected males, CI creates a selective pressure for the spread of *Wolbachia* [84]. The spread of *Wolbachia* in mosquito populations is crucial, because incomplete wMel *Wolbachia* coverage in the *Ae. aegypti* population leaves the potential for DENV and ZIKV transmission. In the 2011 releases of wMel-infected *Ae. aegypti* in two suburbs of Cairns, Far North Queensland, Australia, the infection frequency approached 100% in the suburb Yorkeys Knob (area of 614 houses) and reached 90% in the suburb Gordonvale (area of 668 houses) after just five weeks with weekly releases of 7500 females per suburb. However, after six weeks the frequencies fell to 95% and 81% in the suburbs, respectively, leaving a portion of the population with the ability to transmit dengue [105]. Collections from two communities near Cairns in 2012 and 2013 showed long-term average *Wolbachia* infection frequencies of >95%, although the frequencies briefly fell as low as 80.3% and 82.6% in the suburbs Yorkeys Knob and Gordonvale, respectively. In Yorkeys Knob, *Wolbachia* infection was distributed evenly throughout the community, whereas there was significant geographical clustering of uninfected individuals in Gordonvale [145]. The incomplete *Wolbachia* coverage was suggested to be due to immigration of uninfected mosquitoes from outside the release area, cryptic breeding sites, or other environmental
phenomena such as “larval curing” (loss of *Wolbachia* infection during larval development) [145]. However, the occurrence of larval curing in mosquitoes has been poorly defined to date. Specifically, little is known about the temperature thresholds for *Wolbachia* during mosquito development or whether any potential curing persists after temperatures return to normal. Understanding larval curing in *wMel*-infected *Ae. aegypti* has important applications, as lower *Wolbachia* levels in adults might have downstream impacts on cytoplasmic incompatibility [200-207] (although in *D. simulans* between-strain differences in CI are not explained by *Wolbachia* density [170]), maternal transmission [208, 209], and pathogen inhibition [91, 157, 170, 210, 211].

I investigated the effects of high temperatures during egg and larval development on laboratory-reared *wMel*-infected *Ae. aegypti* using fluctuating daily temperatures that simulate the real-world conditions of a heatwave in Cairns, Australia. My results have implications for the projected spread of *wMel* *Wolbachia* through *Ae. aegypti* populations and for the vector competence of *wMel*-infected *Ae. aegypti* under different environmental conditions.

### 3.2. Methods

#### 3.2.1. Ethics statement

Blood feeding of mosquito colonies using human volunteers was performed in accordance to the QIMR Berghofer Human Research Ethics Committee permit QIMR HREC361. Written informed consent was obtained from all volunteers who participated in the study.
3.2.2. Mosquitoes

Mosquitoes were taken from a Wolbachia-free *Ae. aegypti* colony (“Cairns” line) started from eggs collected in Cairns, Australia, in January 2015 and from a colony of *w*Mel-infected *Ae. aegypti* (“*w*Mel” line) started from eggs collected in suburbs of Cairns in April 2015. The colonies were maintained in separate, identical climate-controlled rooms at 27 ± 1°C and 70 ± 10% relative humidity with a 12:12 hour light:dark cycle and crepuscular periods. Eggs were flooded in aged (≥ 48 h) tap water and allowed to hatch naturally. Larval stages were reared under a controlled density (< 200 larvae per tray) in trays with 3 L of aged tap water. Larvae were fed on ground TetraMin tropical fish food (Tetra, Germany). Pupae were transferred into cages measuring 40 × 40 × 30 cm for adult emergence. Colonies were maintained with a population size of > 500 individuals per generation. Adult mosquitoes received 10% sucrose solution *ad libitum*, and females were blood-fed on a human volunteer for 15 min every 7 d. The *w*Mel-infected *Ae. aegypti* colony was regularly screened for *Wolbachia* using PCR of the *wsp* gene from the time of establishment [152]. Prior to the start of the experiments, my screening showed that the colony was completely infected with *Wolbachia*.

For the experiments, eggs were collected from *Ae. aegypti* *w*Mel (F₁₆ and F₁₇ generations used) and *Ae. aegypti* Cairns (F₁₈ and F₁₉ generations) colonies at 8:30 A.M. following the first night of oviposition. Eggs were counted under a stereomicroscope at 23°C and were separated into batches of approximately 600 eggs. Each batch was placed inside a dry paper towel, which was folded and placed next to a damp paper towel inside an open plastic bag. Egg bags were placed inside their corresponding environmental chambers at the coldest point of the temperature cycles, which was 20°C for the control
condition and 30°C for the treatment condition. Eggs were left to mature for 48 h, and then batches of approximately 150 eggs were flooded in 500 mL aged tap water in plastic trays (183 × 152 × 65 mm). Four replicate trays were used per treatment group. From the day of hatching until pupation, ground TetraMin tropical fish food (Tetra, Germany) was administered daily at the coldest point of the temperature cycles using the “medium” diet described by Hugo et al. [147]. Pupae were transferred into 1-L plastic containers with mesh tops, and emerging adults were given 10% sucrose solution *ad libitum*. Adult females were aspirated out at 0–2 days post-emergence and at 4–7 days post-emergence. They were frozen at −20°C until processing.

### 3.2.3. Environmental treatments

I tested the effect of high temperatures during egg and larval development on *Wolbachia* levels in *Ae. aegypti* wMel adult females in two replicate experiments: Each replicate experiment compared various heatwave temperature regimes applied during particular periods of immature mosquito development that varied in duration and stage of onset. The temperature profiles I used simulated observed temperatures during average and extreme conditions in Cairns, Queensland. The Australian Bureau of Meteorology defines a heatwave as “a period of at least three days where the combined effect of excess heat and heat stress is unusual with respect to the local climate” [212]. I designed the treatment temperature profile to surpass the severe daily mean temperature threshold of 30.4°C for Cairns, which is based on temperature data from 1958 to 2011 [212]. Both treatment and control temperature profiles followed a truncated sinusoidal progression during the day and exponential decrease at night, representing a profile of daily temperature variation [213]. The shapes of the profiles were the same for each condition, but the profile was raised or
lowered to adjust the mean temperature (Fig 3.1). Experiments were conducted in two environmental chambers (294-L Panasonic MLR-352H-PE and MLR-351H, Gunma, Japan). Nine treatment groups were exposed to fluctuating heatwave temperatures between 30°C and 40°C for varying durations beginning at various life stages. Controls consisted of wMel Ae. aegypti and wildtype Cairns Ae. aegypti exposed to diurnal temperature fluctuations between 20°C and 30°C. Transfers between environmental chambers were made at the coldest point of the temperature cycles (20°C for the control condition and 30°C for the treatment condition) in order to minimize the likelihood of heat shock. As illustrated in Fig 3.2, treatment groups exposed to high temperatures beginning from early embryogenesis (eggs at ≤ 15 hours post-oviposition) lasting three, five, or seven days are denoted by “E3,” “E5,” and “E7.” Groups exposed to high temperatures beginning at the immature larval stages (1st/2nd instars) lasting three, five, or seven days are denoted by “I3,” “I5,” and “I7.” Groups exposed to high temperatures beginning at more mature larval stages (3rd/4th instars) lasting three, five, or seven days are denoted by “M3,” “M5,” and “M7.” Prior to the two studies, a pilot study was conducted to determine differences in means for a range of onsets and durations (Fig 3.3).

Data loggers, both factory installed and independent HOBO data loggers (Onset, Cape Cod, MA), recorded light intensity and temperature variation. Actual water temperatures in the control chamber were within 1.00°C of the programmed air temperature throughout the duration of the experiments. This was also the case in the treatment chamber, except during the coldest periods, when water temperature was as much as 2.93°C lower than the programmed air temperature.
3.2.4. *Wolbachia* density in adult females

*Wolbachia* densities within individual adult females were determined by quantitative PCR. The head was removed from each frozen adult female before DNA extraction. Genomic DNA was extracted using QuickExtract DNA Extraction Solution (Epicentre Technologies Corporation) as per the manufacturer’s instructions and was diluted 1:10 in purified water. Multiplex qPCR was performed, amplifying the target *Wolbachia*-specific *wsp* gene and the somatic *Actin5c* gene, which acted as a reference gene to standardize for mosquito body size (*wsp* F: 5′–CATTGGGTGTGTGTGTGTGTGTGTG–3′, R: 5′–ACACCAGCTTTTACTTGACCAG–3′, *Actin5c* F: 5′–GACGAAGAAGTTGCTGTGCTGTTTG–3′, R: 5′–TGAGGATACCCACGCTTGCTGC–3′) [155-157]. qPCR reactions were performed in 10 µl total volume containing 5.0 µl Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen), 1 µM of each primer, and 2 µL of DNA template. Cycling was performed using a RotorGene 6000 system (Corbett Research) with the following program: 95°C for 2 min, 50°C for 2 min, and 50 cyclic repeats of 95°C for 10 s, 52°C for 10 s, and 72°C for 20 s. This was followed by a standard melt analysis to confirm that only the expected product had been amplified. Quantification cycles (Cq) values were calculated using the Comparative Quantification algorithm in the RotorGene 6000 software (Corbett Research). Repeat reactions were performed with samples for which the duplicate Cq values differed by more than 0.75. Quantification cycles (Cq) were normalized by taking into consideration the different amplification efficiencies of the *wsp* and *Actin5c* genes, and *Wolbachia* to host genome ratios were calculated using Q-Gene [158].
3.2.5. *Wolbachia* visualization in mosquito ovaries

Fluorescence *in situ* hybridization (FISH) was carried out using a *Wolbachia*-specific 16S rRNA probe [91]. Three freshly collected adult females (legs and wings removed) from each treatment group were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer overnight and were transferred to 70% ethanol. Bodies were embedded in paraffin wax and sectioned with a microtome. Slides were dewaxed with two successive xylene washes for 10 min, two successive 5-min washes with 100% ethanol, and two successive 5-min washes in 95% ethanol. Slides were hybridized with the *Wolbachia*-specific *W2* probe (5´–CTTCTGTGAGTACCGTCATTATC–3´) [91] conjugated on the 5´ end to the fluorescent probe Alexa Fluor 488 (Molecular Probes, Inc). Slides were left in a dark humidity chamber at 37°C overnight and washed briefly in 1× saline sodium citrate (SSC) buffer + 10 mM dithiothreitol (DTT) at room temperature, then two 15-min washes in 1× SSC + 10 mM DTT at 55°C, two 15-min washes in 0.5× SSC at 55°C, a 10-min wash in 0.5× SSC + 10 mM DTT + 4’,6-Diamidino-2-phenylindole (DAPI) (0.01 mg/50 mL) at room temperature, and then a final 10-min wash in 0.5× SSC + 10mM DTT at room temperature. Slides were washed briefly with distilled water and mounted with Vectashield Hard Set mounting medium (Vector Laboratories, Burlingame, CA). Slides were allowed to dry in a refrigerator overnight. Images from all sections were captured with GE DeltaVision Core Deconvolution Microscope (GE) equipped with an Olympus X181 inverted microscope using an Olympus 20X/0.75 U Apo 340 lens or an Olympus 10X/0.40 D Plan Apo UV lens and a Photometrics Cool Snap HQ CCD camera. Images were acquired at a resolution of 1024 x 1024. DAPI excitation was 390/18nm and emission collection was 435/48 nm with 0.2 s exposure (5% ND filter). AlexaFluor 488 excitation
was 475/28 nm and emission was 523/36 nm with a 0.15 s exposure (32% ND filter). Images were reformatted using SoftWorx (Enterprise Softworks (Pty) Ltd.) and were cropped and standardized for contrast using Adobe Photoshop CS6 (Adobe Systems, Inc.).

### 3.2.6. Body size

To determine the effect of the heat treatments on adult body size, the left wing of six females from each treatment group was removed and dry mounted on a slide. The distance from the axial notch to the wing tip, excluding the fringe scales, was used as a proxy for body size [147, 214].

### 3.2.7. Statistical analysis

All analyses were performed in R [215] and GraphPad Prism v. 6 (GraphPad Software, San Diego, California, USA). Normality and homogeneity of variances within treatments were tested using Shapiro-Wilk and Bartlett’s tests, respectively. Log10-transformed *Wolbachia* densities were used for all analyses. A two-way blocked analysis of variance (ANOVA) was performed to determine the effects of treatment and collection time point and their interaction on *Wolbachia* density. Replicate was included as a blocking factor to account for any variation between the two experiments. An analogous two-way blocked ANOVA was performed to determine the effects of treatment group and collection time point and their interaction on body size. Pair-wise *post-hoc* comparisons between treatments and controls and between collection time points were made for both ANOVAs, and *P* values were adjusted for multiple comparisons using Tukey’s honest significant difference test. Differences were considered significant if adjusted *P* values were < 0.05. A nonlinear regression was performed using ordinary least squares fit for each stage of onset at the two collection time points to determine relationships between the heat
treatment duration and *Wolbachia* density. Sum of squares F-tests were used to determine significant differences in slopes and y-intercepts.

### 3.3. Results

#### 3.3.1. *Wolbachia* density in adult females

I found significantly lower *Wolbachia* densities relative to *w*Mel controls in 0–2 d-old females emerging from eight of the nine treatments (Fig 3.4), with only the mature instar treatment lasting three days (M3) showing no significant reduction. *Wolbachia* levels in the 0–2 d-old females that were exposed to 30–40°C for seven days starting at the egg stage (E7) were less than 0.1% of *w*Mel control densities (Fig 3.4). Both treatment group and collection time point were significant predictors of *Wolbachia* density ($F(10, 362) = 197.34, \ MSE = 55.24, \ P < 0.001$ and $F(1, 362) = 397.21, \ MSE = 111.20, \ P < 0.001$, respectively). Compared with the 0–2 d adult collection time point, 4–7 d-old adult females in all treatment groups except the *w*Mel control group and the M3 group had higher *Wolbachia* levels, with adults from three-day treatments (E3, I3, and M3) showing *Wolbachia* densities that were not significantly different from *w*Mel-infected controls (Fig 3.4). The *Wolbachia* levels in 4–7 d-old adults from the six other treatments remained significantly lower than in *w*Mel-infected controls. There were inverse relationships between the duration of heat treatment and *Wolbachia* density and for all stages of onset; however, the relationships differed significantly both in their slopes and y-intercepts ($F(5, 305) = 3.68, \ P = 0.003$ and $F(5, 305) = 2.79, \ P = 0.02$, respectively) (Fig 3.5). Duration of heat exposure had the greatest impact on *Wolbachia* density in emerging females when high temperatures began in the 3rd/4th instar stages. At 4–7 days of age the impact of heat duration on density was most pronounced when high temperatures began at the egg stage.
3.3.2. *Wolbachia* visualization in mosquito ovaries

I also investigated whether I could visualize reductions in *Wolbachia* levels in the ovaries of adult mosquitoes after exposure to high temperatures during development. Using FISH I visualized very low levels of *Wolbachia* in the ovaries of 0–2 d-old E7 females (Fig 3.6 B). I also noticed that the E7 ovaries were much less developed than in controls, a possible consequence of the heat exposure. In 4–7 d-old E7 females (Fig 3.6 D), *Wolbachia* remained at very low levels compared with 4–7 d-old wMel-infected controls (Fig 3.6 C).

3.3.3. Body size

I found a significant effect of treatment group on wing length ($F(10,71) = 13.70$, $MSE = 0.32$, $P < 0.001$) and of the treatment group–collection time point interaction ($F(9,71) = 2.81$, $MSE = 0.07$, $P = 0.007$). Collection time point and replicate were not significant predictors ($F(1, 71) = 0.22$, $MSE = 0.005$, $P = 0.64$ and $F(1, 71) = 1.21$, $MSE = 0.03$, $P = 0.27$, respectively). Treatment groups E7, I5, I7, M3, M5, and M7 were all significantly smaller than wMel controls (Fig 3.7). There was no significant difference in wing length between wMel controls and Cairns controls.

3.4. Discussion

I found that when *Ae. aegypti* infected with the wMel strain of *Wolbachia* were exposed to daily fluctuating temperatures of 30–40°C during early development, the emerging females had reduced *Wolbachia* levels compared with controls. The most affected group consisted of mosquitoes exposed to high temperatures starting at the egg stage and lasting for seven days (E7). In E7 emerging females, mean *Wolbachia* levels were less than 0.1% of the levels of wMel controls. Loss of *Wolbachia* density from a subset of the mosquito population may be a concern for *Wolbachia*-based dengue and Zika...
control efforts in regions where the aquatic habitats of juvenile *Ae. aegypti* can reach extremely high temperatures. It has previously been shown that different *Wolbachia* strains attain different infection densities and that density is correlated with the level of virus inhibition [155, 157, 170, 210]. The relationship between wMel density and DENV and ZIKV inhibition can be assumed from near complete blockage of these viruses in *Ae. aegypti* harboring dense wMel infections [97, 99, 102], but the relationship has not been specifically defined. A recent study found that exposure of adult wMel-infected *Ae. aegypti* to 28°C ± 4°C beginning at 5–8 d of adult age was associated with reduced *Wolbachia* densities; however, there was no interaction between the reduced densities and DENV infection, dissemination, or transmission [183]. Eggs and larvae exposed to high temperatures in my study produced adult *Ae. aegypti* with very low *Wolbachia* densities; therefore, the level of pathogen inhibition in adult mosquitoes that were subject to impacts of heat exposure during early development deserves investigation. The partial recovery of *Wolbachia* density by 4–7 days of age suggests that any impacts of heat exposure during mosquito development on subsequent virus inhibition may be attenuated with age.

This study is the first to investigate the duration and timing of heatwave conditions in relation to immature development of mosquitoes infected with *Wolbachia*. To achieve this I simulated normal and heatwave conditions based on temperature data from a city selected for *Wolbachia* biocontrol. I found an inverse relationship between the duration of heat exposure and *Wolbachia* density in adult females, raising the possibility that longer periods of heat might be capable of clearing *Wolbachia*. The slope of this relationship varied by the stage of heat onset and by the age of adult females collected. Duration of heat exposure had the greatest impact on *Wolbachia* density in emerging females when high
temperatures began in the 3rd/4th instar stages; however, the impact of heat duration on density at 4–7 days of age was most pronounced when high temperatures began at the egg stage. In addition to reducing bacterial densities, high temperatures resulted in smaller adult body sizes, with more prominent effects in the later stages of heat onset and the longer durations. This is likely due to the known inverse relationship between larval rearing temperature and adult body size [216]. I controlled for the effect of body size by standardizing Wolbachia density measurements with the host gene Actin5c.

Loss of Wolbachia density in response to heat has also been reported in T. urticae [191] O. scapulalis [217], D. simulans [195], D. bifasciata [194], Ae. albopictus [199], the predatory mite Metaseiulus occidentalis [218], and the wasp Leptopilina heterotoma [219]. The mechanism behind the loss of Wolbachia in response to high temperatures is not fully understood, but deformation of the Wolbachia cellular membrane could be a contributing factor [220]. My FISH visualization confirms the loss of Wolbachia from the ovaries of mosquitoes exposed to high temperatures. Partial recovery of Wolbachia in the ovaries after the mosquito returns to normal temperatures suggests that Wolbachia replication continues even after the ovaries are fully developed. It is uncertain whether replication continues throughout the female lifespan and at what age Wolbachia densities would be restored to control levels in heat-exposed females.

My results support the notion that wMel has a more restricted thermotolerance than its mosquito host Ae. aegypti. Loss of thermotolerance in insect symbionts can be due to point mutations that occur as the symbiont co-evolves with the host [221]. In the case of the obligate symbiont of aphids Buchnera aphidicola, a point mutation affecting heat-shock protein transcription leads to death of the symbiont following a heat treatment [222].
Compared with other symbionts of insects, \textit{wMel} has experienced far less reductive evolution, as evidenced by its large genome with very high levels of repetitive DNA and mobile DNA elements [223]. Because of the low mutation rate of \textit{wMel} [223], loss of thermotolerance is less likely than for other symbionts [224]. If reductive evolution of \textit{wMel} does occur, then rearing \textit{wMel}-infected \textit{Ae. aegypti} under constant temperatures in the lab might accelerate loss of \textit{wMel} thermotolerance. More studies are needed to understand the co-evolution of \textit{wMel} and \textit{Ae. aegypti}.

\textit{Wolbachia} may hold the potential to reduce and even eliminate dengue and Zika transmission in endemic areas. The advent of a promising control tool for dengue fever and Zika could not have come at a better time, as currently many tropical countries have no options to control the massive arbovirus outbreaks they experience. The strategy of releasing \textit{wMel}-infected \textit{Ae. aegypti} is being tested in dengue-endemic regions around the globe, including Australia, Vietnam, Brazil, Indonesia, and Colombia [103], although substantial epidemiological data is still needed to assess the impacts on dengue and Zika transmission. The importance of measuring \textit{Wolbachia} density in field trials, as opposed to presence or absence of \textit{Wolbachia}, is highlighted by my results and other investigations [155, 201, 204]. I found that the high temperatures that \textit{Ae. aegypti} may experience during early development can attenuate \textit{wMel} \textit{Wolbachia} levels. Consequently, \textit{wMel} \textit{Wolbachia} might be less effective as a dengue or Zika control strategy in regions experiencing periods of extreme heat. If the effectiveness is compromised, increased surveillance and supplementary mosquito control may be required in these regions. Further estimates of \textit{Wolbachia} recovery rates after heat exposure are needed to understand the impacts on DENV and ZIKV inhibition and the spread of \textit{wMel} through naïve \textit{Ae. aegypti} populations.
In summary, I showed that fluctuating daily temperatures of 30–40°C experienced during wMel-infected *Ae. aegypti* egg and larval development significantly reduced *Wolbachia* levels in emerging adult females. However, *Wolbachia* recovered to differing degrees after adults returned to 20–30°C. These findings suggest that the effectiveness of *Wolbachia*-based arbovirus control might be compromised in ecosystems that experience periods of extreme heat, but given that *Wolbachia* levels partially recover after temperatures return to normal, any effects may be temporary. Greater understanding of environmental variables that affect *Wolbachia* can inform release site selection and help to better predict the impacts of *Wolbachia* on arbovirus transmission.
Fig 3.1. Water temperature fluctuations in environmental chambers. Control and treatment chambers are shown in blue and red, respectively. Data loggers were submerged in 500 mL aged tap water in trays (183×152×65 mm) and temperature was recorded every 30 min for the duration of both experiments. Bars denote means and standard errors over days logged.
**Fig 3.2. Experimental Design.** All treatment groups (rows) were transferred to environmental chambers as eggs within 15 hours of being laid, and all developed into adults by the end of the experiment. “wMel” denotes the wMel-infected *A. aegypti* controls, and “Cairns” denotes the wildtype (*Wolbachia*-free) *A. aegypti* controls. For each of the other treatment groups, the letter represents the stage of heat onset, with “E” indicating embryogenesis, “I” indicating immature larvae (1<sup>st</sup>/2<sup>nd</sup> instars), and “M” indicating more mature larvae (3<sup>rd</sup>/4<sup>th</sup> instars). The number represents the number of days the group remained in the high temperature treatment before returning to control temperatures (three days, five days, or seven days). Moving across each row, the cells track the days for each treatment group, with blue cells representing days spent in the control chamber and red cells representing days in the high temperatures chamber. Inverted triangles represent adult collection time points at 0–2 days and 4–7 days after emergence.
Fig 3.3. Pilot study results. Log_{10}-transformed relative *Wolbachia* densities in 0–2 d-old female *A. aegypti* in different treatment groups. Treatments not included in subsequent experiments include the pupae onset stage (P1, P3) and the one-day duration (I1, M1, P1). *Wolbachia* density was measured by qPCR of the *Wolbachia*-specific *wsp* gene and the somatic insect gene *Actin5c*. Displayed values are relative concentrations of *wsp* and *Actin5c* calculated in Q-Gene. Bars denote means bounded by their 95% confidence intervals. Significant differences between treatment groups and the *w*Mel controls are displayed as $P < 0.05$ (*), $P < 0.01$ (**), $P < 0.001$ (***)). Each point represents an individual mosquito.
Fig. 3.4. *Wolbachia* densities in 0–2 d-old (circles) and 4–7 d-old (inverted triangles) female wMel *A. aegypti* exposed to various temperature treatments. “wMel” denotes the wMel-infected *A. aegypti* controls that were not exposed to high temperatures, and for the other treatment groups the letter represents the stage of heat onset—“E” for embryogenesis, “I” for 1st/2nd instars, and “M” for 3rd/4th instars—and the number represents the number of days the group remained in the high temperature treatment. *Wolbachia* density was measured by qPCR of the *Wolbachia*-specific *wsp* gene and the somatic insect gene *Actin5c*. Displayed values are relative concentrations of *wsp* and *Actin5c* calculated in Q-Gene. The horizontal line at $y=10^{-3.552}$ represents the detection limit of *Wolbachia* by qPCR, which was established by the average Cq values for *Wolbachia*-free Cairns *A. aegypti* controls. Bars denote means bounded by their 95% confidence intervals. The lower 95% confidence limit for the 0–2 d-old I5 group ($y=−0.009$) is not shown because it cannot be represented on the log scale. The significance levels of differences between time points are indicated above brackets and between treatment groups and the wMel controls at the top of the graph as $P < 0.05$ (*), $P < 0.01$ (**), $P < 0.001$ (***)). Each point represents an individual mosquito.
Fig. 3.5. Linear regressions of duration and stage of onset. The effect of heat duration during larval development on *Wolbachia* density in adult females by larval stage of onset and adult age of collection. Blue lines represent heat exposure beginning at the egg stage, green lines represent heat exposure beginning in the 1<sup>st</sup> and 2<sup>nd</sup> instar stages, and red lines represent heat exposure beginning in the 3<sup>rd</sup> and 4<sup>th</sup> instar stages. Dotted lines represent females collected at 0-2 d of age, and solid lines represent females collected at 4-7 d of age.
Fig. 3.6. Visualization of *Wolbachia* in ovaries by FISH. Ovaries of *Ae. aegypti* wMel females emerging from the control (A,C) and E7 (B,D) treatment groups, collected at 0–2 d (A, B) and 4–7 d (C, D) after emergence are shown. *Wolbachia* were stained with Alexa Fluor 488 (green) and cell nuclei with DAPI (blue).
**Fig 3.7. Wing length by treatment group.** Significant differences between treatment groups and wMel controls are indicated by asterisks, $P < 0.05$ (*), $P < 0.01$ (**), $P < 0.001$ (***)). Bars denote means bounded by their 95% confidence intervals. Each point represents an individual mosquito.
Chapter 4: Horizon scanning to forecast the future of mosquito-borne disease control

4.1. Background

We are at a crossroads in mosquito-borne disease (MBD) control. The burden of malaria has been reduced dramatically over the past fifteen years [1], with the death rate per 100,000 at risk plummeting by 60% worldwide and by 66% in Africa [2]. However, over the same period the annual number of dengue cases has more than doubled, with over 100 countries now endemic [7]. Mosquito-borne pathogens emerging in new regions have caused explosive epidemics [3, 4, 8]. In 2013, a chikungunya outbreak in Saint Martin quickly spread to 31 other countries and territories in the Americas [8-10]. Most recently, Zika virus in the Americas and the associated microcephaly cases [11] have exposed the dire state of mosquito control around the globe [12]. The potential for pandemic expansion of MBDs has never been more obvious, but political responses are still in disarray.

The World Health Organization (WHO) and several international funding bodies have advocated that control efforts be stepped up to achieve more widespread MBD control and elimination [2, 7], but several challenges stand in the way. Addressing the major ecological and operational challenges in MBD control will require the coordinated effort of researchers, funders, and governments. Strategic definition of priorities both for research and for control will be the determining factor for the coming decades of MBDs. Setting priorities in the realm of MBD control requires the foresight of future opportunities and threats. In many areas of science, statistics and modeling of past events provide probabilistic ranges that can assist in forecasting future events. In cases where enough historical evidence has been amounted to identify causal relationships, and these relationships are not influenced by external factors, it is possible to make short-term
predictions and in some cases, even long-term predictions [111]. Anticipating future opportunities and threats to MBD control when few causal relationships have been detailed and multiple social, economic, and political factors are involved requires an alternative to traditional probabilistic modeling [111]. Horizon scanning—the collective synthesis of observations pertinent to the distant future (10–25 years)—has been used for decades to inform science policy with systematic knowledge about relevant drivers of change and developments [112, 225, 226]. Carried out with the help of a team of experts, the approach involves searching or scanning information at the margins of the known environment and possibly beyond it [227]. Based on the observations gathered in the horizon scan, a list of important trends for decision-making is derived [228].

In addition to its application in government, industry, and the environmental field [229-236], horizon scanning has been used to identify the complex trends underlying the emergence of infectious diseases [237, 238]. In 2006 the UK Office of Science and Innovation Foresight used horizon scanning to carry out the project “Infectious Diseases: preparing for the future,” which sought to analyze future risks of malaria, zoonotic infections, pathogens acquiring resistance, and novel pathogens in the UK and sub-Saharan Africa over the following 10–25 years [237, 238]. For malaria in particular, experts simulated the combined effects of climate change, population growth, and urbanization on the population at risk of *Plasmodium falciparum* malaria in Africa [238]. The emergence of drug-resistant strains in diseases and the spread of diseases to new areas due to increased travel, migration, and trade were identified as important drivers of emerging infectious diseases [237]. In a 2011 horizon scan of infectious disease threats facing the European Union by 2020, drivers relating to globalization and environmental change, social and
demographic change, and health systems were identified. These relatively few applications of horizon scanning to the domain of infectious diseases demonstrate that multiple complex trends underlie the emergence of infectious diseases.

This study represents the first application of horizon scanning to the field of MBD control. The result is a forecast of the next twenty years, including potential drivers of change representing opportunities and challenges in the field. Eight key drivers of change were identified by content analysis of interviews with 25 international MBD experts and of 157 editorial articles in scholarly journals. In addition to the list of key drivers of change, four sets of scenarios for MBD control in twenty years’ time were developed collectively by experts. These drivers of change in MBD control provide a framework on which researchers, funders, and governments can collectively build a global strategy to tackle the greatest challenges going forward.

4.2. Methods

The majority of horizon scans are exploratory and have no hypotheses about the information that will be gathered within a policy domain. Accordingly, I grounded this study in the inductive approach of ethnography, which builds upon the perspectives of the people studied rather than on a priori hypotheses [239]. Ethnography emphasizes a continuous relationship between the researcher and research participants [239]. In contrast to surveys, which are designed by the researchers, an ethnographic interview involves give and take and building upon what has been said in order to break new ground. This means that each ethnographic interview is different to the next, even if the same open-ended questions are used. Horizon scanning does not always apply an ethnographic approach. In some studies expert review has been used to give meaning to issues that were identified in
a web content scan, for instance. In this study I chose to rely on interviews with MBD experts as my primary source of knowledge, with editorial literature content to supplement the interviews. As such, I believed that it was important to adopt an approach that would gain the most value from the knowledge held by these specialists, many of whom have worked in the field of MBD control for over twenty years and hold prominent advisory positions in global fora. For this reason, I chose to apply an ethnographic lens within the framework of horizon scanning.

4.2.1. Journal mining

Journal mining is a useful tool for understanding emerging areas of knowledge. It involves selecting leading journals in the research field, extracting keywords from publications in the leading journals, and creating a hierarchy structure of keywords to help in understanding the research field [240]. Potential issues in MBD control that could impact the next 20 years were identified by scanning a subset of editorial articles in eleven prominent scientific journals that publish literature on MBDs (Table 4.1). Editorial articles were chosen because they act as a platform for experts to debate the issues in the discipline, sometimes extending to related areas such as the social implications of findings [241]. Boolean searches were made using key terms such as “mosquito” and “future” on the full text of editorial articles published between January 1, 2010, and December 31, 2015. Articles of limited geographic focus or specific to one drug therapy or type of vaccine were excluded. Overall, 157 articles were included in the scan. The publication date, title, abstract, and body of the text of each article was copied into RQDA Qualitative Data Analysis software [215]. Author names, contributions and acknowledgements, and any figures or boxes and their captions were omitted.
4.2.2. Participant selection

A convenience sample was chosen in which potential participants in the field of medical entomology or MBD control were referred by peer medical entomologists. I sought to achieve geographical and subject-matter representativeness among participants. Most participants were senior researchers, with an average 31.92 years of experience (SD = 9.65). Fifty international experts were contacted by email (Appendix A), and 25 experts agreed to participate and were interviewed once either in person or via videoconference (Fig 4.1). No participants withdrew from the study.

4.2.3. Interviews with experts

Interviews with experts consisted of one-on-one discussions to identify issues and to explore important driving forces and areas of uncertainty within the field of MBD control [114]. Oral consent to audiotape interviews for notetaking purposes was obtained at the start. The interviews were loosely structured with four open-ended questions to elicit the participants’ opinions on 1) the current state of MBD control, 2) recent developments in the field likely to impact the future, 3) developments that have not been made but are desperately needed for the future, and 4) the biggest potential threats (e.g., ecological, social, or political) for the future. Interviews lasted between 20 and 60 minutes. Discussions often deviated from the four main topics, which allowed researchers to follow other important insights and open new lines of inquiry as participants guided the conversations [242]. Participation did not entail remuneration.

4.2.4. Content analysis

Data were analyzed using content analysis, an established social science methodology concerning the "objective, systematic, and quantitative description of the
content of communication" [243]. Content analysis was used because it enabled us to identify, organize and analyze data under rich themes after which they are described using illustrative quotes grounded in the collected data in support of the analysis to make a meaningful report [244]. First, key concepts were highlighted in all of the editorial articles and interview transcripts and assigned thematic codes. Next, overarching categories were identified by sorting the thematic codes into groups of similar meaning [245]. All content analysis was performed using RQDA [215].

4.2.5. Scenario building

Six experts were asked to participate in the scenario building exercise based on the interest they expressed in their first interviews. Scenarios were generated using the “two axes” method [246]. Broad topics relating to policy making, control approaches, capacity building, funding systems, and researcher interactions along with axes labels resulted from the drivers of change identified through the content analysis. Participants were sent a PowerPoint presentation with double axes templates for each of the broad topics. In one-on-one interviews with participants, we named and detailed four scenarios of the year 2035 for each of the broad topics. Participants elaborated on which events and policies would lead to each of the scenarios. The interviews were not audio recorded. After the exercise, similar scenarios were combined to make four broad categories of double axes scenarios, and the details for each scenario were summarized into three major presentation points.

4.2.6. Ethics approval

The study protocol was approved by the University of Miami Institutional Review Board (IRB ID: 20150049) and the QIMR Berghofer Human Research Ethics Committee and the Safety Committee (P2116) (Appendix B). Informed consent was given in writing
with participants’ signing the Consent Form (Appendix C), in which the study team clearly informed participants about the purpose and procedures of the study, its potential risks and benefits, and plans for the use and protection of materials gathered during the study. In addition to written consent, oral consent to tape record the interview was obtained from each participant. In cases where the participant asked to delete specific information after the interview, those entire sections were erased from my records. Audio recordings were transcribed manually and were de-identified, after which they were erased. Each participant received a random participant ID generated by Excel. Participants were referred to by their ID numbers in all recorded data and never by their actual names.

4.3. Results

Interviews, journal mining, and coding took place over nine months beginning in October 2015. Below I present coded information from interviews and editorial articles summarized as a description of the current state of MBD control, eight drivers of change for the future, and four sets of future scenarios. I presented the findings here to a panel of five senior entomologists in a public forum at the International Congress of Entomology in September 2016 so that the next steps needed to achieve desirable outcomes with respect to the issues presented here could be devised collectively.

4.3.1. The current state of mosquito-borne disease control

A recurrent theme in the interviews was a sense amongst experts that MBD control as it is currently practiced in most of the world is a “disaster.” One participant summed up the views of many participants:

*Where we work, there is so much money being spent on vector control, but we think it is having limited impact.*
A few developed countries like the United States, Australia, and Germany distinguish themselves by their proactive mosquito control. Although mosquito abatement programs in these countries can often drastically reduce mosquito abundances, it is impossible to determine the effects of mosquito control on disease reduction in these settings since MBD transmission is already so limited and sporadic. However, in most of the world, especially in the developing countries that experience the highest burdens of MBDs, the state of MBD control is bleak. As one expert described it:

*If we go to the developing world—that is where mosquito control is what I call "coarse." It is not specific, it is not targeted, it is reactive rather than proactive, it is waiting until epidemics hit. We are always too late. We do not really check whether the tools that we apply still work well, whether there are resistance problems. Management of these programs is poor, and see the results.*

In addition to the contrast in mosquito control between developed countries and the rest of the world, I found a large discrepancy in expert sentiments on the current state of malaria control and the state of arbovirus control. The editorial literature and nearly all experts acknowledged that significant progress has been made over the past decade in reducing the burden of malaria. Conversely, for arboviruses, the consensus is that the control tools in place for the major vectors *Aedes aegypti* and *Ae. albopictus* are not effective in preventing epidemics. The comment below describes this discrepancy:

*The past decade has improved the situation in Africa, in particular for malaria control, but there has really been very little promising headway in control of arboviral diseases. I actually think that the imbalance may shift in the next decade or so, but right now we are not seeing the kinds of improvements associated with*
vector control vis-à-vis malaria as we have seen with dengue or some of the emerging arboviruses such as chikungunya and now Zika.

Another interviewee, when asked about funding and attention for Aedes-transmitted arboviruses, said:

*It has been disastrous. With four hundred million cases of dengue around the world, if anything it shows us that we have done something completely wrong.*

It was also suggested that prior to the recent Zika epidemic in the Americas, Aedes-transmitted arboviruses like dengue, chikungunya, and Zika received relatively little funding and attention. Very few effective control methods exist for the major mosquito vectors *Ae. aegypti* and *Ae. albopictus*, which thrive in urban environments. Consequently, there has never been a successful control program in the world for Aedes-transmitted diseases that has sustained long-term reductions in disease transmission.

The majority of experts interviewed felt that malaria research has received ample funding over the past decade and a half through the Roll Back Malaria initiative. As a result, malaria deaths are now half what they were in 2000 [2]. This success is largely attributed to two simple vector control interventions: insecticide-treated bed nets and indoor residual spraying. However, several experts worry that reducing malaria transmission even further will require additional tools, and the diminishing gains against malaria likely to be experienced in the coming years may lead to donor fatigue. As one expert put it:

*It is really gratifying how well we have done...As we start talking more and more seriously about malaria elimination and malaria eradication...undoubtedly it is going to require more than just bed nets and indoor residual spraying.*
This view was echoed by another expert who stated:

*I think that if donor funding slackens, and it is heading towards that direction, we might find that most countries which are not prepared economically to sustain the interventions might end up with very high disease burdens within a short time if donors pull out or reduce their funding. This is an area that could also be a threat in terms of the future control programs.*

The experts on the whole conveyed that against the rather bleak backdrop of current MBD control, a technological revolution is starting. Many benchtop advances in MBD research are being fuelled by our greater understanding of mosquito, parasite, and bacterial genomics. For the time being, most of these promising technologies remain far upstream of field application. This leaves mosquito control in a state of suspense. While many conventional control tools are quickly losing their effectiveness to insecticide resistance, there is still no epidemiological evidence of the effectiveness of newer technologies. Despite the impressive advances in MBD control being highlighted in the media, mosquito control in most of the world marches on as always. One expert claimed:

*There are some new things out there, but for the most part we are still sort of doing what we have been doing...since World War II.*

Some experts expressed the belief that gathering evidence of the effectiveness of new interventions requires substantial infrastructure for monitoring and surveillance. Such infrastructure they believe is lacking from nearly every endemic setting around the world. Without substantial investment in surveillance systems by national governments and donors, we will continue to apply vector control tools without any evidence of their impact. The comments below reflect the sentiment of many:
Public health authorities do not often have the resources to monitor and evaluate what they do. They just do it. And that means that despite the phenomenal amount of money and effort that goes into mosquito control in urban environments, there is really no careful recording of impact...Most public health authorities I suspect know that what they do is not VERY effective but they hope that it is a little effective. But they have no evidence whatsoever.

There is a lot of make-believe in this and it is very difficult to evaluate the impacts of some of the methods...I think people...more or less are assuming that the more you do of the current methods, the more you are going to succeed. And the methods simply do not work.

4.3.2. Drivers of change

Looking forward, I sought to identify “drivers of change,” issues that are considered to be highly influential in MBD control and are likely to shape the future state of MBDs. Here I describe eight major drivers of change mentioned by multiple experts and described in editorial literature (summarized in Fig 4.2). The drivers are categorized into four broad themes that emerged from the analysis.

4.3.2.1. Ecological drivers

Globalization of vectors and pathogens

In their accounts of the events surrounding globalization of vectors and pathogens, the majority of participants felt that the expansion of Aedes mosquitoes and the pathogens they transmit to new regions has accelerated over the last decades along with globalization. A common view amongst interviewees was that:
As urbanization has expanded in tropical developing countries, so too have the Aedes aegypti and Aedes albopictus populations. The jet airplane provides the mechanism to move these viruses and the mosquitoes around the world, and so you have a constant transport of viruses in humans primarily into new urban areas where there is no effective mosquito control. And this is why we have seen the global spread of these diseases.

A common view amongst experts was that cities sprawling ever closer to forested areas bring populations into contact with mosquitoes that have previously blood fed only on animals, raising the possibility of a number of new zoonotic pathogens that could be transmitted to humans. One expert suggested:

As more and more urbanization [occurs and] deforestation takes place, then you are going to find that people are now coming very close to the forest. So you are going to perhaps get other types of zoonotic infections, perhaps passed from animals to humans by mosquitoes. We don’t know deep in there what the mosquitoes have been doing...We never know what other things will start coming out.

The Zika virus epidemic that started in Brazil last year has called attention to the possibility of the rapid global spread of arboviruses. Most experts agree that behind Zika virus there are several other emerging arboviruses in line. One participant echoed the concerns of many experts:

I am really terrified of this yellow fever outbreak...I think the Zika thing is terrible.
You know, it is like the most horrible horror film, sort of science fiction horror film.
And obviously we have got to do something about it. But I still think that in the end, yellow fever may be the biggest catastrophe of all.

The need to strengthen surveillance and control efforts and coordinate them on a global scale is apparent. However, major barriers to global data exchange include lack of standardization of data collection, protection by Ministries of Health of sensitive patient data, and the fear of economically damaging travel restrictions. As a result, there is currently no global surveillance system in place to track the emergence and spread of mosquito-borne diseases.

*Insecticide resistance*

There is universal alarm among MBD control experts over the quickly diminishing arsenal of effective insecticides. Given accounts of rebounding malaria after the introduction of insecticide-treated bed nets due to insecticide resistance [247], experts suspect that the gains achieved against malaria over the past decade could soon start to reverse. New classes of insecticides and new ways of applying insecticides are clearly needed. One participant explained our losing battle against insecticide resistance:

*With all due respect, we keep searching for new actives, for new insecticides, but why on Earth are we spending so much money, time, and effort on finding new insecticides if we KNOW beforehand that if we start using these insecticides on scale, that sooner or later we will hit the wall of resistance again? We KNOW this. We’ve been there; we’ve done that.*

More targeted methods of applying insecticides could theoretically stave off future resistance. In addition to focal spraying around transmission hotspots, methods that take advantage of mosquito ecology are promising. One participant explained:
The next thing I see on the horizon is...to become much cleverer at exploiting the behavioral ecology of these insects. If we are spraying the inside of the room, then the only thing that I exploit in terms of behavioral ecology is that it will sit on the wall after blood feeding. But there is a lot more. These things, they go out and they feed on sugar, they go out and they mate, they go out and they oviposit.

4.3.2.2. Technological drivers

Biological control

Recent discoveries in biological control provide hope for large-scale MBD control that does not rely on insecticides. Nearly all experts interviewed identified the use of *Wolbachia* bacteria to block arbovirus transmission in the mosquito and the use of genetically modified (GM) mosquitoes to crash mosquito populations as the two most promising biological control technologies. Many experts mentioned that despite the recent review of GM mosquitoes by the US Food and Drug Administration (FDA), convincing the public that GM mosquitoes are safe remains a major obstacle. Consequently, entomologists, science communicators, and science institutions need to take a more public stance on the safety of GM mosquitoes. One participant stated:

*Honestly I think there really has not been a very good effort to educate the public on the relative risk and benefits associated with GM. Unfortunately, when people in the agricultural world rolled these things out, it was not done very well. And we are still suffering from that.*

Rear-and-release approaches like *Wolbachia* and GM mosquitoes involve substantial financial investment for the initial mosquito releases, which may be out of reach for some endemic countries. International donors will likely need to supply the initial
funding, prioritizing locations that experience frequent arbovirus epidemics and failing chemical control. Questions of how biocontrol approaches can be scaled up without the need for persistent expert involvement and can be sustained by communities after releases are critical to paving the way forward. One participant stated:

*One of the problems with the sterile insect technique approach is that once you stop releasing, then the mosquitoes come back. So I think another area that is going to be CRITICAL is the industrialization of mosquito production and release. How do you scale this stuff up? How do you rear and release 100 million mosquitoes a day?*

**Smart surveillance systems**

Surveillance is central to containing MBD outbreaks before they spread and to monitoring the impact of interventions, but it is not done adequately anywhere in the world with the exception of a few developed countries. The result is that arbovirus outbreaks are being handled with blanket strategies such as city- or suburb-wide fogging, and control interventions are being applied without any evidence of their impacts. Insecticides to which there is already a substantial level of resistance in the mosquito population may continue to be used to no avail due to inadequate or absent insecticide resistance surveillance. Overall, this leads to an enormous waste of public resources. One participant explained:

*Too many large-scale control operations go out without any monitoring and evaluation, so millions and millions of bed nets have been distributed without any monitoring and evaluation of whether the resistance status of the mosquito populations has changed in the areas we are distributing the nets. That to me is completely crazy.*
Ideally one day there will be networks of “smart traps” that can be deployed over large endemic areas and that will remotely feed geographically-linked information about vector distributions, pathogen infection rates, and insecticide resistance levels back to decision makers at a central office, who can then decide which interventions to use and exactly where to target them. One participant contemplated these traps:

_Some of these remote trapping things like passive traps...could be useful. It would be really neat if you could have a remote arbovirus STATION that when that mosquito comes and it feeds or whatever, it just detects it somehow and then it sends you an SMS saying, “You’ve got virus”—that would be really cool. I don’t know how far away we are from that. Having remote detecting systems is an OBVIOUS goal that somebody could try to build on with technology._

Finding surveillance technologies that can be cheaply manufactured and can withstand all of the natural elements are key challenges to developing systems that can be used around the world.

4.3.2.3. Political drivers

_Global agendas_

Many of the early successes in MBD control such as the eradication of _Ae. aegypti_ from most of the Americas and the progress against malaria during the early years of the Global Malaria Eradication Program were made possible under the coordination of strong public health institutions like the WHO. Today, due to decreased funding and the fragmentation of member nations’ and donors’ priorities, the WHO is gradually losing its relevance in the global control of MBDs. The multi-year review process for approving new
drugs, vaccines, and control tools has led many stakeholders to see the WHO as slow and bureaucratic [248]. One participant observed:

*WHO has just been responding and not trying to take a longer look and anticipating things like Zika virus or chikungunya virus. It is bureaucratically large and cumbersome, and I do not believe it is a particularly responsive agency. It does have a lot of value, but I do not think that that is one of the strengths of WHO.*

In addition, the global policy recommendations issued by the WHO have been accused of being too general to be applied by national decision makers. One participant stated:

*I would say...forty or more years ago, it [WHO] was pretty strong. It has been pretty weak in the past few decades...I think it is well intentioned, but I don’t think that as an organization it is going to be a leading force in vector control. It is really going to be up to individual governments and unions of governments that are more likely to have a stronger effect.*

Without the strong coordination of a global public health institution, the control of MBDs on a global scale is what one expert described as “messy.” Multiple public and private actors are now involved in tackling MBDs without any coordination of their agendas, so the billions of dollars given to MBD research and control every year are not used synergistically to achieve the maximum benefit. As a result, many proposed MBD control tools never advance beyond the proof-of-principle stage. One participant explained:

*Certainly we have achieved a lot of things...that would not have been possible without external funders coming in, but it really makes organizations beholden to the external funders. And they do not necessarily set the priorities for the organizations. Those are being set by the people who have the money.*
Despite its weaknesses, the WHO is still in the strongest position of any agency to coordinate the fragmented world of MBD control. One participant stated:

There is a lot of work being done in trying to re-evaluate and revitalize the way the WHO interacts with the many players in the field.

Product-driven research

Many experts insist that donors are increasingly emphasizing a product development approach to MBD control research. The growing focus by funders on advanced technology that can be patented has marginalized research on simple interventions and improvements to infrastructure. One participant described a funder’s reaction to the advice that housing improvements be prioritized:

Basically everything that was not lasers and drones, it was rejected. And people were not interested in monitoring mosquito behavior. How do you know if you are killing a mosquito if you don’t know how to monitor a population? There are a lot of really simple things, supposedly simple things—they are obviously not very simple to actually implement, but they do not catch the attention of the funders. They don’t make good sound bites.

Many governmental funding agencies have emulated philanthropists by emphasizing novel product development and requesting business development plans for grant proposals. Consequently, efforts to expand our understanding of mosquito biology and ecology are largely on hold. One participant explained:

There is some Grand Challenge research that is being done that is more exploratory but a lot of the Gates work is product development driven, where a target product profile has been developed and there are some potential products
that meet the target definition and then there is the developmental research to bring them from that concept to delivery.

Assigning intellectual property rights to interventions that are ultimately public goods, especially when they have been financed by taxpayer dollars, raised concern among some experts. In addition, some argued that the competitive attitude spurred by the need to shield ideas and market new technologies could be driving the field in the wrong direction. One participant stated:

One of the issues that one starts to worry about is public ownership of technology...To what extent is research funded by philanthropists or other groups not advancing because it is being restricted? It’s an interesting question, I think.

Moving forward, new collaborative systems of innovation must be promoted by funders in order to harness the creative potential of the entire field towards the development of new technologies rather than vetting researchers against each other.

4.3.2.4. Social drivers

Entomological capacity

The lack of trained entomologists in developing countries is one of the biggest challenges in MBD control. Attempts at promoting capacity building have been made by the WHO, but many experts insisted that such efforts are inadequate. One participant explained:

I have been going to meetings for nearly forty years now...where people have been talking endlessly about capacity development. And there are several problems to that. One is that there is no career structure there for lots of folks, so if you develop capacity, there is nowhere for them to go. And the skill sets that are required are
*NOT properly mapped out either, so that there is lots of basic training done that we keep repeating endlessly. But it is not actually growing a cadre of people on the ground who can actually do the implementation at a higher level.*

Even if more training programs were established, another major hurdle is the lack of career structures in endemic countries to support entomologists. As a result, new entomology graduates from endemic countries often choose to leave their countries or work for private companies. Sometimes even international philanthropic organizations offer salaries within in endemic countries that are significantly higher than what Ministries of Heath and local research institutions can afford to pay. One participant stated:

*Really for most institutions, capacity building has been no more than getting some PhD students through their PhDs...Making sure that jobs remain in country and are as attractive with international rates—that really has not happened at all.*

To stop the brain drain of entomologists in endemic countries, attractive career opportunities must be created at the Ministries of Health and regional vector control programs. In addition, there must be a discussion among international global health actors of appropriate salary ranges for each country.

Even in developed countries, medical entomologists are a “dying breed,” according to some experts. One European participant explained the lack of entomologists in the country:

*If we have a problem with mosquitoes, we have to fly in experts from the outside. That is how pathetic it got. We have lost the expertise.*
Few universities still offer degrees in medical entomology, and single courses in the subject are rarely available. As a result, valuable entomological expertise that took decades to build is quickly being lost. One participant explained the concern:

*Clearly, interest in vector biology has picked up, but you cannot just switch that tap back on immediately. And therefore, finding people who have got real expertise in this sort of area is very difficult.*

The field of medical entomology has failed to capitalize on the recent multiplication of public health degree programs. The study of vector-borne diseases could easily be integrated into these programs as a track that students can choose or as a handful of elective courses. The hands-on learning that is inherent to medical entomology would make it a unique addition to university public health programs.

**Competitive research culture**

A few participants mentioned that the unwillingness of mosquito researchers to collaborate, both with each other and with researchers of other disciplines, is slowing down the gains made against MBDs. One participant explained that the competitiveness in medical entomology is palpable:

*I like competition; don’t get me wrong. I think there is nothing wrong with that. But at the same time, you’ve often got people that are sort of pushing barriers, saying ‘I’ve got the [best] approach,’ ‘No, I’ve got the best approach.’ You get a lot of that in mosquitoes, much more than in other areas...There’s something different about...medical entomology in that it’s much more competitive at some level because the stakes are higher...And I don’t know quite what you do about that...I think there just isn’t that collaborative structure. There isn’t that sense of*
communicating and working out the best way forward. I think that the philanthropists with their big bucks and big checkbooks are problematical. I think the lack of interactions with third world countries where mosquito-borne diseases are the real big problem is problematical as well. I think for those reasons, there is far too much focus on personalities and egos...That really puts back the area. So I’m sad to say it. It’s really a shame, a concern to me.

Clearly the sporadic and limited nature of funding for MBD research is a big contributor to this cut-throat culture. However, there are likely additional stigmas and misconceptions underlying this phenomenon, which does not seem to be a major problem for other areas of entomology or public health.

Many major funders in MBD control research have begun to emphasize the importance of collaborative networks in their grant schemes, which is taken as a positive influence. However, one participant admitted that many collaborations between institutions in developed and developing countries are merely cosmetic:

You know, if you get a small NIH grant for a few years...and your alignment as an academic...is about getting tenure and getting papers. And working in these countries, you would like to do those as quickly and effectively as you can. But actually working with the people is a longer-term proposition...I think that the partnership is often tokenistic and it is really driven by the developed country scientists wanting to get publications.

There is still a long way to go in terms of creating real collaboration among medical entomologists. Many experts are in favor of promoting greater openness among MBD researchers. One participant stated:
Why don’t we get the entire vector community to say, well, we have this proposal...and she is proposing this and she wants to use active ingredient X, but I think if she used active ingredient Z, it’s going to be a lot more impact because of A, B, C, D?...We don’t have that; that is just not in existence. That we as a global community, we help each other in moving our research forward. Everybody does their own thing, protecting, not sharing. Come on. We are living in 2016. Why is that not happening?

4.3.3. Future scenarios

In collaboration with six experts, scenarios of MBD control for the year 2035 were mapped using the double axes method [246]. Four broad topics resulted, encompassing plausible future funding structures, research approaches, policy making, and control methods. Depending on the directions taken on the future for each of these topics, four scenarios were detailed and were given representative names together with participating experts. The key points for each scenario summarize a consensus of how experts visualized each of the scenarios in the year 2035. Double axes with four scenarios described are presented here for each of the four broad topics (Fig 4.3). Experts were not asked to identify an ideal scenario within each of the topics at the time, but the goal of the scenarios is to start a discussion among researchers, funders, governments, and global institutions about the direction in which we would like to MBD control to progress over the next two decades and to identify key steps that must be taken in the next 5–10 years to achieve ideal outcomes.
4.4. Discussion

This study demonstrates that entomological expertise has a fundamental role to play in MBD priority-setting exercises. Horizon scanning was chosen as a foresight methodology for this study given the lack of priority setting exercises in MBD control on which to base hypotheses. Given that it relies on a convenience sample of expert participants, horizon scanning is biased by the culture of the study group, in this case mosquito biologists. Therefore, the conclusions here are offered primarily as a foundation for future studies. The insights of other stakeholders in MBD control, including donors, Ministries of Health, epidemiologists, social scientists, and vector control personnel were not represented in this study but certainly deserve further investigation.

The main theme that emerged from my horizon scanning exercise is that MBD control experts are deeply concerned about the future state of MBDs. Experts were nearly unanimous in the belief that something must drastically change if we are to prevent complete control failure in the near future due to insecticide resistance. Pyrethroids are currently the only class of insecticides approved for use on bed nets, and 27 African countries have already detected pyrethroid resistance [249]. Experts emphasized that greater focus must be given to mosquito ecology if future progress is to be made against MBDs. Traditionally, research on mosquito ecology has been neglected by funders despite its importance to MBD control and elimination [110]. Nevertheless, a few promising control approaches based on mosquito ecology are in development. Auto-dissemination of the larvicide pyriproxyfen takes advantage of oviposition behavior such that gravid females pick up insecticide on their legs while laying eggs and transfer it to new larval habitats [250]. Attractive toxic sugar baits capitalize on the natural sugar feeding behaviors to crash
mosquito populations [251]. The administration of endectocides such as ivermectin to humans or livestock ensures that mosquitoes will die shortly after ingesting a blood meal [252]. Such alternatives to traditional indoor insecticide applications are critical for eliminating malaria and controlling other MBDs, but many experts agree that mosquito ecology remains a neglected area of research [110], so the development of new application methods is slow.

One alternative to insecticides supported by many experts is the use of the endosymbiotic bacterium Wolbachia in Ae. aegypti to block pathogens, which raises the possibility of stopping dengue and Zika virus transmission cycles across entire cities [91, 96, 97, 102]. Trial releases have been made in several countries, although epidemiological evidence is still being gathered [103]. As further discoveries are made about the mosquito microbiome [132, 133, 142, 168, 253], new endosymbiont-based approaches are likely to be tested. Another promising alternative to insecticides is based on releasing insects carrying a dominant lethal (RIDL) gene developed by the British biotech company Oxitec [77]. A RIDL strain of Ae. aegypti called OX513A, branded the “friendly mosquito,” was tested in Grand Cayman in 2009 [77], and field trials are in progress in several locations in Brazil. Targeted gene editing made possible by the CRISPR-Cas9 system will accelerate the genetic engineering of mosquitoes to render them infertile or refractory to pathogens [254-256]. Scaling up “rear-and-release” approaches such as Wolbachia and GM mosquitoes in disease-endemic countries remains a major challenge. Laboratory infrastructure to accommodate the mass rearing of mosquitoes is lacking in most developing countries, so the use of pop-up facilities or modular shipping-container-type laboratories for rearing could be a temporary alternative [257].
One major concern identified by the horizon scan is the lack of a coordinated, global response to *Aedes*-transmitted arboviruses despite the magnitude of the threat they pose. Brief waves of funding have been the typical response to *Aedes*-transmitted disease incursions, a phenomenon that one participant named “disease *du jour* syndrome.” However, between crises there is no ongoing funding akin to the Global Fund to develop control infrastructure and conduct applied research on *Aedes*-transmitted diseases in order to sustain the progress made during these brief funding booms. Several experts expressed their concern that without global coordination against *Aedes* spp., yellow fever might spread uncontrollably in coming years. Fatality rates as high as 75% have been reported in hospitalized patients, and the current global supply of vaccines is insufficient to control it if it were to spread [13]. Due to the extensive geographical ranges of *Ae. aegypti* and *Ae. albopictus*, surveillance systems that are integrated on a global scale are needed to serve as early warning systems to provide evidence for targeted control efforts and prevent the spread of arbovirus pandemics. Critical infrastructure must be prioritized to prevent the spread of future MBD pandemics, including laboratories, rearing facilities, and comprehensive surveillance systems. A new alliance of stakeholders, sustainable funding mechanisms, and an integrated surveillance system must all be established on a global scale if any future progress is going to be achieved for *Aedes*-transmitted diseases.

As MBDs become an increasingly global issue, strategic global coordination of funding and MBD control efforts is essential. The number of players involved in MBD control is now greater than ever, and their multiple agendas must be pointed in the same direction in order to achieve the maximum benefit of their resources. This will involve a certain dose of humility for all of those involved: researchers, governments, NGOs, global
institutions, and businesses. The WHO is in the best position of any of these actors to coordinate the sea of mixed agendas, but doing so will take substantial reform within the WHO itself and a new vision of its role in global MBD control going forward. In a field that has traditionally been reactive, it is increasingly clear that strategic planning for the future is the only way to turn the tide against MBDs. As such, it is my hope that this horizon scanning exercise will be one of many proactive initiatives to be carried out collaboratively within the field of MBD control. My intention is that the ideas presented here will serve as a discussion platform for building future alliances and strategies.
Table 4.1. Articles included in horizon scan. Editorial-type articles of eleven major journals publishing on MBDs were scanned for potential drivers of change in MBD control.

<table>
<thead>
<tr>
<th>Journal</th>
<th>Types of articles included</th>
<th>Number of articles scanned</th>
</tr>
</thead>
<tbody>
<tr>
<td>The American Journal of Tropical Medicine and Hygiene</td>
<td>editorial, perspective</td>
<td>7</td>
</tr>
<tr>
<td>Annual Review of Entomology</td>
<td>review</td>
<td>9</td>
</tr>
<tr>
<td>Journal of Medical Entomology</td>
<td>forum</td>
<td>4</td>
</tr>
<tr>
<td>The Lancet (all)</td>
<td>editorial &amp; comment</td>
<td>11</td>
</tr>
<tr>
<td>Malaria Journal</td>
<td>opinion, editorial, commentary</td>
<td>34</td>
</tr>
<tr>
<td>Nature (all)</td>
<td>news &amp; views, comments and opinion, editorial</td>
<td>17</td>
</tr>
<tr>
<td>Parasites &amp; Vectors</td>
<td>editorial</td>
<td>1</td>
</tr>
<tr>
<td>PLoS Neglected Tropical Diseases</td>
<td>policy platform, editorial, viewpoints, expert commentary</td>
<td>27</td>
</tr>
<tr>
<td>Science</td>
<td>perspective, review, editorial, policy forum</td>
<td>17</td>
</tr>
<tr>
<td>Trends in Ecology and Evolution</td>
<td>opinion</td>
<td>12</td>
</tr>
<tr>
<td>Trends in Parasitology</td>
<td>opinion</td>
<td>18</td>
</tr>
</tbody>
</table>
Fig 4.1. Characteristics of study population. Twenty-five experts in mosquito-borne disease control were interviewed. The continents where participants worked, the disease systems on which they focused, and the number of years since their first publications are shown.
Fig 4.2. **Drivers of change in mosquito-borne disease control.** Drivers were identified by content analysis of 157 editorial articles and interviews with 25 mosquito-borne disease control experts.
**How will funding structures support research?**

**Scenario 1: Fixed budget**
- Government allocates a fixed percentage of GDP to basic research and global institutions provide funds for key questions
- Collaborative networks receive 10+ year grants to support broad research areas
- North-South partnerships are formed between developed and developing country institutions to distribute capacity and resources

**Scenario 2: Development pathway**
- Multiple innovators receive 10+ year grants to collaboratively take an idea all the way to application
- Funders consult independent think tanks of experts from multiple disciplines to prioritize the most promising innovations
- Industry partners provide expertise and resources in kind during development

**Scenario 3: Disease du jour**
- When a new crisis or trendy idea emerges, all research efforts are diverted to work on it, putting progress in other areas is put on hold
- Single research groups receive short-term funding for science that stops short of implementation
- Researchers are pressured to produce results quickly at the cost of scientific rigor

**Scenario 4: Picking winners**
- Innovations that are revolutionary and have patent potential are supported by funders
- Single research groups are awarded short-term funding to provide efficacy data for chosen interventions
- After trials in endemic countries are finished, disease burden returns to what it was, i.e. the "trampoline effect"
How will we approach research?

**Scenario 1: EVImalaR**
- There is long-term funding for the pursuit of key questions determined by a consortium of experts
- Partners from multiple disciplines and from both developed and developing countries are involved in entire research process
- Data is open access and is summarized for national programs to use

**Scenario 2: IVCC**
- Key interventions are developed using long-term funding committed by donors
- A consortium of researchers work towards a common goal and forego IP by sharing data with national control programs
- Experts from business, economics, and social science are employed to help translate technologies for target markets

**Scenario 3: Nobel prize**
- Funding is awarded to driven individuals with the best track records
- Partnerships are made with key individuals based on reputations and connections or with developing country partners to boost perceived impact
- Science progresses in sporadic leaps, which may eventually translate into new applications

**Scenario 4: Venture capitalism**
- Industry partners supply funding for the development of certain innovations but insist on IP agreements
- Several competing research groups work on similar technologies simultaneously
- Products are taken to market and are either purchased by those who can afford them or fail due to insufficient need or perceived utility

**Axes:**
- Collaborative vs. Competitive
- Researcher Interactions vs. Motivation
- Knowledge-driven vs. Product-driven
Who will drive vector control policy globally and locally?

**Scenario 1: Task force**
- Global institution has personnel on the ground helping governments and reporting back
- A range of policy options are provided based on local context
- If there is insufficient local capacity, policies stay at the theoretical stage

**Scenario 2: Vertical control**
- There is entomological capacity at the Ministry of Health, regional, and community levels
- The government commits to funding part or all of the control program for several years and is not fully reliant on donors
- A nation-wide surveillance system is in place to direct interventions

**Scenario 3: Magic bullet**
- Representatives of philanthropist organizations help countries create plans in line with philanthropists’ global objectives
- New technologies are tested in endemic countries for epidemiological outcomes
- Financial support is conditional on use of specific tools

**Scenario 4: Adaptive elimination**
- Donor support of infrastructure, capacity building programs, and key entomological personnel
- Field research informs choice of interventions from an array of tools supported by donors
- Monitoring systems are established at the community level to evaluate impact of interventions
Fig 4.3. *Scenarios in MBD control for the year 2035.* Four broad topics of scenarios that resulted from discussions with six experts are displayed as sets of double axes. For each set, the term directly on each axis represents the facet within each broad topic being considered, and the axes labels represent dichotomies for each facet. Each scenarios was given a representative name and three major points that describe how experts visualize the scenario in the year 2035. The four broad topics cover A. how funding structures will support MBD research, B. how MBD will be approached, C. who will drive vector control policy globally and locally, and D. which types of interventions will be used.
Chapter 5: Summary and conclusions

5.1. Overview of key findings, limitations, and implications

In this study I sought to provide an ecological forecast for the proposed biocontrol tool Wolbachia by characterizing Wolbachia infections in natural mosquito host populations and within the microbial communities of mosquito ovaries. I found that after polymorphic populations of the saltmarsh mosquito Culex sitiens and the container breeder Aedes notoscriptus were colonized from field populations, their Wolbachia infection frequencies remained relatively constant over time. This finding is contrast to existing theoretical models predicting the spread of Wolbachia in arthropod populations, which suggest that depending on the balance of fitness effects caused in the host by the bacteria, Wolbachia should either increase to fixation or completely disappear from arthropod populations [116, 119, 121]. It is also in contrast to the observed exponential increase of Wolbachia infection frequency in newly infected Ae. aegypti populations [97]. As such, this important finding expands our understanding of Wolbachia ecology in mosquito populations.

The persistence of polymorphia with respect to Wolbachia in caged Cx. sitiens and Ae. notoscriptus populations gives a greater understanding of the unexpected intermediate infection frequencies which have also been described in field populations (Hugo et al., manuscript in preparation). Polymorphic populations of other arthropods have been described, in which some members are infected with Wolbachia and others are not. For instance, Wolbachia is found at low frequencies in natural populations of Drosophila simulans in Eastern Australia [127] and in East African and West African D. simulans populations [129]. In addition, marked fluctuations in Wolbachia infection frequencies
have been described in natural populations of *D. melanogaster* [128]. This study represents the first description of polymorpha in regards to *Wolbachia* infection in mosquito populations. Due to the limits of standard PCR for characterizing the *Wolbachia* infection frequencies, I was unable to determine whether infections persist in the apparently uninfected individuals at very low levels. The presence of faint bands on gels at the position where the *wsp* gene would appear during the PCR-based *Wolbachia* screens of the colonies and the variety of *Wolbachia* densities I found in *Cx. sitiens* ovaries support this hypothesis. Further characterization of *Wolbachia* infection frequency fluctuations over time using quantitative PCR is needed.

I did not identify any particular bacterial species affecting the levels of *Wolbachia* infection in mosquito ovaries, so it is possible that other mechanisms like incomplete maternal transmission or weak cytoplasmic incompatibility could explain the maintenance of stable equilibria of *Wolbachia* infections in *Cx. sitiens* and *Ae. notoscriptus* populations. Due to challenges with paired mating experiments in these species, I was unable to characterize maternal transmission and cytoplasmic incompatibility. In future studies, forced paired mating could be used as an alternative method to study these parameters. It is also possible that the stable infection frequencies I observed in these species are a result of host-symbiont co-evolution. A large body of literature suggests that host populations that acquire a heritable symbiont like *Wolbachia* can undergo unique patterns of genome evolution [122]. As symbiotic partners evolve together, an irreversible codependence can form resulting in modified responses in the host-symbiont system, such as suppressed or modified immune responses in the host or decreased environmental tolerance of the symbiont [125, 126]. It could be that throughout the course of evolution with *Wolbachia*
in *Ae. notoscriptus* and *Cx. sitiens* populations, some of the fitness effects caused by *Wolbachia* such as cytoplasmic incompatibility have been dampened in favor of increased tolerance by the hosts. If this is the case, it would explain the incomplete penetrance of *Wolbachia* in these species. If the stable equilibria of *Wolbachia* I observed in *Cx. sitiens* and *Ae. notoscriptus* do prove to be an example of host-symbiont co-evolution, the dynamics in these population could serve as an ecological forecast for the expected co-evolution in wMel-infected *Ae. aegypti*.

I also described distinct patterns of *Wolbachia* densities between the natural host species *Cx. sitiens* and the novel host species *Ae. aegypti*. Whereas *Ae. aegypti* follow a clear “all-or-none” pattern of infection, *Cx. sitiens* show a variety of densities at which *Wolbachia* can persist. This could be due to genetic differences in the strains of *Wolbachia* [170], or it could be due to differences in the host immune systems. Given that I found a low abundance of microbiota in *Ae. aegypti* ovaries, one hypothesis is that the innate immune system of *Ae. aegypti* is less activated than the immune systems of mosquito species with more complex microbiomes. If this is the case, the reduced immunity of *Ae. aegypti* could partially explain its enhanced suitability as a vector of multiple arboviruses.

I identified a panel of core bacterial species inhabiting the ovaries of *Cx. sitiens* and *Ae. aegypti*. In *Cx. sitiens* ovaries, bacteria in the phyla Proteobacteria and Bacteroidetes predominated. Prominent species included *Wolbachia*, *Acinetobacter rhizosphaerae*, *Sphingomonas yabuuchiae*, *Ralstonia* spp., *Pseudomonas* spp., and *Methylobacteriaceae* spp. After *Wolbachia* was excluded from the analysis, there was a similar abundance of these species among all *Cx. sitiens* samples, suggesting that bacterial interactions within the ovaries are not responsible for limiting the levels of *Wolbachia*. I showed that apart
from *Wolbachia*, Bacillaceae-related bacteria and *Pseudomonas* spp. dominate the *Ae. aegypti* ovary microbiome. The taxonomic composition was very similar across all *Ae. aegypti* samples after excluding *Wolbachia*, suggesting that unlike in the mosquito midgut, the bacterial diversity in *Ae. aegypti* ovaries does not vary by sampling location [141]. My results are comparable to those of other mosquito microbiome studies in *An. gambiae* and *An. coluzzii* in Burkina Faso [168], suggesting that the primers used in my study successfully amplify many common mosquito ovary endosymbionts. Due to the lack of a negative control for the DNA extraction kit during the sequencing of *Cx. sitiens* ovaries, I am not confident that some of bacterial species identified for these samples were not due to contamination. However, the low number of reads in the negative control used during the sequencing of *Ae. aegypti* ovaries implies that contamination during DNA preparation was minimal. The absence of *Spiroplasma* spp. from my samples is a notable difference between my study and microbiome characterizations in *Ae. sollicitans* [171], *Ae. stricticus* and *Ae. vexans* [172], *Cx. annulus* [173], *Cx. tritaeniorhynchus* [174], *An. gambiae* and *An. coluzzii* [168]. Although I did not find *Spiroplasma* spp. in *Ae. aegypti* ovaries, it is possible that they may occur in other *Ae. aegypti* tissues, such as the hemolymph or midgut [179]. The panel of bacterial symbionts I identified in *Ae. aegypti* ovaries could serve as a reference for future studies on the microbiota of the species and provide candidates for MBD control through the use of genetically modified bacteria.

I also aimed to contribute to an ecological forecast for *Wolbachia* by investigating environmental variables accounting for the low *Wolbachia* levels in individual mosquitoes. Using an experimental approach to test the effects of heat exposure during larval development in caged populations of wMel-infected *Ae. aegypti*, I found that emerging
female wMel-infected *Ae. aegypti* exposed to daily fluctuating temperatures of 30–40°C during early development had reduced *Wolbachia* levels compared with controls. Notably, in females exposed to high temperatures starting at the egg stage and lasting for seven days, mean *Wolbachia* levels were less than 0.1% of the levels of wMel controls. My result is supported by the only other study of heat impacts on *Wolbachia* in *Ae. aegypti*, which found that exposure of adult wMel-infected *A. aegypti* to 28°C ± 4°C beginning at 5–8 d of adult age was associated with reduced *Wolbachia* densities [183]. However, the eggs and larvae exposed to high temperatures in my study produced much lower *Wolbachia* densities in adult *Ae. aegypti* than those densities resulting from heat exposure during the adult stage [183]. Loss of *Wolbachia* density in response to heat has been reported in other insects, including *T. urticae* [191] *O. scapulalis* [217], *D. simulans* [195], *D. bifasciata* [194], *Ae. albopictus* [199], the predatory mite *Metaseiulus occidentalis* [218], and the wasp *Leptopilina heterotoma* [219]. However, this study is the first to investigate the duration and timing of heatwave conditions in relation to immature development of mosquitoes infected with *Wolbachia*. Although I do not know whether the heatwave conditions I simulated are representative of the temperatures experienced in *Ae. aegypti* larval habitats due to a lack of temperature data from the field, the inverse relationship I found between duration of heat exposure during larval development and *Wolbachia* density in adult females raises the possibility that long periods of heat might be capable of clearing *Wolbachia* in field wMel-infected *Ae. aegypti*.

My findings have implications for *Wolbachia* as a biocontrol tool, given that it has previously been shown that *Wolbachia* density in mosquitoes is correlated with the level of virus inhibition [155, 157, 170, 210]. The finding that *Wolbachia* density partially
recovers by 4–7 days of age in absence of heat suggests that any impacts of heat exposure during mosquito development on subsequent virus inhibition may be attenuated with age. The partial recovery of *Wolbachia* levels that I observed in the ovaries after mosquitoes returned to normal temperatures suggests that *Wolbachia* replication continues even after the ovaries are fully developed. Future experiments should investigate the limits of *Wolbachia* recovery to determine the age in heat-exposed females at which *Wolbachia* densities would be restored to control levels. Overall, my results support the notion that *wMel* has a more restricted thermotolerance than its mosquito host *A. aegypti*. Given that *wMel* has experienced far less reductive evolution than other symbionts [223] and has a low rate of genetic mutation [223], loss of thermotolerance is not likely [224]. However, more studies are needed to track the co-evolution of *wMel* and *Ae. aegypti*.

In light of my finding that exposure to high temperatures during larval development can attenuate *wMel* *Wolbachia* levels in adult *Ae. aegypti* females, it can be surmised that the use of *wMel* *Wolbachia* might be less effective as a dengue or Zika control strategy in regions experiencing periods of extreme heat. If the effectiveness of *Wolbachia* against pathogens is compromised even temporarily, increased surveillance and supplementary mosquito control may be required in these regions. Greater understanding of heat exposure and other environmental variables that could potentially affect *Wolbachia* is needed to inform release site selection and accurately predict the impacts of *Wolbachia* on disease transmission.

In addition to building an ecological forecast for the future of *Wolbachia*, I sought to develop a policy forecast for the next twenty years of mosquito-borne disease control using the methodology of horizon scanning. By involving multiple subject experts in
horizon scanning exercise, I was able to gather opinions on MBD control that may have been too new or too political to be expressed in traditional outlets. As a result of my content analysis, I was able to describe the current state of MBD control, to identify eight drivers of change likely to impact the future of MBD control, and to develop four sets of scenarios for the future of MBDs. The general impression I got during my interactions with MBD control experts is that experts are deeply concerned about the future state of MBDs, and they believe that something must drastically change if we are to prevent complete control failure in the near future due to insecticide resistance. I discovered that the human capacity within the field of MBD control is dwindling and that career structures both in developing and developed countries have been unsupportive of the long-term, locally-relevant research needed in endemic countries. In light of my findings, it is my belief that more supportive career structures for MBD control researchers must be developed if we do not want to risk losing the contribution of their expertise, which is desperately needed to prioritize future MBD control efforts.

My finding that expert opinions on the state of mosquito-borne control are more pessimistic for arboviral diseases than for parasitic diseases draws attention to the urgent need for a coordinated, global response to *Aedes*-transmitted arboviruses given the magnitude of the threat they pose. Although malaria control has received the ample support of funders like the Global Fund and the Bill and Melinda Gates Foundation, no comparable funding exists for prevention and control of *Aedes*-transmitted diseases like dengue, chikungunya, Zika, and yellow fever. In addition to lack of stable funding, most countries lack the surveillance systems necessary to monitor *Aedes* populations and arbovirus transmission. Therefore, the need to form a global funding body for the control of *Aedes*—
transmitted diseases and the need to establish a global system for their surveillance emerged from my research.

In light of increasing insecticide resistance around the globe, my identification of new technologies likely to drive the future of MBD control provides an important basis for setting the research agenda for the development of new control tools. *Wolbachia*-infected *Ae. aegypti* were identified as a promising new technology that could eventually interrupt dengue and Zika virus transmission [91, 96, 97, 102]. Trial releases at small sites in Australia, Vietnam, Brazil, Indonesia, and Colombia have already been conducted [103]. Applications of *Wolbachia* for malaria lag behind those for dengue and Zika, but as further discoveries are made about the mosquito microbiome [132, 133, 142, 168, 253], new endosymbiont-based approaches are likely to be tested. Another promising candidate for biological control that I identified in the horizon scan is the genetic modification of mosquitoes spearheaded by the British biotech company Oxitec. Field trials using Oxitec’s “friendly mosquito” OX513A *Ae. aegypti* are in progress in several locations in Brazil and are planned for the Florida Keys. Convincing the public that GM mosquitoes are safe and seeking community as well as regulatory approval for releases of GM mosquitoes are major obstacles that must be overcome in the next few years. Experts mentioned that both *Wolbachia* and GM involve substantial financial commitment from countries for the initial releases as well as mass rearing facilities and advanced monitoring systems, which may put these biocontrol tools out of the reach of many developing countries. Consequently, questions of how biocontrol approaches can be scaled up and can be sustained by communities deserve further investigation. In the future, the development of improved trapping methods [258, 259] and rapid detection tools for *Wolbachia* [260] may reduce the
cost and labor requirements of biocontrol monitoring, making them more accessible to developing countries.

In addition to identifying the widely recognized ecological drivers of change in MBD control, such as increasing insecticide resistance and expanding geographical ranges of vectors and pathogens, I identified several political and social drivers of change that are not commonly described in the literature. The limited, sporadic funding for MBD research and control as a result of global economic recession and changing federal priorities has led to a “disease du jour” phenomenon in MBD research in which the limited funds that remain are often shifted to the most recent MBD crisis. Because researchers must often shift their focus to the disease demanding the most immediate response, there are abrupt discontinuities in MBD research, and as a result, few vector control tools have not been developed over the past two decades. The short-term and sporadic nature of research funding has also led to growing competitiveness among research groups in the field of MBDs. In addition, there is insufficient support to support long-term randomized control trials, which are needed to provide the epidemiological evidence of efficacy for new control paradigms. Another driver of change in the field is the diminishing influence of the WHO is in the global control of MBDs due to decreased funding and fragmentation of donors’ and member states’ priorities. With limited funding for personnel on the ground in endemic countries, the WHO now issues global policy recommendations that are often too general to be of use to National Malaria Control Programs or Ministries of Health. In addition, the WHO is increasingly being regarded as slow and bureaucratic. Therefore, without significant re-structuring, it will be difficult for the WHO to regain the trust of private donors needed to expand its operations [248]. At the same time, multiple new public and
private actors are now involved in MBD control without much coordination of their agendas. The prioritization by philanthropic organizations of “sexy” science and their desire for large returns puts pressure on researchers and skews MBD control research towards the development of advanced technologies regardless of their appropriateness for developing countries. Consequently, it is important that the WHO engage private and public stakeholders in order to coordinate the fragmented world of MBD control.

Overall, the important findings presented in this work constitute both an ecological forecast for the future of *Wolbachia* and a policy forecast for the future of MBD control. It is my hope that they will provide the basis for future ecological and policy studies that can inform a more strategic approach to MBD control.

### 5.2. Policy implications

My findings are relevant to a variety of considerations for policy making, a few of which I describe here.

In light of my findings that *Wolbachia* infection frequencies can persist at intermediate frequencies in mosquito populations and that *Wolbachia* can be cleared to some extent by exposure to high temperatures during mosquito larval development, comprehensive monitoring systems for *Wolbachia* should be established and maintained in areas where wMel-infected *Ae. aegypti* have been released for disease control. Any temporary reductions in *Wolbachia* coverage, such as those that might occur following a heat wave, could leave a proportion of the *Ae. aegypti* population susceptible to arbovirus infection, creating the possibility of disease transmission. Continuous monitoring of *Wolbachia* infection frequencies in *Ae. aegypti* will ensure that any drops in coverage are quickly detected and that appropriate supplementary control measures are used.
The lack of trained entomologists in developing countries is one of the biggest challenges in MBD control, and it deserves the immediate attention of policy makers. Although the WHO has provided recommendations for capacity building programs [261, 262], entomological capacity building is still not prioritized by many large MBD control funders, and as a result, trained entomologists are severely lacking from most of the world. The main focus of policy makers for now should be establishing career structures for entomologists so that the incentives to stay in MBD control are available. Also, the skill set needed by national coordinators in order to carry out entomological surveillance and vector control should be defined so that future training programs can be tailored to the most relevant areas of knowledge. Finally, better metrics must be developed to determine whether capacity building programs are effective.

As MBDs become an increasingly global issue, policy initiatives to better coordinate global funds and MBD control efforts are needed. The multiple agendas of the researchers, governments, non-profit organizations, global institutions involved in MBD control must be harmonized in order to achieve the maximum benefit of their resources. Based on my findings, it is my belief that the WHO is in the best position of any agency to coordinate these mixed agendas and to develop a single, strategic approach, but doing so will require a new vision of the WHO’s role in global MBD control going forward.

5.3. Future directions

Future studies should expand on the ecological mechanisms responsible for polymorphia in regards to *Wolbachia* and elucidate the role of mosquito immune systems and virus-bacteria interactions in regulating *Wolbachia* densities. Greater understanding of the ecological barriers to high *Wolbachia* infection frequencies in mosquito populations
and high *Wolbachia* densities in mosquito ovaries is needed to predict the spread and pathogen interference effects of *Wolbachia* when introduced to new mosquito populations for disease control.

Approaches to enhance collaborations among MBD researchers and to provide open access to research data should also be explored. One possibility is a formal commitment by researchers to making protocols and trial data openly accessible, but first the implications of such a commitment on intellectual property, publishing, and career advancement must be detailed. An exploration of the attitudes of scholarly journal editors must be made to know whether journals would be willing to pledge their support for an open research platform in order to make it a sustainable option for researchers. Finally, more research is needed on how real-time access to data would be translated into faster and better decision making in endemic countries.

Future foresight studies like the horizon scanning exercise I conducted are needed to set global priorities for the future of MBD control. Addressing the major challenges facing MBD control will require the strategic definition of priorities by researchers, funders, and global institutions both for research and for control, but doing so will first require foresight of future opportunities and threats in MBD control. Although horizon scanning has been used for decades to provide systematic knowledge and to develop science policy in the environmental field [229-236, 263], this valuable tool has rarely been applied to field of MBDs. The drivers of change and the set of future scenarios that I identified with experts can serve as a platform for future research on the policy implications of these issues. Finally, as the factors underlying MBD control continue to evolve, updated
horizon scans will be needed in the future in order to create new policy options based on the most current body of knowledge.
WORKS CITED


21. Gorgas W: **Sanitary conditions as encountered in Cuba and Panama and what is being done to render canal zone healthy.** *Med Rec* 1905, **10**.


59. IVCC [http://www.ivcc.com/]


63. Vanderplank F: Experiments in the hybridisation of tsetse-flies (Glossina: Diptera) and the possibility of a new method of control *Trans R Entomol Soc Lond* 1947, **98**:1-18.


65. Runner G: Effect of röntgen rays on the tobacco, or cigarette beetle and the results of experiments with a new form of röntgen tube. USDA; 1916.

66. Knipling EF: Sterile-male method of population control successful with some insects, the method may also be effective when applied to other noxious animals. *Science* 1959, **130**:902-904.

67. Muller HJ: The production of mutations by X-rays. *Proc Natl Acad Sci USA* 1928, **14**:714.


70. Knipling E: **Possibilities of insect control or eradication through the use of sexually sterile males.** *J Econ Entomol* 1955, 48:459-462.

71. Baumhover AH: **A personal account of developing the sterile insect technique to eradicate the screwworm from Curacao, Florida and the southeastern United States.** *Fla Entomol* 2002, 85:666-673.


94. Suh E, Dobson SL: **Reduced competitiveness of Wolbachia infected *Aedes aegypti* larvae in intra-and inter-specific immature interactions.** *J Invertebr Pathol* 2013, **114**:173-177.


98. Turley AP, Zalucki MP, O'Neill SL, McGraw EA: **Transinfected Wolbachia have minimal effects on male reproductive success in *Aedes aegypti*.** *Parasit Vectors* 2013, **6**:36.


102. Dutra HLC, Rocha MN, Dias FBS, Mansur SB, Caragata EP, Moreira LA: **Wolbachia blocks currently circulating Zika virus isolates in Brazilian *Aedes aegypti* mosquitoes.** *Cell Host Microbe* 2016.

103. **Progress** [http://www.eliminatedengue.com/progress]


121. Turelli M: **Cytoplasmic incompatibility in populations with overlapping generations.** *Evolution* 2010, **64**:232-241.

122. Bennett GM, Moran NA: **Heritable symbiosis: the advantages and perils of an evolutionary rabbit hole.** *Proc Natl Acad Sci USA* 2015:201421388.

123. Wilson AC, Duncan RP: **Signatures of host/symbiont genome coevolution in insect nutritional endosymbioses.** *Proc Natl Acad Sci USA* 2015:201423305.


125. Feldhaar H: **Bacterial symbionts as mediators of ecologically important traits of insect hosts.** *Ecol Entomol* 2011, **36**:533-543.


APPENDIX A: Recruitment email
Dear Dr.

I hope this message finds you well. I'm a PhD student working with Dr. John Beier at the University of Miami in Florida and with Dr. Greg Devine at QIMR Berghofer in Brisbane, Australia. We're conducting a policy study called "Horizon scanning to map the future of mosquito-borne disease control," in which web content analysis and the opinions of 30-50 experts worldwide will be used to explore how vector-borne disease control might develop over the coming twenty years in changing environmental, socioeconomic, and political contexts.

Given your many years of experience with vector-borne disease epidemiology and ecology, you've been recommended to me by one or more senior researchers as an expert in the field and as someone who is equipped to identify long-term trends and future developments in vector-borne disease control. As such, I'd like to invite you to participate in the Horizon Scanning study, which will consist of two 30-minute interviews via Skype or in person over the next 13 months. In these interviews we will discuss your views on the future of vector-borne disease control. Please find more information about the study attached. Should you decide to participate, there is a consent form attached for you to complete and return to me. If you have any questions or concerns, please feel free to contact me.

I look forward to chatting with you.

Kind regards,

Jill Ulrich

*This study has been approved by human research ethics committees at the University of Miami (IRB ID: 20150049) and at QIMR Berghofer (P2116).
APPENDIX B: Ethics approvals
APPROVAL

March 26, 2015

Gina Maranto
Ungar 230 J, 1365 Memorial Drive
Coral Gables, FL 33124
305-284-8519
Fax: g.maranto@miami.edu
g.maranto@miami.edu

Dear Ms. Gina Maranto:

On 3/25/2015, the IRB reviewed the following submission:

<table>
<thead>
<tr>
<th>Type of Review:</th>
<th>Initial Study</th>
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<tbody>
<tr>
<td>Title of Study:</td>
<td>Horizon scanning to map the future of mosquito-borne disease control</td>
</tr>
<tr>
<td>Investigator:</td>
<td>Gina Maranto</td>
</tr>
<tr>
<td>IRB ID:</td>
<td>20150049</td>
</tr>
<tr>
<td>Funding:</td>
<td>University of Miami</td>
</tr>
<tr>
<td>Documents Reviewed:</td>
<td>*Horizon Scanning Study Summary</td>
</tr>
</tbody>
</table>

The IRB approved the study from 3/25/2015 to 3/24/2018 inclusive. Before 3/24/2018 or within 45 days of the approval end date, whichever is earlier, you are to submit a completed Continuing Review to request continuing approval or closure.

If continuing review approval is not granted before the expiration date of 3/24/2018 approval of this study expires on that date.

To document consent, use the consent documents that were approved and stamped by the IRB. Go to the Documents tab to download them.

NOTE: Translations of IRB approved study documents, including informed consent documents, into languages other than English must be submitted to HSRO for approval prior to use.
In conducting this study, you are required to follow the requirements listed in the Investigator Manual (IRP-103), which can be found by navigating to the IRB Library within the IRB system.

Should you have any questions, please contact: Vivianne Casarese, Sr. IRB Regulatory Analyst, (phone: 305-243-6713; email: vcasarese@med.miami.edu)

Sincerely,

[This is a representation of an electronic record that was signed electronically and this page is the manifestation of the electronic signature]

Amanda Cordero-Rojas, MPH, CIP
Director
Regulatory Affairs & Educational Initiatives
Human Research

Personnel

Q1: Research Personnel
Select all staff involved in the project and their proposed role. QIMR Berghofer HREC should be notified via Modified Application of all personnel changes.

<table>
<thead>
<tr>
<th>Name</th>
<th>Emergency Contact</th>
<th>Role</th>
</tr>
</thead>
<tbody>
<tr>
<td>Jill Ulrich</td>
<td></td>
<td>Researcher conducting all of the interviews and hosting the expert panel.</td>
</tr>
</tbody>
</table>

Q1.1 Other Staff
Please provide details on other staff.

Prof. Gia Maranto, University of Miami. External supervisor and PI for University of Miami IRB. Contact: Tel: +1.305.284.8519, g.maranto@miami.edu

Basic Details

Q1.2 Is this a Clinical Trial?
- Yes
- No

Q1.3 Is QIMR Berghofer the primary assessment/approving body for the HREC application?
- Yes
- No

Q1.3.1 Approving Body Details
Please provide details on the primary approving body.


Q2 Disclosure of commercial or other interests of researchers. No

Q2a Please list all other institutions involved in this project. Not answered
Q3
Experimental Plan and Procedures.

1) Project Title

"Horizon scanning to map the future of mosquito-borne disease control"

2) Objectives

Purpose: To explore how mosquito-borne disease control might develop over the coming two decades in changing environmental, social, economic, and political contexts.

Specific Aim 1: To describe the current scientific landscape in mosquito-borne disease control and to detect potential drivers of change in the field over the next 20 years.

Objective 1a: To use web content analysis to identify themes indicating potential drivers of change in the control of mosquito-borne diseases.

Objective 1b: To assess the importance of the potential drivers of change through expert analysis.

Objective 1c: To identify other potential drivers of change in unstructured interviews with experts.

Specific Aim 2: To make sense of the potential drivers of change alone with experts by predicting the drivers of changes' influence on mosquito-borne disease control and considering a range of possible future states.

Objective 2a: To map the potential drivers of change according to their influence on mosquito-borne disease control.

Objective 2b: To consider a range of possible future states and to explore the possible consequences of each.

Specific Aim 3: To facilitate a forum in which experts describe a vision of the preferred future for mosquito-borne disease control in two decades time (the "success scenario") and identify the key steps needed to reach it.

Objective 3a: To stimulate discussion in the forum about the potential drivers of change and possible futures described by participants in one-on-one interviews.

Objective 3b: To encourage experts to describe the Success Scenario of mosquito-borne disease control in twenty years time through discussion.

Objective 3c: To encourage experts to construct a list of key steps that must be taken by researchers to reach the Success Scenario.

3) Background

Substantial progress against mosquito-borne diseases over the last two decades made possible by mosquito control efforts and antimalarial combination therapies is encouraging for the near future (Djoumass, Kampani, Shattock, & Greenwood, 2010). The decline of the global malaria burden has created the momentum to bolster control efforts in order to achieve world-wide disease elimination. Between 2000 and 2013, deaths from malaria decreased by 47% worldwide and by 59% in Africa (World Health Organization, 2014). As of December 2014, nineteen countries were in the malaria pre-elimination or elimination phase, and seven in the prevention of re-establishment phase (World Health Organization, 2014).

Despite the increasing resolution of researchers and funding organizations, the push to control mosquito-borne diseases is hampered by the reality that vector-based approaches are gradually losing their effectiveness. Mosquito resistance to at least one insecticide has been reported in thirty-nine countries, and thirty-nine countries have reported resistance to two or more insecticides (World Health Organization, 2014). For the past 40 years, the strategy of space spraying with ultra-low volume (ULV) insecticides has been used for vector control despite their inability to suppress vector populations for more than a few days or weeks (Audet, Becker, Carroll, & Brown, 1987; Castles, Arndorfer, Hambly, Najera, & Rector, 1995; Dunn, Lentz, Smith, & Franks, 1999; Light, Dunn, & Good, 2011). Similarly, adulticides are unlikely to substantially reduce the numbers of chironomids mosquitoes during an outbreak (Peel et al., 2013). In malaria-eliminating countries, the traditional vector control tools of insecticide-treated nets and indoor residual spraying no longer protect the highest risk populations, adults who work outdoors away from home and use asymptomatic positive reservoirs (Collier et al., 2013). New solutions will be needed to fight mosquito-borne diseases in the future. The effects of climate change will leave populations more vulnerable to vector-borne diseases in the coming decades (Butin, 2006). At the same time, booted technology for surveillance and modelling could enhance preparedness for disease
(Dhiman, Fried, Dhillion, & Dash, 2011; Ezen & Ezen, 2011). Despite this changing landscape, experts in mosquito-borne disease control have not yet taken a systematic approach to forecast and prepare for the future. Horizon scanning is a foresight tool used to think about, debate, and shape the future in a systematic way (van der Heijden, 2005b). In the last decade, it has been used by many businesses, national governments, and non-government organizations to predict societal and environmental needs as well as to forecast emerging science and technology (Hafez & Sutherland, 2010; Sutherland & Woodroffe, 2009; van der Heijden, 2005a). Scanning for what might happen in the future, including threats and opportunities, is a critical component of science policy. Looking for early warnings and finding blind spots in scientific knowledge can reduce the likelihood of unnecessary health or environmental harm caused in the future (Gerrens et al., 2005).

Despite its wide application in government, business, and industry, horizon scanning is only beginning to be applied to the environmental field. It is expected that horizon scanning will be useful for a host of ecological and environmental issues, as horizon scanning involves looking for trends and opportunities, and many environmental issues have components of both (Sutherland & Woodroffe, 2009). Horizon scanning can inform science policy by providing more systematic knowledge about relevant drivers of change and developments (Hafez & Sutherland, 2010; Sutherland, Pickover, Rassia, Policy, & Rassia, 2013). The objective is to assist decision-makers to develop strategies that are sufficiently flexible to remain robust in a range of possible plausible futures (Sutherland & Woodroffe, 2009). This study represents the first application of horizon scanning in the field of mosquito-borne diseases. The results of the study will be a forecast of the next twenty years of mosquito-borne disease control, including potential drivers of change that could represent opportunities and challenges in the field. Collectively, experts will develop a vision of a "Success Scenario" for mosquito-borne disease control in twenty years, and they will identify steps that can be taken to reach this scenario. It is hope that the research process employed in this study will help experts to identify blind spots and gaps in scientific knowledge so that research in the near future can be directed towards maximizing the opportunities and reducing unnecessary health or environmental harm in mosquito-borne disease control. Ultimately, it is hoped that this study will foster collaboration and a sense of unified purpose among experts in the field of mosquito-borne disease control.

4) Inclusion and Exclusion Criteria
To be included, participants must work in the field of entomology or mosquito-borne disease control and be considered to be an "expert" by at least one of their peers. It is essential that all participants spoke English fluently so that the interviewer could understand their views and accurately convey them. Participants may be excluded if there are too many willing respondents for the amount of time or funds to devote. In this case, exclusion would only occur after the funds were exhausted. Participants may also be excluded if the interviewee feels uncomfortable working with them or if the interviewer feels uncomfortable with them, given that the chronological method is dependent on trust in communication.

5) Procedures Involved
Horizon scanning involves a collective sense-making by a team of experts in which observations that could be pertinent to the future are collected and synthesized, and based on these observations, implications for decision-making are defined (Sutherland, Sølvig, Oceanic, & Wilburton, 2012). Horizon scanning can be divided chronologically into three main phases: (1) mapping signals, (2) mining zones, and (3) backcasting (Sutherland & Woodroffe, 2009). The methods employed for horizon scanning in this study are based on the taxonomy of horizon-scanning methods used in identifying and prioritizing future possible issues described by Sutherland and Woodroffe and also on the methods used by the International Council for Science (ICSU) in their 2011 Foresight Analysis publication (ICSU, 2011; Sutherland & Woodroffe, 2009). The ICSU Foresight Analysis was chosen as a model study because of its similar purpose to this study, namely, to explore the vision for the desirable long-term evolution of international science and to determine the organization's role in achieving such a vision (ICSU, 2011).

Phase One: Sensing Signals
Potential drivers of change of mosquito-borne disease control over the next 20 years will be identified by a) web content analysis and b) interviews with experts.

Web Content Analysis

Content analysis is an established social science methodology concerned broadly with "the objective, systematic, and quantified description of the content of communication" (Berger, 2004). For the purpose of this study, it will involve scanning a subset of science blogs, science Twitter feeds, science Reddit posts, and Opinion-type articles in scholarly journals (for example, the Journal of Medical Entomology publishes Editorial articles and the Malari Journal publishes Commentary, Editorial, and Opinion articles).
for key themes. Random sampling from a subset of these media, accessed from hosting services, is common practice in web content analysis given the unmeasurable extent of the Internet (Herring, 2010). Eligible blogs, Twitter feeds, and Reddit posts will not necessarily be written by experts, as the intention of this component of the research strategy is to incorporate the voices of the greater society before analyzing these themes with experts. Ultimately, the results are seen as research that might provide a strong influence on the direction of mosquito-borne disease control in the future. Inclusion of peer-reviewed, Opinion-type scholarly articles for the identification of potential drivers of change that may be new or too nuanced to have been noticed by non-experts yet, as well as informal content, will be included in analysis extending to related areas such as the social implications of findings (Bolzman, 2007). Hence, they are essential for identification of potential drivers of change in the field.

Themes will be identified by looking for topics that recur regularly in the subset of blogs, Twitter feeds, Reddit posts, and Opinion-type articles (Ryan & Bernard, 2003). Once key words related to mosquito-borne diseases and their control have been identified in the texts, a Key Words in Context (KWIC) approach will be used to systematically search the corpus of each text to find all instances of each key word or phrase used. Each time an instance is found, a copy of it and its immediate context will be recorded. Themes will be identified by sorting excerpts into groups of similar meaning (Ryan & Bernard, 2003). All text content analysis will be performed using RQDA, a freeware package for qualitative data analysis.

First round of interviews with experts

Spending time in interviews with experts will consist of one-on-one discussions to identify issues and to explore important driving forces and areas of uncertainty (Sutherland & Woodroffe, 2009). All interviews will be audio taped unless the participant requests otherwise. These interviews will be mostly unstructured, using an ethnographic methodology. Ethnography involves an inductive analysis building upon the perspectives of the people studied rather than on a priori hypotheses. Ethnography emphasizes continuous relationships between the researcher and research participants. The Heritage Anthropological Association (2004) states that questions cannot be established prior to the ethnographic experience because ethnography is an iterative process. Therefore, the first part of the interview will gather basic data on the participant's professional and scientific background. No personal biographical information will be solicited. Later discussion topics will likely include the present state of mosquito-borne disease control, recent developments in the field, likely future developments, and potential threats in the future. Discussion may deviate from these topics, as other important insights can be gained by letting participants guide the conversation. After the discussion, themes gathered from the web content analysis will be presented to the participants, and he or she will be encouraged to reflect on these themes and to give his or her opinion about their relevance as potential signals. At the end of the interview, the participant will be thanked for his or her insights and will be reminded of the upcoming phase in the research process, namely, a second interview and an expert forum.

After each interview, the study team will transcribe the audio recording into a Word document. After all interviews have taken place, thematic analysis of the transcripts will be conducted using the RQDA package in R.

Phase two: Mapping sense

In the same-mapping phase, experts will participate in the second round of one-on-one interviews, and they will be asked to: a) map the potential drivers of change identified in phase one according to their influence on mosquito-borne disease control and b) consider a range of possible future states and explore the possible consequences of each.

Second round of interviews with experts

Interviews will be audio taped and will be conducted in a similar manner as in Phase One; however, they will be slightly more structured based on the goals of mapping potential drivers of change and constructing potential scenarios. At the beginning of the interview, the discussion from the Phase One interview will be recapitulated. Next, the potential drivers of change that were identified from all of the Phase One interviews will be presented to the participant in small note cards. There will also be cards with arrows. The participant and the interviewer will discuss and map these drivers of change according to the participant's view on their relationship to mosquito-borne disease control (positive or negative influence indicated by arrow direction; important or not important indicated by distance from the center card "mosquito-borne disease control"). A photo will be taken of the resulting system map. During the second part of the interview, the participant will be asked their about a time two decades in the future, to consider a range of possible future states (scenarios) of mosquito-borne diseases given the potential drivers of change, and to explore the possible consequences of each. At the end of the interview, the participant will be thanked for his or her insights and will be reminded of the upcoming phase, an expert forum.

After each interview, the study team will transcribe the audio recording into a Word document. After all interviews have taken
place, the system maps and the scenarios derived from the photos and transcripts will be consolidated. Graphic design software will be used to depict the system maps and the scenarios in understandable, visually pleasing graphics.

Phase Three: Backcasting

A script for the initial panel questions will be written based on the potential drivers of change and potential futures described by participants in Phases 1-2. These questions will be reviewed by the participants one month before the conference so that they will not feel nervous or uncomfortable. The graphics depicting the system maps and visions from Phase Two will be presented on a large screen to serve as discussion aids. Once the initial panel questions have finished, the experts on the panel will be encouraged to ask each other questions and to begin to construct the Success Scenario through discussion. The researcher will make notes about the Success Scenario on a dry-erase board as the experts discuss. After approximately 15 minutes of discussion or once a Success Scenario has been constructed, the experts will be asked to help construct a list (also recorded by the researcher on the dry-erase board) of key steps that must be taken by researchers to reach the Success Scenario. After 10 minutes of discussion or once the list is thought to be complete by the panelists, the forum will open up to the audience for their comments on both the Success Scenario and the list of key steps.

The expert forum will be witnessed by conference participants and will be video recorded. The forum will own the rights to the video, but it is hoped that an agreement can be reached to make a final, edited version of the video to post on the Internet for open access.

The results of all three phases will be published in a scientific journal. The publication will relate expert views on the next two decades of mosquito-borne disease control. The views will be compiled and will be de-identified.

1) Data and specimen banking

De-identified data in the form of Word documents and photos (system maps) will be backed for future reference on a hard drive that can be accessed by members of the Mosquito Control Laboratory at GMR Bergoder. For persons outside of the Mosquito Control Laboratory to access the data, he or she must email the Lab Head and request permission. All audio recordings will be deleted. The video recording will be owned by the International Congress of Entomology. The rights to disseminate this video have yet to be negotiated with the ICE.

7) Data Management

Each participant will receive a random participant ID generated by Excel. A password-protected Excel file of the participant IDs will be stored with the other data as described below. Only the study team will have access to the participant ID Excel file. The participants will be referred to by their ID numbers in all recorded data and never by their actual names.

Interviews will be audio taped to provide a supplement to written field notes and in order to review them for my understanding. To protect confidentiality, tapes will be marked with a pseudonym and only the study team will have access to the transcripts. Audio recordings will be transcribed following the interview, and then the audio recording will be deleted. Handwritten field notes will have the date and time of the interview but no other identifying details. The handwritten field notes will be stored in a locked cabinet at GMR Bergoder when not in use, and only the study team will have access to the cabinet. Digital notes will be stored as Word documents. The digital photos of system maps taken during the Phase Two interviews will be stored as pages. The digital video of the expert forum will be stored for editing. All data will be stored in a password-protected folder on a hard drive at GMR Bergoder. Only the study team will have access to the folder. This folder and all of its contents will be deleted after the publication of the manuscript.

No statistical analysis of the data will take place, but all content analysis will be done on de-identified data in the REDCap package in Excel.

8) Roles to Subjects

This research involves the observation and interaction with adult participants in ordinary life and poses risks not greater than those inherent in everyday life.
ordinarily encountered in daily life.

It is not necessary to the project that participants reveal any sensitive personal information, and it will not be questioned for. There is a possibility that the participant might volunteer or reveal such information in the course of interviewing. Any personal information disclosed by the participant that I consider sensitive will not be included in the research output.

All data will be kept anonymous, minimizing the risk that information could be identifiable to the participant. In addition, information that could be identifiable despite anonymity because of its specificity to the participant's position or experience will only be used with a second check of consent, which will be obtained orally at the time of the interview or written over the phone/videoconference. The data that this consent is obtained will be recorded in the notes.

6) Potential Benefits to Subjects

There will be no financial or material benefits to the participants. However, the research may help participants to record their thoughts at a particular point in time, to gain insights into their needs and priorities, to foster research ideas, to promote understanding of processes of change through time, and to assess particular challenges facing researchers and society. The interviews may also affirm the value of the participant's experience and knowledge by peers and may be rewarding experiences (ANTHROPOLOGY ASSOCIATION, 2004).

10) Vulnerable Populations

This research does not involve individuals belonging to vulnerable populations.

11) Seating

All research will take place mostly in Australia, where Jill Ulrich is a visiting PhD student. Jill Ulrich will conduct interviews in person at the participants' home institutions, which may be in several cities in Australia, or via Skype (videoconference).

In-person interviews will take place in the participants' office or laboratory, at professional meetings or conferences, in public places such as restaurants and cafes, or in the participants' home upon invitation.

12) Resources Available

A mix of ethnographic methods and web content analysis will be provided by professors at the Jayne and Leonard Abess Center for Ecosystem Science and Policy at the University of Miami. Travel funding will also be provided by the Abess Center. Participant recruitment and meeting arrangements will be supervised by Dr. Lisa Hugo at the QIMR Berghofer Research Center.

Dr. Hugo has an extensive network of research connections including experts in mosquito-borne disease control, particularly Australian experts involved in World Health Organization research.

13) Prior Approvals

The study protocol will be reviewed by the QIMR Berghofer Human Research Ethics Committee and the Safety Committee, who must approve the study before research can commence. Interviews and travel plans will be approved by Dr. Lisa Hugo at the QIMR Berghofer Research Center.

14) Recruitment Methods

Ethnographic research depends upon a convenience sample where participants are chosen based on their professional culture knowledge of a particular topic and a community recognized proficiency in the culture. Potential participants will be referred by experts who are knowledgeable about mosquito-borne disease control and are involved in research new mosquito-borne disease control methods. First contact with potential participants will be made via email or phone conversation, in which the study team will discuss the project and explain the interview goals and process before asking for participation. The study team will stress that participation is completely voluntary. If the expert agrees to participate, the study team will email the Consent form and a complete, signed version will be requested via email or phone before the first one-on-one interview. The study team will work most closely with key informants who exhibit the most extensive knowledge of the opinions of the community of experts.

While this work does not require a specific number of participants, the study team expects to interview between 20-30 experts. Participants will not be paid.

15) Local Number of Subjects

The study team expects to interview 20-30 experts. Approximately 10-20 of the participants will be from Australia, given that it is
more convenient for the interviewer to meet with them in person. The other 12 participants will be from other countries.

16) Confidentiality

As described above in the "Data Management" section, to protect confidentiality, audio tapes will be sealed with a pseudonym and will be transcribed and de-identified shortly after the interview. Interviews will be transcribed, and the audio recording will be deleted. Only the study team will have access to the data, which will be password protected and stored on encryped drives. After the project, members of the Macquarie University or QMRR staff will have access to the de-identified data, and only the dataset will be able to grant permissions for outsiders to access the data.

17) Provisions to Protect the Privacy Interests of Subjects

As described above in the "Data Management" and "Confidentiality" sections, participants will not be asked to divulge sensitive or private information. The names and identities of participants will be confidential. Also, if the participant feels uncomfortable about something that has been said, he or she may ask that certain topics be omitted from the data after the interview has taken place.

18) Consent Process

Informed consent will be given in writing with participants' signing the Consent Form (see attached Consent Form). The Consent Form will be emailed to each participant and a complete, signed version will be requested via mail or email before the first one-on-one interview. The interview will clearly inform participants about the purpose, procedures, study, and benefits. Plans for the use and protection of medical data will be stated during the interview. The names and identities of participants will be confidential. In cases where the participant asks to delete information, the study team will respect his or her wishes according to the following guidelines. If the speaker asks that what they say remain confidential before they speak, the interviewer will stop recording. If the speaker asks that the discussion of certain topics be omitted after the interview has taken place, the study team will delete these sections from the tape and from the notes.

In addition to the Consent Form, consent will be obtained at the time of the interview or later over the phone/videoconference. If information is recorded that could be identifiable despite anonymity because of its specificity to the participant's position or experience, the date that the consent is obtained will be recorded in the notes.

The participant may withdraw from the study at any time. In this case the participant's wishes regarding the information already collected will be respected. If the participant asks to delete information, all of that participant's information will be deleted from the tape and from the notes.

19) Process to Document Consent in Writing

Informed consent will be given in writing with participants' signing the Consent Form (see attached Consent Form). The Consent Form will be emailed to each participant and a complete, signed version will be requested via mail or email before the first one-on-one interview.

Q3a
Statistical Justification

Please state in 1 to 2 lines the hypothesis to be tested, indicate the expected number of subjects in each group if not already mentioned, and state the basis for this number being sufficient for the purposes of this study (unless it is a pilot study).

Harbison sampling is a qualitative data collection method that does not require a specific number of participants for representativeness. It was chosen over a survey or quantitative approach because it is a hypothesis-generating rather than hypothesis-driven approach. The sample size was therefore determined by the time and resources available.
C4
Detail the methods and personnel used for the collection of samples. Each participant will receive a random participant ID generated by PracLab. A password-protected Excel file of the participant ID will be stored with the other data as described below. Only the study team will have access to the participant ID Excel file. The participants will be referred to by their ID numbers in all recorded data and never by their actual names.

Interviews will be audio taped to provide a supplement to written field notes and in order to review them for the researcher’s understanding. To protect confidentiality, the tapes will be marked with a pseudonym and only the study team will have access to their contents. Audio recordings will be transcribed following the interview, and then the audio recording will be deleted. Handwritten field notes will have the date and time of the interview but no other identifying details. The written field notes will be stored in a locked cabinet at QMRR. In Bangkok when not in use, and only the study team will have access to the cabinet. Digital notes will be stored as Word documents. The digital photos of system maps taken during the Phase Two interviews will be stored in a hard drive at QMRR. Only the study team will have access to the folder. This folder and all of its contents will be deleted after the publication of the manuscript.

C5
Outline any aims on the participants for the purpose of this project. No statistical analysis of the data will take place, but all content analysis will be done on de-identified data in the REDA package in IL.

De-identified data in the form of word documents and photos (system maps) will be backed up for future reference on a hard drive that can be accessed by members of the Mosquito Control Laboratory at QMRR Bangkok. For a few weeks at the Mosquito Control Laboratory to access the data, he or she must ask the Lab Head and request permission. All audio recordings will be deleted. The video recording will be owned by the International Congress of Entomology. The rights to disseminate the video have yet to be negotiated with the ICE.

C6
Describe the protocols for research conducted on minor/persons with impairments and/or persons receiving highly dependent care. Some aspects outside of Australia will be included as participants in the study, as this is an international study of mosquito-borne disease control experts. The same protocol will be used with participants in other countries as were those in Australia.

QA.1
Select participants involved in this project.

C7
What are the provisions for consultation and approval for research being performed on collectives (e.g., Aboriginal and Torres Strait Islander Peoples). Not answered.

Epidemiological
Q9
Who are the target subjects for the research and how will they be selected? Participants must work in the field of entomology or mosquito-borne disease control and be considered to be an “expert” by at least one of their peers.
It is essential that all participants speak English fluently so that the interviewer may understand their views and accurately convey them.
Participants may be excluded if there are too many willing respondents for the amount of time or funds to travel. In the case of a participant who would only accept if the funds were exhausted. Participants may also be excluded if the interviewer feels uncomfortable working with them or if the interviewer sees that they are uncomfortable with me, given that the ethnographic method is dependent on trust in communication.

Q10
Describe how risks to the patient will be managed during and after the study, including the provision of counseling or briefing services.
This research involves the observation and interaction with adult participants in ordinary life and poses no greater than those ordinarily encountered in daily life.
It is not necessary to the project that participants reveal any sensitive personal information, and it will not be questioned by me. There is a possibility that the participant might volunteer or reveal such information in the course of interviewing. Any personal information obtained by the participant that is considered sensitive will not be included in the research output.
All data will be kept anonymous, minimizing the risk that information could be identifiable to the participant. In addition, information that could be identifiable shall be anonymized because of its specificity to the participant’s position or experience, which will only be used with a second check of consent, which will be obtained orally at the time of the interview or later over the phone/videoconference. The data that this consent is obtained will be recorded in the notes.
There will be no financial or material benefits to the participant. However, the research may help participants to record their thoughts at a particular point in time, to gain insight into their needs and priorities, to foster research ideas, to explore understanding of processes of change through time, and to assess particular challenges facing researchers and society. The interviews may also offer the value of the participant’s experience and wisdom by peers and may be recorded experiences (American Anthropological Association, 2009).

Q1. Detail the use of “Identified,” “Potentially Identifiable” and “De-Identified” samples or data for use in the research and the permissions obtained for their use. Not answered.

Q1.1
Does this research involve use or disclosure of information from a Commonwealth Agency?
- Yes
- No

Q1.2
Detail the use of human tissue samples used in the research, which have not been collected by informed consent. Not answered.

Q1.3
Describe the storage, retrieval and disposal of samples and data used in the project. De-identified data in the format of word documents and photo (system maps) will be stored for future reference on a hard drive that can be accessed by members of the Mosquito Control Laboratory at QMHR Berhampur. For someone outside of the Mosquito Control Laboratory to access the data, he or she must email the lab head and request permission. All audio recordings will be deleted. The video recording will be owned by the International Congress of Entomology. The rights to disseminate the video have yet to be regulated with the ICE.
Q1.4
Describe the selection, management, and monitoring of treatments that will be required during the study.

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APPENDIX C: Horizon Scanning Consent Form
Horizon scanning to map the future of mosquito-borne disease control

University of Miami
CONSENT TO PARTICIPATE IN A RESEARCH STUDY

“Horizon scanning to map the future of mosquito-borne disease control”

The following information describes the research study in which you are being asked to participate. Please read the information carefully. At the end, you will be asked to sign if you agree to participate.

PURPOSE OF STUDY:
You are being asked to participate in a research study. The purpose of this study is to explore how mosquito-borne disease control might develop over the coming two decades in changing environmental, social, economic, and political contexts.

You are being asked to be in the study because you are considered by your peers to be an expert in mosquito-borne disease control.

PROCEDURES:

Phase 1: Spotting signals

You will participate in a one-on-one, informal interview. The interview will take place in a setting of your choosing or (only if necessary) via Skype. The interview will be audio taped but only for note-taking purposes. The tape will never be made public. The interview will last approximately 30 minutes.

The Phase 1 interview will follow this rough structure:

1) You will be asked briefly about your professional and scientific background. No personal or biographical information will be solicited.

2) We will discuss the present state of mosquito-borne disease control, recent developments in the field, likely future developments, and potential threats in the future.

3) You will be shown themes that are potentially important to the future of mosquito-borne disease control, themes that were identified by web content analysis of science blogs, science Twitter feeds, science Reddit posts, and Opinion-type scholarly articles.

4) You will be asked to reflect on these themes and to give your opinion about their relevance to the future of mosquito-borne disease control.

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Horizon scanning to map the future of mosquito-borne disease control

Phase 2: Making sense

You will participate in a second one-on-one, informal interview. The interview will take place in setting of your choosing or (only if necessary) via Skype. The interview will be audio taped but only for note taking purposes. The tape will never be made public. The interview will last approximately 30 minutes.

The Phase 2 interview will follow this rough structure:

1) We will briefly recap the topics we discussed in the Phase 1 interview.

2) You will be shown note cards with “potential drivers of change in mosquito-borne disease control.” These drivers of change are themes that were distilled from interviews with all 20-30 experts in the study. They are factors that are considered important to the future of mosquito-borne disease control by experts.

3) We will discuss and map the drivers of change with arrows according to their direction and effect size on mosquito-borne disease control. A photo will be taken of the resulting system map.

4) You will be asked to think about a time two decades in the future, to construct a range of possible future states (scenarios) of mosquito-borne diseases given the potential drivers of change, and to explore the possible consequences of each.

5) We will discuss your plans to attend the International Congress of Entomology in September, 2016.

Phase 3: Backcasting

Phase 3 will take place in an expert forum, namely, an organized panel discussion led by me at the XXV International Congress of Entomology (ICE) in Orlando, Florida, during September 25-30, 2016. A small group of experts interviewed in Phases 1-2 will be asked to participate in the panel discussion. Due to time and travel constraints, not all experts are expected to attend the conference. Also, the conference organizers will limit the number of panel members, so not all participants from Phases 1-2 will be asked to participate in the panel discussion. The panel discussion will be witnessed by conference participants and will be video recorded. The conference will own the rights to the video, but it is hoped that an agreement can be reached to make a final, edited version of the video to post on the internet for open access.

If you are asked to participate in the panel and you accept, one month before the conference, you will be mailed a script of questions that will be asked of you during the panel. You will be asked to approve these questions or to make changes.

At the panel session, graphics depicting the system maps and scenarios that we constructed in Phase 2 will be presented on a large screen to serve as discussion aids. The panel discussion will proceed as follows:
Horizon scanning to map the future of mosquito-borne disease control

1) Each panelist will be asked about some of the potential drivers of change and possible futures he or she described in Phases 1-2.

2) Panelists will then be encouraged to collectively construct a "Success Scenario" of what mosquito-borne disease control could look like in twenty years. I will be taking notes about the Success Scenario on a dry-erase board as you discuss.

3) Panelists will be asked to collectively construct a list of key steps that must be taken by researchers to reach the Success Scenario.

4) The forum will open up to the audience for their comments on both the Success Scenario and the list of key steps.

The length of time you are expected to participate in the study is 17 months. You are asked to participate in two one-on-one interviews (30 minutes each) on dates most convenient for you and to possibly take part in an expert panel at the ICE in September, 2016 (approx. 1 hour).

RISKS AND/OR DISCOMFORTS:
We do not anticipate you will experience any personal risk or discomfort from taking part in this study. However, there is a minimal chance that you might experience one of the following:

- Disclosure of unwanted information: Should you disclose of information that you later decide should not be shared, you may request to delete this information from the records at any time. Sensitive personal information will never be solicited during the interviews. Information that could be identifiable to you despite anonymity because of its specificity to your position or experience will only be used with a second check of consent, which will be obtained orally at the time of the interview or later over the phone/videoconference.

- Audio: If you feel uncomfortable being audio taped, it can be turned off at your request.

BENEFITS:
No direct benefit is anticipated for your participation in this study.

CONFIDENTIALITY:
If you agree to participate in the study, you will receive a random participant ID generated by Excel. A password-protected Excel file of the participant IDs will be stored with the other data as described below. Only the study team will have access to the participant ID Excel file. You will be referred to by your participant ID in all recorded data and never by your actual name.

Interviews will be audio taped to provide a supplement to written field notes and in order to review them for any understanding. To protect confidentiality, the tapes will be marked with a pseudonym and only the study team will have access to their contents.
Horizon scanning to map the future of mosquito-borne disease control

Interviews will be transcribed shortly after the interview, and then the audio recording will be deleted. Handwritten field notes will have the date and time of the interview but no other identifying details. The written field notes will be stored in a locked cabinet at QIMR Berghofer when not in use, and only the study team will have access to the cabinet. Digital notes, digital photos, digital video, and all other data will be stored in a password-protected folder on a hard drive at QIMR Berghofer. Only the study team will have access to the folder. After the publication of the manuscript, the de-identified data will be kept in on a hard drive accessible only by members of the Mosquito Control Laboratory at QIMR Berghofer. The Lab Head will have the authority to grant permission to outsiders to access the data. Your name will never appear in any of the archived data.

You may refuse to be audio recorded before, during, or after any interview. In cases where you ask to delete information, the study team will respect your wishes according to the following guidelines: If you ask that what you say remain confidential before you speak, the study team will stop recording; if you ask that the discussion of certain topics be omitted after the interview has taken place, the study team will delete those entire sections on the tape and from my notes.

Unfortunately, even if you want your views to be identified by your name in the final publication, this will not be possible.

The investigators and their assistants will consider your records confidential to the extent permitted by law. The U.S Department of Health and Human Services (DHHS) may request to review and obtain copies of your records. Your records may also be reviewed for audit purposes by authorized University or other agents who will be bound by the same provisions of confidentiality.

**COSTS:**
There are no costs associated with your participation in this study. Participation in the ICE panel is voluntary, and we understand if you are unable to make it to the conference. Even if you are invited to participate in the panel discussion, you must still pay the standard conference registration fee. Unfortunately, we are unable to offer financial assistance for fees associated with conference attendance.

**COMPENSATION:**
There will be no compensation for your participation in this study.

**RIGHT TO DECLINE OR WITHDRAW:**
Your participation in this study is voluntary. You are free to refuse to participate in the study or withdraw your consent at any time during the study. As the investigator, I reserve the right to remove you without your consent at such time that I feel it is in the best interest for you.
If you are an employee or student at the University of Miami, your desire not to participate in this study or request to withdraw will not adversely affect your status as an employee or grades at the University of Miami.
Horizon scanning to map the future of mosquito-borne disease control

CONTACT INFORMATION:
The study team will gladly answer any questions you may have concerning the purpose, procedures, and outcome of this project and can be contacted any time before or during the study. You may reach our study team contact in Australia, Jill Ulrich, at +61 7 3362 0124 or by email at Jill.Ulrich@qimrberghofer.edu.au. You may reach our study team contact for the United States, Gina Maranto, at +1 (305) 284-8519 or by email at g.maranto@miami.edu. If you have questions about your rights as a research subject you may contact Human Subjects Research Office at the University of Miami, at +1 (305) 243-3195.

PARTICIPANT AGREEMENT:
I have read the information in this consent form and agree to participate in this study. I have had the chance to ask any questions I have about this study, and they have been answered for me. I am entitled to a copy of this form after it has been read and signed.

Printed Name of Participant

Signature of Participant ................................... Date

________________________
Jill Ulrich
Printed Name of Person Obtaining Consent

Signature of Person Obtaining Consent ................................... Date

Revised 3/24/15