Studies on *Aedes polynesiensis* introgression and ecology to facilitate lymphatic filariasis control

A thesis submitted to the University of Oxford for the
degree of Doctor of Philosophy in Zoology

Limb K. M. Hapairai

Wolfson College

2013

Abstract

The mosquito *Aedes polynesiensis*, a member of the *Aedes scutellaris* complex, is the main vector in the South Pacific region of the *Wuchereria bancrofti* parasite, the causative agent of lymphatic filariasis (LF), and is also a major nuisance biter. Decades of Mass Drug treatment (MDA) have not been successful in elimination LF. Two non-vector species in the *Ae. scutellaris* complex were introgressed with *Ae. polynesiensis* to attempt to obtain lines that would produce cytoplasmic incompatibility (CI) with wild populations and / or LF-refractoriness. Despite selection of progeny from *Brugia*-challenged, non-infective females at each backcross, no refractory line was acquired. However, three lines from crosses between aposymbiotic *Ae. polynesiensis* and *Ae. riversi* displayed CI and male mating competiveness suitable for the purpose of population suppression using the incompatible insect technique (IIT). A population study was conducted of potential release sites and the evaluation of monitoring tools for *Ae. polynesiensis* on Moorea and Tetiaroa, French Polynesia. There was no evidence of active migration between selected islets on the atoll of Tetiaroa, suggesting it is a suitable site for field releases of CI males. The BioGents Sentinel trap was shown to be an efficient and convenient trap suitable for *Ae. polynesiensis* monitoring. The effects of temperature and larval density on life-table parameters relevant to IIT were examined, including: larval survivorship, developmental time to pupation, male to female ratio, male pupae yield, male size and adult male survival. These findings were used to design and conduct a 14-week field experiment testing CI male strain against an isolated population, using optimized rearing conditions. Approximately 8000 males were released weekly on motu Onetahi, Tetiaroa atoll. Significant sterility was induced by *Wolbachia* in the targeted female population, supporting the development and scale-up of this approach toward *Ae. polynesiensis* nuisance and LF transmission reduction.

Acknowledgments

First, I would like to thank my supervisor Dr Steven Sinkins for his supervision, guidance, and for giving me the opportunity to complete my Dphil studies at the University of Oxford. I would like to thank my co-supervisor Dr Hervé Bossin from the Institut Louis Malardé (ILM) who hosted me in this lab and allowed me to discover the fascinating world of medical entomology. I also thank Dr Patrick Tahiata Howell, Director of ILM, for his constant encouragements throughout my studies.

I am very grateful to the medical entomology team at ILM. I thank particularly, my teammate Michel Ayo Cheung Sang for giving me his trust and loyalty. Mauruuru ia oe! Jerome Marie, Tuterarii Paoafaite, and Michel Germain for their assistance with the many weeks of fieldwork and for creating wonderful working atmosphere. I thank Catherine Plichart for her helping me with molecular biology.

Part of the work presented here would not have been possible without my collaborators. I thank Dr Huang Phuc for sharing his knowledge on filarial work and his indefatigable work ethics, and Dr Hayley Joseph for allowing me to collaborate with James Cook University. I also thank Dr Zakaria Kambris for reminding to have a balanced life as a scientist. I thank Dr Eric Chambers for reading and providing comments to my thesis. I thank Prof Charles Godfray for his assistance with the examination. I thank Dr Pricille Frogier at the Delegation de la Recherche de la Polynésie Française for awarding me the funds that made the thesis possible.

Finally I would like to acknowledge the support from my family and friends around the planet. I thank my parents for their moral support and reminding me of the importance of long term goals. Last I thank my wife for her skills in pupae picking, BG trapping and mosquito sorting, also for the many weekends spent at the lab, and for taking care of our little family while I was gone catching mosquitoes. Mauruuru!

Contributions

I declare that all the work presented in this thesis is my own original research that with the following acknowledgements as part of several collaborative research efforts:

- Chapter 2: Dr Huang Kim Phuc did the initial interspecies crossing of *Aedes polynesiensis* with *Aedes riversi*

- Chapter 3 and 5: Michel Cheong Sang, Tuterarii Paoafaite, Michel Germain, Jerome Marie and Herve Bossin provided assistance for fieldwork.

- Chapter 4: Jerome Marie recorded daily mortality for 30°C.

Table of Content

Chapter 1: General Introduction and Background 7

1.1 Mosquitoes in French Polynesia 7

1.1.1 Life cycle 8

1.1.2 *Aedes polynesiensis* 11

1.2. Pathogen transmission by *Ae. polynesiensis* 16

1.2.1 Lymphatic filariasis in French Polynesia 16

1.2.2 Pathology 19

1.2.3 The *Aedes scutellaris* complex 20

1.3 Control of Lymphatic Filariasis 23

1.3.1 Mass Drug Administration 23

1.3.2 Vector control 25

1.4 Specific Research and Development Activities 28

1.4.1 *Wolbachia* 28

1.4.2 Refractoriness 30

1.4.3 Ecology of *Aedes polynesiensis* 30

1.4.4 Rearing and release scaling up 32

1.5 Conclusion 33

1.6 Aims of the thesis 35

Chapter 2: Investigating cytoplasmic incompatibility and LF refractoriness in *Aedes polynesiensis* 36

2.1 Introduction 36

2.2 Objectives 38

2.3 Materials and Methods 39

2.3.1 Mosquito species and strains 39

2.3.2 Antibiotic Treatment 40

2.3.3 *Wolbachia* screening 40

2.3.4 Experimental crosses 41

2.3.5 *Brugia pahangi* challenges 42

2.3.6 Male Fitness 43

2.3.7 Statistical analysis 44

2.4 Results 45

2.4.1 Antibiotic treatment 45

2.4.2 Creation of lines 45

2.4.3 *B. pahangi* susceptibility 48

2.4.4 Egg hatch 50

2.4.5 Male survival and wing lengths 51

2.4.6 Male competitiveness and suppression assay 52

2.4 Discussion 54

Chapter 3: Field evaluation of monitoring tools and population studies for *Aedes polynesiensis* 60

3.1 Introduction 60

3.2 Objectives 63

3.3 Materials and Methods 64

3.3.1 Study Sites 64

3.3.2 Mark-Release-Recapture 67

3.3.3 Latin Square Experiment 70

3.3.4 Xenomonitoring 73

3.3.5 Climate and field measurements 73
3.3.6. Data analysis 73
3.4 Results 75
3.4.1 Weather data 75
3.4.2 Mark-Released-Recapture 75
3.4.3 Latin Square Experiment 83
3.5 Discussion 88

Chapter 4: Effect of temperature and larval density on laboratory rearing productivity and male characteristics of *Aedes polynesiensis* 95
4.1 Introduction 95
3.2 Objectives 98
4.3 Materials and methods 99
4.3.1 Mosquito stocks and laboratory rearing 99
4.3.2 Experimental protocol 99
4.3.3 Collection of wild male mosquitoes 100
4.3.4 Male wing length and survival 101
4.3.5 Data analysis 101
4.4 Results 102
4.4.1 Survival to pupae and sex ratio 102
4.4.2 Time to pupation and male proportion on first day of pupation 105
4.4.3. Adult male survival 105
4.4.4. Length of wild vs. laboratory male wings 109
4.5 Discussion 112

Chapter 5: Release of Cytoplasmic incompatible *Aedes polynesiensis*: ranging trial on Tetiaroa 116
5.1 Introduction 116
5.2 Objective 118
5.3 Materials and Methods 118
5.3.1 Migration 118
5.3.2 Male mosquito production 119
5.3.3 Population fertility 121
5.3.4 Population monitoring 122
5.3.5 Male survival and size 123
5.3.6 Data analysis 123
5.4 Results 124
5.4.1 Migration 124
5.4.2 Mosquito production 124
5.4.3 Mosquito population dynamics prior to releases 128
5.4.4 Male survival and population estimate 129
5.4.5 Male size 131
5.4.6 Population fertility 132
5.4.7 Population impact during and after releases 133
5.5 Discussion 136

Chapter 6: Summary and Conclusions 141

Appendix 150

References 153

Chapter 1: General Introduction and Background

Mosquitoes are insects of the family *Culicidae* with over 3200 species worldwide. Some species represent a human nuisance because of the hematophagous behavior of females and also represent a major socio-economical and serious health threat, as they are vectors of diseases such as malaria, dengue, West Nile, chikungunya or lymphatic filariasis.

1.1 Mosquitoes in French Polynesia

Only 15 species of mosquito are represented in all of French Polynesia (Figure 1.1) of which 13 feed on humans (Table 1.1). This relative lack of species richness is explained by its isolation from continental landmasses. This isolation allowed speciation and endemism also seen in other arthropod groups (Craig et al. 2001, Gillespie 2002) with six mosquito species that are endemic to some islands. Before European discovery, only mosquitoes from genera *Culex* (Brunhes and Bousses 2009) and *Aedes* were present, with a probable *Aedes polynesiensis* introduction by the first Polynesians settlers (Belkin 1962). Generally, *Culex* mosquitoes are nocturnal whilst *Aedes* are diurnal or crepuscular (Ingram 1954). The first of the two introduced genera is from voluntary release of *Toxorynchites amboinensis* in 1975, used as a biological control (Rivière et al. 1979b). The second is *Wyeomyia mitchellii,* most likely introduced accidentally through air and sea traffic (Marie and Bossin 2013).

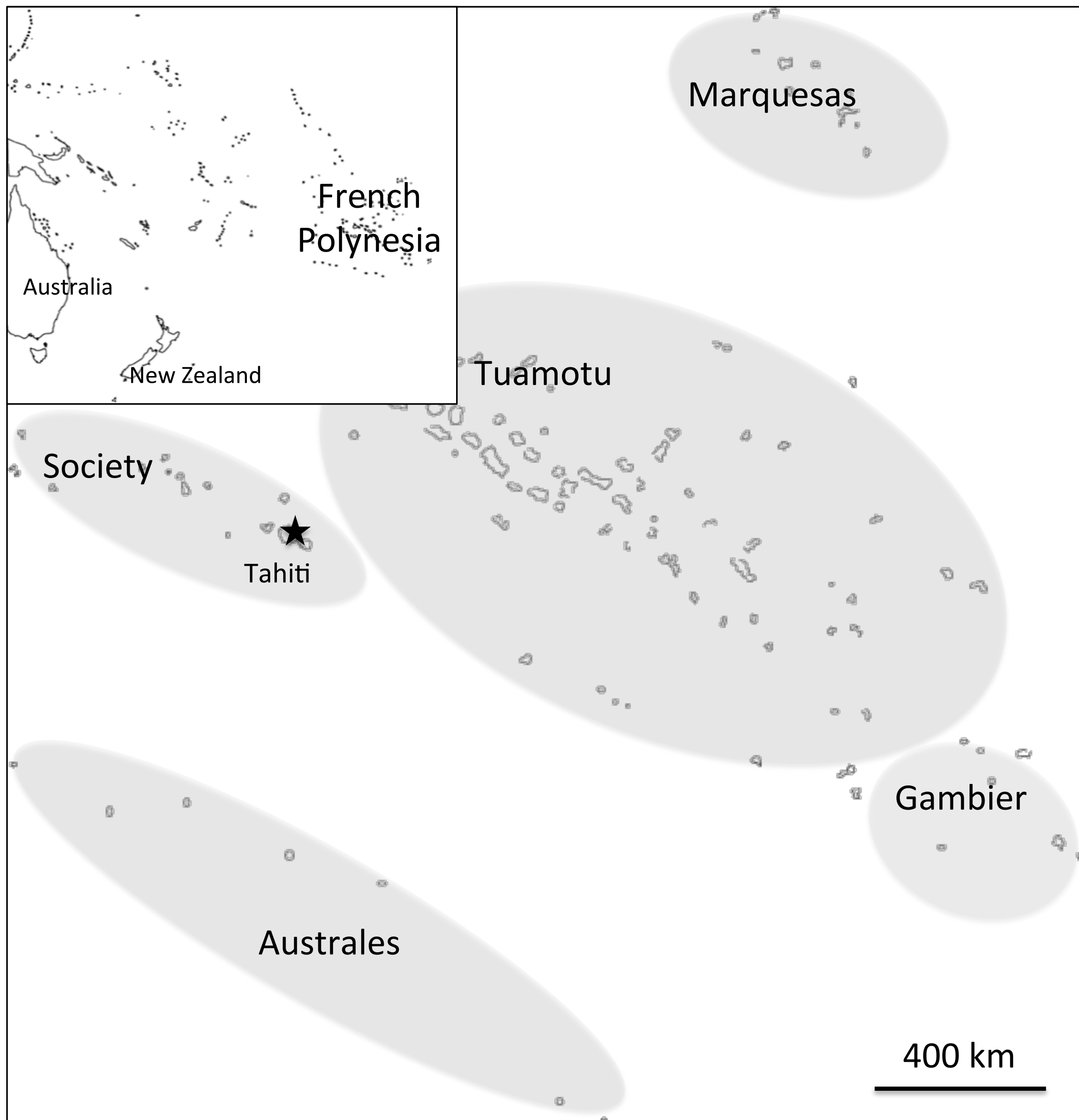


Figure 1.1 Map of French Polynesia. South Pacific regional map added for reference. The islands of Tahiti (☆), Moorea, and Tetiaroa are part of the Society Islands.

1.1.1 Life cycle

The duration of each stage of the life cycle of the mosquito can vary depending on species and developmental conditions such as temperature, predation, nutrient availability, water quality, etc. (Figure 1.2) (Bedhomme et al. 2003, Mercer et al. 2005, Delatte et al. 2009). Generally, after eggs hatch, the aquatic larvae develop through four larval stages during which they consume nutrients, pupate, and the imago emerge around two days later after which they feed on nectar. Copulation occurs shortly after sexual

maturation and subsequently females seek blood meals for egg production. In the case of *Ae. polynesiensis*, aquatic stages can last for 9 to 16 days and adults can live up to 25 to 30 days after laying up to 5 batches with approximately 100 eggs each (Jachowski 1954, Rozeboom 1971).

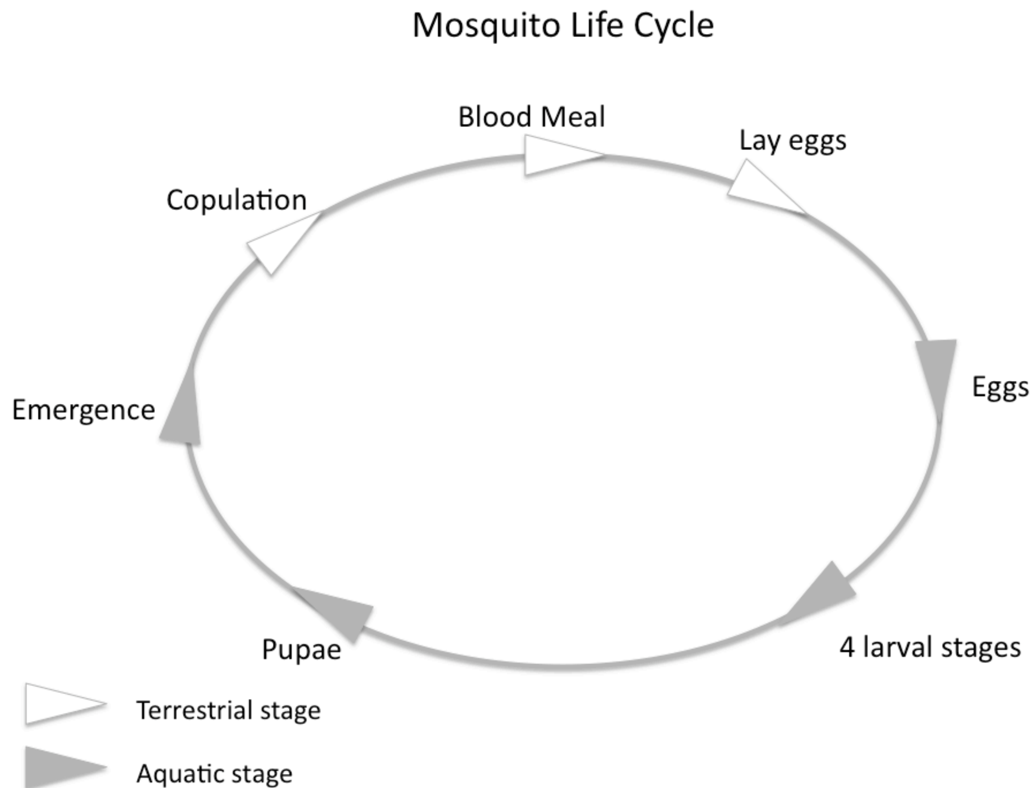


Figure 1.2 Life cycle of mosquitoes. During the terrestrial stage, mosquitoes copulate and females seek a host for a blood meal necessary for development of eggs. Eggs are laid in water containers (artificial and natural) and hatch after maturation. During the aquatic stage, larvae go through multiple larval stages (instars) before pupation and finally emerge.

Table 1. 1. Mosquito species recorded in French Polynesia. Distribution of *Aedes, Culex, Toxorynchites*, and *Wyeomyia* species throughout the archipelagoes of French Polynesia. Selection of stagnant water for breeding site varies between species.

Species	Archipelago of French Polynesia					Preferred breeding site
	Society	Tuamotu	Australes	Gambier	Marqueses	
Aedes aegypti[a,b,c]	x	x	x	x	x	Artificial containers: tyres, tin cans
Aedes edgari	x		x			Wetlands
Aedes nocturnus	x					Water holes
Aedes polynesiensis[a,b,c,d,e,]	x	x	x	x	x	Crab holes, coconuts, artificial containers
Culex annulirostris	x	x	x	x		Clear-water holes
Culex atriceps	x					Coconut, Trees holes
Culex kesseli	x					Pandanus leaves
Culex marquesensis					x	Coconut, rocks
Culex quinquefasciatus[c]	x	x	x	x	x	Septique tanks, brackish water, drums
Culex sechanie	x					Clear water on Mt Marau
Culex roseni	x	x				Intertidal rocks holes
Culex sitiens	x	x				Crab holes
Culex toviiensis					x	River banks
Toxorynchites amboinensis	x			x	x	Artificial containers: tyres, tin cans
Wyeomyia mitchellii [f]	x					Leaves of Alocasia and bromeliades plants

Diseases (including potential) in French Polynesia vectored by mosquitoes:

[a] Dengue fever (Rosen et al. 1954)
[b] Chikungunya fever (Gauzere et al. 2011)
[c] Lymphatic Filariasis (Jachowski and Otto 1952)
[d] Ross River fever (Gubler 1981)
[e] Canine heartworm (Samarawickrema et al. 1992b)
[f] Avian malaria (Marie and Bossin 2013)

1.1.2 *Aedes polynesiensis*

The Polynesian tiger mosquito is a member of the *Ae. scutellaris* complex (Rozeboom and Gilford 1954). The adult is recognized by white markings on the body and legs. The marking is a single white stripe along the thorax at the apex and markings in crescent form on the abdomen (Figure 1.3).

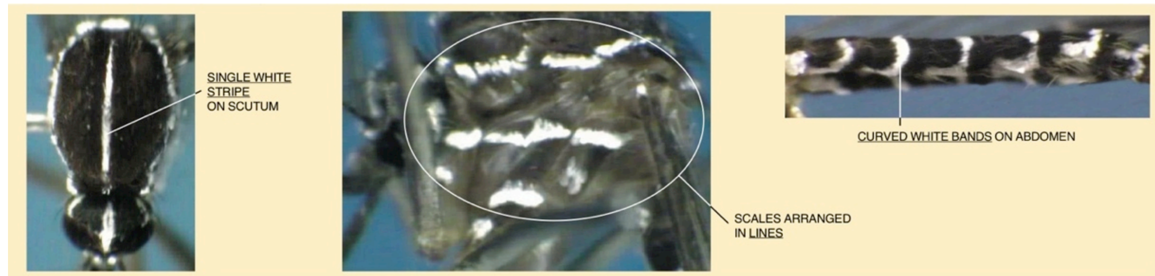


Figure 1.3. Morphology of *Aedes polynesiensis;* dorsal view with a single white strip and lateral view with scales arranged in lines on the thorax, and crescent shapes markings on the abdomen (Media Link Communication Group).

1.1.2.1 Geographic Distribution

Ae. polynesiensis has an extensive geographical distribution throughout the eastern part of the South Pacific. This mosquito species can be found on Pitcairn Islands, Tuamotus-Gambier Islands, Austral Islands, Society Islands, Marquesas Islands, North and South Cook Islands, Samoa, Tokelau Islands, Tuvalu, Wallis and Futuna Island, and Fiji (Iyengar 1955, Rosen 1955, Iyengar 1960).

1.1.2.2 Breeding sites

This species of mosquito can breed in a variety of both natural and artificial water-holding containers (Table 1.1). Physico-chemical characteristics of natural breeding sites can encompass a range of acidity and salinity levels (Riviere et al. 1998b), permanent to ephemeral water containers, and even high nutrient environment (Bonnet and Chapman 1958, Samarawickrema et al. 1993, Riviere et al. 1998a) (Figure 1.4). The mosquito output from different types of breeding containers can vary depending upon

environmental factors (mainly precipitation and temperatures), nutrient availability (Mercer 1999) and predation (Mercer et al. 2005).

Amongst some of the most productive natural containers are coconut fronds and even more importantly rat-eaten coconuts (Riviere et al. 1998a). Rats chew circular openings in the young coconut to eat the meat inside the shell. Once the coconut falls, a period of putrefaction that can last three weeks to months is necessary before mosquito larvae can develop (Figure 1.4). Other water containers are rock holes from lava rocks that hold rainwater (Bonnet and Chapman 1958, Samarawickrema et al. 1993). On atolls, the burrows of the land crab *Cardisoma carnifex* can be a principal breeding site for *Ae. polynesiensis* (Riviere et al. 1998b). Production of larvae in crab burrows is determined by salinity dictated by the depth of the burrow and the level of the water table (fresh water). If the conditions are suitable, breeding sites in crab burrows can be sufficient for maintaining wild populations of *Ae. polynesiensis* despite the removal of all terrestrial water containers (Riviere et al. 1987).

Artificial containers also vary from permanent to temporary water containers from human activity. Such containers include cisterns, rain barrels, tubs and drums which are still common in places where running water is unavailable (Tuamotu) and where irrigation for agriculture is limited (Schmaedick et al. 2008). In French Polynesia, Ministry of Health campaigns also target domestic water containers such as roof gutters, fence pipes, coconut shells, etc. (Bonnet and Chapman 1958).

Figure 1.4. Artificial (Peridomestic litter, tyres, and other containers) and natural (tree trunks, coconut eaten by rats, and crab burrow) breeding sites of *Aedes polynesiensis*.

1.1.2.3 Resting sites

Ae. polynesiensis is broadly considered a forest dwelling species (Samarawickrema et al. 1993, Failloux et al. 1995). Adults have been found resting in dried tree holes and in dried coconut husks (O'Connor 1923). Two studies observed *Ae. polynesiensis* resting on low tree and shrub vegetation but also noted that the species was not seen resting inside human dwellings (Paine 1943, Davis 1949). Similarly *Ae. polynesiensis* was not observed at rest in either European or indigenous type houses. (Jachowski 1954, Ramalingam 1968). Nevertheless, occasionally *Ae. polynesiensis* can be observed around dwellings especially when females are seeking for blood meals in

proximity of piggeries (Rakai et al. 1974) and other domestic animals (Schmaedick et al. 2008). Generally, *Ae. polynesiensis* prefers to rest on the underside of the leaves of vegetation in the proximity of larval breeding sites (Jachowski 1954). On atolls where coconut trees are the main covering, the underside of the leaves of thick *Scaevola fructescens* bushes are preferred by mosquitoes because leaves serve as refuge from the rain, wind and high relative humidity (Rivière et al. 1979a).

1.1.2.4 Dispersal

The distance of dispersal of *Ae. polynesiensis* from its breeding site to resting sites has been crucial for epidemiological evaluations and control strategies. The central dogma of the maximal distance of dispersal is only a short flight of 100 yards (92 m) through the forest (Jachowski 1954). This distance can be affected by many factors such as vegetation, wind, and precipitation (O'Connor 1923, Jachowski 1954) but the mean dispersal distance of *Ae. polynesiensis* is generally considered low (Lardeux 1992).

1.1.2.5 Mating and Host seeking females

After emergence *Ae. polynesiensis* males must wait approximately 24 hours, during which the genitalia mature (rotate 180 degrees), before mating. Females however, are capable of mating shortly after emergence but have a low sexual activity until four hours of age (Eyraud and Quelennec 1976). Once sexual maturity has occurred mosquitoes mate immediately (Eyraud and Quelennec 1976). Mating occurs while mosquitoes are still in flight. However copulation usually is not completed until the male is inverted beneath the female. Males also frequently attempt to copulate around larval sites or while females are seeking a blood meal (Jachowski 1954).

Ae. polynesiensis has a diurnal feeding activity with greater peaks of feeding intensity in the early mornings and afternoons (O'Connor 1923, Jachowski 1954,

Samarawickrema et al. 1987b). The preferred hosts are humans but it can also feed on wild (rats, pigs, and birds) as well as domestic (dogs, sheep, cats) animals (Jachowski and Otto 1952, Ramalingam 1968). The average female will take her first blood meal at three days of age with each blood meal weighing approximately 1.8 mg (Jachowski 1954).

1.1.2.6 Abundance

Mosquito abundance is correlated with seasonal changes, particularly rainfall (Rivière et al. 1979a). Although one study suggests that pupae have the same abundance (productivity) in both wet and dry seasons (Lambdin et al. 2009), density of the vector is affected by seasons (Samarawickrema et al. 1987c). Months where density peaked varied between years but nevertheless depended upon rainfall (Suzuki and Sone 1974, Samarawickrema et al. 1987b). The climate through French Polynesia consists of a relatively "cooler and dryer" season running from May to October with lower precipitation and relative humidity, and during the months of November through April, a "warmer and rainy" season sees higher precipitation and humidity (Météo-France 2010). These are only general trends as the seasonal cycle with two seasons varies according to archipelagoes and years. In the Society Islands where the annual cycle is pronounced, there is strong precipitation during the hot season, but low precipitation during the cool season. Due to its higher elevation (Mount Orohena, Tahiti- 2241 meters elevation), northern and northwestern trade winds affect precipitation heterogeneously around the islands where rainfall is higher along the coast exposed to the trade winds. On the other hand, in the Tuamotu Islands, the two seasons are less distinct from each other compared to the other islands mainly due to its geographical position but also because of the absence of an elevation gradient. Humidity and precipitation are guided solely upon trade winds.

1.2. Pathogen transmission by *Ae. polynesiensis*

Due to its isolation, French Polynesia has been spared from certain wide spread mosquito-borne diseases (malaria, encephalitis, yellow fever, etc.) because of the absence of the vector or the pathogen (Table 1.1). Nevertheless French Polynesia is affected by endemic lymphatic filariasis through the primary vector *Ae. polynesiensis*, and secondarily through *Culex quinquefaciatus* (Rosen 1955, Lardeux et al. 2002c) and potentially through *Aedes aegypti* (Russell et al. 2005a). The other mosquito-borne disease is the dengue virus transmitted primarily by *Ae. aegypti* in urban areas and secondarily by *Ae. polynesiensis* in rural areas (Maguire et al. 1971). The filarial worm, *Dirofilaria immitis,* is also transmitted by *Aedes* species to non-human mammals (Russell et al. 2005a). In the laboratory, *Ae. polynesiensis* has been shown to be a competent vector of Murray Valley encephalitis (Rozeboom and McLean 1956) and Ross River virus (Gubler 1981). It is widely anticipated that the chikungunya virus may soon be introduced to French Polynesia from New Caledonia.

1.2.1 Lymphatic filariasis in French Polynesia

1.2.1.1 Distribution and prevalence

Lymphatic filariasis is a disease caused by filarial nematodes, which infect the lymphatic system. If the infection is left untreated it can develop into various chronic pathologies including elephantiasis. Estimations suggest that over 120 million humans are infected with filarial worms endemically across 80 countries with over 40 million subjected to disability (Chanteau and Roux 2008, Chu et al. 2010, WHO 2012). Over 90% of these infections are from *Wuchereria bancrofti,* found throughout the tropics and some sub-tropical areas worldwide (Michael and Bundy 1997). *Brugia malayi* and *Brugia timori* are limited geographically to Asia and Timor respectively (Melrose 2002).

Throughout the Southeastern Pacific, the geographical distribution of the diurnal subperiodic form of *W. bancrofti* coincides roughly with that of the vector *Ae. polynesiensis* (Macdonald 1976). In French Polynesia, prevalence of filariasis has been noted since the early 50's to be high in the Society islands with up to 30% of the human population infected with microfilariae (mf) (Kessel 1957). This may be explained by the abundance of the vector itself along with a variation in vector competency of *Ae. polynesiensis* for *W. bancrofti* as some strains of mosquitoes from the Society islands allow for the development of greater numbers of infective mf (Failloux et al. 1995). Despite the aggregation of certain islands with higher prevalence, *W. bancrofti* is still found throughout all five archipelagoes of French Polynesia (Mou et al. 2009).

1.2.1.2 Life cycle

The nematode worm *W. bancrofti* develops in various stages in two hosts (Figure 1.5). The first is the human that serves as the definitive host essential for reproduction. The second is the mosquito that serves as an intermediate host for filarial development and vector transmission from infected to uninfected human hosts. A human becomes infected when bitten by mosquitoes harboring infective larvae. These larvae burst from the mosquito proboscis, enter the skin, and migrate to the lymphatic system where they further develop for up to six months to reach the adult stage. Adults are filiform and translucid in color. Males measure between 2 to 6 cm while females and can reach a length of 8 to 10 cm. The adult filarial worms are relatively long lived and can survive for up to 10 years in the human host.

After mating, a single female filarial worm can lay thousands of microfilariae (viviparous), which leave the lymphatic system to spread through the blood system. In the *Ae. polynesiensis*, ingested microfilariae proceed through three stages (L1 to L3)

extending over a 10 to 15 day period of development to reach an infective (L3) stage. The cycle can then repeat itself when a female mosquito seeks a blood meal.

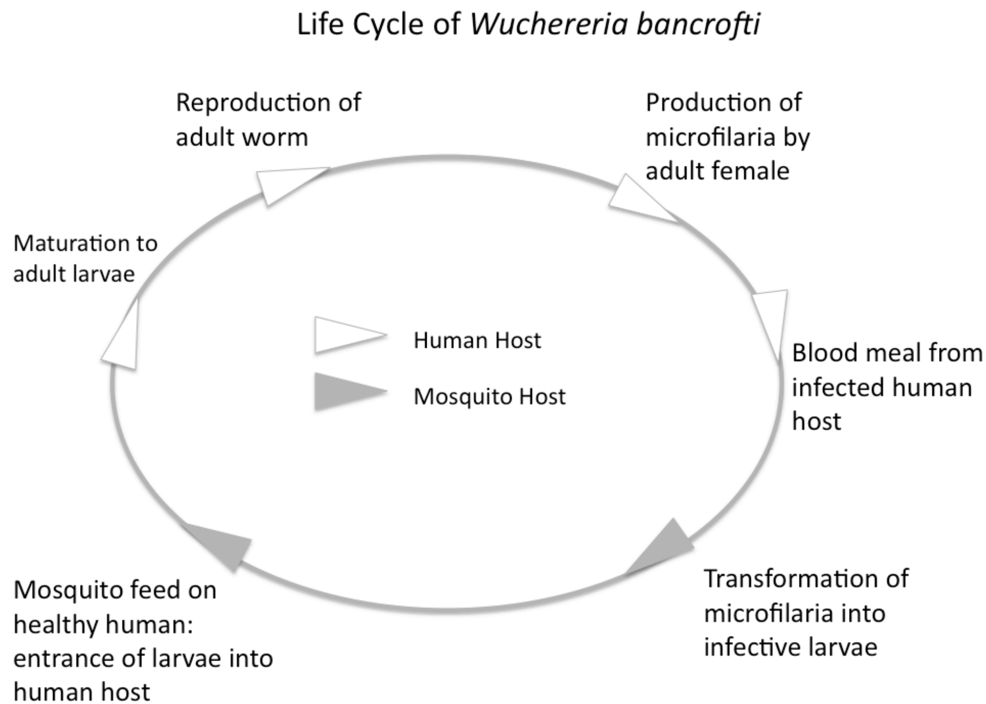


Figure 1.5. Life cycle of *Wuchereria bancrofti* parasite of the human lymphatic filariasis. The parasite has a permanent human host and a temporary mosquito host.

An important characteristic of the relationship between some mosquitoes and their parasites is the tendency for the proportion of mf in the hemocele of the mosquito to decrease when the number of microfilariae (mf) is ingested increases (Bain 1971, Pichon 1974, Bain 1976). This particular phenomenon is called limitation and is the case with *Ae. polynesiensis* and *W. bancrofti* (the opposite being facilitation) (Rosen 1955, Pichon 2002, Snow et al. 2006). This can possibly be explained by a decrease in permeability of the mf ingested through the linings of the stomach in *Ae. polynesiensis* (Brengues and Bain 1972) and consequently allows the mosquito to become a better vector as the mosquito ingests fewer mf (Pichon 2002) (Figure 1.6). This phenomenon makes interruption of the life cycle of *W. bancrofti* particularly difficult (Southgate 1992, Esterre et al. 2001).

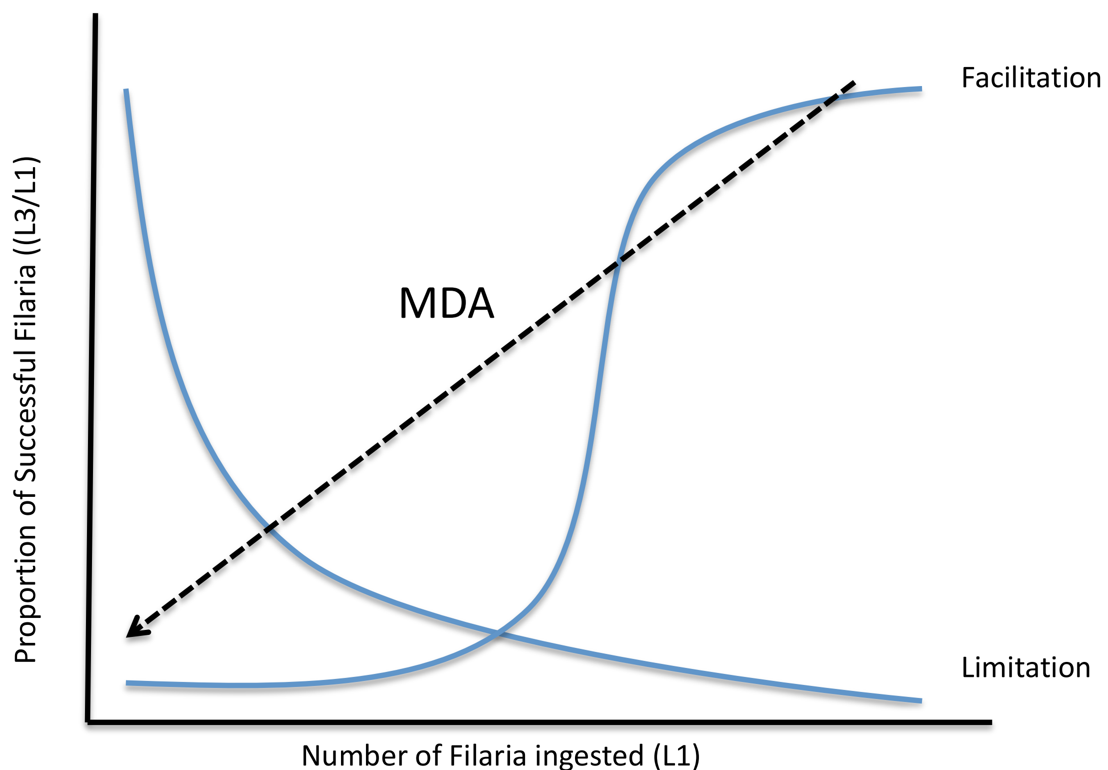


Figure 1.6. Diagram representation of 'facilitation' and 'limitation' of in vectors of filarial. The *Ae. polynesiensis* mosquito exhibits 'limitation' which allows the mosquito to become a better vector as the mosquito ingests fewer mf, such is the case with Mass drug administration (MDA).

1.2.2 Pathology

The asymptomatic phase of filariasis can last for many years without any apparent signs in a patient. This can be the case when filarial worm are of identical sex and cannot reproduce. Later on, a patient usually show signs of the acute phase with repeated high fevers, chills, and painful lymph nodes due to the inflammation of ganglions and lymph vessels (lymphedema) caused by worms in the lymph channel. The chronic phase can be marked by lymphangitis, hydrocele, chyluria, and elephantiasis. Although filariasis is rarely fatal, it triggers a drop in the immune system making the patient more disposed to any other infections. Deformations of body parts not only contribute to functional handicaps, but also to social stigmas and psychological complications.

1.2.3 The *Aedes scutellaris* complex

Over thirty species of mosquitoes constitute the *Aedes scutellaris* complex to which *Ae. polynesiensis* belongs, ranging from continental Asia to French Polynesia (Table 1.2). The first studies observed that although many species of the complex were morphologically similar, not all species were susceptible to the lymphatic parasite (Woodhill 1949, 1950, Backhouse and Woodhill 1954, Rosen and Rozeboom 1954). Differences in the egg hatch rates of the hybrid cross progeny between a few complex members were used to further differentiating species from one another (Tesfa-Yohannes and Rozeboom 1974). Hatch rates varied from high egg hatch in compatible crosses, medium egg hatch in incomplete compatible crosses, and no egg hatches in compete incompatible crosses. (Rozeboom and Gilford 1954, Smith-White and Woodhill 1954, Macdonald 1976, Trpis et al. 1981, Sherron and Rai 1983, Meek 1984, Dev and Rai 1985, Brelsfoard et al. 2008).

Not only did this result in a better understanding of speciation but provided evidence of cytoplasmic incompatibility induced by *Wolbachia* bacteria (Rozeboom 1971), Macdonald (1976) summarized the results of crosses between some species of the complex (Table 1.3).

Table 1.2. Species of the *Aedes scutellaris* complex with their susceptibly to filarial parasites and distribution (Macdonald 1976).

Species		Parasite	Distribution
Aedes	*alcasidi*	Refractory	Philippines
Aedes	*alorensis*	Refractory	Indonesia (Alor Islands)
Aedes	*andrewsi*	Refractory	Christmas Island
Aedes	*aobae*	Refractory	Vanuatu (Banks Island)
Aedes	*guamensis*	Refractory	Marianas Islands
Aedes	*gurneyi*	Refractory	Solomon Islands
Aedes	*hakanssoni*	Refractory	Caroline Islands
Aedes	*hebrideus*	Refractory	Vanuatu (Banks Islands, Torres Islands), Solomon Islands (Santa Cruz Islands), Papua New Guinea (Nuguria Islands, Wuvula Islands)
Aedes	*hensilli*	Refractory	Caroline Islands, Palau Islands
Aedes	*hoguei*	Refractory	Solomon Islands
Aedes	*katherinensis*	Refractory	Northern Australia
Aedes	*malayensis*	Refractory	India (Andaman and Nicobar Islands), Singapore, Malaysia, Thailand, Cambodia, Viet Nam, Taiwan.
Aedes	*marshallensis*	Refractory	Caroline Islands, Marshall Islands, Gilbert
Aedes	*paullusi*	Refractory	Philippines, Malaysia, Indonesia
Aedes	*pernotatus*	Refractory	Vanuatu
Aedes	*quasiscutellaris*	Refractory	Solomon Islands
Aedes	*riversi*	Refractory	Japan (Ryukya Islands)
Aedes	*scutellaris*	Refractory	Indonesia, Papua New Guinea, Northern Australia
Aedes	*scutoscriptus*	Refractory	Micronesia (Caroline Islands)
Aedes	*varuae*	Refractory	Solomon Islands (Santa Cruz Islands)
Aedes	*cooki*	Susceptible	Niue Island
Aedes	*futunae*	Susceptible	Australia (Horne Islands)
Aedes	*kesseli*	Susceptible	French Polynesia (Society and Marquises Islands
Aedes	*polynesiensis*	Susceptible	Fiji, Australia (Horne Islands), Tuvalu, Tokelau, French Polynesia
Aedes	*pseudoscutellaris*	Susceptible	Fiji
Aedes	*rotumae*	Susceptible	Fiji (Rotuma Islands)
Aedes	*tabu*	Susceptible	Tonga Islands
Aedes	*tafahi*	Susceptible	Tonga Islands
Aedes	*tongae*	Susceptible	Tonga Islands
Aedes	*upolensis*	Susceptible	Samoa
Aedes	*horrescens*	Unknown	Fiji

Table 1.3. Summary of interspecific crossing relationship in the *Aedes scutellaris* complex. Compatibility is based on egg hatch of crosses between species of the complex. (MacDonald 1976).

		Male									
		cooki	*hebrideus*	*katherinensis*	*malayensis*	*polynesiensis*	*pseudoscutellaris*	*riversi*	*scutellaris*	*tabu*	*tafahi*
Female	*cooki*				*	#				**	
	hebrideus										
	katherinensis						#		#		
	malayensis	**				*	*		***	#	
	polynesiensis	***			*		#$	#$		***	#
	pseudoscutellaris			#	*	**			#		
	riversi					#					
	scutellaris			***	***		#				
	tabu	***				***					
	tafahi					***					

Legend:

*** high compatibility
** medium compatibility
* low compatibility
# incompatible
$ compatibility remedied with aposymbiotic male

1.3 Control of Lymphatic Filariasis

1.3.1 Mass Drug Administration

The Global Programme to Eliminate Lymphatic Filariasis (GPELF), under the direction of the World Health Organization (WHO), directs endemic countries to implement an annual single-dose, mass drug administration (MDA) using a combination of anti-filarial drugs (Ottesen et al. 1997). Under the auspices of the GPELF, the Pacific Programme (PacELF) was launched in 1999 to specifically target LF elimination as a public health problem in 22 Pacific island countries and territories (PICT) by 2020 (WHO 2012). Elimination would be achieved with annual co-administration of diethylcarbamazine (DEC) with albendazole. These campaigns were succesful in reducing the density of microfilariae (mf) in many PICTs, espcially in French polynesia and Samoa (Suzuki 1975, Kimura et al. 1985). Although MDA is successful in temporarily decreasing the density of mf (Esterre et al. 2001), the efficacy of MDA towards actual elimination of LF in many PICTs is compromised by the biology of the vector (Burkot et al. 2006) and issues with compliance (Joseph et al. 2010).

From the 22 PICT assisted by the PacElf, American Samoa, Fiji, French Polynesia, Kiribati, Papua New Guinea, and Samoa are still implementing or requiring MDA (Table 1.4). The other PICT require only target treatment or implementing surveillance while other requiring no action are PICT that are non-endemic or are no longer considered endemic (WHO 2012). In the six aforementioned PICTs, *Ae. polynesiensis* is the main vector of LF in Fiji, French Polynesia, Samoa and American Samoa. When excluding the population of Papua New Guinea (7,013,829), these four PICTs (1,395,600) represents over 60% of the total population in the South Pacific undergoing PacElf (Table 1.4). For this reason, *Ae. polynesiensis* may pose the greatest

challenge to LF elimination for the PacElf (Burkot et al. 2006). There is a strong necessity for agencies to better coordinate MDA, but in particular a need for effective vector control of *Ae. polynesiensis*.

Table 1.4. Categories of action for 22 Pacific island and territories proposed by the Pacific Programme to Eliminate Lymphatic Filariasis (PacElf) with the estimated population in 2011 (World Bank) and primary vector.

PacElf Categories of action	Countries	Estimated Population	Primary vector
Implementing or requiring MDA	American Samoa	69,543	*Ae. polynesiensis*
	Fiji	868,406	*Ae. polynesiensis*
	French Polynesia	273,777	*Ae. polynesiensis*
	Kiribati	101,093	*Cx. quinquefasciatus*
	Papua New Guinea	7,013,829	*An. punctulatus*
	Samoa	183,874	*Ae. polynesiensis*
Implementing or requiring target treatment	Federated States of Micronesia	111,542	*Cx. quinquefasciatus*
	Marshall Islands	54,816	*Cx. quinquefasciatus*
	New Caledonia	249,000	*Ae. vigilax*
	Palau	20,956	*Cx. quinquefasciatus*
	Tuvalu	9,847	*Ae. polynesiensis*
	Wallis and Futuna	15,289	*Ae. polynesiensis*
Surveillance	Cook Islands	10,900	*Ae. polynesiensis*
	Niue	1,398	*Ae. cooki*
	Tonga	104,509	*Ae. tabu*
	Vanuatu	245,619	*An. punctulatus*
No action needed non-endemic or post-endemic	Guam	182,111	
	Nauru	9,322	
	North Mariana Islands	61,174	
	Pitcairn	67	
	Solomon Islands	552,267	
	Tokelau	1,411	

1.3.2 Vector control

1.3.2.1 Chemical control

Chemical control began alongside MDA with DDT in the early 1950's (Esterre et al. 2005) and is still commonly used predominately in the tourism industry. The application of insecticide is usually achieved through the repeated use of sprays or vapors in attempts to control insect pests including *Ae. polynesiensis*. The cocktail of non-specific insecticides currently includes a range of molecules: organophosphates (chloropryrifos, malathion, temephos, fenitrothion, fenthion), pyrethroids (deltamethrin, cypermethrin, permethrin), and carbamates (propoxur). However, as is the case with many insecticides, resistance to chemical compounds is unavoidable (Failloux et al. 1994).

Attempts to reduce negative effects on non-target species have provided motivation for the use of novel baits or lethal target traps that limit the application of insecticides. The first technique centers upon the ability of *Ae. polynesiensis* to maintain population size despite the lack of terrestrial breeding sites (e.g. coconuts, tree holes, etc). This is because of the capacity for the eggs and larvae to develop underground in the land-crab burrows of *Cardisoma cardifax*. In some places this type of breeding site constitutes the major contribution to the mosquito population. One technique developed for controlling such habitats is the use of insecticide-impregnated baits (chloropyrifos-methyl) (Lardeux et al. 2002b, Lardeux et al. 2002a). The insecticide is simply mixed with attractant so that the crab will collect the baits on the surface and return the baits in the burrow thus delivering the insecticide in the mosquito-breeding site. Baits are currently being evaluated since this method has a particular advantage of not applying insecticide on large surfaces of intertidal zones.

The second technique exploits the resting sites of the mosquitoes. The concept was to impregnate insecticide only on the surface where the mosquito would preferentially rest rather the entire dwelling. Hence, to increase the chances of contact of the mosquito to the target, a dark color cloth (Chambers et al. 2013) impregnated with deltamethrin (non-repulsive) was deployed. Once the mosquito came in contact with the lethal target, it would receive a lethal dose of insecticide.

1.3.2.2 Mechanical control

In French Polynesia, suppression of mosquito breeding sites remains the simplest means of reducing nuisance from mosquitoes around houses: removal of rubbish in the yard, inspection of gutters and septic tanks. However, *Ae. polynesiensis* prefers natural breeding sites reducing the chances of eliminating its breeding. Consequently mechanical control remains very limited for this species. One approach was to spread a layer of polystyrene beads that floated at the surface of the water to prevent surface respiration of the larvae. This could be poured in standing waters and even drinking water wells (Lardeux et al. 2002b) but treatment would be limited to selected breeding sites only.

1.3.2.3 Biological control

In the mid-70's, competitive replacement of *Ae. polynesiensis* with a refractory species *Aedes albopictus* (a species considered harmless at the time) was studied extensively in the laboratory (Gubler 1970c, b, a, Ali and Rozeboom 1971b, a, Gubler 1971, Ali and Rozeboom 1973). Preliminary studies prior to releasing *Ae. albopictus* in the field was encouraging with possibilities of complete replacement of the vector (Rozeboom 1971, Lowrie 1973a, Lowrie 1973b). Field trials for competitive replacement strategy of *Ae. polynesiensis* took place on the atoll of Taiaro, in the Tuamotu Archipelago. *Ae. albopictus* were released but disappeared after 48 months. This strategy

failed most likely due to environmental factors and the failure of *Ae. albopictus* to mate with con-specifics (Rosen et al. 1976). Such a replacement strategy using *Ae. albopictus* today would be unacceptable given what is nowadays known about this mosquito and the viral diseases it transmits (Gratz 2004).

Larval predation has also been of interest particularly for large waters such as ponds and stagnant rivers. Larvivorous fish *Gambusia affinis* and *Poecilia reticulata* and the copepod *Mesocyclops aspericornis* were released into mosquito breeding sites in many islands and atolls. In one study, both fish and copepods were successful in eliminating mosquito larvae in open breeding sites (pond) (Lardeux 1992). Another successful study used both larvivorous fish and copepods in open breeding sites and into land crab burrows (Lardeux et al. 2002b). Although deployment of larvivorous fish and copepods have produced encouraging results, the strategy relies on substantial labor-intensive interventions, making this strategy logistically impractical.

Predatory *Toxorhynchites amboinensis* mosquitoes were introduced in 1975 from American Samoa to control larvae of both *Ae. polynesiensis* and *Ae. aegypti* (Rivière et al. 1979b). The carnivorous larvae of this non-biting giant mosquito feed on larvae found in the same breeding site. *Tx. amboinensis* became temporarily well established in three archipelagoes of French Polynesia and gave a good level of biological control to *Ae. polynesiensis* (Rivière et al. 1979b). However the population of *Tx. amboinensis* remained permanently limited, perhaps because larvae predated upon its own species preventing a wider expansion of the population (Mercer et al. 2005).

1.4 Specific Research and Development Activities

1.4.1 *Wolbachia*

Such scarceness in the development of efficient tools available to control *Ae. polynesiensis* has raised interest in the use of evolutionary genetics to fight vector-borne diseases. One such control approach would use *Wolbachia pipientis*, which is a maternally inherited intracellular bacterium (Hertig 1936, Sinkins 2004). It is capable of interfering with the reproduction of its host by imposing sterility, known as cytoplasmic incompatibility (CI) (Meek 1988). Crosses between members of the *Ae. scutellaris* complex, of which *Ae. polynesiensis* is a member, resulted in partial or even complete sterility of hybrids in the laboratory (Meek 1988), mainly as a result of the incompatibility imposed by the *Wolbachia* present, with which many species in the complex were naturally infected (Wright and Barr 1981, Meek 1984, Behbahani et al. 2005). Unidirectional CI is seen in crosses between *Wolbachia*-infected and uninfected populations, and can also be seen between two *Wolbachia*-infected populations. This type of cross results in a greater number of infected than uninfected progeny and allows *Wolbachia* to increase in frequency over time (O'Neill et al. 1997, Werren 1997, Sinkins and Gould 2006). Bidirectional CI is only seen when two *Wolbachia*-infected populations are crossed and results in no progeny (Figure 1.7). The relative advantage of infection increases as the population frequencies of *Wolbachia* increases; in the case of bidirectional CI whichever strain is in the majority in a mixed population has an advantage, and will thus ultimately replace the minority strain (Sinkins 2004).

Ae. polynesiensis is naturally *Wolbachia*-infected, hence before introgressive hybridization can be carried out, natural *Wolbachia* infections must be cured. For

mosquitoes, this is most commonly done with antibiotic treatment (Laven 1951, Dutton and Sinkins 2005), such as larval treatment using tetracycline (Dean and Dobson 2004).

One study showed that introgressive hybridization and introgression resulted in an *Ae. polynesiensis* modified strain that was infected with *Wolbachia* from *Ae. riversi*. The modified strain was bidirectionally incompatible with the wild strain, resulting in female sterility (Brelsfoard et al. 2008). This study also suggested that because the modified strain was equally competitive with its wild homologue (Chambers et al. 2011), mass releases of modified males could result in elimination of wild type *Ae. polynesiensis* populations (O'Connor et al. 2012).

(A) Bidirectional CI

Female		male	progeny
♀	x	♂	= none
♀	x	♂	= none

(B) Unidirectional CI

Female		male	progeny
♀	x	♂	= uninfected
♀	x	♂	= infected
♀	x	♂	= infected
♀	x	♂	= none

Figure 1.7. (A) Bidirectional cytoplasmic incompatibility. Crosses between two populations with different *Wolbachia*-infections (red and blue). (B) Unidirectional cytoplasmic incompatibility. Crosses between a *Wolbachia*-infected population (red) and uninfected population (black); this pattern can be seen in crosses between populations infected with different *Wolbachia* strains.

1.4.2 Refractoriness

Macdonald (Macdonald 1962) proposed an encouraging technique that would replace a susceptible population by a population that is refractory to microfilaria. Past studies with *Ae. aegypti* demonstrated that susceptibility was linked only to the *fm* gene through simple mendelian inheritance to *B. malayi, B. pahangi, and W. bancrofti* (Macdonald 1962, Macdonald and Ramachandran 1965). Findings within *Ae. aegypti* encouraged the same developments for *Ae. polynesiensis* refractory lines. Further studies on the inheritance of the susceptibility to infection with *B. pahangi* and *W. bancrofti* in the *Ae. scutellaris* group were conducted (Meek and Macdonald 1982). In the *Ae. scutellaris* group, susceptible species were crossed with refractory species to assess inheritance of refractoriness (Meek and Macdonald 1982, Meek 1984). Results however were not as conclusive as in *Ae. aegypti,* with no evidence of a single major gene as method of inheritance in the *Ae. scutellaris* group (Meek and Macdonald 1982); however the studies usually showed dominance of refractoriness.

1.4.3 Ecology of *Aedes polynesiensis*

Previous programs targeting mosquito populations using classical-SIT-like control have generated mixed results (Benedict and Robinson 2003). The first major success was achieved against *Cx. quinquefaciatus* using cytoplasmic incompatibility (Laven 1967). Numerous trials that generated supportive results in the confinement of the laboratory later failed when field evaluated because of insufficient understanding of the ecology of the targeted mosquito (Ferguson et al. 2005). Moreover, proper selection of study sites can drastically change outcomes of the strategy. In one study, immigration of already inseminated females resulted in only partial population suppression instead of elimination (Curtis et al. 1982).

These examples highlight errors that must not be repeated if mosquito *Wolbachia*-based control is to succeed. Pacific island settings can facilitate the implementation of area-wide elimination strategies by targeting naturally isolated mosquito populations with limited immigration (Failloux et al. 1997, Shiu et al. 1997, Behbahani et al. 2005). However, knowledge about relative density, dispersal and flight range of *Ae. polynesiensis* is fragmented at best. In addition, population studies of different island types must be done in order to gather baseline field data over a fairly wide area that can allow for a more accurate understanding of the mosquito/disease population dynamics (Ito and Yamamura 2005).

Knowledge of dispersal and flight range of *Ae. polynesiensis* mosquito is essential for understanding vector-borne transmission dynamics of LF as dispersal influences population dynamics of the vector and the extent to which the parasite will be transmitted to humans (Service 1997). In practice, mosquito dispersal determines appropriate limits necessary to interrupt pathogen transmission (Harrington et al. 2005). Knowledge of the dispersal can also help define future release spatial patterns that must be adopted in order to maximize the spread of released mosquitoes along with subsequent monitoring interventions. Similarly, relative density of *Ae. polynesiensis* within a valley or on an isolated islet is often unknown. Studies investigating the population ecology of the vector are warranted before control programs can be initiated in LF endemics regions.

In addition, the ability to study the ecology of male mosquitoes depends on the efficacy of the sampling tools. In the past, collections of *Ae. polynesiensis* were done using human landing catches (HLC) or human baited collection (HBC) putting the collectors at high risk of infection (Suzuki and Sone 1974, Samarawickrema et al. 1987c, Samarawickrema et al. 1987b, Samarawickrema et al. 1992a). Among the collection techniques investigated more recently for *Ae. polynesiensis* population sampling are the

Fay-Prince trap, the carbon dioxide (CO_2)-baited Centers for Disease Control (CDC) light trap, the Encephalitis Virus Surveillance trap and the BG-Sentinel (BGS) traps of which the BGS showed satisfactory results (Russell 2004, Schmaedick et al. 2008). Investigations into efforts to enhance BGS traps and to test new BG-Mosquitito traps along with the usage of attractants deserve further research. An effective method for sampling *Ae. polynesiensis* (including males) is critical for ongoing surveillance, research (including ecology) and control efforts against filariasis and other mosquito-borne infectious diseases in the South Pacific.

1.4.4 Rearing and release scaling up

One promising approach of vector control is the Incompatible Insect Technique (IIT) suppression strategy for *Ae. polynesiensis* wild population using *Wolbachia*-mediated bidirectional CI (Laven 1967, Sinkins and Godfray 2004, Brelsfoard et al. 2008). IIT and the Sterile Insect Technique (SIT) both have particular advantages: they are species-specific and environmentally non-polluting methods of insect control that rely on the release of large numbers of sterile male insects (Knipling 1955, 1979, Krafsur 1998, Dyck et al. 2005, Bourtzis and Robinson 2006). Importantly, CI *Wolbachia* males do not blood feed, vector diseases, or transmit *Wolbachia*, nor do they establish new populations in the field or transfer genetic material to their environment (O'Connor et al. 2012).

Using the proposed IIT approach, sustained female sterility can theoretically be obtained in field populations by repeated, inundative releases of incompatible males, leading to suppression of local population. To achieve this, large quantity of males must be produced and successfully released in a selected site. Rearing in large numbers have been attempted with difficulties for release of approximately 3800 males/week (O'Connor et al. 2012), a relatively low amount. Larval density and temperature are prime

determinants of male adult size in a mass-rearing framework. Optimizing rearing conditions should favor the shortest developmental period and the lowest mortality. Moreover optimizing larval density and temperature would allow for better sex separation using differences of age at pupation of male and female (Mercer 1999). Despite, the medical importance of *Ae. polynesiensis,* the relationship between rearing conditions and male production has been inadequately studied.

This relationship should first be optimized in laboratory settings in light of being scalable for field releases. For this, multiple intermediate or ranging trials could provide essential insight to a larger suppression study (Harris et al. 2012). Issues range from packaging to transportation of mosquitoes to release sites. There are also concerns about number of workers needed for the production (cost). Production and logistical issues must be resolved for a better chance of an effective strategy. Arguably more important is the biology of released CI males related to male competitiveness and their ability to mate with wild female outside a cage (Chambers et al. 2011). Lessons learned from difficulties encountered during previous male release programs are worthwhile, as this would provide for an improved tool to control *Ae. polynesiensis* and consequently break transmission of LF.

1.5 Conclusion

Decades of MDA treatment have failed to eliminate LF where *Ae. polynesiensis* is the primary vector of the *W. bancrofti* parasite. The negative density-dependence of *Ae. polynesiensis* which exhibits a higher transmission when microfilaremia is low is the hypothesized to contribute to the failure of MDA in certain Pacific island settings. Rather, a program joining MDA and vector control of *Ae. polynesiensis*, is necessary to break transmission of the parasite. Yet, the cryptic behaviour *Ae. polynesiensis* has rendered

available vector control ineffective, raising interest in *Wolbachia*-based approaches using cytoplasmic incompatibility (CI) in an elimination/suppression framework or refractory lines for population replacement. For either suppression or replacement strategies, studies investigating the population ecology of *Ae. polynesiensis* vector for proper sites selection are warranted before releases. An isolated location displaying high mosquito dispersal would present a most suitable site for releases as this would likely determine the outcome of the control program. Effective methods for sampling *Ae. polynesiensis* also need to be assessed to first bolster needed ecological studies including vector migration and dispersal, and subsequent monitoring of the vector population to measure the properties of the release strategy. Furthermore, in the case of suppression, rearing conditions must be optimized in order to obtain large numbers of CI males devoid of female contaminants. Finally, released CI males must be capable of finding and effectively mating with wild females to induce sterility in the wild population for vector suppression and ultimately have an effect on LF transmission.

1.6 Aims of the thesis

There are four aims to this study: the first is to produce lines of *Ae. polynesiensis* demonstrating CI or reduced susceptibility to filarial parasite by introgressive hybridization. The second aim is to characterize a suitable trap for *Ae. polynesiensis* and its ecology on a high island valley and on a low island setting, in order to facilitate future population suppression or replacement strategies, and to assess population size, species distribution, and dispersal at each field site as well as vector transmission (xenomonitoring). The third aim is to study the effects of temperature and larval density on survival of larvae, age at pupation, adult size and adult male survival of laboratory-reared mosquitoes. Finally, findings from the aforementioned aims allowed for an eleven-month field experiment to test an autocidal strategy based on CI males to reduce the fertility of the targeted population.

Chapter 2: Investigating cytoplasmic incompatibility and LF refractoriness in *Aedes polynesiensis*

2.1 Introduction

The lack of efficient tools in the arsenal available to control *Ae. polynesiensis* has raised interest in the use of genetic or *Wolbachia*-based approaches. Differences in *Wolbachia* infections and geographical distribution have been studied between species of the *Aedes scutellaris* group (Meek 1984). Widely distributed throughout the South Pacific, *Ae. polynesiensis* is infected with an A-supergroup *Wolbachia* type (Behbahani et al. 2005). *Ae. riversi* found only in Japan on the Ryukyu Archipelago (Huang 1972) has been collected as far north as Tsuchima Island (Mogi 1976). *Ae. katherinensis* is naturally found in Northern Australia (Darwin) and along with *Ae. riversi* is not a vector of diseases.

Reciprocal crosses of *Ae. riversi* with *Ae. polynesiensis* were observed to be completely sterile because *Ae. riversi* is infected with a B-supergroup *Wolbachia* type which causes bidirectional cytoplasmic incompatibility (CI) (Macdonald 1976, Dean and Dobson 2004). *Ae. katherinensis* is not *Wolbachia*-infected and therefore exhibited only unidirectional CI in crosses with *Ae. polynesiensis* - females of the latter mated with *Ae. katherinensis* males resulted in normal numbers of viable eggs but the reciprocal cross of *Ae. katherinensis* females with *Ae. polynesiensis* males was completely sterile (Meek and Macdonald 1982). Subsequently, antibiotic treatment of *Wolbachia* permitted crosses of aposymbiotic *Ae. polynesiensis* males with *Wolbachia*-infected *Ae. riversi* and naturally aposymbiotic *Ae. katherinensis* (Trpis et al. 1981, Dean and Dobson 2004).

Given that laboratory hybridization of *W. bancrofti* susceptible *Ae. polynesiensis* with non-susceptible *Ae. riversi* and *Ae. katherinensis* (Meek and Macdonald 1982) is

possible, it was conjectured that it could be possible to create lines of *Ae. polynesiensis* that displayed reduced susceptibility to the parasite. Moreover, studies in *Ae. aegypti* presented encouraging results suggesting a single gene was involved in inheritance of refractoriness (Macdonald 1962, Macdonald and Ramachandran 1965).

Introgressive hybridization of *Ae. riversi* and *Ae. katherinensis* females with aposymbiotic *Ae. polynesiensis* males was revisited in this study with the aim of developing alternative vector control strategies. The first approach is the IIT suppression strategy for *Ae. polynesiensis* populations using a bidirectional CI line for suppressing populations exploiting differences in *Wolbachia*-infection (Laven 1967, Brelsfoard et al. 2008). Ideally this line would exhibit identical genetic material as *Ae. polynesiensis* with the exception of being infected with a different strain of *Wolbachia*. Moreover sufficient male mating competitiveness of this line must be maintained compared to wild *Ae. polynesiensis* as IIT hinges upon releases of incompatible males being able to compete against wild males for their wild female counterparts (Dobson et al. 2002). Finally this CI line must also show equal vector competency so as to not increase the risk of LF in the eventuality of an accidental females release.

A second approach is the same introgressive hybridization to generate lines with reduced filarial susceptibility as a means to disrupt the disease transmission cycle by replacing natural mosquito populations with a line displaying reduced susceptibility (Macdonald and Ramachandran 1965). For this line, selection is done on females that do not get infected by the parasite, subsequently backcrossing these selected females (refractory-dominant) with wild type males. This line must first exhibit reduced susceptibility to the parasite. If possible, it should also be infected with an alternate *Wolbachia* strain to allow replacement through bidirectional CI (Sinkins and Gould

2006). *Brugia pahangi* was used as it has been shown to be an acceptable model for studies of *Wuchereria* susceptibility (Meek and Macdonald 1982).

2.2 Objectives

The overall objective is to produce and characterize *Ae. polynesiensis* lines displaying CI and reduced susceptibility to filarial nematode parasites that may be utilized in eradication or replacement strategies respectively, and in the process to obtain further information on the inheritance of susceptibility to filarial nematodes in *Ae. polynesiensis*.

The specific aims are to produce by repeated backcrossing (introgressive hybridization), and characterize:

1) A line of *Ae. polynesiensis* for use in replacement strategies, that is infected with *Wolbachia* from *Ae. riversi* and that is refractory to, or shows reduced susceptibility to, filarial nematodes, by incorporating filarial challenge and selection of individuals showing no filarial worm development as parents for each subsequent generation.

2) A line of *Ae. polynesiensis* for use in replacement strategies that shows reduced filarial susceptibility as above, but has been produced by introgressive hybridization and selection with *Ae. katherinensis* instead of *Ae. riversi, Ae. katherinensis* may be more closely related to *Ae. polynesiensis* based on higher hatch rates in previous crossing experiments, and the resulting line could therefore be more fit / competitive than line 1.

3) Lines of *Ae. polynesiensis* that are incompatible with wild populations, for use in IIT strategies, that is infected with *Wolbachia* from *Ae. riversi*, by repeated backcrossing for complete genome replacement but without incorporating selection for reduced filarial susceptibility. The fitness of this line can be maximized by avoiding the bottlenecks associated with filarial nematode challenge and selection each generation.

2.3 Materials and Methods

2.3.1 Mosquito species and strains

Aedes polynesiensis:

- *Ae. polynesiensis* Afareaitu (APA)- Collected in December 2010 from the Afareaitu Valley (17°33'00.94" S 149°49'09.07" W).
- *Ae. polynesiensis* Maupiti (APM)- Collected in 2004 on the island of Maupiti (16°26'14.00" S 152°15'23.00).
- *Ae. polynesiensis* Maupiti Tetracycline treated (APMT)- Aposymbiotic strain of APM treated with tetracycline antibiotic to remove the *Wolbachia* bacteria.
- *Ae. polynesiensis* Tetiaroa (APT)- Collected in December 2010 from the Tetiaroa atoll (17°33'00.94" S 149°49'09.07" W).
- *Ae. polynesiensis* Tetiaroa (APTT)- Aposymbiotic strain of APT treated with tetracycline antibiotic to remove the *Wolbachia* bacteria.

Aedes katherinensis (AK): Collected in Australia in 2009.
Aedes riversi (AR): Collected in Ryukyu Archipelago (Japan) in the 1990s.

Eggs were hatched using deoxygenated water with yeast for a more synchronized hatch. Larvae were fed on fish food pellets, and reared in plastic trays containing tap water (approximately 2 L). Pupae were then collected and placed into multiple 100ml bowl containing water until emergence. Once emergence occurred, a 10% sucrose solution was provided as an energy source. Approximately one week after emergence mosquitoes were fed on sheep's blood with rabbit skins mounted on the Hemotek artificial feeding system (Gerberg 1970). Eggs were collected on humid filter paper (Whatman, Whatman International, Maidstone, England).

Hatch rate was measured at 48 hours after eggs were submerged. Larvae and eggs were then counted under a dissecting scope. Number of larvae and eggs were compared to calculate hatch rate. Either individual female egg paper or colony egg papers were used depending upon eggs available.

2.3.2 Antibiotic Treatment

Ae. polynesiensis is infected with an A-supergroup *Wolbachia* type whilst *Ae. riversi* with a B-supergroup *Wolbachia* type which causes CI (Macdonald 1976, Dean and Dobson 2004). Nonreciprocal fertility is seen in crosses with *Ae. katherinensis* which is an aposymbiotic species. Hence, before cross mating between *Ae. polynesiensis* and *Ae. riversi* or *Ae. katherinensis* even if all three species are morphologically similar (part of the *Aedes scutellaris* complex), antibiotic treatment of *Ae. polynesiensis* was necessary to overcome sterility of hybrid progenies (Trpis et al. 1981).

Antibiotic treatment was attempted with the first larval (L1) stages with tetracycline or Rifampicin. This was achieved by hatching eggs and placing L1 larvae in antibiotic (50µl/ml) treated (buffered pH 7) water for four days after which mosquitoes were allowed to develop in clean water. Adults were also fed 1 mg/ml of antibiotic in a 10% sucrose solution over five generations (Otsuka and Takaoka 1997, Dean and Dobson 2004). To confirm success of antibiotic treatment, *Wolbachia* screening was performed as described below.

2.3.3 *Wolbachia* screening

To determine infection status of *Wolbachia* of various species of mosquitoes and between strains of cross mating progenies, DNA was extracted using the Livak buffer protocol of Collins et al. (Collins et al. 1987). Mosquitoes were placed in 1.5-mL and grinded in 50 µL Livak buffer (1.6 ml 5M Nacl, 5.48g sucrose, 1.57g Tris, 10.16ml 0.5M EDTA, 2.5ml 20% SDS, pH 8) after which 50 µL was added to rinse the pestle. Tubes were incubated at 65°C for 30 min followed by a brief centrifugation to collect condensation (about 20 sec). 14µl 8M K-acetate was added and mixed before incubation on ice for 30 min. The tubes were then centrifuged for 20 min at 4°C at max speed and

the supernatant was transferred into new tubes. 100% ethanol (200µl) was added to the tubes and mixed by inversion before being placed at -20°C for 15 min. The tubes were again centrifuged at 4°C for 20 min before discarding supernatant. The pellet was rinsed with 250µl ice-cold 70% ethanol and centrifuged for 5 min. The supernatant was again discharged and pellet was allowed to dry on bench for one hour after which the pellet was resuspended in water (50µl).

Polymerase chain reaction (PCR) amplification of the *wsp* gene using appropriate primers (Zhou et al. 1998) was carried out in 25µl reaction volume: 2µl buffer, 0.5µl dNTPs, 1.5µl 25 mM MgCl2, 1µl forward and reverse primers and 0.05 U/ml Taq DNA polymerase using the following thermal cycler conditions: 95°C, 5 min (1 cycle); 94°C, 1 min; 55°C, 1 min; 72°C, 1 min (35 cycles); and 72°C for 10 min (1 cycle).

2.3.4 Experimental crosses

Crosses between *Ae. riversi* (AR) or *Ae. katherinensis* (AK) females and APMT males were conducted in cages. Cage-mating consisted in placing males in excess (4:1) to the number of virgin females in a 30cm X 30cm X 30 cm rearing cages. Two matings were performed on APMT ♂ X AK ♀ crosses with approximately 100 and 200 females per cage.

Both cage matings (as above) and forced (induced) mating was carried out for AR and APMT (Gerberg 1970). Mosquitoes over 72hrs of age were prepared for mating. Females AR were anesthetized by exposure to diethyl ether until females were immobilized and placed on the dorsum on filter paper. Males were decapitated after which wings and legs were removed. Each male was mounted in dissecting needles through the thorax. Contact between female and male was done under the dissecting microscope with female being placed on its ventral side with its head directly away from

the male. Insemination was completed only after the male claspers released the female terminalia. Females were then placed into 30X30X30 cm rearing cages.

After cross mating (cage or artificial mating), introgression was performed by backcrossing hybrid females (F1) with APMT males over a number of generations. PCR was performed regularly to confirm for the presence of *Wolbachia* in ARxAPMT progeny or the absence of the bacteria in AKxAPMT progeny.

2.3.5 *Brugia pahangi* challenges

Challenges with AR progeny were performed beginning with the first generation of backcrosses and for the AK progeny at the second backcross generation. After each challenge with *B. pahangi*, dissection of females was performed to assess for parasite infection of the individual females. This was done by observing the presence of parasites in mosquitoes 14 days after the infectious blood meal (details given below). Only offspring from females that were not infected with the parasite were used to found the next generation. Worm concentrations used are described in Table 2.1.

B. pahangi filarial nematode worm challenges were done by membrane feeding set at 37º C. Hare/rabbit skin was used as membrane to increase feeding with the membrane placed horizontally to obtain a homogenous density of worms suspended in blood freely. *B. pahangi* worms were obtained from University of Glasgow where they were maintained in Mongolian jirds. On arrival they were centrifuged for 4 min at 2000 g to remove the shipping medium and then suspended in sheep's blood to various concentrations. Care was taken to homogenize worm density in blood every 10-15 minutes during mosquito feeding by lightly agitating the Hemotek feeder. Fed females were separated into 50 ml cylindrical ovicups. In each ovicup, females were allowed to lay eggs on humid filter paper and fed on 10% glucose solution on a cotton wick and re-fed every 48hrs until dissections.

Generally, five females per cage were dissected immediately after blood meals to ensure that filarial nematodes had been ingested. From day two to day 13, a few females from each cage were also dissected to monitor development of the parasite. Finally, fourteen days after worm challenge, all remaining females were sacrificed and dissected under a dissecting microscope.

Mosquitoes were anesthetized with CO_2, and their wings and legs removed. The head, thorax and abdomen were dissected mounted on a glass slide with PBS. Tissues were gently separated and the proboscises were opened with dissecting needles. Larval stage and number of larvae were counted with respect to the location in the mosquito (i.e. proboscis, head, thorax and abdomen).

2.3.6 Male Fitness

Survival comparisons were measured by loading 15x15x15 cm cages with 15 males or females. Mosquitoes were provided with a 10% sucrose solution and mortality was recorded. For wing lengths, photographs were taken using the Leica EZ40 (EZ4D, Leica Microsystems GmbH, Wetzlar, Germany) and measured using the ImageJ software (http://rsbweb.nih.gov/ij/) as described in Rasband (2004). To this end, the right wings were removed as close as possible to the thorax using forceps. Wings were mounted on to a glass slide and fixed with a cover slip to press the wing flat against the slide. The length between the auxiliary incision (A_1) and the tip to the wings (between R_3 and R_{4+5}) was measured on a calibrated dissecting microscope (Figure 2.1). Wing length comparison were done for APr, BC9 and APT with 20 males each.

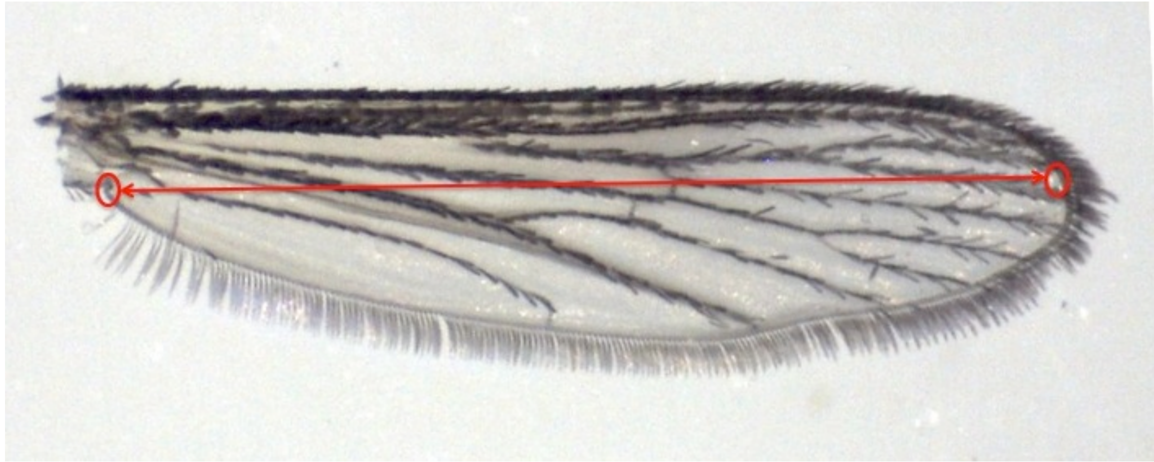


Figure 2.1 Photograph of *Ae. polynesiensis* wing. Distance measured for wing length is between auxiliary incision (A_1) and the tip to the wings (between R_3 and R_{4+5}).

For comparison of male competitiveness for APk and APr, a total of 50 males were released into a 30X30X30 cm rearing cages with varying wild:modified male ratios; 1:0, 1:1, 0:1. i.e. 25 wild: 25 modified for ratio 1:1. After a 24hr-resting period, a total of 50 virgin females were released into the cage containing the fifty males. For BC9, cage suppression assay was conducted using 1:1 and 1:40 ratios i.e. 50 wild and 200 BC9 for ratio 1:40. Males were also allowed a resting period before 50 virgin females were released.

Mosquitoes were allowed to mate for at least three days after which a blood meal was provided. Females were individualized into similar 50 ml ovicups as above. After eight-day maturation period of the eggs, individual ovicups were flooded with deoxygenated water and vacuumed to assess hatch rate as described above.

2.3.7 Statistical analysis

The numbers of infected females of hybrid and backcross progenies were compared to the numbers infected APMT females (control) using Chi-square (X^2) goodness of fit. Male mating competitiveness was also analysed with a X^2 goodness of fit comparing the departure from a 1:1 ratio between the observed and expected number of

hatching broods. Hatch rates of backcross progenies were compared with wild type Afareaitu strain using Kruskal-Wallis test. Survival of each progeny was also compared with either APMT or APT stains using the Log-rank estimator. Analysis of Variance (ANOVA) was used to compare differences in male wing lengths followed by Tukey's multiple comparison test. Compatible female egg hatch from male competitiveness was subject to Kruskal-Wallis Test. Statistical analysis was done using GraphPad Prism version 5.0 (GraphPad Software Inc., La Jolla CA)

2.4 Results

2.4.1 Antibiotic treatment

High mortality was observed in tetracycline treatment of the larvae as shown in previous studies (Otsuka and Takaoka 1997). Rifampicin treated larvae had a lower mortality rate but antibiotic was not successful in removing *Wolbachia*. Adult treatment with 1 mg/ml in a 10% sucrose solution with tetracycline over five generations effectively removed *Wolbachia* from APT. This aposymbiotic strain of *Ae. polynesiensis* is APTT. Treatment of the APA was discontinued as treatment did not provide for an aposymbiotic strain after seven generations.

2.4.2 Creation of lines

From inter-species crossing and selection, four lines were selected:

1. The APr line is infected with *Wolbachia* from *Ae. riversi* with an estimated 98.4% *Ae. polynesiensis* nuclear genetic background after five generations of backcrossing, and displays bidirectional CI when crossed with wild type A-subgroup *Ae. polynesiensis* (Figure 2.2). It is also selected for reduced susceptibility to *B. pahangi*. This line resulted from crosses of AR females with

APMT males, with filarial challenge applied each generation. There were great difficulties with simple cage mating. Dissection of a few females showed that in cage mating, AR ♀ females had little to no sperm in the spermathecae and consequently had lower numbers of eggs. Observation of F1♂ testis showed reduced numbers of sperm and distortion of spermatozoid typically with three times greater head size and a reduced tails lengths (¼ the size of normal size tails). Furthermore, eggs showed greatly reduced viability despite APMT being aposymbiotic and subsequent backcrossing produced no hatching eggs (Table 2.2). To overcome this, forced mating was performed from APMT ♂ X AR ♀, F1 ♀, and BC1 ♀ crosses. Forced mating resulted in greater hatch rates (Table 2.2). Forced mating was not necessary from BC2 onwards and simple cage mating was resumed. Levels of filarial susceptibility were observed to be erratic at five backcrosses generations, thus part of the line (the other is BC11) was allowed to intercross afterwards.

2. The BC11 line originated from the sister line of APr and thus displays the same bidirectional CI, but with >99.9% *Ae. polynesiensis* nuclear genetic background. This line resulted from being backcrossed for 11 generations with APMT without selecting for susceptibility to parasite infection (Figure 2.2). Following the backcrosses, the line was allowed to intercross afterwards.
3. The BC9 line originated from the APr line and thus displays the same bidirectional CI as line BC11. This line resulted from being backcrossed nine generations, of which four generations with APTT, without selecting for susceptibility to parasite infection (Figure 2.2). Following the backcrosses, the line was allowed to intercross afterwards. As with BC11, this line contained

around 99.9% of *Ae. polynesiensis* genetic background with of both Maupiti (APMT) and Tetiaroa (APTT) parents.

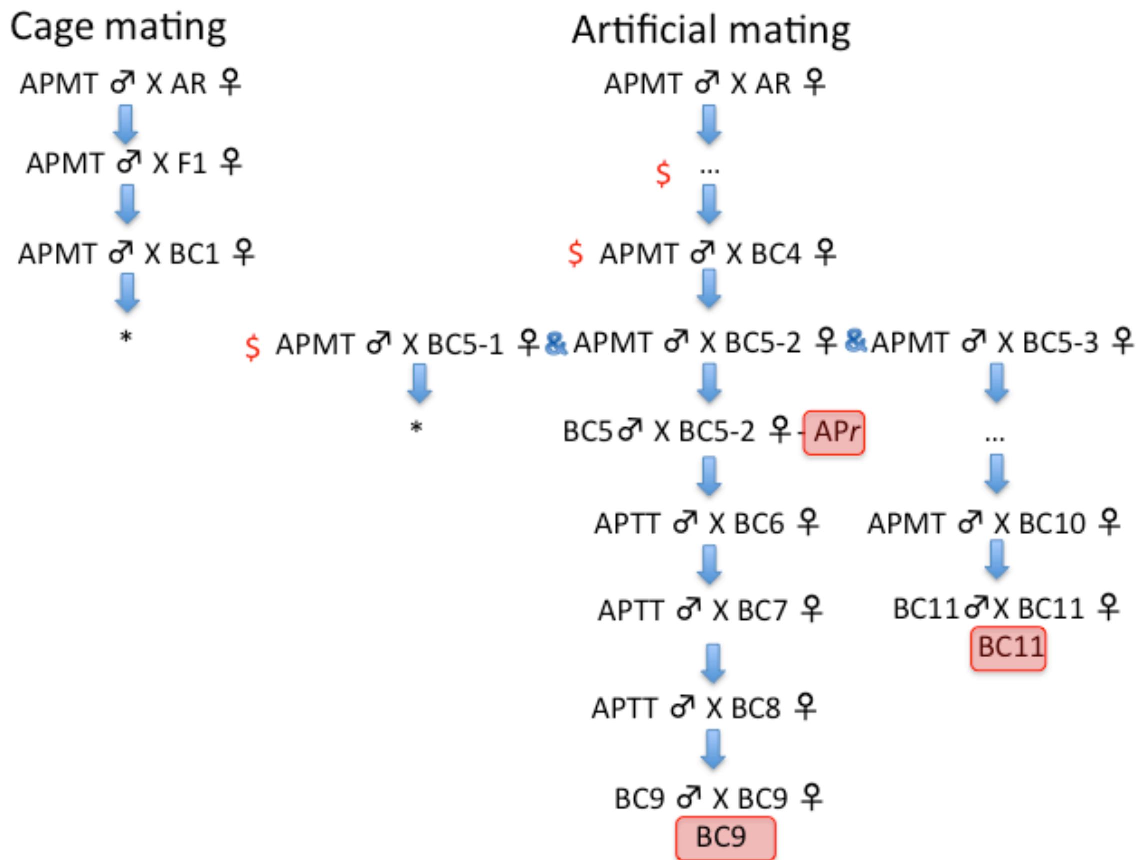


Figure 2.2 Cage and artificial mating of *Ae. riversi* and *Ae. polynesiensis*. Creation of APr, BC11, BC9 lines through initial inter-species (APMT and AR) crossing and selection against susceptibility to B. pahangi (F1 to BC5). BC9 line was created by backcrossing with APTT to add *Ae. polynesiensis* from Tetiaroa background. * lines lost through bottleneck. The '$' indicates a generation at which selection for reduced susceptibility was performed.

4. The APk line was selected for reduced susceptibility at each backcross and is not *Wolbachia* infected thus displaying only unidirectional CI (Figure 2.3). This line resulted from crosses of AK females with APMT (aposymbiotic) males in a cage mating environment. Successful mating for these first crosses was observed only after seven and nine days as opposed to 24 hours in normal laboratory conditions with *Ae. polynesiensis* (Eyraud and Quelennec 1976). This type of delay may be due to pre-mating control mechanisms which supports the distinction of the two species (Meek and Macdonald

1982) but verifies that the two species are closely related. Individuals showed hybrid characteristics with *Ae. polynesiensis* scale-patterns and rather larger size individuals (similar to AK). Size decreased after backcross 1 (BC1). F1 progeny female was backcrossed for four generations with APMT male and allowed to intercross afterwards.

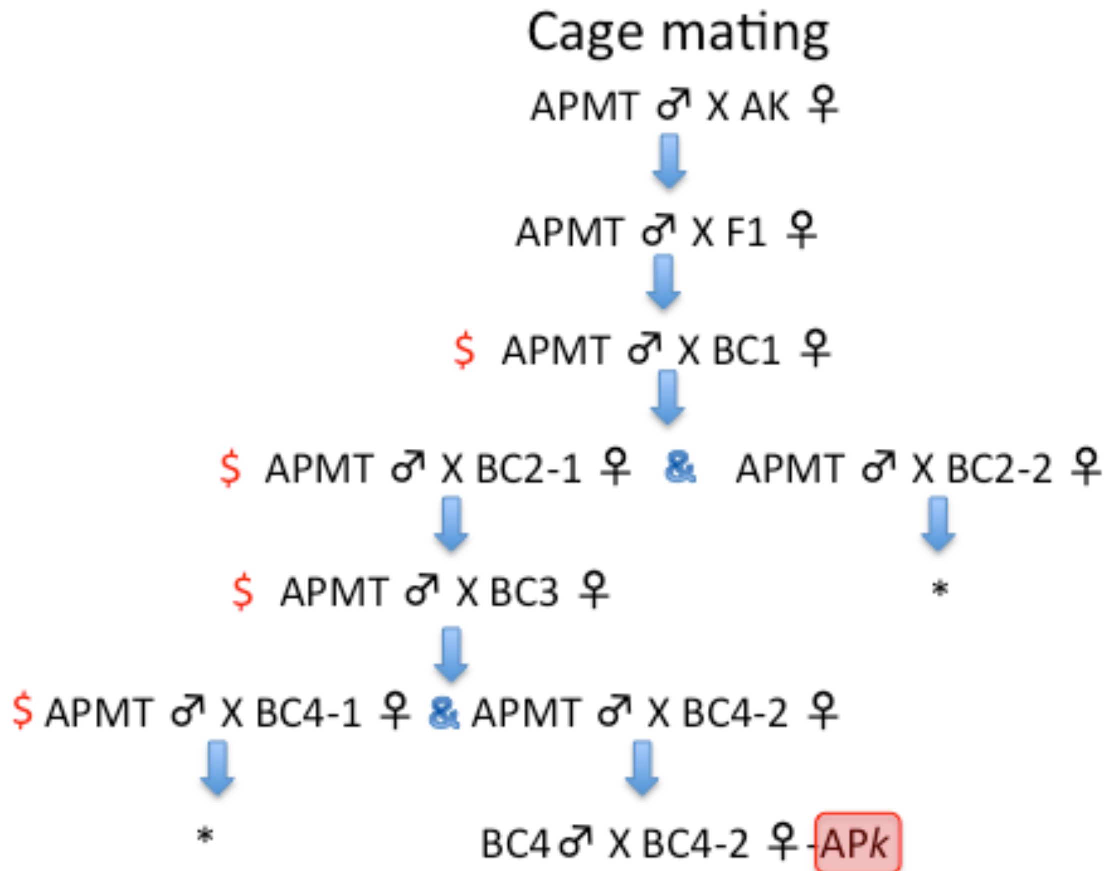


Figure 2.3. Cage mating of *Ae. katherinensis* and *Ae. polynesiensis* Creation of the APk line through inter-species (APMT and AK) crossing and selection against susceptibility to *B. pahangi*. * lines lost through bottleneck. The '$' indicates a generation at which selection for reduced susceptibility was performed.

2.4.3 *B. pahangi* susceptibility

Individuals showing no filarial worm development were selected as parents for each subsequent generation. A female with no worm infection however does not necessarily gmean that it is genetically refractory. Although dissections done immediately following filarial nematode challenges consistently displayed proper infection (100% infected), differences in quality (health) of *B. pahangi* worms may have resulted in some

worms simply dying instead of females actually being able to clear themselves of worm parasites. Also important to note was that the concentration of worms used was higher than naturally occurring *W. bancrofti* microfilarial levels (10-fold) in the human populations in the South Pacific. This was deemed necessary to increase probabilities of infecting mosquitoes at each worm challenges. For evaluation, APr was challenged with 2 mf/ml and was observed with 90% mosquitoes not being infected (Table 2.1).

APr and APk lines showed reduced susceptibility to *B. pahangi* when compared to APMT strain (control) at every worm challenge. For the APk line the reduced susceptibility was significant compared to APMT at BC1 and BC2 progenies but was not significant in subsequent progenies. The APr line was only significantly different to APMT at F1 hybrid and BC2 progenies. The mean number of L3/infective mosquito for APMT ranged from 1.8 to 3.5 (with 20 mf/ml) and was not significantly different from APr and APk progenies.

Table 2.1. Susceptibility to *B. pahangi* infections in developed *Ae. polynesiensis* strains. Strains were hybrids and backcross progenies with *Ae. katherinensis* (APk) and *Ae. riversi*. (APr). Parasite (mf) concentration ranged from 2-20 mf/ml for blood meal. The number and percentage of infective mosquitoes (L3) with significance from susceptible APMT stains are given (X^2 test).

Line	Progeny	mf/ml	# Repetition	N	# Infective	% Infective	X^2
APk	BC1	20	2	70	27	39%	*
	BC2		2	57	19	33%	*
	BC3		1	37	35	95%	NS
	BC4		1	16	10	63%	NS
APr	F1	15	2	89Ψ	0	0%	*
	BC1		1	5	1	20%	NS
	BC1	20	1	10	2	20%	NS
	BC2		6	45	5	11%	*
	BC3		1	130	107	82%	NS
	BC4		1	57	47	82%	NS
	BC5	2	1	10	1	10%	NS
	BC5	20	1	75	28	37%	NS

Ψ 28 were females fed twice.
* X^2 significantly different from the expected compatibility, P-value <0.05.

2.4.4 Egg hatch

Theoretically, crosses would have *Ae. polynesiensis* background at each backcross- F1: 50%, BC1%:75%, BC2:87.5%, BC3:93.75%, BC4:96.87% (APk), BC5:98.43% (APr), BC9:>99%. Generally, the egg hatch rate for APk and APr increased at each backcross as more genetic background from aposymbiotic *Ae. polynesiensis* is introduced. For both *Ae. katherinensis* and *Ae. riversi* lines, there seems to be a strong bottleneck effect at B1 (and BC2 *Ae. katherinensis*) progeny (hatch rate >11%), not accounting for over half of females that did not lay eggs. The general trend of egg viability showed that after the bottleneck, the egg hatch recovered to normal hatch rates. The APk (BC4 intercross) line had a 73. 89% hatch rate compared to 9.15% hatch rate at BC2. Similarly, the APr (BC5) line displayed an increased hatch rate (57.97%) from BC1 (6.92%). The BC11 line had a hatch rate of 71.03%. Similarly, the BC9 line created with APTT background in April 2012 had a hatch rate of 42.9%.

Table 2.2. Hatch rate of APk and APr lines. *Ae. polynesiensis* developed strains of hybrids and backcross progeny with *Ae. katherinensis* (APk) and *Ae. riversi.* (APr, BC11 and BC9).

Line	Mating	Progeny	N	# Eggs	%Hatch Rate	%SEM
APk	Cage	F1	4	4032	20.36%	7.82%
		BC1	7	7671	10.08%	2.46%
		BC2	9	2972	9.15%	2.68%
		BC3	23	873	33.78%	1.70%
		BC4	8	1775	19.29%	6.22%
		APk[a]	25	1460	73.89%	6.57%
APr	Cage	F1	1	3000	0.50%	n/a
		BC1	1	18	0.00%	n/a
	Artificial	F1	3	1218	11.60%	2.20%
		BC1	3	2267	6.92%	1.27%
		BC2	10	620	23.88%	6.60%
		APr[a]	8	5797	57.97%	8.41%
		BC11[a]	3	2933	71.03%	9.68%
		BC9[b]	5	828	42.99%	9.93%

[a] Line created and hatch rate measured in July 2010

[b] Line created in April 2012

2.4.5 Male survival and wing lengths

Survival assays (Figure 2.4 a-b) for APr, APk, and APMT (control) showed no significant difference for survival of females (Log-rank, *P*=0.25). However APr males suffered from significant mortality compared to APk and APMT (*P*=0.03). The projected survival curves support the hypothesis that *Ae. katherinensis* is more closely related to *Ae. polynesiensis* than *Ae. riversi.*

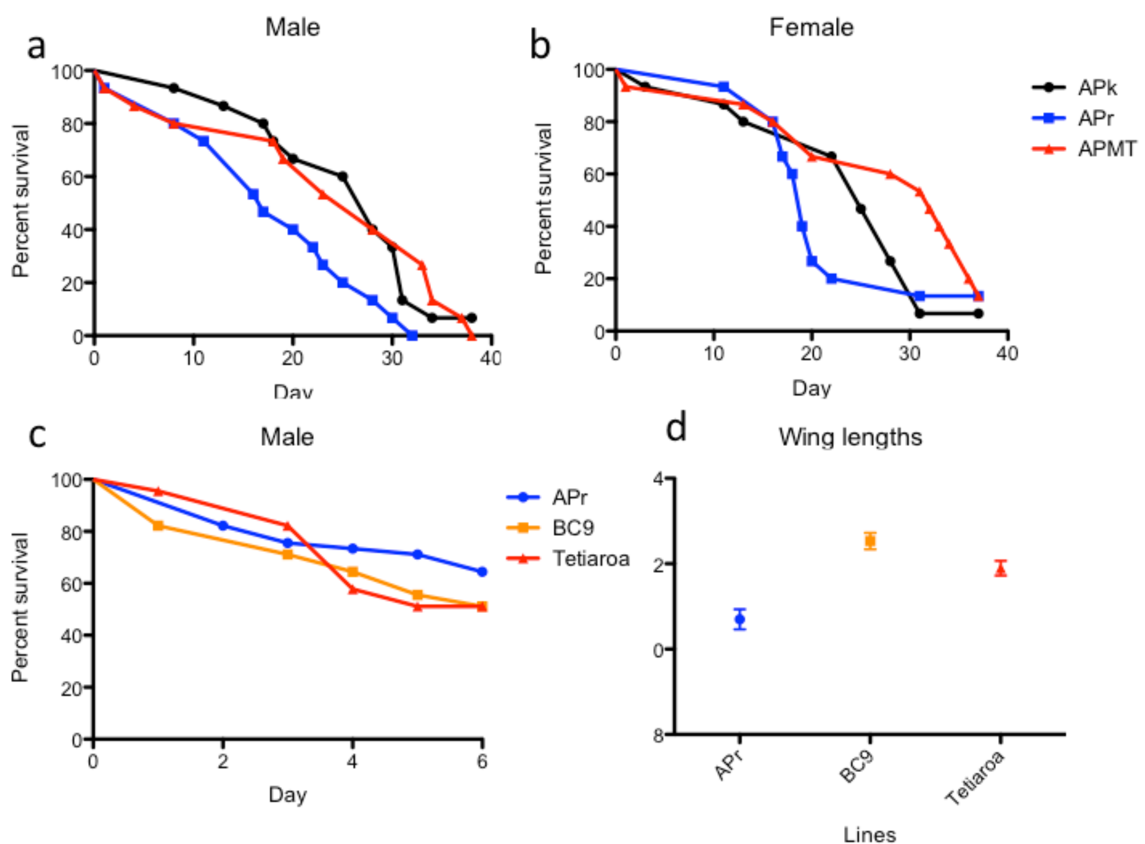


Figure 2.4. Survival curves of selected lines. Male and female total percent survival curves for APk, APr*,* and APMT lines (a-b) were mortality was taken until all mosquitoes died. Male survival curve (c) and mean with 95% CI of wing lengths (d) for APr, BC9, and APT.

In the anticipation of a weekly male release, male survival was compared between APr and BC9 with wild strain (APT) over seven days. This last comparison showed no significant difference for survival of males (Log-rank, *P*=0.44). From larvae reared in the same conditions, average male wing length for APr, BC9 and APT were 2.07mm, 2.25mm and 2.19 mm respectively (Figure 2.4). ANOVA (n=20 each) showed significant

difference in wing lengths between the three lines (*F*=93.3, *df*= 2, *P*<0.001). Tukey's multiple comparison test determined that average wing lengths were: BC9>APT>APr.

2.4.6 Male competitiveness and suppression assay

When APk males were in competition with APM males for APMT females, crosses of APk male with APMT females were compatible (eggs hatched) while crosses of APM males with APMT females were incompatible. For analysis the following rates were used: compatible hatch >62.9% (lowest male and female APMT cross hatch rate), and incompatible <62.9%. Two replications showed that 54% and 56% respectively of the crosses were compatible (when intermediate were removed). Assuming equal male competitiveness, goodness of fit test showed no significant difference (X^2, P=0.93) between expected rate (50%) and observed rate for the 25:25 cages (Table 2.3).

Table 2.3. Male mating competitiveness of the APk line. APk males were in competition with APM males for APMT females in cage trial. The mean and SEM hatch rate is for compatible broods.

	Percent compatible[β]	Compatible	Incompatible	Mean hatch	SEM
APk rep1	54.84%	17	19	86.59%	3.17%
APk rep2	56.25%	18	17	91.23%	2.71%
Control	100%	26	0	91.02%	1.99%

[β] Assuming equal male competitiveness, goodness of fit test showed no significant difference between expected rate: 50% for APk repetition and 100% for the control.

For the APr line, all 37 broods from the 0:50 cages (APA:APr) resulted in no egg hatch confirming cytoplasmic incompatibility when two *Wolbachia* types are crossed (Figure 2.5). From the 50:0 cages, three broods (10%) did not hatch which may be explained by natural sterility or unmated females. Therefore for the 25:25 cages the expected proportion of broods that would not produce egg hatch was expected to be at 45%, assuming equal mating competitiveness. In the 25:25 cages, there was no significant

difference between the observed and expected number of hatching broods for the 25:25 cages (X^2, P>0.99). The observed brood hatch rate decreased from 90% to 0%, inversely proportional to the number of APr males present (R^2>0.99).

The hatch rate of compatible brood for the 50:0 and 25:25 cages were 80% and 81% respectively. If females used sperm from multiple males, the expected hatch rate would be lower in the 25:25 cages. A comparison of egg hatch rate from theses females did not differ significantly (Kruskal-Wallis, P=0.71) supporting the hypothesis that females utilized sperm from only one male.

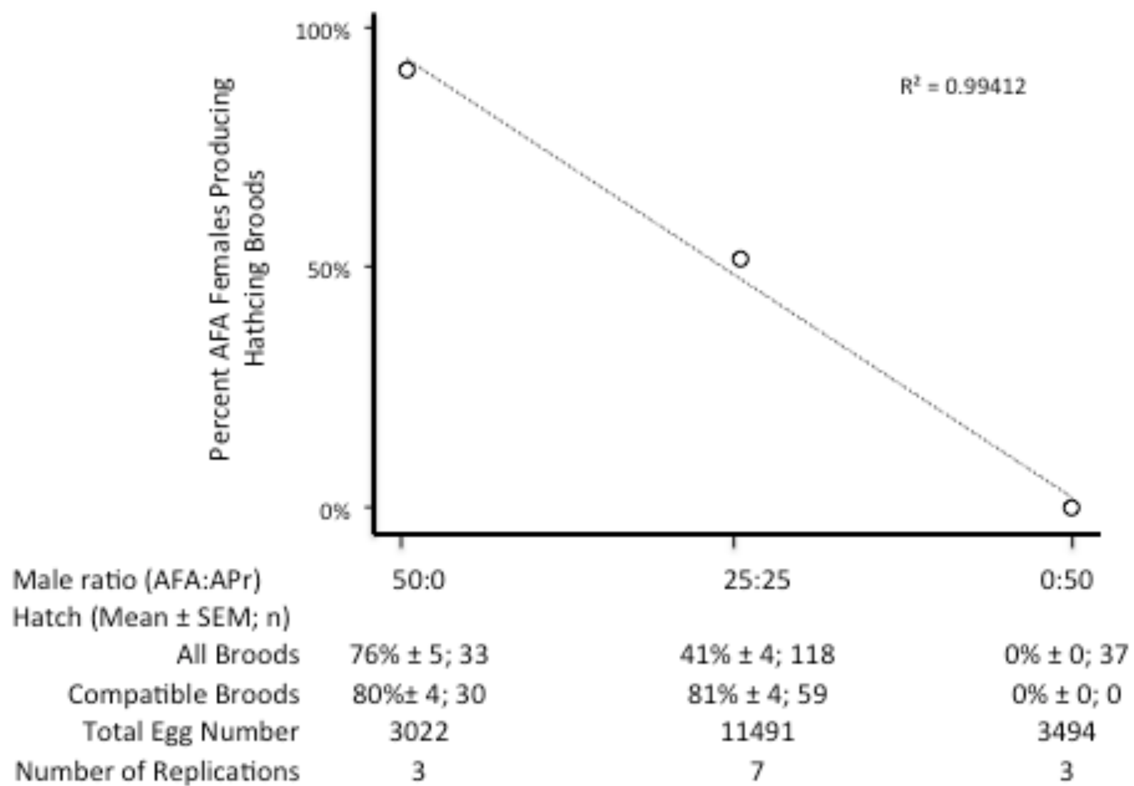


Figure 2.5. AP*r* male mating competitiveness assay. Females were considered to have produced a hatching brood when eggs hatch was >0%. Circles indicate the mean number producing hatching broods for each male ratio. Solid line is the predicted values of compatibly mated brood calculated assuming equal competitiveness of APA and APr males. R^2 value is fitted to the observed values. All broods are the average egg hatch resulting from both compatible and incompatible broods.

For BC9, cage suppression assays using 1:1 and 1:40 ratios i.e. 50 wild and 200 BC9 for ratio 1:40 were conducted in three replications (Table 2.4). From the 50:50 ratio,

the percentage of compatible broods ranged from 31% to 7% and for the 50:200 ratio 17% to 7%. Assuming equal mating competitiveness, there was no significant difference between the observed and expected number of hatching broods except for replication 2 and 3 for 50:50 (X^2, P<0.01).

Table 2.4 Suppression assay using BC9. Females were considered to have produced a hatching brood when egg hatch was >0.3%. The number of successful females that laid eggs from 50 virgin females in cages loaded with 50:50 or 50:200 APT and BC9 males. Hatch is the average % egg hatch resulting from both compatible and incompatible broods with SEM.

Rep	No. female	Percent Incompatible	Incompatible	Hatch	SEM
		50:50			
1	32	31%	10	79%	9%
2	28	14%*	4	48%	19%
3	14	7%*	1	87%	n/a
		50:200			
1	30	17%	5	88%	4%
2	30	7%	2	62%	3%
3	11	9%	1	87%	n/a

* X^2 significantly different from the expected compatibility, P-value <0.05.

2.4 Discussion

Local elimination of LF could in theory be achieved by replacing the vector species with a refractory population of *Ae. polynesiensis* or by suppressing the vector population for an extended period; both strategies should be capable of disrupting the transmission of *W. bancrofti* within the community. For a replacement strategy, the strains would ideally be fully refractory to the parasite as found in *Ae. aegypti* where refractoriness is complete and influenced by simple mendelian inheritance (f^m) (Macdonald 1962, Macdonald and Ramachandran 1965). From this study, differences in susceptibility to *B. pahangi* of backcross progeny compared to a susceptible strain were significant for BC1 and BC2, and only FI from introgressed *Ae. katherinensis* and *Ae. riversi* lines respectively. From BC1 (*Ae. riversi*) and BC3 (*Ae. katherinensis*) onward,

despite selection of non-infected mosquitoes in preceding worm challenges, females did not display significant reduction in susceptibility (number of infective L3 larvae) compared to the control strain. Although challenges of APk and APr lines with *W. bancrofti* at levels of microfilaremia found in the community would provide a more accurate degree of susceptibility, results from such challenges would probably be similar to *B. pahangi* challenges found in this study. Although prior cross experiments demonstrate that the *Wuchereria* refractoriness is dominant, introgressive hybridization crosses with *Ae. katherinensis* and *Ae. riversi* suggest either an incomplete dominance for the refractoriness phenotype, or that the selection process was not sufficiently robust, in other words the percentage of females with no developing *Brugia* that were not genetically refractory was simply too high to allow the refractory phenotype to be selected. Obtaining refractoriness in *Ae. polynesiensis* from crossing with species from the *Ae. scutellaris* complex is technically extremely challenging, and thus other methods should be considered.

One of the methods that may provide the basis of a replacement strategy of *Ae. polynesiensis* is from *Wolbachia* infection itself, since some strain-host combinations have shown to up regulate the innate immune system (Kambris et al. 2009). This last study demonstrates that the presence of *w*MelPop introduced into *Ae. aegypti*, by means of embryonic microinjections from *Drosophila*, inhibits the development of *B. pahangi* (Kambris et al. 2009). Moreover *w*MelPop infected *Ae. aegypti* also demonstrate refractoriness to dengue and chikungunya virus (Moreira et al. 2009) suitable for field releases in northern Australia resulting in its successful establishment of wMel (Hoffmann et al. 2011). Creation of an *Ae. polynesiensis* line infected with *w*MelPop to reduce transmission of LF would be very attractive, particularly since *Ae. polynesiensis* is a vector of dengue virus and potentially chikungunya virus (Rozeboom et al. 1954).

Recent studies of *Ae. polynesiensis* infected with *w*AlbB from superinfected *Ae. albopictus* (refractory to LF), demonstrate the ability of the MTB strain to reduce the number of infective (L3) *B. pahangi* larvae (Andrews et al. 2012). One key factor for transmission is directed by the productivity of infective larvae in sufficient numbers, knowing that less then 20% of *W. bancrofti* larvae will mature and leave the mosquito (Ramachandran and Zaini 1968) and less than 6 % will be able to enter the final human host (Zielke 1976). A modest reduction in susceptibility of the mosquito population to be released may therefore be sufficient to break the transmission cycle. Although the MTB strain is not refractory, its reduced susceptibility may arguably be sufficient to break the transmission of *W. bancrofti* if indeed *B. pahangi* hold as a suitable model for parasite infections.

Lymphatic filariasis elimination through novel vector control may be achieved through releases of CI males to either obtain permanent elimination of the vector species or transient elimination for a period that extends beyond the lifespan of adult *W. bancrofti.* To that end, characterised CI lines from this study have potential for releases. The APr line in cage populations showed equal male mating competitiveness to wild males and also demonstrates strong CI when mated with wild *Ae. polynesiensis* due to the presence of an incompatible *Wolbachia* strain. The BC9 line also showed the ability to suppress wild types in cages and has the advantage compared to BC11 (and APr) of the added Tetiaroa background (APTT). This may be reflected in larger wing lengths for BC9 compared to APr with lengths closer to that for APT. Routine collection of field mosquitoes followed by tetracycline treatment for subsequent backcrosses into existing CI lines to diversify genetic background would avoid negative effects related to bottlenecks from inbreeding and provide desirable male characteristics essential for IIT (i.e. size, hatch rates, mating competitiveness). Introduction of genetic backgrounds

closer to that found in wild populations would overcome the reduction of fertility for a number of generations after interspecies introgression explained by chromosomal defects. These defects are too often intensified in laboratory settings where high degree of inbreeding is observed. In addition, because minor levels of genetic variability in *Ae. polynesiensis* in the Society Islands, backcrossing with aposymbiotic *Ae. polynesiensis* from the target population would allow the CI line to accumulate an allelic distribution similar to the target population (Brelsfoard and Dobson 2012).

In the context of IIT, it is important to know whether CI males are competitive and whether the females of the vector species are monoandrous (inseminated by a single male) or polyandrous (inseminated by multiple males) (Curtis 1985). This laboratory study and that of Brelsfoard et al (2008) supports the hypothesis that females utilized sperm from only one male based upon comparison of hatch rates of compatible broods in cages. Studies using microsatellite of field collected females have showed a varying degree of polyandry in vector species from 2.5% of *An. gambiae* in Mali (Tripet et al. 2003) to 25% of *Ae. albopictus* on La Reunion Island (Boyer et al. 2012). The frequency of multiple inseminations in vector species is vital in determining the success of SIT male programs. There is a general lack of baseline field ecological data for *Ae. polynesiensis* compared to other mosquitoes, particularly concerning behaviour including dispersal, survival, and mating.

The transition from laboratory to an area-wide control program represents a significant challenge. Semi-field systems (SFS) are defined as enclosed environments, situated within the natural ecosystem of the target disease vector and exposed to the local ambient conditions (Knols et al. 2003, Ferguson et al. 2008). Studies using SFS for the CP strain have provided essential safety and male mating information as an intermediate step to an area-wide control strategy (Chambers et al. 2011). Pacific island settings can

facilitate the implementation of area-wide elimination strategies by providing naturally isolated mosquito populations with limited immigration. The same CP strain was released in small numbers on a islets (motu) of Raiatea in a pilot field trial for proof of concept and in the process allowed description of pertinent life-history and mating competitiveness which could not be as accurately determined in laboratory or SFS settings (O'Connor et al. 2012). In the context of SIT, further ranging trials are often not only needed to describe ecological parameters but also answer essential logistical questions related to mass production and male mating competitiveness. This was seen for SIT of *Ae. albopictus* in Italy where laboratory tests and initial pilot studies (Bellini et al. 2007) were followed by multiple ranging field trials on sexing of males and sexual performance of release males to estimate the effort required to obtain suppression for the local population (Bellini et al. 2013).

Before releases using the aforementioned CI lines, vector competency must be measured in wild females in the event of an accidental release of females. Although this would be unlikely lead to replacement of the wild population, CI lines should not demonstrate greater parasite competency, as this could forfeit the purposes of releases by increasing transmission. Vector competency was not assessed for the CP strain; APr females showed equal vector competency to *B. pahangi* compared to the wild-type APMT. Future efforts must define vector competency of released lines to *W. bancrofti* relative to wild type mosquitoes. Ideally comparisons would be done to collected mosquitoes from the intended release location to account for natural variations in vector competency of *Ae. polynesiensis* (Failloux et al. 1995).

Using CI lines in an IIT framework may be suitable tool against LF but may also address mosquito-induced nuisance. In French Polynesia, tourism is the main economic income. A majority of hotels house their guests in open bungalows with open-air

common areas (lobby, dinning, pool, etc). A common complaint from guests during daylight hours is the nuisance caused by diurnal *Aedes* species (Bossin and Hapairai 2012). Since these hotels are in rural areas or on motus, the main target of intense insecticidal spraying has been for *Ae. polynesiensis.* Used as part of an integrated approach, releases of CI males would provide an effective solution as males do not bite and would, if successful matings occur, eliminate biting female offspring from these matings. Moreover, *Wolbachia*-based elimination is an alternative to chemical control that provides the added value to hotels following the increasing demands for "eco-tourism" and sustainable development.

The non-transgenic, *Wolbachia*- bacterial elimination strategy provides a socially acceptable alternative to transgenic approach (i.e. RIDL) that requires appropriate positioning of government regulations (Harris et al. 2012). An simple explanation to decision makers stating that many arthropods were naturally infected with *Wolbachia* (Werren 1997) successfully granted ILM importation permits of B-type *Wolbachia* infected mosquito into French Polynesia intended for field releases. To this date, despite persuasive results obtained in other countries such as Brazil, Cayman Island, and Malaysia (Lacroix et al. 2012), transgenic mosquitoes have not received approval for importation into French Polynesia first aimed at confined laboratory evaluation before field releases. The demonstration of an epidemiological impact on LF transmission and economical advantages through vector control would likely encourage the development and implementation of sustainable vector control approaches to other regions in the Pacific where *Ae. polynesiensis* is present.

Chapter 3: Field evaluation of monitoring tools and population studies for *Aedes polynesiensis*[1]

3.1 Introduction

Knowledge of the dynamics of adult *Ae. polynesiensis* populations and patterns of mosquito dispersal and migration is fragmented at best (Lardeux et al. 1992). This lack of baseline ecological data has impeded the used of SIT and IIT in large-scale application against mosquito vectors (Benedict and Robinson 2003, Ferguson et al. 2005). Population size and density estimates in particular are critical to measure efficacy of intervention strategies involving releases of modified or *Wolbachia*-transinfected mosquitoes into the field. Understanding spatial distribution patterns is also vital to optimize release and monitoring efforts during implementation of control programs. Mark-release-recapture (MRR) techniques have been successfully applied to the study of *Aedes* mosquito populations in the field. Recent applications have monitored population dynamics of *Ae. albopictus* in relation to chikungunya virus transmission in Rome, Italy (Marini et al. 2010), *Ae. aegypti* for dengue transmission in Queensland, Australia (Russell et al. 2005b) and the preliminary field testing of genetically engineered sterile males in Pahang, Malaysia (Lacroix et al. 2012). MRR experiments were also recently used with success to study the adult population dynamics of *Ae. polynesiensis* (Mercer et al. 2012b).

The assessment of vector-borne disease transmission risk and the field evaluation of IIT rely strongly on the ability to estimate the size, density, distribution and dispersal

---

[1] Parts of this chapter have been published in the Journal of Medical Entomology:

- Population studies of the filarial vector *Aedes polynesiensis* (Diptera: Culicidae) in two island settings of French Polynesia 50, 965-976.
- Field evaluation of selected traps and lures for monitoring the filarial and arbovirus vector, *Aedes polynesiensis* (Diptera: Culicidae), in French Polynesia. 50, 731-739.

capacity of adult mosquito populations (Morrison et al. 2008). Moreover, diagnostic tools are required to assess the status of LF in countries that have transitioned to a post-MDA surveillance phase or are still implementing preventive chemotherapy. The detection of microfilaria in human or vector populations is considered an important test, complementary to the monitoring of filarial antigenemia, to assess the efficiency of LF elimination programs (Ottesen 2006, Ramzy et al. 2006, Weil and Ramzy 2007). PCR assays have been developed (Rao et al. 2006) that are highly sensitive and specific for the detection of *W. bancrofti* DNA in human blood samples as well as in mosquito vectors (Williams et al. 2002, Plichart et al. 2006, Boakye et al. 2007, Farid et al. 2007, Plichart et al. 2007). When parasite prevalence is low, large numbers of mosquitoes are required to detect any infections by xenomonitoring. Consequently, sampling methods must be sensitive in capturing mosquitoes at low densities (Chambers et al. 2009) and compatible with cost-effective implementation of multiple traps over extended geographic areas to ensure collecting sufficiently large numbers.

While numerous methods to sample adult mosquitoes exist, most are unsuitable for *Ae. polynesiensis* because of their limited sensitivity for capturing adequate numbers of this species (Suzuki and Sone 1974, Samarawickrema et al. 1987a, Samarawickrema et al. 1992b, Lardeux et al. 1995, Russell et al. 2005a). Among the collection techniques investigated for *Aedes* population sampling are the Fay-Prince trap, the carbon dioxide (CO_2)-baited Centers for Disease Control (CDC) light trap (Schmaedick et al. 2008) and the CDC Backpack aspirator (Williams et al. 2006) used to sample typical *Ae. aegypti* harborage sites both indoor and around houses. The BG-Sentinel (BGS) trap (BioGents GmbH, Regensburg, Germany) has shown potential for sampling adult *Ae. polynesiensis* populations in American Samoa and French Polynesia (Schmaedick et al. 2008, Chambers et al. 2009, Mercer et al. 2012a, Mercer et al. 2012b). Although the human

landing collection method is variable in catch rates due to differences in the human bait attractiveness, this method has been used for adult *Ae. polynesiensis* sampling (Russell 2004, Russell et al. 2005a). However, safety concerns related to the occupational risk of exposure to vector-borne diseases make human bait collections (HBC) undesirable for *Ae. polynesiensis* monitoring particularly in LF endemic areas or during periods of arbovirus transmission. Unlike human landing catch and HBC which are influenced by the variable performance and attractiveness of operators and can be impractical in a variety of environments, urban and natural (Silver 2008), the BGS trap provides a standardized collection method. Collections with a CDC backpack aspirator of host seeking mosquitoes attracted to an operator are comparable to human landing collections (Schoeler et al. 2004), but none of the previous studies described above have compared the sampling efficiency of the BGS against the HBC using the CDC backpack aspirator for collecting *Ae. polynesiensis.*

In order to facilitate the development and evaluation of novel control strategies, the population size, migration, and dispersal patterns of *Ae. polynesiensis* were examined in Moorea island and Tetiaroa atoll. The frequency of filarial parasites in the Hotutea mosquito population was also investigated to assess the risk of LF transmission in the valley. Also, two models of Biogents traps for monitoring populations of adult mosquitoes were monitored again in Moorea and Tetiaroa atoll. The evaluation included the BGS trap and the more recently commercialized and cheaper BGM trap with and without BG-lure or carbon dioxide (CO_2) plus octenol as an attractant. CO_2, which is typically supplied via dry ice, gas cylinders or propane combustion is expensive and often difficult to procure in often remote tropical islands settings. The use of yeast-sugar fermentation, a comparatively inexpensive and convenient source of CO_2 (Smallegange et al. 2010), was investigated in combination with octenol. The efficacy of collections from

each of these trapping devices was compared to the HBC method using a CDC backpack aspirator to determine their potential as sampling alternatives.

3.2 Objectives

The overall objective was to characterize the population ecology and compare monitoring tools for future *Wolbachia*-based vector control involving either a replacement with refractory lines or elimination of a vector population using CI lines (i.e. APr).

The specific aims were to estimate population size and dispersal using MRR and evaluate trapping methods in Latin square experiments (LSE) on two typical island settings:

1) On Moorea, in the Hotutea valley, the aim was to estimate population sizes and densities assuming an open population, and describe the distribution and dispersal capacity of adult mosquito populations at various elevations. Traps with lures were evaluated for monitoring adult populations of mosquitoes in the Hotutea and Atiha valleys:

- BGS trap with BG-Lure or CO2 and Octenol
- BGM trap with BG-Lure or CO2 and Octenol
- HBC

2) On Tetiaroa, on Motu Auroa, the aim was to assess migration levels flanking motus, estimate population sizes and densities, and describe the distribution and dispersal capacity of adult mosquito populations. Traps with lures were evaluated for sampling adult populations of mosquitoes on motus Tiaraunu and Hiraanae:

- BGS trap with BG-Lure or CO2 and Octenol
- HBC

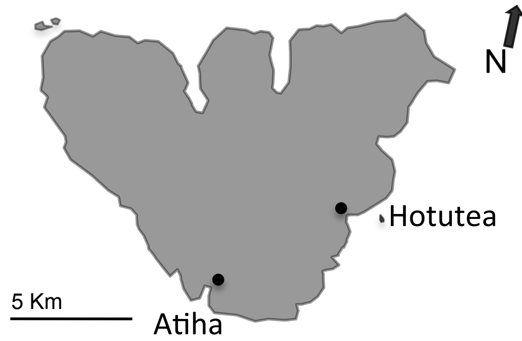
3.3 Materials and Methods

3.3.1 Study Sites

3.3.1.1 Moorea

The first MRR study was conducted in the Hotutea valley (17°33'00.94" S 149°49'09.07" W) in the district of Afareaitu (volcanic island). The valley is flanked by steep volcanic hills on the north, south, and west sides (Figure 3.1). Likely sources of mosquito immigration would come from the inhabited coastal area directly facing the Hotutea valley. Most human dwellings are found near the coast at low elevation (from shoreline to 60 meters above sea level). These dwellings range from small (5 m x 5 m) wooden/tin houses to large public buildings (gymnasium). The valley includes 198 households, a stadium, public hospital, schools, grocery stores, and many small annexes. Vegetation in inhabited areas of the valley (low and mid elevation) typically displays mango, breadfruit and coconut trees with many ornamental plants. In uninhabited areas (high elevation) the vegetation is typical of subtropical forest found on Pacific high islands. Water streams are covered by Tahitian Chestnut *Inocarpus fagifer*, with short *Hibiscus tiliaceus* understory. This understory progressively becomes the primary cover further away from the main stream. At higher elevation, the slopes are covered with sword grass *Miscanthus floridulus* and ferns.

Trap evaluation in Moorea was done by replicating the LSE in the Hotutea valley and Atiha valley (17º33'15.00" S 149º49'27.00") districts on the windward side of the island (Figure 3.1). The main breeding containers were rat-chewed coconuts, abundant on the ground and domestic water containers.



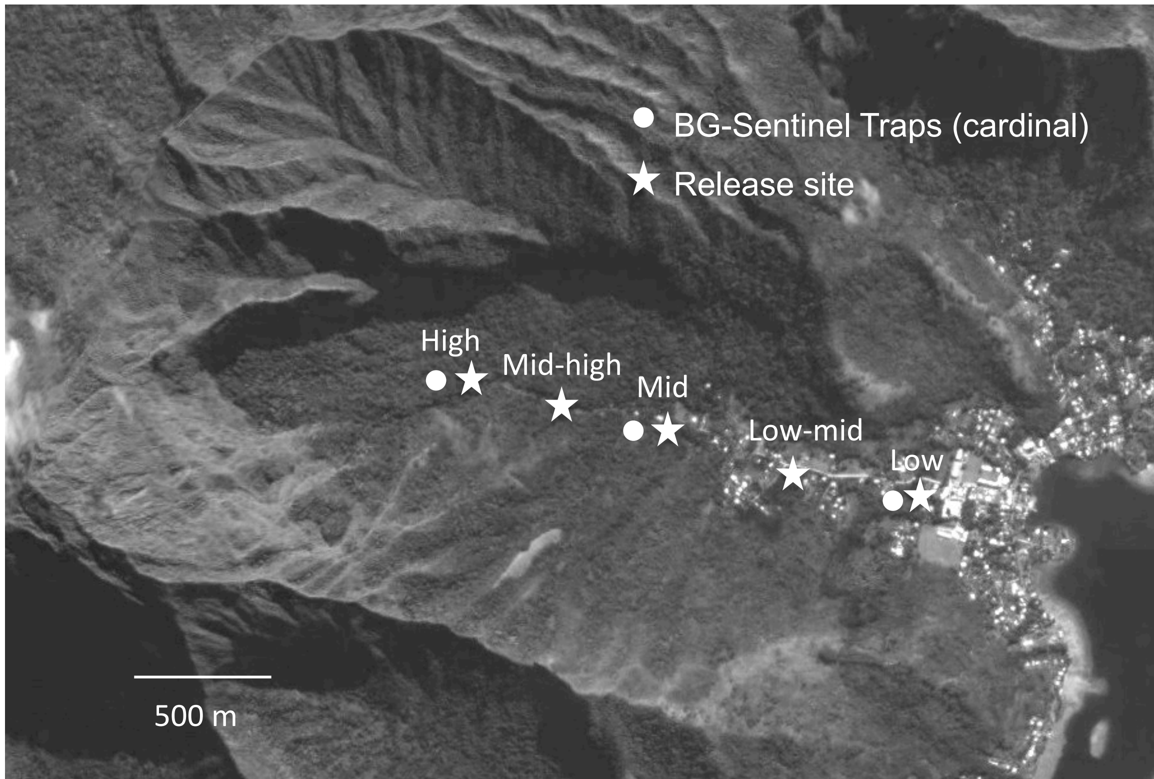


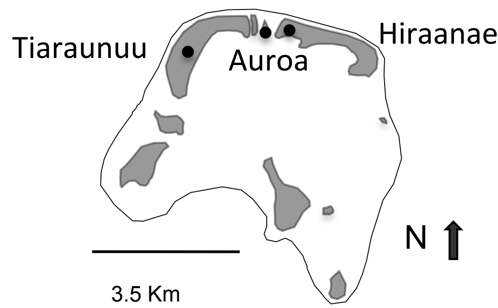
Figure 3.1. Map of LSE experiments on Hotutea and Atiha Valleys on Moorea. Aerial view of the Hotutea valley for the MRR experiment. Mosquito releases were conducted at five release sites (stars). Release sites are separated by approximately 500 m. Mosquito sampling was done at low, mid, and high elevation (circles) using a set of up to 16 baited BGS traps distributed along the four cardinal directions from the point of mosquito release.

3.3.1.2 Tetiaroa

The second MRR study site is the atoll of Tetiaroa (17°00'54.77" S 149°35'04.00" W) located 54 km north of Tahiti. This low coral island, which stretches over 2.3 square miles (6 square km) is divided into 13 motus (islets) of varying surface areas sometimes separated by large stretches of water. One of these islets is motu Auroa

located on the north end of Tetiaroa (Figure 3.2). This motu is around 165 m wide and 430 m long for an area of approximately 3.2 hectares and its maximum elevation is 3 m above sea level. This motu and its neighboring islets (motu Tauini and motu Hiraanae) were selected for the study. The stretch of water separating Auroa from Tauini (west) and Hiraanae (east) is 77 m and 190 m respectively (Figure 3.2). These motus were similar in vegetation and topography to motu Auroa. Tropical "Alizés" trade winds generally blow from east to west. Vegetation on the north end of the motu is essentially composed of shrubs *Pemphis acidula*, with coconut palms *Cocos nucifera*, *Guettarda speciosa* and *Pisonia umbellifera* on the rest of the motu.

On Tetiaroa, trap evaluation was conducted by replicating the LSE on Tiaraunu (16º58'23.00" S 149º33'56.00") and Hiraanae (16º58'25.00" S 149º33'17.00"). The main water-holding containers found were rat-chewed coconut shells that were abundant on the ground.



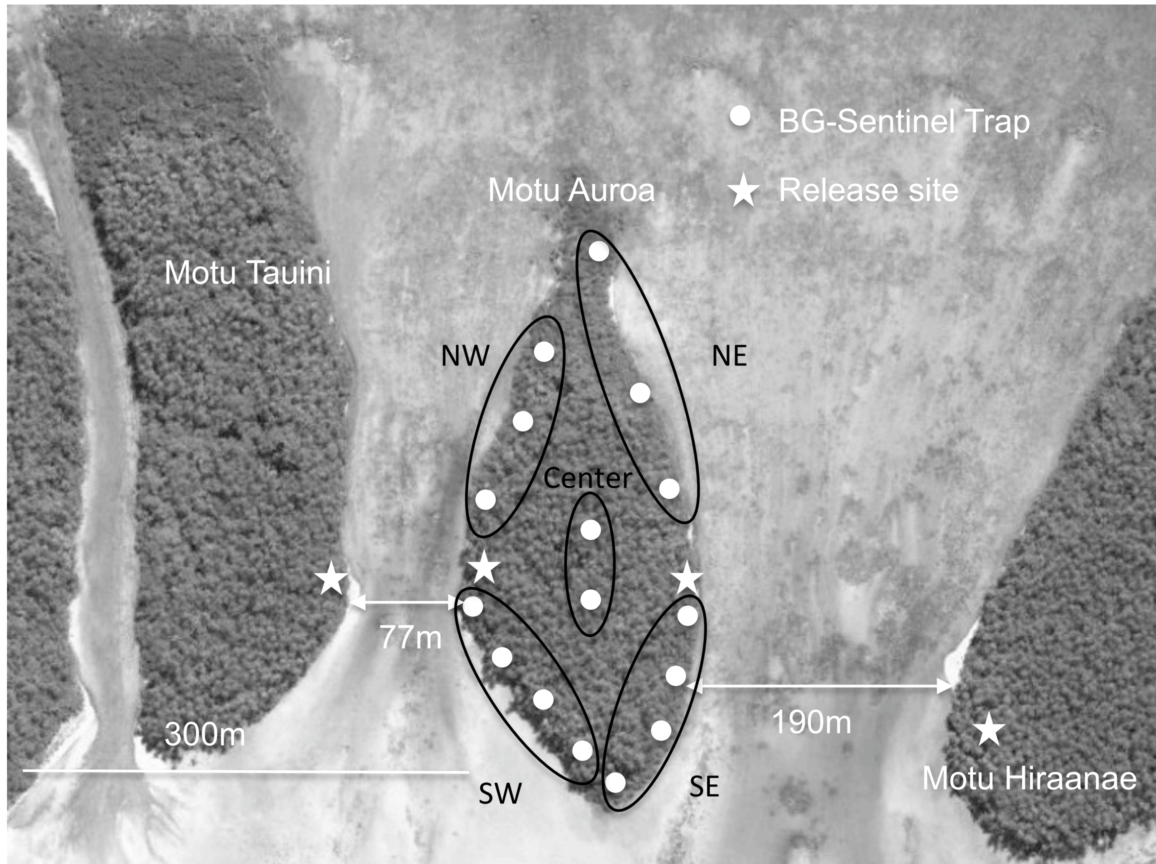


Figure 3.2. Map of LSE experiments on motu Tiaraunuu and Hiraanea. Aerial view of motu Auroa on the atoll of Tetiaroa. Mosquito releases were conducted at four release sites (stars): one site on each side of motu Auroa (WA and EA), and one site on each shoreline of the islets neighboring motu Auroa (WT and EH). Collection of mosquitoes on the periphery and at the center of motu Auroa was done using 16 baited BGS traps (white circles) partitioned into four quadrants (NW, NE, SW, SE) and a center area (black ellipses).

3.3.2 Mark-Release-Recapture

Ae. polynesiensis colonies from Hotutea and Tetiaroa were established and amplified (F3). For MRR, eggs were hatched under vacuum at each generation. After 48 hours, 200 larvae were distributed into each larval pan (30 cm x 20 cm x 7 cm) containing

1.5 L of tap water. Liver powder (MP Biochemicals, Solon, OH) was provided ad libitum (approximately 600 mg of powder supplied over the entire larval cycle). Sex separation and initial count was done at the pupal stage prior to emergence.

3.3.2.1 Mark/Release

Laboratory reared male and female mosquitoes were allowed to emerge into respective cages (30cm x 30cm x 30 cm) each containing ≈700 individuals. Once emerged, adults were provided a 10% sucrose solution as energy source and the cages were placed in 25-30°C and 65-80% RH holding conditions. Adult cages were covered and transported to each study site. To mark mosquitoes, fluorescent powders (Day Glo, Switzer Brothers, Cleveland OH) of various colors were applied using a 250 ml polyethylene wash bottle (Thermo Fisher Scientific, Pittsburg, PA) covered with a 100μm mesh cloth (Figure 3.3, a). Fluorescent powder was applied through the cage screen by brief squeezes of the wash bottle and mosquitoes were released within minutes (Figure 3.3, b). Mosquitoes were three to four days old and sugar-fed at time of release (host seeking). Marked and non-marked male and female specimens were held for control in separate cages under laboratory conditions and survival was recorded for 12 days.

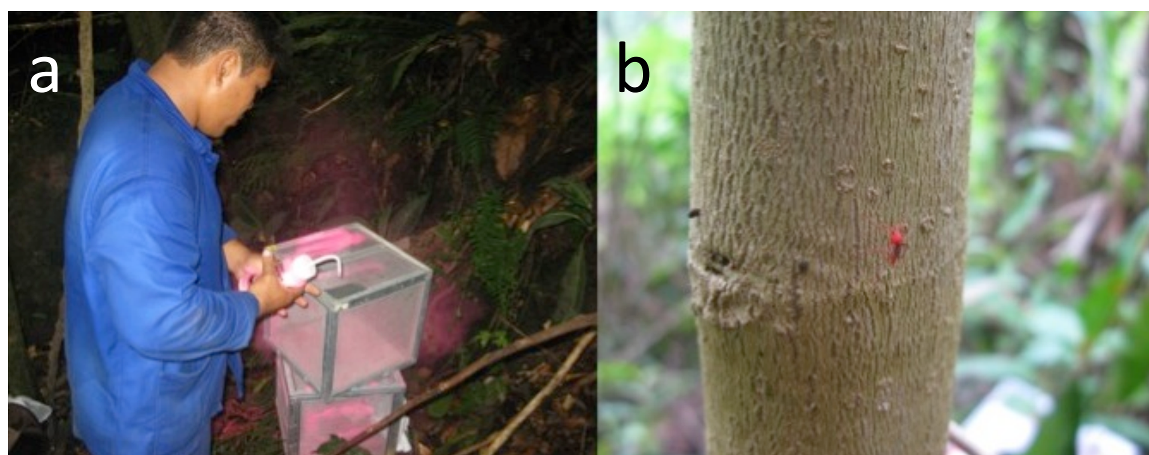


Figure 3.3. Mosquito dusting for MRR. Caged mosquitoes were dusted using Dayglo® fluorescents powder (a). Following dusting, mosquitoes were released (b).

For Hotutea, cohorts marked with fluorescent powders of different colors were released at stations each separated by 500 m, selected along an altitudinal transect (Figure 3.1, Table 3.1). The low elevation (17°33'3.74"S 149°47'45.28"W) was at 18 m above sea level 500 m away from the coastal line, low-mid elevation (17°32'59.90"S 149°48'2.49"W) at 30 m, mid elevation (17°32'56.64"S 149°48'17.00"W) at 65 m, mid-high elevation (17°32'51.90"S 149°48'34.94"W) at 110 and the high elevation (17°32'48.33"S 149°48'52.49"W) at 215 m. Males were marked and released at all five points over a two-hour period. Females were released similarly with the exception of the lower two release sites considered too close to human dwellings for female releases.

For Tetiaroa, mark and release was done on motu Auroa to measure intra-motu dispersal and on the shorelines of its two neighboring islets motu Tauini and motu Hiraanae, to investigate the potential for inter-islet mosquito migration (Figure 3.2). Powders of different fluorescent colors were used to distinguish between four release sites: one site on the west shoreline of motu Tauini (WT), one on the east shoreline of motu Hiraanae (EH) and two release sites along the west and east shorelines of motu Auroa (WA and EA respectively).

3.3.2.2 Recapture

One day after release, mosquitoes were sampled daily for five consecutive days using BGS traps baited with BG-Lure (Biogents, Regensburg, Germany). Traps were powered by 12-V (7 amp) non-spillable batteries, charged in parallel in groups of four at 24 hr intervals (Lacroix et al. 2009).

3.3.2.3 Sampling routine

Mosquitoes were collected at three of the five release sites (1,000 m apart) in the Hotutea valley at low, mid and high elevations (Figure 3.1). Up to 16 BG sentinel traps

were placed at each of the three sampling elevations: four traps along each cardinal direction (north, south, east, west) at 25 m, 50 m, 100 m, and 250 m away from the point of release (Figure 3.1). Hence, the Mid50E sampling refers to BGS trap located 50 m east of the mid elevation release point. The approximate sampling area was of 1.9 hectare for low and mid, and only 1.0 hectare for high elevation because the 250 m sampling locations on the north and west were inaccessible (cliffs). In total, three sampling zones and 46 BGS traps were used in the Hotutea experiment. This release-recapture design allowed measuring potential dispersal at distances up to 2,000 m from the release point. For Tetiaroa, recapture was done using 16 BGS traps distributed across motu Auroa (Figure 3.2). The mean distance between release (WA, EA) and recapture sites (BGS traps) was measured to check the significance of distance differences. There was no significant difference in the mean distance (t = 0.3199, df = 30, P = 0.751) separating WA (119.7 m, SEM ± 13.01 m) and EA (125.3 m, SEM ± 11.83 m) from the BGS traps.

3.3.3 Latin Square Experiment

3.3.3.1 Carbon dioxide production

CO_2 was generated by mixing dry instant yeast -*Sacchoromyces cereviviae* (Fermipan red, Casteggio Lieviti srl, Casteggio, Italy), powdered sugar (Chelsea, Auckland, New Zealand) and tap water in 5L plastic bottles. Yeast-produced CO2 was delivered to the trap CO_2 intake using a silicone tubing connection. The average volume of CO_2 produced from a range of sugar concentrations was first estimated (Smallegange et al. 2010) before conducting mosquito sampling tests in semi-field conditions. Smallegange et al. (2010) suggested that 35 g of yeast in 2.5 L of tap water produced the most carbon dioxide. In the present experiment, the same amount of yeast was mixed with either 600 g, 700 g, 800 g or 900 g of sugar. CO2 yield was measured one hour after mixing and again 24 hours later.

3.3.3.2 Sampling devices

The BGS and BGM traps were evaluated against the HBC using a battery powered modified CDC backpack aspirator (model 1412, John W. Hock Company, Gainesville, FL) collection. HBC was used with a 2-person operating team involving a static human volunteer acting as bait and an aspirator operator, both wearing long trousers and shirts for protection from mosquito bites. The CDC backpack aspirator was used to capture approaching mosquitoes attracted to human bait similar to human landing collections. HBC were conducted for 15 min (average suction airflow 13.0 m/s) for each 24hr collection period.

The BGS trap (Figure 3.4. a) was developed primarily to collect adult *Ae. aegypti* (Krockel et al. 2006, Williams et al. 2006, Maciel-de-Freitas et al. 2007) and *Ae. albopictus* (Farajollahi et al. 2009). This sampling device was also shown to collect adult *Ae. polynesiensis* quite effectively (Schmaedick et al. 2008, Mercer et al. 2012a, Mercer et al. 2012b, Hapairai et al. 2013).

The BGM trap (Figure 3.4. b) offers a combination of visual cues and a blend of chemical attractants released using an airflow mimicking convection currents created by a human body (similar to the BGS) in a more compact and light design. A significant difference of the BGM trap is that mosquitoes must first pass through the fan blades before being captured in the net. To prevent ant infestation, BGS and BGM were suspended from a tree branch 20-30 cm above the ground as *Ae. polynesiensis* flies low and has a propensity to bite around the ankles. Engine grease was applied on the strings used for trap suspension and on the CO_2 tubing to prevent predation of collected mosquitoes by ants (Figure 3.4).

Figure 3.4. BG Sentinel and BG Mosquito trap connected to 12V battery. CO_2 was produced using 5L bottle containing a mixture of yeast, sugar and water.

All traps and the CDC backpack aspirator were powered using 12-V, 20Ah (Fullriver, Guang Zhou City, China) batteries, charged (Oz-charge, Fairfield, Australia) in parallel in groups of two at 24-h intervals. The suction power of all sampling devices was measured at start and end of each mosquito collection using a wind meter (Model 3000, Kestrel, Champlain, NY). Batteries powering BGS and BGM traps were placed on the ground next to the traps (Figure 3.4) and replaced after each 24hr collection period. Collected mosquitoes were identified to species using a microscope (LEICA-EZ4D) and species keys (Belkin 1962) before male and female specimen were counted for calculation of sex ratio.

3.3.3.3 Sampling routines

On Moorea and initially on Tetiaroa, trap evaluation used a randomized 5 X 5 LSE design. Treatments consisted of one of each BGS, BGS+L, BGM, BGM +L, and BGS or BGM plus CO_2 and octenol (BGS+C/O, BGM+C/O respectively) against the HBC with each treatment rotated daily amongst stations.

3.3.4 Xenomonitoring

The presence of *W. bancrofti* in the mosquito population was assessed only for valleys on Moorea since there are no human hosts living on sampled motus on Tetiaroa and therefore no expected parasite transmission. DNA from pools of *Ae. polynesiensis* females collected in this valley were extracted using the Qiagen DNeasy kit protocol (Qiagen, Hilden, Germany). The purified DNA was used in a real-time polymerase chain reaction with the LDR primers (Rao et al. 2006) mixed with Sybr Green (Bio-Rad, Hercules, CA) using the iCycler (Model 170-8731, Bio-Rad, Hercules, CA) according to manufacturers instructions and a protocol employed in a previous study (Chambers et al. 2009).

3.3.5 Climate and field measurements

All climatic data were recorded using an automated weather station (Model U30 Hobo, Pocasset, MA). GPS locations were measured using a Garmin 78S model (Garmin International, Inc., Olathe KS). Elevation data were cross-referenced to Google maps.

3.3.6. Data analysis

Statistical analysis was done using GraphPad Prism version 5.0 (GraphPad Software Inc., La Jolla CA). Comparisons between numbers of captured mosquitoes were transformed as $\log_{10}(x+1)$ to correct for lack of normality and unequal variances in the raw data. Treatments were compared to each other using analysis of variance

(ANOVA) and mean separation by the Tukey's multiple comparison test. Pairwise comparisons of dispersal distance between elevations for males and females were calculated using Student's t-tests. The likelihood-ratio, G-test for goodness of fit was used to compare male;female ratio of the BGS+L and HBC which measured the departure from a 1:1 expected ratio.

3.3.6.1 Population estimates

Population estimates in Hotutea valley were calculated using the revised Jackson's positive method assuming open population using the equation: $y_i = (R_i *100*100)/(M_c*C_i)$ (Trpis et al. 1995, Silver 2008). The recapture rate is the proportion (percent) of marked mosquitoes recaptured over the total number of marked mosquitoes released. The number of unmarked (U) and recaptured (R) female mosquito was used to calculate the corrected recaptures (y) and total captured (T). The Adjustment of marked recaptured (M) for each day was calculated by subtracting the total captured of previous collection. Data were computed into the equation Po=(100*100)/ao-M, with ao=1.25 for males at low elevation and 1.73 and 2.29 for females at mid and high elevations respectively.

On motu Auroa, population aggregation was analyzed by allocating traps to quadrants: NW and NE (3 traps each), SW and SE (4 traps each), and 2 traps at the center. Population estimates were calculated using the Lincoln-Peterson Index with subtraction of marked mosquitoes released and it's variance using equation: $P = (an/r)-a$, and $varP = [a^2n(n-r)]/r^3$ (Lincoln 1930, Silver 2008). The standard error was calculated as the square root of the variance (Costantini et al. 1996).

3.3.6.2 Xenomonitoring

Estimates of lymphatic filariasis (LF) prevalence through xenomonitoring was calculated using the PoolScreen (v. 2.02) software (Department of Biostatistics and

Division of Geographic Medicine, University of Alabama at Birmingham, USA) which, provided maximum likelihood estimates (MLE) with 95% confidence intervals based on the likelihood ratio method.

3.4 Results

3.4.1 Weather data

High precipitation was observed on Moorea (Hotutea valley) with >160 mm during the five days of the MMR study (May 4-9, 2011). This resulted in low insolation (54.97 W/m^2) and high relative humidity (93.21%). The average temperature recorded in this valley during the study was 22.67°C with prevailing south-west winds blowing from the coast into the valley. During the course of the MRR study in Tetiaroa (April 12-17, 2011), weather records indicated a higher average temperature (27.63°C), greater insolation (159.69 W/m^2), and lower relative humidity (77.11%) and precipitation (7 mm). The trade winds (Alizés) pattern typical of the dry season in the tropics came from the east. Trap evaluation in Moorea and Tetiaroa was done by replicating the LSE from November 7th to 12th and 23rd to 26th, 2011 respectively.

3.4.2 Mark-Released-Recapture

3.4.2.1 Abundance and Distribution

A total of 7,005 males and 5,682 females *Ae. polynesiensis* were marked and released at five locations in the Hotutea valley (Figure 3.1 and Table 3.1). There was no significant difference in survival between marked and non-marked mosquitoes held in control cages (log-rank, $P = 0.25$). Mosquitoes were collected at three of the five release sites at low, mid and high elevations (Figure 3.5 a, b and c respectively). A total of 175

male and 409 female *Ae. polynesiensis* (including 12 marked males and 191 marked females), 48 male and 66 female *Ae. aegypti*, and 7 male and 21 female *Cx. quinquefasciatus* were collected from all three sampled elevations. There were significant differences in the number of both *Ae. polynesiensis* males ($F = 5.114$, df = 2, $P = 0.010$) and females ($F = 8.440$ df = 2, $P < 0.001$) at each of the three elevations. Pairwise comparisons for *Ae. polynesiensis* showed that the number of males at mid elevation was significantly higher than at low elevation ($P < 0.05$); other comparisons were not significantly different. Pairwise comparisons showed that there was significantly more *Ae. polynesiensis* females at mid elevation than at low or high elevation ($P < 0.05$). There was no difference in the number of *Ae. polynesiensis* females between low and high elevation. *Ae. aegypti* males were collected only at low elevation while *Ae. aegypti* females were collected at both low and mid elevation; no *Ae. aegypti* were collected at high elevation. Comparisons for *Ae. aegypti* females showed that low and mid elevation collections were significantly different ($t = 4.863$, df = 30, $P < 0.0001$). *Cx. quinquefasciatus* was not collected at high elevation. Comparison for *Cx. quinquefasciatus* at low and mid elevation was not significant for either males ($t = 1.118$, df = 29, $P = 0.27$) or females ($t = 0.998$, df = 29, $P = 0.32$).

On the Tetiaroa atoll, a total of 2,286 males and 1,731 females *Ae. polynesiensis* were marked and released (Table 3.1). Mosquitoes marked and released on WA and EA (Figure 3.6 a and b respectively) of motu Auroa were recaptured using 16 BGS traps. A total of 150 males and 872 females *Ae. polynesiensis* were collected on this motu including 7 marked males and 255 marked females. None of the mosquitoes marked and released on the shores of the neighboring Tauini and Hiraanae islets were recaptured on motu Auroa. There were significant differences in the number of males captured ($F = 8.204$, df = 4, $P = 0.0026$) in each of the five sampling sections (i.e four quadrants and

center) but these differences may be attributed to the overall low number of captures. There were no significant differences in the number of females between the five sampling sections ($F = 2.976$, df = 4, $P = 0.068$).

Figure 3.5 Distribution of traps and numbers of marked and released *Ae. polynesiensis* specimens recaptured in the Hotutea valley: (a) low elevation, [18 m], (b) mid elevation [65 m], and (c) high elevation [215 m]. Stars indicate release points and circles sampling locations. Values in meters refer to the distance of each sampling location to the release point. The numbers separated by a coma in the circles indicate the number of marked and recaptured males and females respectively. At low elevation only recaptured marked males are indicated as no females were released at this site.

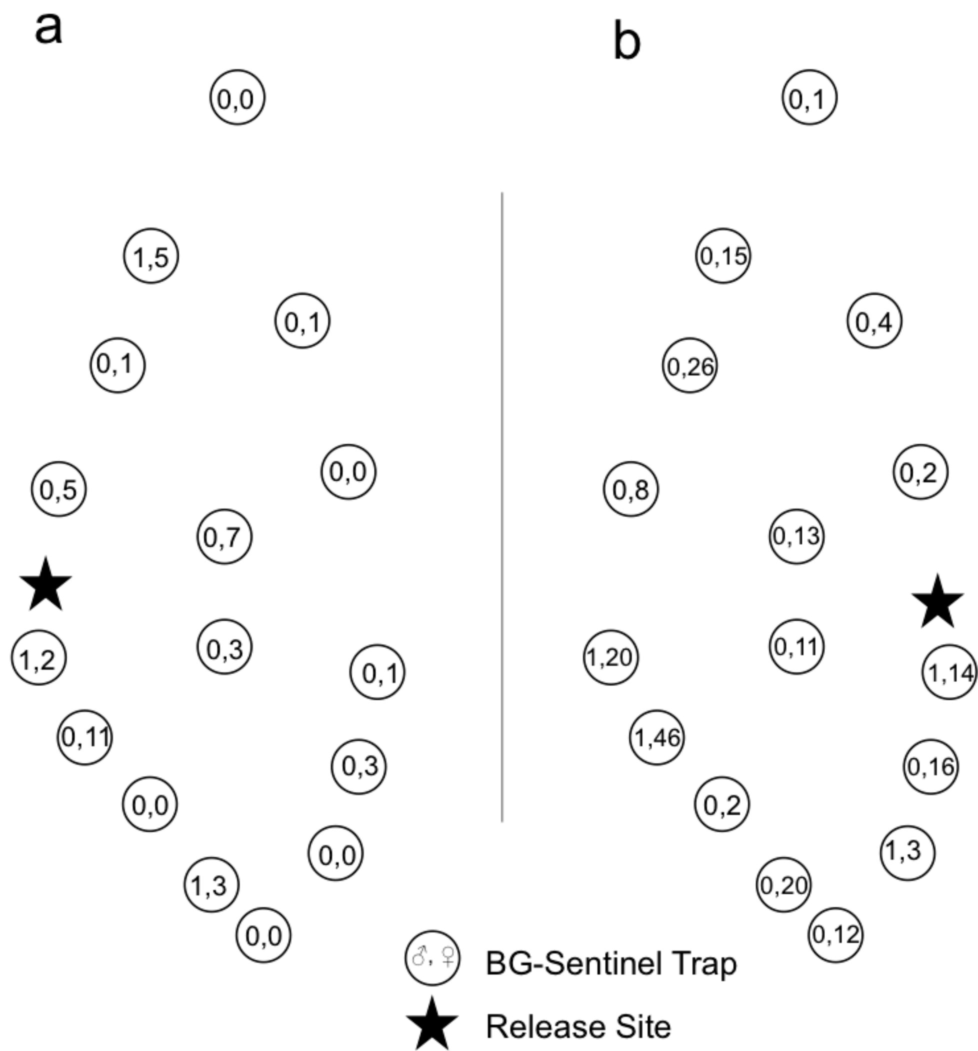


Figure 3.6. Distribution of traps and numbers of marked and released *Ae. polynesiensis* specimens recaptured on motu Auroa: (a) mosquitoes marked with yellow fluorescent powder, released on the west shore and recaptured throughout the motu, (b) mosquitoes marked with orange fluorescent powder, released on the east shore and recaptured throughout the motu. Stars indicate release points and circles sampling locations. The numbers separated by a coma in the circles indicate the number of marked and recaptured males and females respectively.

Table 3.1. Collected mosquitoes for MRR. Experiments were conducted in Moorea (Hotutea valley) and Tetiaroa atoll, French Polynesia

Site	Sex	Location	Elevation	Released	Number of BGS	Recaptured	% Recaptured	Unmarked *Ae. polynesiensis*	*Ae. aegypti*	*Cx. quinquefasciatus*
Hotutea	♂	Low	18 m	2149	15	5	0,23	16 a	48 a	5 a
		Low-mid‡	30 m	1615	0	0	0	N/A	N/A	N/A
		Mid	65m	902	16	0	0	95 b	0 a	5 a
		Mid-high‡	110 m	1207	0	0	0	N/A	N/A	N/A
		High	215 m	1132	14	7	0,62	64 ab	0 a	0 a
	♀	Low	18 m	0	15	N/A	N/A	79 a	61 a	14 a
		Low-mid	30 m	0	0	N/A	N/A	N/A	N/A	N/A
		Mid	65m	1919	16	133	6,93	277 b	5 b	7 a
		Mid-high‡	110 m	1860	0	10*	0,54	N/A	N/A	N/A
		High	215 m	1903	14	58	3,05	53 a	0	0
Tetiaroa	♂	West Auroa	Sea level	460	16	3	0,65	143	0	0
		East Auroa	Sea level	405		4	0,99			
		Motu Tauini	Sea level	667	0	0	0	N/A	0	0
		Motu Hiraanae	Sea level	754	0	0	0	N/A	0	0
	♀	West Auroa	Sea level	448	16	42	9,38	617	0	0
		East Auroa	Sea level	435		213	48,97			
		Motu Tauini	Sea level	442	0	0	0	N/A	0	0
		Motu Hiraanae	Sea level	406	0	0	0	N/A	0	0

* Specimens recaptured at Mid and High elevation
‡ Mark-Release only
a, b: For each species, means in the same column followed by the same letter are not significantly different (Tukey's multiple comparison test, $P = 0.05$ on $\log_{10}(x+1)$ transformed trap catches).

3.4.2.2. Dispersal

In the Hotutea valley, the average distance of dispersal for males at low and high elevation were 45.0 m (SEM ± 5 m) and 39.28 m (SEM ± 10.7 m) respectively, with no significant difference between the two elevations (t = 0.430, df = 10, P = 0.6812). Average distance of dispersal for females at mid and high elevation were 43.6 m (SEM ± 3.55 m) and 52.58 m (SEM ± 4.98 m) respectively, also with no significant difference (t = 1.422, df = 189, P = 0.1566). Ten of the 191 recaptured females (5% of total) released at the mid-high elevation were caught at significantly greater distances: two females at mid100E (690 m), two females at mid50E (640m), one female at mid100N (570 m), one female mid250N (530 m), three females at high50S (490 m), and one female at high250E (200 m). Average distance of dispersal of these ten females was 534 m (SEM ± 45.82 m).

On motu Auroa, the average dispersal distance of mosquitoes marked and released on WA was 106.6 m (SEM ± 61.5 m) for males and 122.5 m (SEM ±18.9 m) for females. From EA the average dispersal distance was 108.75 m (SEM ± 54.37 m) for males and 125.23 m (SEM ± 8.58 m) for females. There was no significant difference in the dispersal of males released from either shores (t = 0.044, df = 5, P = 0.96). Difference in distance of dispersal was however significant for females (t = 5.244, df = 5, P <0.0001).

3.4.2.3. Population estimates

For the Hotutea valley, population estimates were calculated using recaptured marked specimens across five sampling days using the Jackson's positive method (Table 3.2) (Mercer et al. 2012b). The estimate of population size (*Po*) for males at high elevation was 6,859 (6,800 males/hectare). For females, the population size estimate was 3,870 (2,000 females/hectare) at mid elevation and 2,457 (2,400 females/hectare) at high elevation.

Table 3.2. Population estimate in Hotutea valley. Data for calculation of estimate using Jackson's Positive method.

Location	Day released	Adjustment of Marked recaptured (*M*)	Total recaptured (*T*)	Unmarked captured (*U*)	Marked recaptured (*R*)	Corrected recaptured (*y*)
♂ High	0	1132	-	-	-	-
	1	1132	49	46	3	0.541
	2	1129	2	2	0	0.000
	3	1129	4	2	2	4.429
	4	1127	3	2	1	2.958
	5	1126	13	12	1	0.683
♀ Mid	0	1919	-	-	-	-
	1	1919	152	116	36	1.234
	2	1883	96	47	49	2.711
	3	1834	94	59	35	2.030
	4	1799	62	51	11	0.986
	5	1788	6	4	2	1.864
♀ High	0	1903	-	-	-	-
	1	1903	34	22	12	1.855
	2	1891	13	4	9	3.661
	3	1882	24	11	13	2.878
	4	1869	32	13	19	3.177
	5	1850	10	3	7	3.784

For motu Auroa, the Lincoln-Peterson Index, which subtracts marked individuals over time was used to estimate the size of the closed population (Mercer et al. 2012b). Two estimates were calculated for the male and female population using release-recapture data. The total male population was estimated at 21,926 individuals (SEM ± 12,791; 6,852 males/hectare) and 14,478 individuals (SEM ± 7,340; 4,524 males/hectare) from WA and EA respectively. Total female population estimates were 6,581 individuals (SEM ± 1,050; 2,056 females/hectare) and 1,260 individuals (SEM ± 100; 394 females/hectare) from WA and EA respectively (Table 3.3). The female population estimates for the motu differed by ≈5.2x between the two cohorts.

Table 3.3 Lincoln-Peterson Index with approximate density of mosquitoes on motu Auroa.

Side released	Sex	Lincoln-Peterson Index [a]	SEM	Approximate Density [b]
West Auroa	♂	21926	12791	6852
East Auroa		14478	7340	4524
West Auroa	♀	6581	1050	2056
East Auroa		1260	100	394

[a] Lincoln-Peterson Index with a subtraction of the number marked mosquitoes.
[b] Estimated number of mosquitoes per hectare

3.4.2.4. Xenomonitoring

A total of 63 pools of female mosquitoes were analyzed by PCR: 14 pools collected at the low elevation (n=79), 36 pools at the mid elevation (n=268, 9 females were removed because of missing body parts), and 14 pools at the high elevation (n=53). Three pools turned out positive by PCR for *W. bancrofti*, one from the low elevation and two from the mid elevation. Poolscreeen estimates indicated Maximum Likelihood of Infection in the Hotutea vector population of 0.77% (0.15% - 2.23%)

3.4.3 Latin Square Experiment

3.4.3.1 Carbon dioxide production

The mix generating the most CO_2 after one hour was 35g yeast + 700g sugar in 2.5 L of water (Table 3.4) with an average of 104.93 ml/min (S.D.± 13.60 ml/min). Consequently, this yeast-sugar solution was used in traps in combination with octenol. Yeast-production of CO_2 decreased on average by 61% after 24 hours regardless of the sugar quantity used.

Table 3.4. Carbon dioxide average flow rate (ml/min) produced under semi-field conditions by different yeast-sugar solutions. Averages are based on measurements taken at either 1hr or 24hr after mixing the 35 g yeast with various quantities of sugar in 2.5 L of tap water. Measurements were done outdoor in a shaded area, during the day.

	Average CO_2 production (ml/min± S.D.)	
Sugar (g)	1 hour	24 hours
600	100.67 ± 5.85	42.04 ± 4.03
700	104.93 ± 13.60	40.40 ± 8.17
800	92.71± 33.59	35.27 ± 7.05
900	91.31 ± 26.66	39.59 ± 4.49

3.4.3.2 Trap comparison

The average number of females collected with the HBC method was 33.67 (S.D. ±13.38) mosquitoes in Tetiaroa and 35.8 (S.D ±34.70) mosquitoes in Moorea with most females collected on the fly. The average number of females collected with BGS and BGM traps in the respective islands were not significantly different from HBC (Table 3.5).

On Moorea, collections were conducted close to human dwellings near densely forested areas to increase the chance of sampling both aedine species. BGS traps + BG Lure collected more male and female *Ae. polynesiensis* and *Ae. aegypti* mosquitoes than their BGM counterparts. For *Ae. polynesiensis*, BGS+L or BGS+C/O collected significantly more females than BGM+L (F=4.676, df=4, P=0.003). ANOVA and mean separation by Tukey's multiple comparison test showed no significant differences. For *Ae. polynesiensis* males, number of individuals collected did not differ between collecting methods (F=1.321, df=4, P=0.276). Trapping differences were also not significant for *Ae. aegypti* on Moorea for males (F=0.660, df=4, P=0.622) or females (F=0.575, df=4, P=0.682).

The trapping study in Tetiaroa was designed to follow the same experimental protocol as in Moorea, comparing Biogents traps with HBC. However, on the first day of

the experiment the electrical wires connecting the Biogents traps to the batteries were destroyed by rats in three of the four BGM traps (75%) and one of the four BGS traps (25%), thus preventing a complete 24hr period collection sample. Rat-induced damages of BGS traps were prevented by placing the battery and electrical wires inside the trap. No such solution was applicable to the lighter, conical design of BGM traps. After three consecutive days of sampling, five out of the twelve BGM traps (41%) had undergone electrical wire damage by rats. Consequently, the BGM traps had to be removed from the Tetiaroa study and the experiment adjusted to a randomized 3X3 Latin Square (BGS+L, BGS+C/O, and HBC). BGS+L collected more *Ae. polynesiensis* males and females than BGS+C/O or HBC. Male collections were significantly greater with BGS+L than with the HBC sampling method (F=4.233, df=2, P=0.034). For females the observed difference was not significant (F=1.748, df=2, P=0.207)

Table 3.5. Mean *Ae. polynesiensis* and *Ae. aegypti* mosquitoes (mean ± S.D) captured per treatment on Moorea and Tetiaroa.

Island	Treatment[1,2]	N[3]	*Ae. polynesiensis*		*Ae. aegypti*	
			♂	♀	♂	♀
Moorea	BGS+L	10	4 ± 3.46 a	35.2 ± 24.91 ab	13.00 ± 18.90 a	4.90 ± 8.27 a
	BGS+C/O	10	10.4 ± 12.20 a	95.70 ± 129.06 ab	8.00 ± 13.03 a	3.80 ± 5.33 a
	BGM+L	10	2.8 ± 2.82 a	9.40 ± 6.15 c	6.30 ± 9.83 a	1.6 ± 2.17 a
	BGM+C/O	10	2.00 ± 1.89 a	15.90 ± 21.54 b	5.7 ± 11.90 a	3.1 ± 7.41 a
	HBC	10	3.70 ± 7.35 a	35.80 ± 34.7 b	4.10 ± 7.17 a	1.40 ± 1.71 a
Tetiaroa	BGS+L	6	12.00 ± 10.55 ab	228.67 ± 292.82 a	NR	NR
	BGS+C/O	6	5.17 ± 4.67 bc	102.33 ± 76.79 a	NR	NR
	HBC	6	0.67 ± 0.82 c	33.67 ± 13.38 a	NR	NR

[1] Collection periods for BGS and BGM treatments were 24-h and 15 minutes for HBC.

[2]BGS+L: BG-Sentinel trap with BG-lure; BGS+C/O: BG-Sentinel trap with CO_2 plus octenol; BGM+L: BG-Mosquitito trap with BG-lure; BGM+C/O: BG-Mosquitito trap with CO_2 plus octenol; HBC: Human bait collection using a CDC backpack aspirator

[3] N is the total number of days sampled for each treatment

NR: Not relevant, *Ae. aegypti* is absent from Tetiaroa

a, b, or c: For each species on a given island, means in the same column followed by the same letter are not significantly different (Tukey's multiple comparison test, $P = 0.05$ on $\log_{10}(x + 1)$ transformed trap catches).

3.4.3.3 Male: Female ratio

Ae. polynesiensis male:female ratios were calculated for Moorea, and Tetiaroa (Table 3.6). Ratios were female biased in all locations where BGS+L and HBC were tested. The G-test for goodness of fit established that female biases were significant for all locations. Comparatively *Ae. aegypti* male:female ratios were male biased for both BGS+L and HBC (Table 3.6). This male bias was significant at both sampled locations for the BGS+L.

Table 3.6. G-Test for goodness of fit of male:female ratio for *Aedes polynesiensis* and *Aedes aegypti* comparing BGS+L to HBC at various locations.

Species	Island	Location	Treatment	n[1]	Average male:female ratio	G-test	p-value
Ae. polynesiensis	Moorea	Afareaitu	BGS+L	5	0.08	172.08	<0.001
		Atiha	BGS+L	5	0.15	233.02	<0.001
		Afareaitu	HBC	5	0.06	237.25	<0.001
		Atiha	HBC	5	0.12	533.66	<0.001
	Tetiaroa	Tiaraunu	BGS+L	3	0.06	1052.84	<0.001
		Hiraanea	BGS+L	3	0.04	379.35	<0.001
Ae. aegypti	Moorea	Afareaitu	BGS+L	5	3.42	26.63	<0.001
		Atiha	BGS+L	5	2.17	117.18	<0.001
		Afareaitu	HBC	5	3.50	0.19	0.66
		Atiha	HBC	5	1.50	1.08	0.30

[1] n is the number of days sampled per treatment at each location

3.4.3.4 Xenomonitoring

A total of 107 pools of mosquitoes were analyzed by PCR: 66 pools were from Atiha (n=1256), and 41 pools from Afareaitu (n=735). In Atiha, two pools originating from the same trapping station were PCR positive for *W. bancrofti*. In Afareaitu, three pools from two different sampling stations were PCR positive for *W. bancrofti*. LF transmission prevalence was estimated for Atiha and Afareaitu at 0.17% (0.02%-0.59%) and 0.435% (0.08%-1.26%) respectively with a 95% confidence interval. There was no significant difference in the number of positive pools collected using either trap.

3.5 Discussion

The study provides a better understanding of the population dynamics, migration and dispersion patterns of the medically important *Ae. polynesiensis* mosquito species at two island settings typical of the Society Islands in French Polynesia. MRR experiments have previously been used with success to study the population dynamics of *Ae. polynesiensis* on a motu in the lagoon of Raiatea in the Society Islands (Mercer et al. 2012b). Dusting did not appear to be detrimental to either male or female *Ae. polynesiensis* in view of the high initial flight out of the cage, survival of controls, and rapid dispersal across the release area (valley and motu). Survival of marked and unmarked control individuals was satisfactory. Marked females were recaptured from all cardinal directions at both sites suggesting mixing with the field population. Although males accounted for a comparatively low but significant proportion of trap catches relative to females, marked males were poorly recovered compared to marked females. Recapture rates of marked males on Auroa were comparable to those reported on Toamaro (Mercer et al. 2012b). Together these data suggest a) that *Ae. polynesiensis* males are relatively short-lived in the field and b) that a more efficient male trapping system is warranted. Lincoln-Petersen female population estimates varied greatly for motu Auroa. In nature, reported parity rates are high for *Ae. polynesiensis* (Russell et al. 2005a) with ≈80% parous females caught in BGS traps (Mercer et al. 2012b). Moreover, it is estimated that a relatively high proportion of females are actively seeking a blood meal at any given time (Mercer et al. 2012b). Under these assumptions, the female population estimates for the Hotutea valley and motu Auroa could, conservatively, be doubled. Population estimates for males calculated for both study sites may not be very accurate owing to the low recapture rate.

As expected, the abundance of the rural species *Ae. polynesiensis* was the highest inside the Hotutea valley while the urban species, *Ae. aegypti* was more abundant closer to the costal area. Both species were found at the middle of the valley where dwellings were spread out. The presence of *Aedes* vectors throughout the entire valley depicts a high risk of exposure to infectious bites across the valley. Although BGS traps were used to measure dispersal of *Ae. polynesiensis*, with the majority of traps being positioned away from homes, the presence of LF from xenomonitoring of some collected mosquitoes suggest a relatively high transmission rate of LF across the Hotutea valley. Wide distribution of the vector and presence of LF suggest the population replacement could be a successful control strategy as gene transfer is high, thus encouraging the development of *Ae. polynesiensis* LF refractory lines.

Dispersal patterns among *Aedes* mosquito species may influence disease transmission dynamics. Dispersal is influenced by local variables such as availability of suitable habitats for development, exposure to wind, and vegetation. As observed in the present study, the conditions in the valley and on the motu influenced dispersal in different ways. The average dispersal distance measured for both males and females was significantly greater on the motu than in the valley. Maximum dispersal distances were undoubtedly constrained by the relatively limited dimensions of the motu. Dispersal on the motu may have been influenced by additional factors including the type and density of vegetation, and the more direct exposure to prevailing winds compared to the valley. Although host-seeking females *Ae. polynesiensis* occasionally venture on the shoreline of highly infested motus to secure a blood meal (personal observations), *Ae. polynesiensis* is not thought to frequently cross open spaces, preferring to disperse through the vegetation instead. None of the individuals released on the shorelines of either islets flanking motu Auroa were recaptured on Auroa, thus supporting this hypothesis. Similarly, those

females which dispersed over significantly greater distances in the Hotutea valley did so through the relatively thick vegetation present between the mid and high release/sampling zones. Early field ecology studies estimated the *Ae. polynesiensis* maximum dispersal range at 100 yards (92 m) (Jachowski 1954). The measurements presented above indicate a greater dispersal capacity (690 m).

The absence of recaptured marked mosquitoes from neighboring islets demonstrates the relative isolation of motu Auroa. The stretch of water separating the islets seems sufficient to limit the migration of a mosquito between the two populations during a relatively short period. Although recent genetic studies suggest a uniform population structure of *Ae. polynesiensis* of an island (Brelsfoard and Dobson 2012), for the purpose of a population suppression study, the limited migration of isolated islets such found on Tetiaroa would be more than sufficient.

A critical parameter worth measuring in anticipation of a release program is the dispersal capacity of a target mosquito population in order to assess likely invasive pressures (Hendrichs et al. 2005). On one hand, mosquitoes displaying a highly dispersive behavior are advantageous for control strategies involving release of sterile or refractory mosquitoes, as this characteristic will increase their chances of finding and mating with their female counterparts in the field. On the other hand, the greater the dispersal capacity, the greater the risk of immigration to and recolonization of treated areas (Lance and McInnis 2005). In the Hotutea valley, immigration could potentially come from, and be limited to the low elevation zone near the coastal area. The rest of the valley is surrounded by steep hills with characteristics (lower temperature, low vegetation cover, direct exposure to winds) that are less suitable for *Ae. polynesiensis* population maintenance. To overcome immigration issues from costal areas, a “barrier zone” could be prescribed using traditional control methods (Curtis et al. 1982) or by increasing the

release rate of sterilizing males if an SIT strategy is to be conducted (Benedict and Robinson 2003).

This study also provides a comparative examination of three sampling devices and methods for collecting adult *Ae. polynesiensis* in typical island settings of French Polynesia. On both high, volcanic (Moorea) and low, coral (Tetiaroa) island settings, the BGS trap performed very well, demonstrating it is a suitable alternative to the HBC using the CDC backpack aspirator. On Moorea the BGS and BGM traps with either attractant collected male and female *Ae. polynesiensis* in numbers similar to the HBC. On Tetiaroa, the BGS with either attractant collected more mosquitoes than the HBC. Although the HBC allowed for collection of both *Ae. polynesiensis* female and male mosquitoes, the BGS+L collected more male *Ae. polynesiensis* in Tetiaroa than the HBC.

Large-scale surveillance and sampling in remote islands of French Polynesia requires efficient and logistically manageable sampling devices and attractants. HBC, like human landing collections, are labor and time intensive. By comparison, several BGS traps can be set at different, nearby locations in the time required for a single HBC. In addition, a single operator can only do one backpack aspirator collection at a defined time of the day while sets of traps deployed across even distant locations can simultaneously sample mosquitoes during a 24 hr cycle. Collection of *Aedes* mosquitoes is best achieved when they are most active. For *Ae. polynesiensis*, the peaks of host-seeking and blood-feeding activity are usually in the early morning (from dawn to 9:30 am) and late afternoon (from 03:00 pm to dusk) (Jachowski 1954). Logistical constraints may make the HBC difficult to achieve during such narrow time periods, potentially leading to additional sampling biases. Moreover, attractiveness of the human bait will vary from operator to operator thus further increasing the HBC sampling bias. By comparison, BGS traps offer a standardized design, limiting trap-to-trap sampling variations and thus more

consistent sampling outcomes. Collection cycles of 24 hr allow operators to set traps at any time of the day, encompassing an entire diurnal cycle of *Aedes* activity.

The BGS trap has been tested with CO_2 in many studies. Carbon dioxide typically supplied via compressed gas cylinders (Kawada et al. 2007) or using the semi-controlled sublimation of dry ice (Farajollahi et al. 2009, Bhalala et al. 2010) is known to increase catch rates in traps. However, the supply of CO_2 via dry ice, gas tanks or propane combustion is expensive and often difficult to procure in tropical, often remote, Pacific islands settings. The use of yeast-sugar fermentation (Smallegange et al. 2010), investigated here in combination with octenol was a comparatively inexpensive and rather convenient alternative source of CO_2, with the components (yeast, sugar and plastic bottles) being cheap and available locally. The CO_2 produced under semi-field conditions one hour after mixing the optimal yeast:sugar proportion (104.93 ± 13.60 ml/min; mean± S.D.) was lower than the flow rate typically used with mosquito traps but within the flow rate recommended by the manufacturer for BGS (either 70 ml/min or 175 ml/min flow rates depending on the type of Biogents nozzle CO_2 flow restrictor used). However, after 24-hr of yeast-sugar fermentation the CO_2 flow rate from yeast generation had dropped below the minimum recommended flow rate for BG traps (70 ml/min) indicating that yeast generated CO_2 may not be sufficiently reliable for 24 hrs collections under the conditions observed in South Pacific tropical island settings. Another important drawback of using yeast-fermentation as a source of carbon dioxide is that the seasonal fluctuation of the daily average temperature will likely affect the CO_2 flow rate, leading to possible biases in mosquito catch rates which might prevent the use of this method for comparative, long term temporal studies. Turner et al. (2011) recently identified that 2-butanone, a volatile odorant, can trigger a response in *Aedes* mosquitoes that is indistinguishable from that elicited by CO_2. This discovery warrants the comparative

evaluation of 2-butanone formulations vs CO_2 in BGS traps as this compound might provide a compact, and economical lure highly suited for vector research and surveillance in remote locations. One other observed shortcoming of using the CO_2 plus octenol mix compared to the BG-lure was the number of non-target insect species trapped. A large number of *Drosophila* and Noctuidae were collected thus considerably increasing the sorting effort before mosquito species identification could be done.

The setting time and handling ease of BGS vs BGM traps were similar. Although the retail price of the BGM trap is significantly lower than the BGS trap, the condition of mosquitoes collected with BGM traps in this study was generally poor. Wings and legs were usually broken from the thorax, and most specimens had lost their scales, thus making species identification more difficult and therefore time-consuming. Moreover, on all three islands, the BGS traps collected more mosquitoes than their BGM counterparts. Finally, the light, low cost BGM trap design did not cope comparatively as well as the BGS with the rather harsh environmental conditions typically found on low, coral islands settings like Tetiaroa.

The number of *Ae. aegypti* males collected in Moorea was greater than that of females (male bias) for BGS+L and HBC. This is consistent with other studies (Williams et al. 2006, Ball and Ritchie 2010). By comparison, male:female ratios of *Ae. polynesiensis* collected in Moorea using BGS+L or HBC showed an opposite trend, with significantly more females collected than males. Similar observations were made previously with adult *Ae. polynesiensis* collections using BGS (Mercer et al. 2012a, Mercer et al. 2012b, Hapairai et al. 2013). Although differential trophic preferences between *Ae. aegypti* and *Ae. polynesiensis* might play a role, the basis for such differences in male:female collection between the two species remains unknown.

Beyond the logistical challenges associated with an area-wide intervention, the success of a genetics-based control strategy will rely largely on the ability to accurately measure population suppression, i.e. entomological endpoints. The results presented here are relevant to the design and implementation of small-scale open releases as part of an incremental stepwise approach for testing and scale-up of a self-limiting, *Wolbachia*-based suppression strategy. The present findings highlight the importance of establishing baseline population dynamics data prior to area-wide control strategies to minimize the risk of and appropriately manage potential re-infestation events following strategy implementation.

The BGS trap has proved to be a suitable tool for establishing this baseline population dynamics and may also be crucial for subsequent monitoring during and after releases. Naturally contained populations typically found in island settings are better suited for initial release trials to minimize the interference of migration into the treated area. The data presented here suggests that motus of Tetiaroa are suitable sites for initial releases of CI males for a suppression or replacement strategy, since there is no evidence of migration between motus.

Chapter 4: Effect of temperature and larval density on laboratory rearing productivity and male characteristics of *Aedes polynesiensis*[2]

4.1 Introduction

The IIT requires a highly efficient sex separation method to avoid the accidental release of females, which is obviously essential if population elimination is the aim. The recent *Ae. polynesiensis* IIT trial conducted in Raiatea relied upon standard laboratory rearing protocols and mechanical sex separation techniques combined with visual sorting of release individuals to safeguard against female release (O'Connor et al. 2012). While appropriate for small feasibility trials, manual inspection is unlikely to be cost effective at a larger scale.

Production of large numbers of sterile male mosquitoes devoid of contaminating females has been a major hurdle for numerous SIT control strategies (Benedict and Robinson 2003, Benedict et al. 2009). The number of males required for SIT is high, largely exceeding the standard rearing capacity of most mosquito laboratories (Bellini et al. 2007). Various biological, genetic, and transgenic approaches have been developed for stringent male-female separation of some species considered for SIT (Papathanos et al. 2009). Although particularly suited for high throughput separation, genetic or transgenic approaches are not yet available for certain *Aedes* species like *Ae. polynesiensis* or the regulatory approval for their use may not yet be resolved in certain countries. Sex separation of these species is based therefore on biological methods that exploit naturally occurring differences between males and females, such as pupal sexual dimorphism or

[2] Parts of this chapter are in press for Acta Tropica Issue entitled "Biology and behaviour of male mosquitoes in relation to new approaches to control disease transmitting mosquitoes": Limb K. Hapairai, Jérôme Marie, Steven P. Sinkins, and Hervé C. Bossin. Effect of temperature and larval density on laboratory rearing productivity and male characteristics of *Aedes polynesiensis* (Diptera: Culicidae). *Wolbachia* SIT Special Issue

delayed female pupation. These biological methods are naturally prone to variation. Under standard laboratory conditions existing rearing protocols and tools require regular adjustment limiting their use for small- to mid-scale operations. Other general concerns with these systems include the rather inefficient male recovery and the concomitant selection of small males for release that may display reduced field performance (Papathanos et al. 2009). For example, the sieving technique used as part of a long-term SIT program targeting *Ae. albopictus* (Skuse) in Italy allowed the recovery of 15-29% of total male pupae usable for irradiation and field release (Bellini et al. 2007, Bellini et al. 2013). These limits highlight the need improve rearing and sexing protocols for large-scale SIT or IIT operations.

Male production and mating competitiveness in the field are critical parameters for the success of SIT. These parameters and the stringency of separation between male and female pupae are determined in part by rearing conditions. Rearing conditions should be selected to achieve fast and synchronous immature development, high male yield, large adult body size and long survival under field conditions. Better size separation and greater delay in female pupation may also be achieved with consistent rearing conditions. The effects of immature rearing conditions on adult life history traits has been investigated for several mosquito vector species (Mercer 1999, Gimnig et al. 2002, Bayoh and Lindsay 2003, Ng'habi et al. 2005, Benedict et al. 2009). Studies have shown that larval density exerts a strong effect on both time to pupation and adult body size (Gomulski 1985, Agnew et al. 2000, Macia 2009). High larval densities may decrease the time to pupation but decrease synchronicity in pupation. They also increase growth rate but generate smaller, short-lived adults particularly when food supply was limiting (Nayar 1969, Reisen et al. 1984, Mercer 1999). Rearing temperature also has an effect with optimal temperatures resulting in higher larval (Bayoh and Lindsay 2003) and pupal

(Lyimo et al. 1992) survival rates. Larger mosquitoes tend to survive better than smaller ones (Reisen et al. 1984, Packer and Corbet 1989). Older, larger males have the greatest mating success (Mahmood and Reisen 1982, Bock et al. 1983, Ponlawat and Harrington 2009, Maiga et al. 2012). Synchronous larval development is also important to ensure predictable pupal output and subsequent male releases. Success requires an understanding of individual variability and the development of protocols that enhance product quality and production efficiency.

In addition to vectoring *W. bancrofti*, *Ae. polynesiensis* is of veterinary importance as it is also the primary vector *Dirofilaria immitis*, the canine heartworm parasite (Chambers et al. 2009). Despite the medical and veterinary importance of *Ae. polynesiensis* in the Pacific and the need for sustainable control strategies against this vector, only limited data are available regarding the influence of rearing conditions on male development. Mercer (1999) examined the effect of larval density on *Ae. polynesiensis* development by testing three larval densities (100, 200, and 400 larvae/pan) with a fixed amount of diet (500 mg of liver powder in 1.5 L of water). The comparison of the mean adult dry weight of specimens collected in the field with adults reared at different densities in the laboratory indicated that *Ae. polynesiensis* field populations develop under food-limited conditions. The study also revealed that *Ae. polynesiensis* responds to intraspecific larval competition by producing small adults over extended developmental periods. Finally, pupal wet weights, adult dry weights, and adult wing lengths are equally acceptable measures of *Ae. polynesiensis* mosquito size for vector and fecundity studies.

In this study, the influences of two rearing variables were examined on several life history traits critical for the success of *Ae. polynesiensis* SIT/IIT. A range of temperatures and larval densities was tested to determine the optimal rearing conditions for the

development of a standardized rearing protocol likely to improve the efficacy of *Ae. polynesiensis* vector control programs that rely on the release of incompatible or sterile males.

3.2 Objectives

The overall objective was to measure the effect of temperature and larval density on life history of *Ae. polynesiensis* in an IIT strategy. The specific aims were to identify a range of rearing conditions at which production is optimized for large adult male cohorts whilst reducing potential female contaminant to:

1) Identify conditions that favour high male pupae production testing a range of rearing temperatures (20°C, 25°C, 27°C, and 30°C) and larval densities (50, 100, 200, and 400 larvae/L). The survival of L1 larvae to pupal stage and sex ratio of theses surviving pupae were measured to identify optimal temperature, density and interaction of both temperature and density

2) Identify rearing conditions that would facilitate further sorting of male pupae from female pupae with aforementioned optimal temperatures and densities. The period of time separating male pupation from female pupation, as well as day of pupation, was measured for each temperatures and density.

3) Identify rearing conditions that would provide male sizes comparable to that of wild males with suitable adult survival rate with aforementioned temperatures and densities. Wing lengths of laboratory-reared mosquitoes were measured and compared to wild mosquitoes from Atimaono. Adult male survival was measured daily in a cage assay under laboratory conditions.

4.3 Materials and methods

4.3.1 Mosquito stocks and laboratory rearing

All experiments were done using a strain of *Ae. polynesiensis* (colonized for >15 generations) originally collected from the Hotutea valley, Moorea (17°33'00.94" S 149°49'09.07" W). The insectary was equipped with an air conditioner (model Splitair, Galanz, Guangdong, China) and a portable humidifier (model CMP-3, Bionaire, Montreal, Canada) for constant climate control.

4.3.2 Experimental protocol

Mosquito rearing using four larval densities (50, 100, 200, and 400 larvae/L) was done either in the insectary (25°C, ±0.5°C) or in an environmental test chamber (model MLR-351H, Sanyo, Watford, United Kingdom) for the other three tested temperatures (20°C, ±0.2°C; 27°C, ±0.2°C, and 30°C, ±0.2°C). Relative humidity in the insectary and the test chamber was set at 70-90%. The 12 on 12 off light cycle inside the chamber matched the natural photoperiod in the insectary. A total of 16 treatments were conducted and each treatment was replicated four times. Climatic parameters in the insectary and the test chamber were recorded and verified throughout the experiment using a data logger (model Hobo U12-012, Onset, MA, USA).

Eggs were placed under vacuum for one hour to synchronize hatch. Less than three hours later, L1 larvae were distributed to the set densities in 24 x 16.5 x 5 cm plastic pans (water surface area of 396 cm^2 with water depth of 2.5 cm) containing one litter of distilled water. Feeding was done daily *ad libitum* equivalent to approximately 1.6 mg of liver powder provided per larvae until pupation. Details of rearing treatments are given in Table 4.1. Rearing pans were examined daily for pupae at approximately the same time. These were removed on the day they formed, sorted by sex and counted manually under a

dissecting microscope (EZ4D model, Leica Microsystems GmbH, Wetzlar, Germany) to establish larval survivorship and sex ratio. Male pupae from each temperature/density cohort were allowed to emerge in separate cages (30 x 30 x 30 cm cage, Bioquip, Rancho Dominguez, CA, USA). One day after emergence, 20 males were selected randomly from each treatment and their wing length was measured. The remaining males were used to monitor their survival.

Table 4.1. Tested rearing temperatures and larval densities

Rearing Temperatures (°C)	No. replicates each	No. larvae/L	No. larvae per unit of water surface[a] (larvae/cm^2)	No. larvae per unit of water volume (larvae/ml)	Water vol. per larva (ml)	Approx. liver powder/L[b] (mg)
20, 25, 27, and 30	4	50	0.13	0.05	20	80
		100	0.25	0.1	10	160
		200	0.51	0.2	5	320
		400	1.01	0.4	2.5	640

[a] water surface area measured 396 cm^2 with a depth of 2.5 cm for a total volume of [b]1000ml.
[b] an approximate of 1.6mg of liver powder/larvae was provided *ad libitum* throughout larval development.

4.3.3 Collection of wild male mosquitoes

Wild pupae were collected for comparison purposes from natural containers, mainly rat-chewed coconuts at Atimaono, commune of Papara, Tahiti (17º46'03.00 S 149º26'58.00 W). One collection was done during the dry season (September 2011) and another during the wet season (January 2012). Pupae were transported to the ILM research station and allowed to emerge in plastic containers under standard insectary conditions. To compensate for location effects, *Ae. polynesiensis* mosquitoes were also sampled on the grounds of the ILM Medical Entomology Research Laboratory, Paea (17º43'48.00 S 149º34'44.00 W) using BGS traps placed in shaded areas. Sampling consisted for the most part, in four-hour collections conducted weekly from October 2009

to September 2011. Mosquitoes from these two geographic locations were identified to species, sexed and the wing length of *Ae. polynesiensis* males was measured as described below.

4.3.4 Male wing length and survival

Wings were photographed using a dissecting microscope (EZ4D) with a micrometer (Wild-Heerbrugg, Switzerland) for calibration and measured to the nearest 0.01 mm as in Chapter 2. Mosquitoes with wings not completely sclerotized were not used for measurement. Twenty specimens from each treatment were randomly selected for wing measurements.

Additional pans were necessary for larval densities 50 and 100 to generate sufficient material for wing measurement and adult male survival. To monitor survival of adult males, up to 15 male mosquitoes (< 24h-old) from each larval rearing density were placed into 15 x 15 x 15 cm cages (Bugdorm, Megaview Science, Taichung, Taiwan). Four replications were done for each larval density and temperature. Cages were placed in the insectary and provided with a 10% sucrose solution using coffee filter wicks renewed weekly. Mortality was recorded daily until all mosquitoes died.

4.3.5 Data analysis

The effects of rearing temperature and larval density on pupae survival, male to female ratio, laboratory mosquito wing lengths, and age at pupation were evaluated with a two-way ANOVA. Wing-lengths from laboratory and wild specimens were compared using one-way ANOVA and mean separation by the Tukey multiple comparison test. Comparison of wing lengths from wild specimens collected at two different seasons was done using the Student t-test. The likelihood-ratio G-test for goodness-of-fit was used to test departures from 1:1 male to female ratios at pupation for each treatment. Mortality

was recorded daily and survival was estimated using the log-rank test (X^2). Statistical analysis was done using GraphPad Prism version 5.0 (GraphPad 2010).

4.4 Results

4.4.1 Survival to pupae and sex ratio

4.4.1.1 Survival to pupae

Average larval survivorship in all treatments ranged from 26.5% ± 4.2 SEM (27°C, 100 larvae/L) to 62% ± 4.5 SEM (20°C, 50 larvae/L) (Figure 4.1). Temperature, density, and their interaction had a significant effect on survival to pupae (Table 4.2). Survivorship for each temperature was the highest when larvae were reared at 200 larvae/L except at 20°C.

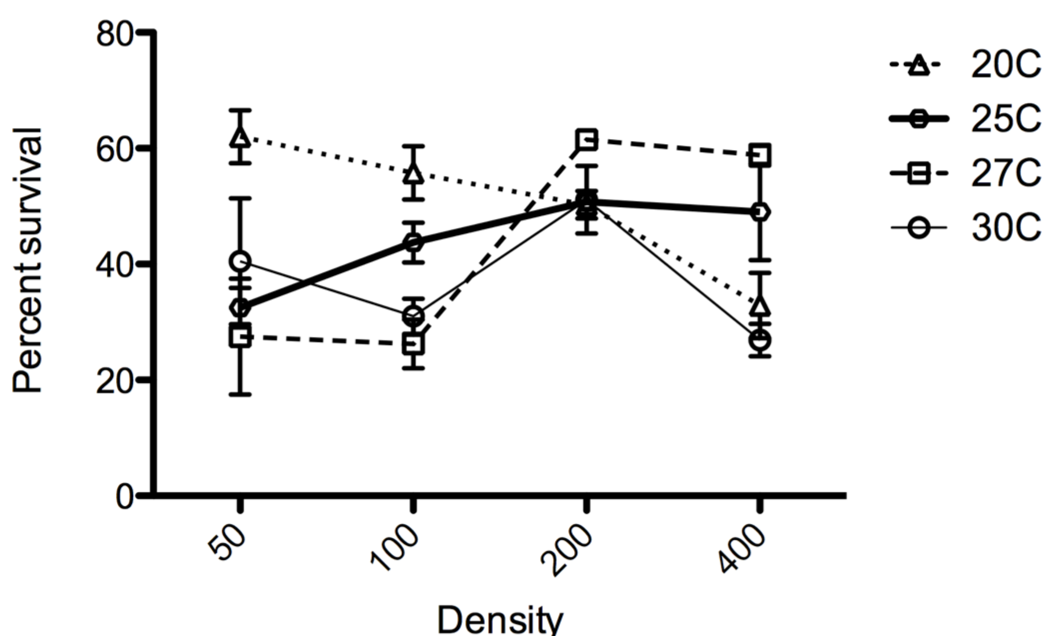


Figure 4.1. Larval survivorship. Mean percent survival and SEM of larvae reared under laboratory conditions at various densities and temperatures. Percent survival represents the number of L1 larvae successfully developing to pupae. This was calculated for each treatment by subtracting the sum of observed pupae to the known number of larvae.

4.4.1.2. Sex ratio

Pupal sex ratio was male biased at most rearing temperatures and densities (Table 4.3). Temperature and the interaction with density at which larvae were reared had a significant effect on the observed male pupae bias (Table 4.2). Male to female ratio was lowest when reared at 20°C and 100 larvae/L and highest when reared at 25°C and 50 larvae/L (Figure 4.2).

Table 4.2. Analysis of variance table of temperature and density.

Statistics	Source[a]	Pupae survival	Male:female ratio	Age at pupation male	Age at pupation female	Lab male wings length
Sum of squares	Residual	0.56	38.65	5.37	12.44	0.74
	Temperature	0.13	18.02	2.56	2.56	0.89
	Density	0.2	1.42	19.03	19.03	0.05
	Interaction	0.65	15.58	2.54	2.54	0.05
F ratio	Temperature	3.79	6.01	56.67	91.1	105.7
	Density	5.84	0.47	3.98	0.33	5.62
	Interaction	6.21	1.73	2.54	0.76	1.89
P-value	Temperature	*	***	***	***	***
	Density	***	NS	**	NS	***
	Interaction	***	*	*	NS	*

[a] Degrees of freedom: Residual= 48, Temperature=3, Density=3, and Density x temperature interaction=9.
P-value are * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, NS not significant.

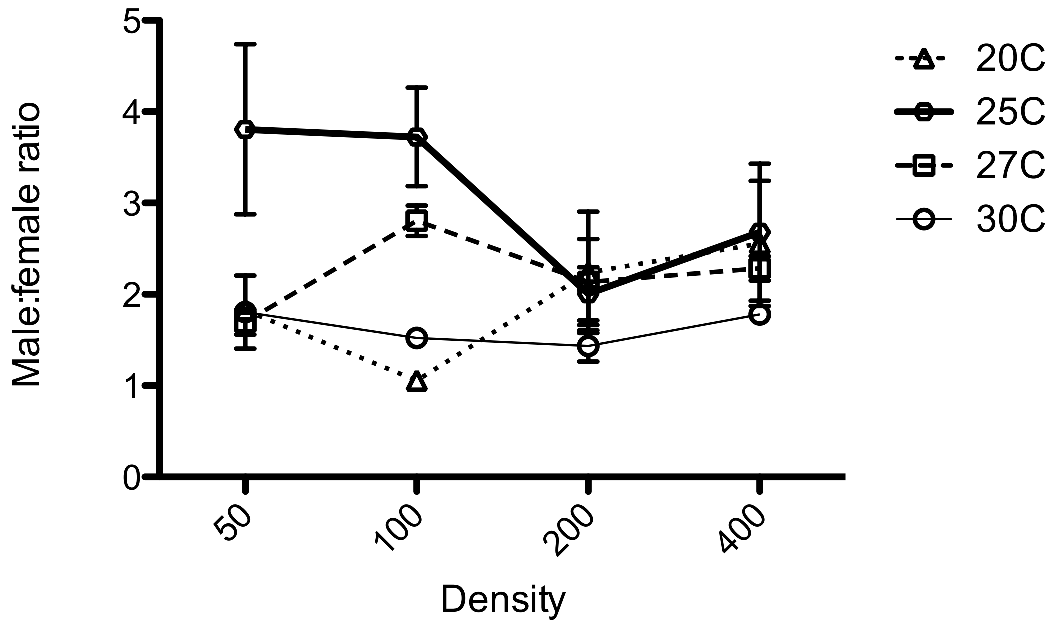


Figure 4.2. Sex ratio at pupation. Mean and SEM of male per female pupal ratio reared under laboratory conditions at various densities and temperatures.

Table 4.3. Male:female ratio reared at different temperatures and densities. Mean and SEM of male per female ratio with G-test for goodness-of-fit.

Temperature	Density	Mean ratio	SEM	Total G-value	Total *P*-value
20	50	1.828	0.094	10.812	*
	100	1.055	0.082	1.215	NS
	200	2.241	0.665	62.354	***
	400	2.557	0.686	90.345	***
25	50	3.808	0.932	21.356	***
	100	3.725	0.538	59.396	***
	200	2.007	0.291	46.634	***
	400	2.681	0.749	130.675	***
27	50	1.696	0.138	3.291	NS
	100	2.805	0.167	24.017	***
	200	2.136	0.471	66.705	***
	400	2.285	0.132	147.506	***
30	50	1.807	0.401	3.345	NS
	100	1.525	0.072	5.697	NS
	200	1.436	0.170	17.003	**
	400	1.783	0.084	34.516	***

Significant different from a 1:1 male to female ratios, *P*-value are * $P<0.05$, ** $P<0.01$, *** $P<0.001$, NS not significant

4.4.2 Time to pupation and male proportion on first day of pupation

Time to male pupation decreased as temperature increased for both males and females (Figure 4.3). Mean male age at pupation ranged from 5.7 days at 27°C and 200 larvae/L to 7.6 days at 20°C and 50 larvae/L. Female mean age at pupation ranged from 6.5 days at 27°C and 200 larvae/L to 9.5 days at 20°C and 100 larvae/L. The effects of density, temperature, and their interaction were statistically significant for males (Table 4.2). For females, only temperature had a significant effect on age at pupation (Table 4.2).

Male yield on first day of pupation ranged from 1.1 % of total male pupae produced at 20°C and 100 larvae/L to 46.9% at 25°C and 50 larvae/L (Table 4.4). Male pupation was delayed by one day when reared at 30°C and 50 larvae/L. When all densities were considered, the fraction of males produced on the first day of pupation at 25°C was significantly higher than for any other temperature (F = 7, df = 3, P = 0.004 and Tukey's comparison Test).

4.4.3. Adult male survival

Log-rank test of adult male survival revealed no significant differences between larval densities when reared at 20°C (X^2=0.862, d.f.=3, P = 0.834) or 30°C (X^2=5.482, d.f.=3, P = 0.139 (Figure 4.5a and 4.5d). However, significant differences in adult male survival were observed between larval densities at 25°C (X^2=15.29, d.f.=3, P = 0.001) and 27°C (X^2=12.49, d.f.=3, P = 0.005) (Figure 4.5b and 4.5c).

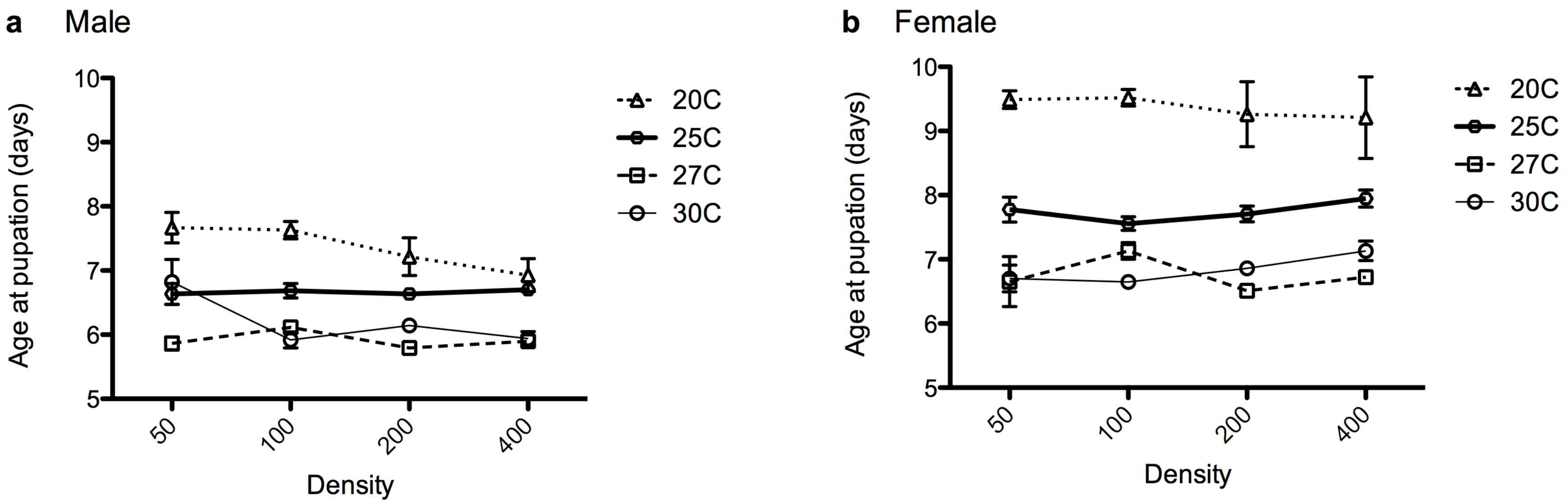
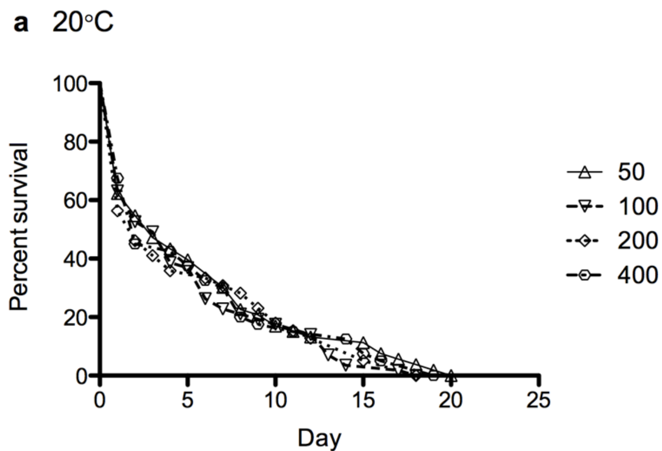


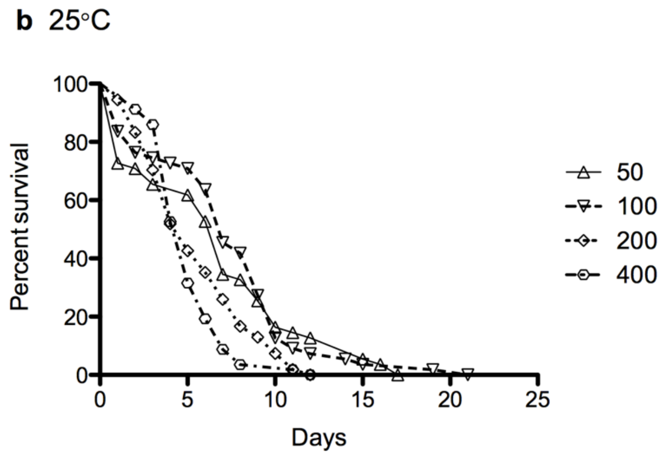
Figure 4.3. Time to pupation. Mean age at pupation and SEM of males (a) and females (b) measured at every 24-hr interval (day) for each treatment.

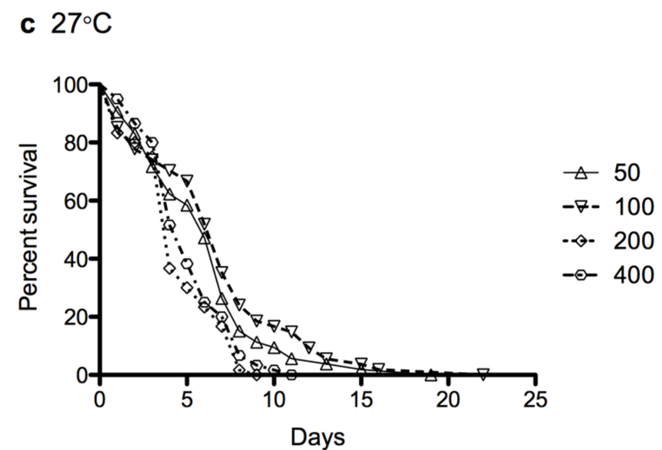
Table 4.4. Summary of parameters relevant to male rearing productivity and male field performance. The number of surviving males 6 days after emergence (*S*) was calculated by multiplying the mean male yield on 1st day of pupation (*P*) by the percent male survival 6 days after emergence (*s*). $S = s \times P$

Temperature (°C)	Density	Percent of larval survival	Days to pupation	Percent of female pupae on 1st day	Mean male yield on 1st day pupation *P*	Percent of total male pupae recovered on 1st day	Male:female mean age difference at pupation	Median survival of adult male (day)	Percent male survival 6 days after emergence *s*	Number of surviving males 6 days after emergence *S*
20	50	62%	10	0	1	5%	1.8	3	40%	0.4
	100	56%	10	0	3.55	12%	1.9	3	26%	0.9
	200	50%	10	2.1	23.27	35%	2	2	33%	7.7
	400	33%	10[a]	0.6	40.96	46%	2.3	2	33%	13.5
25	50	33%	6	0	5.86	47%	1.1	7	53%	3.1
	100	44%	6	0	14.74	43%	0.9	7	64%	9.4
	200	51%	6	0	29.69	45%	1.1	5	35%	10.4
	400	49%	6	0	55.95	43%	1.2	5	19%	10.6
27	50	28%	5	0	1.94	23%	0.8	6	47%	0.9
	100	26%	5	0	0.51	3%	1	7	52%	0.3
	200	62%	5	0	24.87	31%	0.7	4	23%	5.7
	400	59%	5	0	35.11	22%	0.8	5	25%	8.8
30	50	41%	6	0	4.71	39%	0	6	48%	2.3
	100	31%	5	0	3.48	19%	0.7	6	49%	1.7
	200	51%	5	0	5.04	8%	0.7	6	43%	2.2
	400	27%	5	0	15.39	22%	1.2	8	70%	0.7

[a] 1.1% of males pupated on day 9.







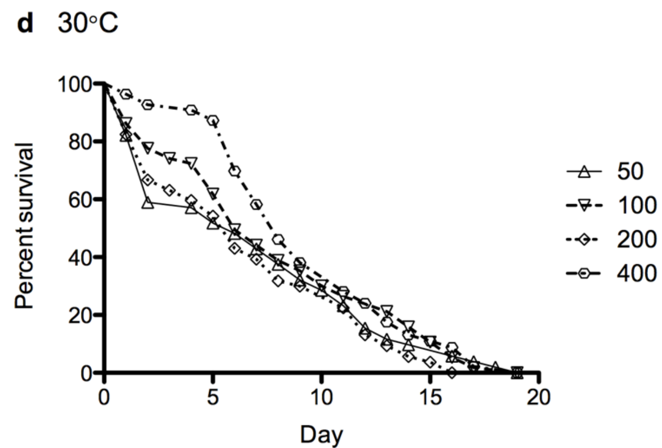


Figure 4.4. Adult male survival. Survival curves of adult males collected from each treatment and held in cages in the insectary: (a) 20°C, (b) 25°C, (c) 27°C, and (d) 30°C. Survival was measured daily until all mosquitoes were dead.

4.4.4. Length of wild vs. laboratory male wings

The size of *Ae. polynesiensis* males was analysed for seasonal trends. Wing lengths from wild *Ae. polynesiensis* male mosquitoes collected in Paea and Atimaono during the wet (December-March) and dry (April-November) seasons were normally distributed (Figure 4.5a). Mean male wing lengths during the wet and dry seasons were 2.129 ± 0.014 SEM (n = 110) and 2.132 mm ± 0.013 SEM (n = 109) respectively. The mean length of all wild male wings measured (2.13 ± 0.009 SEM, N = 219) was used as a benchmark for comparison with laboratory reared specimens.

The length of wings from laboratory-reared mosquitoes generally decreased as the temperature increased (Figure 4.5b). Wings were the shortest for mosquitoes reared at 30°C and 50 larvae/L (2.036 mm ± 0.017 SEM) and the longest at 20°C and 100 larvae/L (2.23 mm ± 0.0186 SEM). The effects of density, temperature, and their interaction were statistically significant only for males (Table 4.2). Wing lengths of male mosquito cohorts reared at 20°C were not significantly different from each other (F = 5.63, df = 4, P = 0.0002) (Table 4.5). Within these cohorts, larval densities of 100 and 200 larvae/L produced male mosquitoes with significantly larger wing lengths than wild males. Wing lengths of male mosquito cohorts reared at 30°C were also not significantly different from each other (F = 6.04, df = 4, P = 0.0001). Within these cohorts, larval densities of 50 and 400 larvae/L produced male mosquitoes with significantly smaller wing lengths than wild males (Figure 4.5 and Table 4.5). By comparison, significant wing lengths differences were observed between male mosquito cohorts reared at 25°C (F = 3.85, df = 3, P = 0.01). Within these cohorts, males produced at a larval density of 50 larvae/L had significantly smaller wings than those produced at 400 larvae/L. Similarly, significant wing lengths differences were observed between male mosquito cohorts reared at 27°C (F = 3.91, df = 3, P = 0.01). Within these cohorts, males produced at a larval density of 100

larvae/L had significantly smaller wings than those produced at 200 larvae/L. No significant wing length differences were observed between laboratory males reared at 25°C or 27°C and wild males (Figure 4.5 and Table 4.5).

Table 4.5. Wing length of wild and laboratory mosquitoes. Mean wing length and SEM of wild male mosquitoes collected in the field vs laboratory male mosquitoes reared according to different temperature/larval density treatments.

Treatment	Temperature (°C)	Density	Mean wing length (mm ± SEM)[*] [‡]
Wild	Variable	Variable	2.13 ± 0.0009a
Laboratory	20	50	2.20 ± 0.018a
		100	2.24 ± 0.018a*
		200	2.23 ± 0.013a*
		400	2.18 ± 0.018a
	25	50	2.14 ± 0.018a
		100	2.19 ± 0.013ab
		200	2.18 ± 0.010ab
		400	2.20 ± 0.0007b
	27	50	2.11 ± 0.016ab
		100	2.12 ± 0.008a
		200	2.15 ± 0.007b
		400	2.14 ± 0.008ab
	30	50	2.05 ± 0.013a*
		100	2.07± 0.011a
		200	2.05 ± 0.009a
		400	2.05 ± 0.013a*

[*] For each wing length of a given temperature/density treatment, means in the same column followed by the same letter are not significantly different from each other (Tukey's multiple comparison test)

[‡] For each wing length of a given temperature/density treatment, means in the same column followed by the same symbol are significantly different from mean wing length of wild males (Tukey's multiple comparison test)

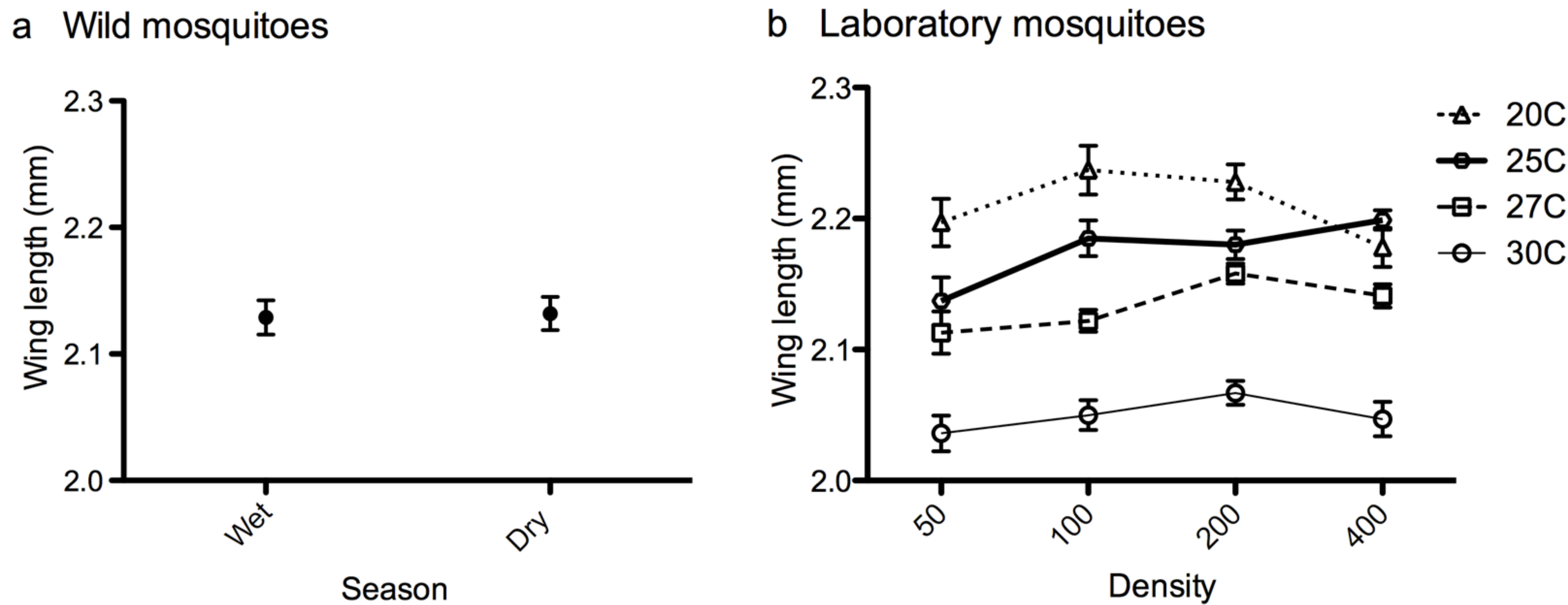


Figure 4.5. Wing length of wild and laboratory mosquitoes. Mean wing length and SEM of (a) wild male mosquitoes collected during wet and dry season, and (b) laboratory male mosquitoes collected from each temperature and larval density treatment.

4.5 Discussion

In this study, the effect of two rearing variables, temperature and larval density, were measured on several life history traits critical for the success of *Ae. polynesiensis* SIT/IIT i.e. larval survivorship, male:female ratio, time to pupation, and male yield with respect to mass-production and wing length and survival rate with regards to male performance. Besides the development of an appropriate CI-generating strain (see Chapter 2), the success of *Ae. polynesiensis* IIT at an operational level will require the optimization of a number of rearing and male performance parameters. For mass production, rearing conditions should be selected to allow short developmental time to pupation, synchronous pupation, and high adult male yield. These conditions would ensure maximum productivity while keeping production cost as low as possible. Importantly, male mosquitoes produced under such selective rearing conditions must display optimal mating competitiveness in the field to ensure the success and operational applicability of IIT.

In this experimental design both diet concentrations and larval densities increased by the same multiple (2-fold) resulting in the same amount of diet per larva in treatments examining different larval densities reared at different temperatures. As a consequence diet at low larval concentrations is relatively dispersed, and feeding to repletion might take more time and energy for these larval cohorts. Future studies on larval rearing should consider the addition of minerals, vitamins, yeast, and other sources of proteins. This could be compared to the natural breeding sites of *Ae. polynesiensis* such as highly nutritious coconuts. The goal here is to find a blend of nutrients that produce large quantities of quality mosquitoes at a lower cost.

Production of large numbers of sterile male mosquitoes free of female contaminations has been a major hurdle for numerous SIT/IIT control strategies (Benedict et al. 2009). Current trends are geared towards genetic manipulation of mosquitoes for the development of transgenic lines available for malaria, dengue and chikungunya vectors (Curtis et al. 1976, Catteruccia et al. 2005, Phuc et al. 2007, Papathanos et al. 2009). However, for *Ae. polynesiensis*, genetically modified mosquito sexing lines are not available thus leaving mass production in an IIT strategy dependent on biological separation exploiting the naturally occurring differences between males and females.

An empirical approach to larviculture was used to improve existing rearing and sex-separation procedures and develop a standardized laboratory protocol. As demonstrated in this study, temperature and larval density significantly influenced some or all of these parameters. The optimization of rearing conditions is thus a balance between critical productivity and male performance parameters. Conditions tested in this study allowed the identification of sets of temperatures and larval densities delivering high male yield and little or no female contamination on the first day of pupation (Table 4.4). Seven of the sixteen treatments tested resulted in the recovery, on the first day of pupation of 35% to 47 % of all male pupae produced, with the highest recovery rates generally obtained at a rearing temperature of 25°C. Overall, these treatments allowed the recovery of male *Ae. polynesiensis* pupae with an efficiency greater than that previously described for other Aedine mosquitoes (Medici et al. 2011, Bellini et al. 2013). *Ae polynesiensis* thus appears to be a species particularly amenable to biological and mechanical sex separation. Moreover, four of those treatments (200 and 400 larvae/L at 20°C and 25°C) yielded the highest number of male pupae (23.27, 40.96, 29.69 and 55.95 pupae respectively) offering prospects of adequate productivity when considering mass production. While the highest difference in mean age at pupation between male and

female was seen at 20°C (1.8 to 2.3 days), rearing at this relatively low temperature greatly increased the mean time of larval development (10 days), a feature less desirable in the context of mass production where production time should ideally be kept to the minimum. Likewise, cohorts of male pupae collected on first day of pupation were contaminated with some females and adult male survival rate was the lowest making treatments of 200 and 400 larvae/L at 20°C inadequate for male production, sex separation and field performance. By comparison, rearing treatments at 25°C and larval densities of 200 and 400 larvae/L provided conditions highly suited for IIT (Table 4.4). Short development time (6 days to pupation) with a useful difference in mean male:female age difference at pupation > 1day, allowing the recovery on the first day of pupation of nearly half of total males produced without female contamination, equal mean wing length with wild males, and relatively high numbers of adult males surviving at least six days after emergence, corresponding to the period of highest male sexual activity in some Aedine species (Boyer et al. 2011).

Laboratory rearing at 25°C at ILM, Tahiti where the temperature averages 26°C annually would minimize the cost for climate control thus reducing the unit cost of sterile males. Mosquito rearing at high (30°C) and low (20°C) temperatures would be particularly costly and thus inadequate for scaled-up operations. Moreover, when compared to wild mosquitoes, male mosquitoes reared at 25°C or 27°C exhibited wing lengths similar to mosquitoes collected in the wild ($t=0.16$, $d.f= 87$, $P=0.87$). Overall, standardized production at 25°C would facilitate implementation of IIT in most insectaries.

While competitiveness assays using incompatible *Ae. polynesiensis* males have been conducted (Chambers et al. 2011), the factors influencing male mating competitiveness are still not fully understood. The mating ability and reproductive

potential of *Ae. polynesiensis* was studied to some extent in the 1970s to understand the rapid displacement of *Ae. polynesiensis* by *Ae. albopictus* (Gubler 1971, Ali and Rozeboom 1973). More recent Aedine studies indicate that some males are more successful than others in mating with their female counterparts and that male age and size might be important factors (Ponlawat and Harrington 2009, Boyer et al. 2011). Further studies will be initiated to identify determinants of *Ae. polynesiensis* male sexual performance that are of importance to IIT.

Overall, this empirical approach allowed the development of a standardized laboratory protocol (rearing at 25°C at a larval density comprised between 200 and 400 larvae/L) well suited for the production and sorting of males only, displaying characteristics (wing length, adult survival) considered desirable for competitive field performance. This rearing protocol will be used as an internal standard in further comparative (mass) rearing tests to evaluate new rearing systems, alternate diet formulations and feeding regimes specifically designed for male mosquito mass production for the purpose of SIT/IIT.

Chapter 5: Release of Cytoplasmic incompatible *Aedes polynesiensis*: ranging trial on Tetiaroa[3]

5.1 Introduction

Although MDA is successful in temporarily decreasing the density of mf (Esterre et al. 2001), its capacity in achieving LF elimination is compromised in many Pacific island countries and territories (PICTs) by the biology of the *Ae. polynesiensis* (Ichimori 2001, Burkot et al. 2006). The pattern of negative density-dependent transmission may contribute to the failure of LF elimination programs (Pichon 2002). Various attempts have been made to control *Ae. polynesiensis*, but so far none have been completely successful (Burkot and Ichimori 2002). Considering the wide range and large number of available breeding sites, coupled with the often rugged and inaccessible terrain of most PICTs, it is not feasible to eliminate all potential breeding sites (Samarawickrema et al. 1992a, Riviere et al. 1998a, Lardeux et al. 2002b, Young 2007).

A novel vector control strategy similar to the sterile insect technique (SIT) was previously described in Chapter 2. Unlike classical SIT strategies that rely upon chemicals or radiation to sterilize males, the proposed method takes advantage of the cytoplasmic incompatibility (CI) induced by *Wolbachia* (Sinkins and O'Neill 2000). Consequently, this approach renders mosquitoes infected with different *Wolbachia* types reproductively incompatible (Werren 1997). Field surveys to date have indicated that wild *Ae. polynesiensis* are infected with a single *Wolbachia* type (Dean and Dobson 2004,

---

[3] Parts of this chapter are in manuscript form for PLoS Neglected Tropical Diseases: Limb K. Hapairai, Michel A. Cheong-Sang, Steven P. Sinkins, and Hervé C. Bossin. Release of Cytoplasmic incompatible *Aedes polynesiensis* males: ranging trial on Tetiaroa, French Polynesia.

Behbahani et al. 2005, Plichart and Legrand 2005). Studies under laboratory (Brelsfoard et al. 2008) and semi-field conditions (Chambers et al. 2011) supported the *proof of concept* of using CI for population suppression. A field trial was recently conducted on the island of Raiatea (O'Connor et al. 2012), with an average of 3800 males/week released over multiple weeks with the logistical issues related to production and sorting of males from females.

For this study, the objective was to produce larger numbers of males from the APr strain, an *Ae. polynesiensis* CI line that was characterized in Chapter 2. Larval temperature and density were managed in a large-scale production setting using the optimized rearing protocol developed in Chapter 4. The initial objective was to produce and release 6000 males each week. Male mosquitoes that would be released would also need to be free of potential female contaminants at the time of release. Releases were conducted on motu Onetahi for treatment and Tiaraunu for control on the atoll of Tetiaroa. Tetiaroa and its numerous islets were selected for the closed population dynamics characterizing its motus (Chapter 3). An additional objective was to demonstrate the induction of sterility into a wild female population using the APr strain. In total 14 weeks of APr male releases were completed on Tetiaroa and egg hatch from wild females was checked weekly to measure the effectiveness of the strategy. Logistical aspects were also addressed to overcome issues related to field releases in remote islands, which are common in PICT. This study was intended to serve as a ranging trial before the implementation of a larger-scale population suppression trial on Onetahi.

5.2 Objective

The overall objective was to produce and release 6000 males/weeks of the APr strain that displays CI when mated with wild females on Onetahi and in the process measure the changes in fertility of the wild population.

The specific aims were:

1) Measure potential migration of males from release motu Onetahi to the closest motu Honuea located at 200m apart using MRR.

2) Scale up production of males using parameters defined in Chapter 4 to 6000 males/week while measuring the number of females contaminants removed before each release.

3) Measure the quality of the males produced by comparing their wing length and adult survival to that of their wild and Tetiaroa strain counterparts respectively.

4) Measure and compare female fertility on treatment motu Onetahi and control motu Tiaraunu before, during and after release to monitor impact of APr males releases.

5) Monitor *Ae. polynesiensis* population on Onetahi throughout the study and verify cohorts of field collected females for *Wolbachia*-type infection by PCR for accidental female releases.

5.3 Materials and Methods

5.3.1 Migration

Ae. polynesiensis-Tetiaroa colonies were established by collecting specimens using a backpack aspirator (John W. Hock Company, Gainesville, FL), amplified and maintained as in Chapter 3. Resulting eggs were hatched under vacuum, larvae were fed on Liver powder (MP Biochemicals, Solon, OH), and placed into a 200 ml bowl containing water until emergence in cages as in Chapter 3. Once emergence occurred, a

10% sucrose solution was provided for energy source and cages left at the ILM Insectarium until release. For MRR, fluorescent-powder marked males were released on the northeast shoreline of Onetahi. Males were recaptured using four BGS traps immediately after releases for five days as in Chapter 3 on the southwest shoreline of Honuea (Figure 5.1).

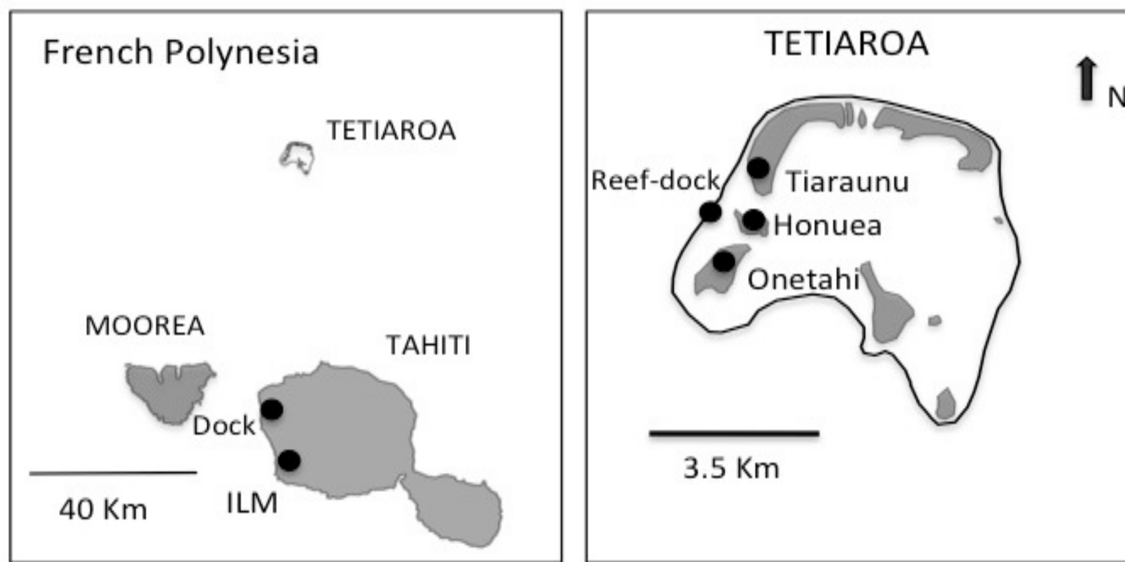


Figure 5.1. Map of Tetiaroa, French Polynesia. The Tetiaroa atoll is located north of Tahiti where mosquitoes were reared at ILM in Paea, Tahiti. Mosquitoes were first transported by land to the dock of the Intercontinental Hotel in Faaa, Tahiti and shipped by boat to the reef-dock on Tetiaroa before entering the lagoon to reach motu Onetahi.

5.3.2 Male mosquito production

Mosquitoes were reared at the ILM Insectarium in a 4x4x2 m room (Table 5.1). Conditions were created to match as closely as possible those described in Chapter 4 using a standard split air conditioner rather than an incubator. Synchronized egg hatch was induced using vacuum in multiple Erlenmeyer flask. Immediately after hatch, approximately 200 L1 larvae were distributed per larval pan (30x20x7 cm) each containing 1.2 L of tap water. Food (calf liver powder) was provided ad libitum (approximately 500 mg of powder/pan spread over the larval cycle) as in Chapter 4.

On the first day of pupation, pupae and larvae were collected and separated using a Hock sorting device (John W. Hock company, Gainesville, FL). Male pupae were further sorted according to size based on sexual dimorphism (Focks 1980) using this same device. Male pupae were allowed to emerge in groups of 500 in 30X30X30 cm aluminum cages (Bioquip, Rancho Dominguez, CA, USA). Following emergence, dead pupae and adults were removed and counted on the day preceding the release (Table 5.1). Individuals in each cage were examined visually to remove any potential female contaminant and a 10% sucrose solution was provided.

Cotton cloth was used to cover cages to avoid insolation during the seven-hour transfer. Mosquito cages were transferred from the Tahiti laboratory to the release site on Tetiaroa (an early morning one-hour car ride from laboratory to dock followed by a three-hour open ocean boat ride from Tahiti to Tetiaroa) (Figure 5.1). Since there is no pass to enter the atoll of Tetiaroa reef, cages were transferred by small vessels over the reef-dock before arriving on the lagoon side of Tetiaroa. Cages were loaded on another barge until Onetahi. Upon arrival, cages were loaded on a 4x4 vehicle and taken to the release stations. Each cage was placed on a roofed, ant-proof release platform protecting the APr male mosquitoes from rain and direct sunlight. This platform was made by welding a 50cm piece of metal on one end to an automobile rim that was used as a base. A 45 x 50 x 50 cm plywood was mounted to the other end of the piece of metal creating an elevated platform. Another similar size plywood was mounted 45cm above the (bottom) support plywood as to provide covering for cages. Engine grease was applied generously on the 50cm piece of metal to prevent ants from climbing into the cages. The transportation routine generally began at 4:00 am with all male releases completed before 11:00am. Males were four days old at time of release.

Up to 17 release stations were selectively distributed across motu Onetahi. The stations were more concentrated in areas where BG-mosquito catch rates were highest (Figure 5.2). Cages were left opened on the release platforms allowing mosquitoes to fly away. The actual number of males released was calculated for each cage by subtracting the number of dead mosquitoes that did not fly out of the cage, the number of dead pupae, dead adults, and females removed from the cage before release to the total number of pupae inserted into the cage for emergence.

5.3.3 Population fertility

Fertility in wild females was monitored at both treatment and control sites. Monitoring was performed every two weeks from April to August (eight measurements). Once releases began, fertility was measured weekly on the day of release. Post-release measurements began four weeks after last release and were conducted every other week from January to March (three measurements).

Fertility was measured by blood feeding field-collected females and placing them individually into oviposition tubes with 10% sucrose solution. Wild females were collected south of the airstrip (central portion) on the treated site at various sampling locations across the highly vegetated portion of the motu where mosquitoes where most abundant (Figure 5.2). Recovered individual egg batches were hatched after embryo development by simple flooding in yeast water. Hatch rates were determined by examining egg status under a dissecting microscope (Leica EZ4D, Leica Microsystems GmbH). To ensure accurate hatch rates, the insemination status of females which egg batch did not hatch was determined. Spermathecae were dissected, crushed on a microscope slide in PBS and examined for the presence of sperm under a compound microscope (Chambers et al. 2011). Egg batches from non-inseminated females were removed from the data set.

5.3.4 Population monitoring

Adult populations at both treatment (Onetahi) and control (Tiaraunu) sites were monitored before, during and after male releases.

1) Before releases, the distribution of *Ae. polynesiensis* on motu Onetahi was examined over time using three consecutive 24-hour collections in May, July, and August 2012. To that end, an array of 37 BGS traps lured with BG-lure (Biogents, Regensburg, Germany) was deployed across the motu and five traps were used to monitor the mosquito population at the control site (Chapter 3). Collected mosquitoes were counted and the wings of *Ae. polynesiensis* males were measured whenever possible. Five BGS traps were placed also on Tiaraunu for comparison.
2) During releases, monitoring was done weekly for 20 hours using 12 BGS traps on Onetahi and three traps on Tiaraunu. BGS traps on Onetahi were placed in the central and southern portions of the motu (Figure 5.2)
3) After releases, monitoring was conducted every other week until complete female fertility was recovered. The network of BGS traps used was the same as the release period.

Collected mosquitoes from BGS traps were killed using chloroform and transported to ILM where specimens were sorted and identified. Females specimen were preserved at -20°C for later PCR evaluation to measure accidental release of APr female onto Onetahi.

PCR detection of *Wolbachia* was done using the 136F/691R wsp primers to detect A-type and 81F/522R wsp primers to detect B-type *Wolbachia* (Zhou et al. 1998, Plichart and Legrand 2005, Brelsfoard et al. 2008) DNA extraction was performed on pools of 20 female mosquitoes without heads, using the Qiagen DNeasy kit (Qiagen, Valencia, CA). Elution was in 200µl and 5 µL DNA was used for PCR as in Chapter 3.

5.3.5 Male survival and size

To measure daily survival of males, a semi-field male survival cage assay was performed on ILM field station property. The released strain was compared to F1 male progeny from Tetiaroa field collected females. To measure survival, up to 100 male mosquitoes were placed into 30 x 30 x 30 cm cages. Four cage replicates were done and repeated three times. Cages were provided with a 10% sucrose solution using coffee filter wicks that were renewed weekly. Cages were placed on frame in order to prevent ant predation under tarpaulin to protect from rainfall similar to Chambers (2011). Mortality was counted daily.

To measure survival of released males after one week, two MRR experiments were performed on week 8 and week 10. To mark mosquitoes, fluorescent dust (Day Glow, Switzer Brothers, Cleveland OH) was applied as previously described in Chapter 3. Marked and released mosquitoes were recaptured in BGS traps within the next 24 hrs as described above, and a week later.

The length of BGS-collected wild male mosquitoes intact wings was compared to that of randomly selected laboratory-reared APr male mosquitoes. Right wing length was measured to the nearest 0.01 mm from the auxiliary incision to the tip using photographs taken (LEICA EZ4D) with a micrometer (Wild-Heerbrugg, Switzerland) for calibration then measured using the ImageJ software (Rasband 2004) as in Chapter 2.

5.3.6 Data analysis

Difference in female fertility between Onetahi and Tiaraunu was calculated using the Fisher Exact test. Analysis of Variance (ANOVA) was used to compare wing lengths of wild collected males and produced males. Daily survival of cages males was estimated using the log-rank test with surviving males being censored for analysis. Comparison of

wing length from laboratory and wild specimen was done with a Student t-test. Regression analysis was used to measure the slope of the transformed $\log_{10}(X+1)$ raw data. Statistical analysis was done using GraphPad Prism version 5.0 (GraphPad 2010). The wild male population released (MRR) was estimated using the modified Peterson Index formula P=[(an)/r]-a (Silver 2008).

5.4 Results

The study was conducted over an eleven-month period. Pre-release population fertility was conducted at two weeks intervals from April to August 2012. A total of 14 weekly-releases were performed from September to December 2012. Three post-treatment population sampling were done in January and February 2013.

5.4.1 Migration

The dominant wind direction during recapture was east to west (May 4-9, 2011). A total of 28 males and 102 females were collected over a five-day period from the four traps on motu Honuea. None of the 686 marked males that were released on Onetahi were collected across the 200 m sea-channel on Honuea.

5.4.2 Mosquito production

The insectary was maintained throughout the production period at 25.0 °C (±3°C) and between 75 % and 95% relative humidity. Temperature in the insectarium was controlled using a standard split air-conditioner (model Splitair, Galanz, Guangdong, China) in an effort to create temperature conditions similar as in Chapter 4 and air was also humidified (model Vapadisc 777, Villeneuve la Garenne, France) for adult mosquitoes. Direct sunlight was screened by shades covering windows. Activities of mosquito production are summarized in Table 5.1.

Colony amplification for egg production was done during the first five weeks. From week 6, approximately 80,000-100,000 eggs were obtained per week from ten colony cages initially containing approx. 600 female and 300 male pupae each. These female pupae were collected on the second day of pupation (Table 5.1) and males from the first day of pupation. Mosquitoes in the colony cages were one week old before being blood fed four times per week for two weeks (total of 28 days) after which mosquitoes were discarded. Females were allowed to lay eggs on wet filter paper replaced twice per week. Eggs that were at least five days old were hatched simultaneously under vacuum.

During the first three weeks of the scaling phase, pupae were gathered from the first and second day in order to increase the number of released males. After this initial phase, only males from the first day were processed using 200 pans/week. This number of pans allowed of production of over 12,000 male pupae/week. Attempts were made to transfer all produced males to Onetahi, however logistical constraints limited the number of transferrable release cages and therefore the number of males transferred to Onetahi (ca. 8500 pupae). Given this limitation, mosquito production was adjusted from 200 pans/week to 130 pans for week 8 through week 13, and 150 pans for week 14.

To measure the efficiency of mechanical sorting based on male-female sexual dimorphism using the John Hock sorter, the number of sorted females pupae was compared to the number of removed adult females in release cages using a mouth aspirator. On week 5 and 6, female pupae separated from males using the John Hock sorter were counted (males approximated) to 187 females/10,900 males and 190 females/12,000 males. From the 8,500 pupae that were placed in cages for each week (Table 5.2), a total of four adult females were collected using the mouth aspirator (two females per week). Mechanical sorting based on dimorphism allowed removal of approximately (2/187 females and 2/190 female) 99% of females.

Table 5.1. Schedule for three persons involved in laboratory production and field release of CI male mosquitoes on Tetiaroa. Activities for laboratory are given in numbers (1 to 8) and field in letters (a to h):

1) Hatch eggs
2) Distribute L1 pupae into pans
3) Feed larvae
4) Separate male pupae for release
5) Count male pupae and place in cages
6) Separate females for colony
7) Remove females from holding cages
8) Feed adult colony

a) Transport CI males to Tetiaroa
b) Release CI males
c) Collect and feed blood wild females
d) Monitor population with BGS traps
e) Feed adult mosquitoes
f) Identify specimen collected in BGS traps
g) Hatch eggs for fertility measurement
h) Count egg hatch

	Activities*			
	Laboratory		Field	
Day	Person 1	Person 2	Person 2	Person 3
Thursday	1,2,3	1,2,3	-	-
Friday	3	2	-	-
Monday	3	3	-	-
Tuesday	4	-	-	-
Wednesday	5	-	-	-
Thursday	5 &1,2,3	5 &1,2,3	-	-
Friday	6 & 3	6 & 3	-	-
Monday	7 & 3	7 & 3	-	-
Tuesday	8 & 4	-	a,b,c,d	a,b,c,d
Wednesday	8 & 5	-	c,d,e	c,d,e
Thursday	5 &1,2,3	5 &1,2,3	-	e,f
Friday	6 & 3	6 & 3	-	e,f
Monday	7 & 3	7 & 3	-	e,f
Tuesday	8 & 4	-	a,b,c,d	a,b,c,d
Wednesday	8 & 5	-	c,d,e	c,d,e
Thursday	5 &1,2,3	5 &1,2,3	-	g,h & e,f
Friday	6 & 3	6 & 3	-	h & e,f
Monday	7 & 3	7 & 3	-	e,f
Tuesday	8 & 4	-	a,b,c,d	a,b,c,d
Wednesday	8 & 5	-	c,d,e	c,d,e
Thursday	5 &1,2,3	5 &1,2,3	-	g,h & e,f
Friday	6 & 3	6 & 3	-	h & e,f

*Numbers and letters of the same color are activities for the same release

L1 larvae were not counted individually as in Chapter 4 as this method would have been too time consuming (12-15mins/pan). Instead, groups of approximately ten larvae were suctioned and distributed in each pan twenty times (total 200 larvae) allowing

for a comparatively faster distribution of larvae (1-2mins/pan). Verification in a few randomly selected seeded pans indicated a range between 200 and 300 larvae per pan.

Fourteen releases were performed with an average of 7465 male mosquitoes released each week and a total of 104,518 male mosquitoes released (Table 5.2). The threshold was obtained at around 8000 males/week after the fourth week utilizing 70 man-hours for each production batch (Table 5.1). After mechanical pupae sorting, a total of 285 contaminating adult females were removed during the entire period from all cages intended for release with an average of 99.7% male purity before visual control (Table 5.2). This average may have been improved by avoiding collections of male pupae on the second day of pupation when females began pupation.

Table 5.2. Weekly production of APr male cages and female contaminant. Pupae were counted and placed in cages before emergence. Prior to male releases, cages were controlled visually. Adult females if any were removed and counted. This value was deducted from the number of pupae to calculate the number of males effectively released.

Week	# Pupae in Cages	# Removed females	% Removed females	# Released males
1	4942*	10	0.20%	3006
2	7000*	122	1.70%	5855
3	6000*	2	0.00%	5709
4	8500	15	0.20%	8195
5	8500	2	0.00%	8141
6	8500	2	0.00%	8073
7	8500	22	0.30%	8127
8	8500	0	0.00%	7574
9	8500	34	0.40%	8015
10	8500	0	0.00%	8129
11	8500	28	0.30%	8126
12	8500	3	0.00%	8110
13	8500	45	0.50%	8222
14	9500	0	0.00%	9236
Total	112442	285	0.30%	104518

* Collected and males sorted on first and second day of pupation.

5.4.3 Mosquito population dynamics prior to releases

The *Ae. polynesiensis* distribution was not homogenous across motu Onetahi (Figure 5.2):

- Northern portion: Very few *Ae. polynesiensis* mosquitoes were collected on this part of the motu in May, July and August. It is very likely that the airstrip provided an artificial barrier sufficient to prevent mosquitoes from reaching the, rather barren, northern tip of the motu. The new airstrip was built to run parallel to the dominant trade wind (East) creating an airstream with no vegetation for mosquitoes to rest.
- Central portion: The number of mosquitoes collected was the highest along the central portion of the motu in May, July, and August collections. Adult resting sites were numerous in this comparatively densely vegetated area. In some places, a depression in the ground favored the soil to saturate with the water table after heavy rainfall. These damp conditions are favorable to the development of *Pisonia umbellifera*, and *Hibiscus tiliaceus*. Moreover, occasional searches for potential larval development sites revealed the presence of large numbers of natural containers (rat eaten coconuts, leaf axils) but also numerous domestic breeding containers (building materials, cistern, unmaintained swimming pools) in this central area.
- Southern portion: The number of mosquitoes collected in the southern portion decreased over from April to August. Vegetation cover was less important with some coconut trees that provided less suitable resting sites for mosquitoes.

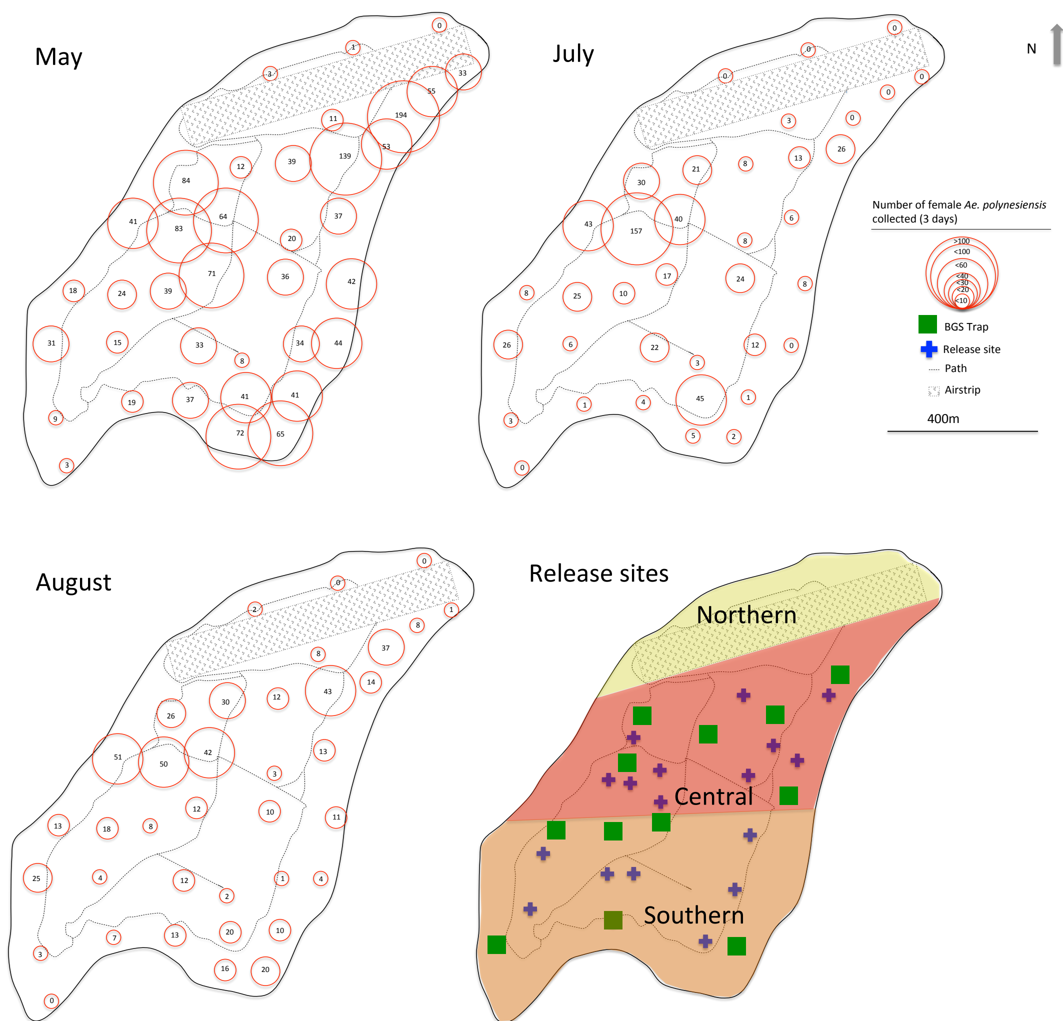


Figure 5.2. Map of mosquitoes distribution on motu Onetahi before releases. Circles represent the total number of *Ae. polynesiensis* female specimens collected over three days in the months of May, July and August using the BGS traps with BG-Lure. The distribution *Ae. polynesiensis* across motu Onetahi is heterogeneous. Mosquito density is high in the central portion (red), intermediate in the southern portion (orange) and low in the northern portion (yellow) of the motu.

5.4.4 Male survival and population estimate

From semi-field cage studies, release-strain male survival was higher than that of the Tetiaroa males (Figure 5.3). The survival curves were significantly different between the release-strain males and F1 Tetiaroa males (Log-Rank; X^2=50, P<0.0001). The

median survival was also higher for release-strain males with 10 days compared to 4 days for Tetiaroa males.

MRR experiments of APr males on Onetahi show evidence of survival of released male mosquitoes in between releases. On week 8, a total of 7574 APr males were marked and released across the 17 stations and sampling using the network of 12 BGS traps described above was launched. Within 20hrs following the release, 174 males (both marked and non-marked) were collected in BGS traps of which 61 males were marked (recapture=35%). On week 9, a total of 8015 APr males (not marked) were released, 116 males were collected of which 21 were marked males released on week 8 (recapture = 13%). MRR was repeated on week 10 with recapture rate of 22% (Table 5.3). On week 11, a total of 8126 APr males (not marked) were released and 131 males were collected of which 17 were marked males released on week 10 (recapture=18%). The average rate of males recaptured one week after release is 15.5%.

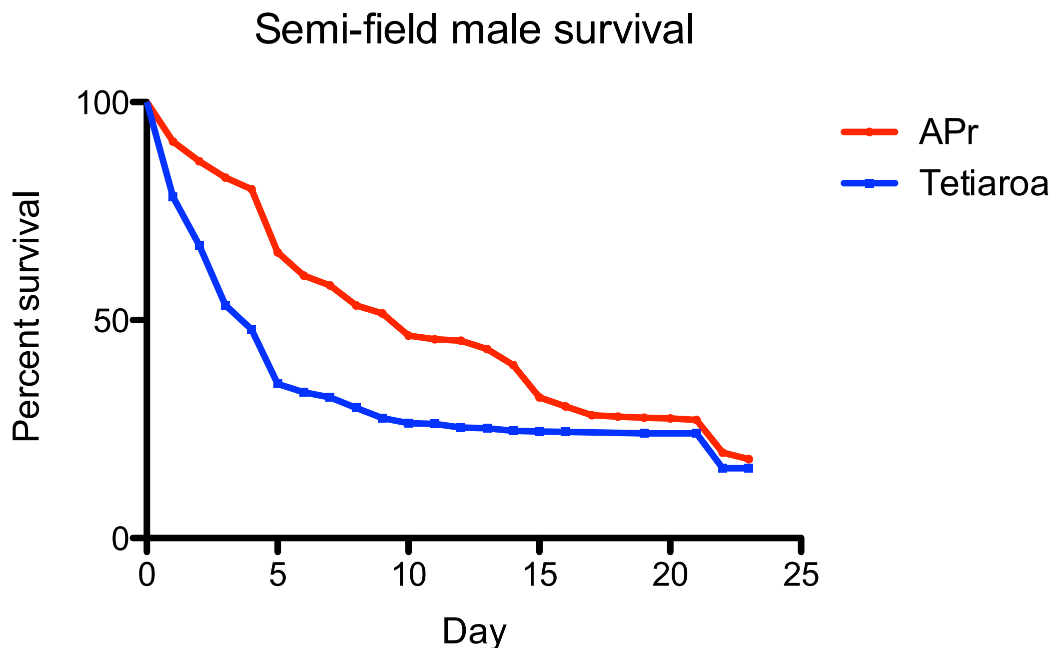


Figure 5.3. Semi-field male survival. Survival curves of APr (red) and Tetiaroa (blue) males in semi-field cages.

Using a modified Peterson Index, the wild male population was estimated with formula P=[(an)/r]-a (Silver 2008). From males collected on the day of release, the wild male population was estimated at 14,031 (week 8) and 28,122 males (week 10) or 189 males/ha and 380 males/ha respectively (Table 5.3). This figure is probably an overestimate of the actual size of the wild male population, given that some of the non-marked males captured on week 8 and week 10 were actually composed of released males carried over from the previous week (approximately 15.5%). Conservative estimates given for week 8 and 10 would approximate a 1:2 ratio release-strain males per wild male mosquitoes.

Table 5.3. Mark-Release-Recapture of males experiment on Onetahi. APr-strain males were released and collected on week 8-11, with males marked on week 8 and 10. Released males were collected in BGS trap for 20 hours either immediately or seven days later. The wild male population was estimated using the modified Peterson index.

Week	# APr released	# APr marked	# Total captured	Collection after release	# Marked	Recapture rate	Wild population estimate
8	7574	7574	174	1-20hrs	61	35%	14031
9	8015	0	116	7days	21	18%	-
10	8129	8129	165	1-20hrs	37	22%	28122
11	8126	0	131	7days	17	13%	-

5.4.5 Male size

There was no significant difference in wing lengths between laboratory-reared and released APr males (n=112) and wild males collected in the BGS traps (Onetahi n= 160, Tiaraunu n=72) (ANOVA; $P>0.05$). Average wing length of released males was 1.95mm ± 0.005mm (SEM). That of wild collected mosquitoes on Onetahi and Tiaraunu was 1.97mm ±0.01mm and 1.95mm ±0.01mm respectively (Figure 5.4).

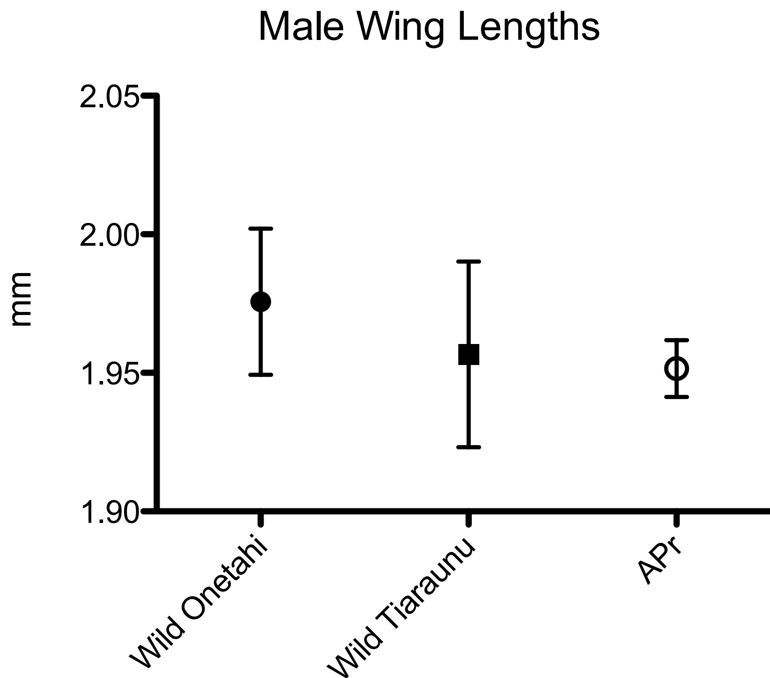


Figure 5.4 Male wings lengths. Mean wing lengths (mm) and 95% CI of wild mosquito collected in BGS traps prior to release on Onetahi and Tiaraunu, and released APr mosquitoes that were produced in the laboratory.

5.4.6 Population fertility

From hatching egg broods, the average female fertility prior to releases was 99.6% ± 0.38% (SEM) on the control site (Tiaraunu) and 99.4% ± 0.57% on the treatment site (Onetahi). Once releases were performed, decrease in fertility was observed beginning week 4 with 88% on Onetahi (females collected at various locations across the central portion) compared to 96% on Tiaraunu. The difference in female fertility between Tiaraunu and Onetahi was significant from week 6 onward until the end of the release period (Fisher's Test, $P<0.05$). Fertility was at the lowest on week 11 with 63% of hatching eggs broods on Onetahi compared to 100% on Tiaraunu. Complete female fertility was recovered on Onetahi a few weeks after the end of the treatments (Table 5.4).

Table 5.4. Female fertility on Onetahi. Percent hatching broods of wild females collected on Tiaraunu (control) and Onetahi (treatment) before, during, and after APr male releases. Lines shaded in gray show measurements (weeks 6-14) where fertility on Onetahi is significantly different from that on Tiaraunu (Fisher's Test).

Release period	Measurement	Tiaraunu	Onetahi	Fisher P-value
Before	1	100%	100%	-
	2	100%	100%	-
	3	100%	100%	-
	4	100%	100%	-
	5	100%	100%	-
	6	97%	100%	0.48
	7	100%	100%	-
	8	100%	95%	-
During	1	100%	98%	0.47
	2	100%	100%	-
	3	100%	100%	-
	4	96%	88%	0.61
	5	98%	93%	0.62
	6	100%	84%	0.03
	7	100%	83%	0.02
	8	98%	65%	<0.01
	9	100%	84%	0.02
	10	100%	74%	<0.01
	11	100%	63%	<0.01
	12	100%	84%	0.01
	13	100%	89%	0.03
	14	97%	73%	<0.01
After	1	100%	100%	-
	2	100%	100%	-
	3	100%	100%	-

5.4.7 Population impact during and after releases

The slope of the regression line of monitored population during the release weeks (shaded in gray in Figure 5.5, a) on Onetahi was -0.0002 ± 0.014. This slope was not significantly different from non-zero (F<0.001, P-value=0.98) but poor goodness of fit ($R^2<0.01$). During the same period on the control motu Tiaraunu the slope measured -0.072 ± 0.016. This slope was significantly different from non-zero (F=0.64, P-value=0.001) and goodness of fit with $R^2=0.64$. Significant precipitations were observed

only at the beginning of the month of December and continued throughout to February (Figure 5.5, c).

All 360 females collected in BGS traps from weeks 8-11 and 13-14 were screened for *Wolbachia* type by PCR. Controls included Tetiaroa (A-type) and APr (B-type) for positives, APTT (aposymbiotic) for negative, and a pool of 19 A-type females and a single B-type female. PCR confirmed infection with wild A-type *Wolbachia* of analyzed females collected on Onetahi.

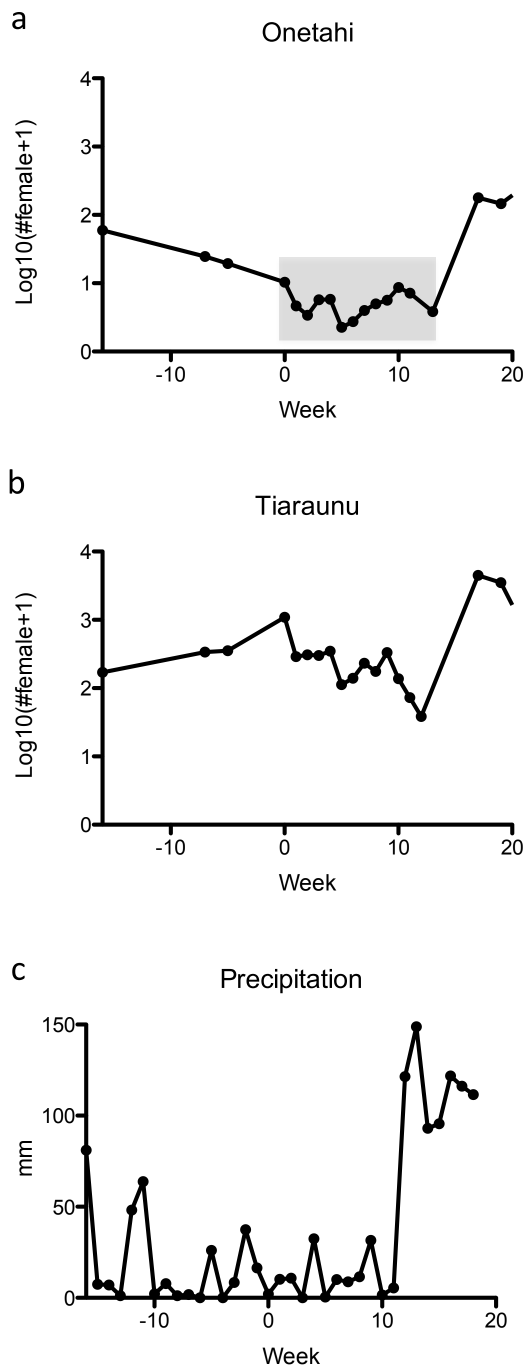


Figure 5.5. Population monitoring on Onetahi and Tiaraunu. Mosquito populations were monitored using BGS trap over a 36-week period. Collection in BGS traps were transform $\log_{10}(x+1)$ for representation on Onetahi (a) and Tiaraunu (b). The weeks shaded in gray show the period that CI males were released. Weekly precipitation (c) is given in mm of average rainfall/week. A significant increase in weekly precipitation starting on week 12/13 with over 150mm/week preceded the increase in population on both Onetahi and Tiaraunu.

5.5 Discussion

The MRR study between Onetahi and Honuea corroborated well with previous studies showing limited active migration of *Ae. polynesiensis* between relatively close motus. On the atoll of Rangiroa in the Tuamotu Archipelago, Lardeux et al 1991 (Capture-MRR) did not observe active migration across a 30m channel between the two motus of Terei'a (Lardeux 1991). More recently, the MRR study in Chapter 3 conducted in Tetiaroa between motu Auroa and its two flanking motus Hiraanae and Tauini, respectively 190m and 77m apart from Auroa, also showed no evidence of active migration. None of the males marked and released on motu Onetahi were recaptured 200m away on motu Honuea. However, these studies measured migration over a relatively short time scale (3-5 days), which does not preclude much longer-term natural and/or human induced migration. A significant weather event (cyclone "Oli") which occurred during releases in the O'Connor et al study (2012) arguably contributed to reduction of the wild population on Toamaro. High winds and inundative sea waves brought by this cyclone may have contributed to the emigration of mosquitoes off of Toamaro. During the present (four month) release trial, the assumption of low immigration risk of wild *Ae. polynesiensis* unto Onetahi and emigration of CI males unto surrounded motu is sound.

Potential immigration of mosquitoes to Onetahi would likely come from the average twice/day private aircrafts departing from the Faaa International airport located in an urbanized area. However, such flights would probably favor introduction of the urban mosquito *Ae. aegypti* (currently absent from Tetiaroa). For this purpose, fumigation of aircraft using insecticide should be prescribed as a standard protocol for future increasing flights to Tetiaroa. Human-assisted, inter-motu mosquito migration is

furthermore improbable as human movements on the atoll is strictly regulated and was limited during the trial to a couple scientists going between motu Onetahi and Tiaraunu each week with a dinghy or kayak.

Rearing at 25.0 °C with 200 larvae allowed for collection of up to 47% of all males on the first day of pupation assuming a 1:1 male to female ratio (Chapter 4). Considering this yield, the release of 6000 males would require the production of 12,756 males using a total of 128 pans at 200 larvae/pan (i.e. 100 male pupae/pan). Although not directly measured in this study, first day pupation yielded ca. 8500 pupae from 130 pans (Table 5.2). This approximates between 65% and 43% males recovered from pans loaded with 200 and 300 L1 larvae/pan respectively. This high recovery rate for *Ae. polynesiensis* produced by optimizing rearing conditions is encouraging compared to only 10-15% males recovered with *Ae. albopictus* in Italy (Bellini et al. 2007).

In the O'Connor et al study (2012), an average of 3800 males/week were transported to the release site but the sorting process (multiple handling of adult males multiple times) affected their survival at the time of release (unpublished data): Individuals for release were first separated by sex at the pupal stage using a mechanical sorter (separation exploiting sexual size dimorphism). This first step removed approximately 90% of females. The remaining 10% of female contaminants were removed manually after anesthetizing pools of adult mosquitoes with chloroform. For production and release, two researchers and five technicians were required for an average of 3800 males/week. By comparison, the standardized rearing and separation protocol used here greatly improved the speed and productivity of the operation with 99% of females contaminants removed after the first separation step (combining time to pupation and mechanical sorting). This difference allowed for much quicker removal of the few potential female contaminants. Although comparison is arguable, for production, release,

and monitoring (female fertility and BGS trap), one researcher and two technicians handled an average of 7465 male/week (Table 5.1). The absence of female pupae on the first day of pupation observed in Chapter 4 was not reproduced in this study, probably because of fluctuations in temperature (22-28°C) and with variation in developmental time related to variation of larval density (200-300 larvae/pan). This study provides a procedure for rearing and sorting *Ae. polynesiensis* males in larger numbers which, with further improvements, may be scalable to semi-industrial production levels allowing the implementation of future large pilot suppression operations.

Although larger number of males could have easily been produced with the existing manpower, this objective was not pursued due to the subsequent constraints related to the shipment of cages to the release sites using existing logistics (boat). Logistics in this study allowed for routine shipment and releases of adults in the same day of up to 17 cages (ca. 500 mosquitoes each). The addition of two cages for a total of 19 cages on the last week as part of a demonstration to the Hotel owner was unfavorably high for routine releases. Preparation of release-strain male mosquitoes for transport was minimal. Future work will need to focus on the improvement of shipping methods to decrease volume of material to be released thus allowing the transfer of much larger numbers of males mosquitoes without affecting their mating competitiveness. Transfer of specimens at the pupal stage would easily reduce the volume of material transported. However, there is the risk of accidental females being released, as the visual adult screening of large adult numbers would not be performed. To avoid the release of fertile females, pupae might simply be exposed to low sterilizing doses of radiation before their transport and release. Irradiation at low level is of particular interest because it does not affect the fertility nor the competitiveness of released males (Brelsfoard et al. 2009).

Prior to CI male releases, fertility on Onetahi and Tiaraunu was nearly 100%. Once release began, fertility decreased on Onetahi while fertility on Tiaraunu remained the same. Fertility was significantly reduced beginning on week 6 and subsequent weeks with the lowest fertility measured on week 11 with 63% (Table 5.4). In the period following the end of the treatment, fertility on Onetahi quickly returned to the level of the control motu Tiaraunu. This result confirms that reduction in fertility on motu Onetahi was induced by the CI males mating with wild females rather than by natural fluctuation of fertility in the wild population. Moreover, semi-field survival studies and MRR experiments of released males demonstrate that release-strain males represent at least 15.5% of all males from week to week, a period during which CI males mate with wild females.

Monitoring of the wild population on Onetahi showed a non-significant decrease in the number of wild females during the 14 week-releases. This lack of significant reduction in the target population may be explained by the relatively low 1:2 CI:wild males ratio achieved during the treatment period (Table 5.3). Other factors may also have contributed such as harborage, potentially from eggs laid at an earlier period. Altogether, viable eggs from fertile females were in sufficient numbers to palliate for the unfertile eggs from females that mated with CI males. The trend of the population on Onetahi was further confounded toward the end of the study when heavy rainfall arrived later in the season than expected (Figure 5.5). The maximum length of time of which a viable egg of the *Ae. polynesiensis* will hatch in field-condition is unknown. In laboratory conditions, observed shelf life is one month (50% hatch rate) with only an anecdotal three-month-old batch of eggs that hatched (>1%). Further studies on the half-life of eggs are warranted in laboratory but unquestionably in field conditions, as this would provide inference on the duration of the treatment period required to achieve population elimination.

This ranging trial was conducted in a location with no conventional control for *Ae. polynesiensis*. Insecticide spray and breeding site removal throughout Onetahi might help reduce the size of the initial target population prior to releases of CI males. Rat control would possibly rid Onetahi of most new breeding sites essentially formed by rat-chewed coconuts that are omnipresent on the islet. The combined use of conventional (insecticidal) control and IIT as part of an area-wide integrated pest management would greatly reduce the number of CI males required to achieve population suppression on Onetahi.

This study demonstrated the scalability of the rearing method developed with the potential for CI male mass-production. In this study fertility was significantly impacted by releasing approximately 1:2 released:wild male ratio. To achieve suppression or elimination of LF vector, sustained inundative releases in excess of 5:1 to 10:1 CI:wild male ratios might be necessary. Furthermore before further releases of CI males are contemplated, studies on the viability of eggs and integration of conventional control and IIT are desirable. The significant sterility induced by CI in the targeted female population strongly supports the development and scale up of suppression/elimination strategies to control *Ae. polynesiensis*.

Chapter 6: Summary and Conclusions

Lymphatic filariasis (LF), a disease that can lead to profound psychological and social stigma is the primary cause of disability in the South Pacific. In the absence of suitable macrofilaricidal drugs, LF elimination strategy currently relies on repeated MDA. Microfilaricidal compounds are distributed to treat the entire at risk population throughout the region with the goal of breaking the transmission cycle by maintaining microfilariae levels in the blood below that required to sustain transmission (Ottesen et al. 1997). MDA campaigns have been applied repetitively since 1949 in French Polynesia with current treatments involving the annual distribution of Notezine® and Zentel®. These campaigns have resulted in the significant reduction in LF prevalence over time (Esterre et al. 2001, Chanteau and Roux 2008) and the decline of the disease's most severe forms. However, despite decades of MDA, LF sentinel sites demonstrate a failure to achieve LF elimination thus revealing that active transmission of LF is still occurring throughout the country (Mou et al. 2009). This failure is related to the biology of *Ae. polynesiensis* the principal LF vector in many countries in the region.

In contrast with other vectors (e.g., Anophelines), *Ae. polynesiensis* displays a pattern of negative density dependent transmission, which makes it a particularly efficient LF vector in areas of low-level microfilaremia, a situation created by years of MDA (Southgate 1992, Pichon 2002). The understanding of the vector's biology implies that MDA alone is insufficient to break the LF transmission cycle (Burkot et al. 2006). Unfortunately, the inaccessibility of *Ae. polynesiensis'* breeding sites, its cryptic behavior and diurnal nature has prevented its effective control solely using conventional methods (insecticides, breeding site treatments, etc.). The paucity of tools in the arsenal available

to control *Ae. polynesiensis* has driven an increased interest for adapting demonstrated SIT concepts in an IIT framework using *Wolbachia*-mediated bidirectional CI (Brelsfoard et al. 2008).

In this thesis, *Wolbachia* was investigated and evaluated in laboratory and field settings with the goal of providing a suitable tool to control *Ae. polynesiensis* as part of an integrated strategy to combat LF in the South Pacific. Chapter 2 describes the introgressive hybridization of vector aposymbiotic *Ae. polynesiensis* with two species of the *Ae. scutellaris* complex. Subsequent backcrossing was used to produce and characterise *Ae. polynesiensis* lines displaying CI and reduced susceptibility to filarial nematode parasites that may be utilized in elimination or replacement strategies respectively, and in the process characterise the inheritance of susceptibility to filarial nematodes in *Ae. polynesiensis*. Prior cross experiments suggested that the *Wuchereria* refractoriness is dominant, so the initial intent was to produce a line refractory to the *W. bancrofti parasite* using *B. pahangi* as a model (Macdonald 1976). Refractory lines would be produced by selecting the progeny from non-infective females at each backcross. However, results from vector competency of produced lines introgressed with either non-vector *Ae. riversi* and *Ae. katherinensis* to the *B. pahangi*, suggest an incomplete dominance of genes for a refractoriness phenotype and / or imperfect selection. Possibly future refractory *Ae. polynesiensis* work should be with infection of *Wolbachia* known to up regulate the innate immune system using embryonic microinjection, as performed in *Ae. aegypti* (Kambris et al. 2009).

Although the initial intent was not achieved, three lines exhibiting strong CI from crosses between *Ae. polynesiensis* and *Ae. riversi* were generated. Eggs from crosses between males infected with B-supergroup *Wolbachia* type and wild type A-supergroup *Wolbachia* females produced results similar to the previously described CP strain

(Brelsfoard et al. 2008). The difference from the CP strain is that selection was done at each backcross progeny of non-infected female from vector competency of the refractoriness study. The APr (*Ae. polynesiensis*+riversi) line resulting from this thesis demonstrates an equal vector competency to *B. pahangi* compared to wild type, an important characteristic in the event of an accidental female release. Assuming the *B. pahangi* model holds, accidental female release would not increase transmission of LF. Also, after initial bottleneck effects from the interspecies cross were overcome, fertility rates, wing lengths, and survival of created CI lines resembled that of wild *Ae. polynesiensis* lines. The BC 9 (backcross 9) line progeny from the APr line was backcrossed with aposymbiotic *Ae. polynesiensis* Tetiaroa for four generations to increase fitness for field releases. Although adaptation to laboratory conditions may provide certain behavioral advantages (mainly artificial membrane feeding), further antibiotic treatment of wild mosquitoes lines should be done and made available for regular and routine backcrossing into existing CI lines. Further introgression would further increase genetic background thus reducing the chances detrimental phenotype to male fitness associated with inbreeding in colony lines.

Cage trials for male mating competitiveness of the APr line and suppression assay with the BC9 line showed that CI males were able to mate with wild type females at the same rate as wild type males thus providing support for release of CI males to sterilize wild females. Although informative, cage trials have many limitations in accurately describing mating behaviour in the wild (Ferguson et al. 2008). For instance the efforts needed for a male to mate in the wild requires it to spend energy traveling before being in proximity of a female, while a male in cage experiments is artificially aggregated with virgin females and provided with an energy source. Future study should identify such factors essential for competitiveness of released males and their ability to copulate with

wild females in the field at a rate comparable if not superior to wild males. For this, investigation of biological factors from field-caught male mosquito must be done particularly on factors that would likely influence longevity and mating competitiveness.

An early hurdle was to find a suitable sampling tool to accurately collect *Ae. polynesiensis* field specimen. The human landing collection and human bait collection (HBC) have been used for adult *Ae. polynesiensis* sampling (Russell 2004, Russell et al. 2005a), these methods show variable catch rates, are unethical, and particularly undesirable. Traps provide an advantage in avoiding unnecessary exposure to infectious bites as well as attractiveness and time biases. In Chapter 3, the efficacy of the BGS and BGM traps for sampling was evaluated against HBC on Moorea and Tetiaroa,. The tested traps were generally successful in collecting female specimens with the BGS trap capturing the greatest number of *Ae. polynesiensis* in both Moorea and Tetiaroa while the BGM trapping was severely hampered by damage from rats in the Tetiaroa experiments. Although this study confirms the efficiency, comparability, and convenience of the BGS trap as safe alternative to HBC, a suitable collection for adult male *Ae. polynesiensis* is critically lacking.

In Chapter 3, MRR studies were conducted to estimate the adult population size, migration, and dispersal patterns of male and female *Ae. polynesiensis*. Dispersal in the Hotutea valley was heterogeneous despite an elevation gradient with two females being collected at 690m away from their release location. This distance is well beyond the central dogma established in the 1950's of 92 yards (Jachowski 1954) putting in doubt the supposed 'short' dispersal and transmission capacity of *Ae. polynesiensis*. This highly exophilic behavior and wider range of dispersal of *Ae. polynesiensis* within the Hotutea Valley would currently better fit a population replacement strategy using a future developed refractory strain discussed in Chapter 2. Moreover, the absence of recaptured

marked mosquitoes on motu Auroa from the neighboring islets (77m and 190m away) demonstrated that mosquito population of this motu is isolated. Male and female mosquitoes released on motu Auroa were recaptured in large numbers in BGS traps placed around the motu demonstrating high dispersal within a motu. This behavior is advantageous for control strategies involving release of males, as this will increase their chances of finding and mating with their female counterparts in the field. Both migration and dispersal suggested that motu type settings such as those found on Tetiaroa are suitable for pilot scale field IIT releases using the APr line.

In Chapter 4, *Ae. polynesiensis* larvae were reared in an incubator under a range of temperatures and larval densities to select fast and synchronous immature development, high male yield, large adult body size and long survival. Generally, the time to pupation decreased with increasing temperatures. Wing lengths showed no significant differences to field collected males except at 20°C and 30°C for some densities which produce larger and smaller wings respectively. Larval density and temperature influenced the proportion of males pupating on first day of pupation with 43% to 47% of total male pupae produced at 25°C. Furthermore, female pupation at this temperature was delayed by at least one day. This delay considerably reduces time spent sorting males from female pupae on the John Hock sorter, since separation would only require sorting male pupae from remaining larvae if done on the first day. This study showed that *Ae. polynesiensis* appears particularly amenable to biological and mechanical sex separation. Altogether, Chapter 4 allowed the identification of rearing conditions for the APr line that could deliver high male yield with essentially no female contamination, adequate adult male size and survival.

Chapter 5 reports the results of the ranging field trial conducted on Tetiaroa. The lack of collection of marked and released mosquitoes on motu Honuea from motu

Onetahi (200m apart) demontrated, as expected, the isolation of motu Onetahi from migration. Prior to releases, population distribution was measured which allowed the positioning of 17 release stations throughout Onetahi according to the higher mosquito densities in the central and southern areas. Production of APr males for field realeases was done at the ILM laboratory using the rearing conditions developed in Chapter 4 (25°C and approximately 200 larvae/pan). After an initial adaptation and colony amplification for eggs production, approximately 8000 males were released weekly. Over the 14-week period, a total of 104,518 CI males were released throughout Onetahi. This sum represents over 93% of the 112,442 male pupae produced for release, the other 7% reflecting natural male mortality from the time of emergence to the time of release (incl., mortality induced by transportation), and 0.3% of female contaminants. The size and quality of the releases are encouraging given that production was done by only two technicians. Scale up of mosquito production for population suppression of a motu like Onetahi should be resonably feasible by increasing the number of mosquito workers and improving current rearing conditions. Moreover, to overcome moratlity induced by transportation, male production for Onetahi could be done on-site in a portable insectarium easily made by adapting shipping containers. This would not only reduce stress on mosquitoes and time for transportation but also remove the dependence on the once per week freight schedule, thus allowing for better males at a higher release frequency.

Baseline fertility rate prior to releases on both treatment (Onetahi) and control (Tiaraunu) motus showed high natural fertility with over 99% fertile females. On Onetahi, female fertility decreased during releases of APr males while fertility on control motu remain unchanged throughout the study. The decreased in fertility on Onetahi was significant within 6 weeks and during all subsequent weeks (through to week 14) with the

lowest fertility rate after 10 weeks at 63% of wild females. Using MRR experiments, male population size estimates suggests an approximate 1:2 CI:wild male ratio. This ranging trial shows that despite a relatively low ratio, released CI males were able to succesfuly find and mate with virgin females demonstrating suitable male mating competiviness of CI male in the presence of wild males. Selected CI colonies, including the APr line, are currently being amplified at ILM for development of mass-rearing protocols in Bellini's laboratory (Centro Agricoltura Ambiente, Crevalcore, Italy), a project supported by INFRAVEC. The goal is to adapt these lines of *Ae. polynesiensis* to mass rearing equipment existing for *Ae. albopictus*. The hope is to optimize production of mosquitoes in existing ILM laboratory or future portable laboratory while achieving required quantities demanded for inundative releases, typically high for SIT.

During the time of the field trial, rat activity was frequently seen and associated with the abundance of chewed coconuts on the ground. These coconuts were most likely the principal breeding sites and egg reservoirs for *Ae. polyensiensis* (Russell et al. 2011). For Onetahi, an initial raticide treatment, and reduction of breeding source would greatly reduce the number of remaining wild eggs and also reduce the number of APr males to be released

Although significant CI-induced reduction in female sterility was observed, the adult population trend observed on motu Onetahi using BGS traps was similar to that of the control motu which both generally followed rainfall fluctuations. This is most likely due to harborage of viable eggs, a vital reservoir for population maintenance. *Ae. polynesiensis* eggs are known to be only viable for less then three months in laboratory but the actual life of eggs on the field is unknown. A modest study addressing the life expectancy of *Ae. polynesiensis* eggs in the field together with a critical observation of

rainfall would have a great impact in determining the duration of future releases to drive the target population to elimination.

The positive outcome and succesful demonstration of fertility reduction from the field trial is encouraging the implementation of a *Wolbachia* population suppression trial. Based on the findings from this thesis, the 'Tetiaroa Aelimin+' program is currently being planned by ILM along with governement and owners of the eco-friendly, Intercontinental hotel "The Brando", the contruction of which is nearing completion on Onetahi. This program aims to provide biological control of *Ae. polynesiensis* mosquitoes in agreement with the resort's stringent ecological ethos. The goal is to initially produce 40,000 males/week essentially by increasing the number of workers (4-5 people), improving existing rearing conditions (INFRAVEC), and using visual verification of mechanical-separated males with an expectation of eliminating the *Ae. polynesiensis* from Onetahi. Weekly releases at this rate would certainly inundate the wild population particularly since contruction of the hotel will continue to reduce available breeding sites by covering the existing land fill and eliminating rats.

At the conclusion of the 'Tetiaroa Aelimin+' program, the findings should then gradually lead to a more ambitious program, aiming at supressing *Ae. polynesiensis* from an isolated high island, such as Maupiti, where transmission of *W. bancrofti* remains (Mou et al. 2009). This small high island (16°27'2.00"S 152°15'37.00"W) has a long history of MDA along with failure to eradicate LF from the small population (Esterre et al. 2001). At this scale, one issue would be unintentionally released females that may result in population replacement instead of elimination (Dobson et al. 2002). As population size decrease due to CI-induced sterility, the probability of establishment of the new *Wolbachia*-infected from released females increases. Although, a degree of female contaminants might be tolerable at the onset of the release effort, the success of

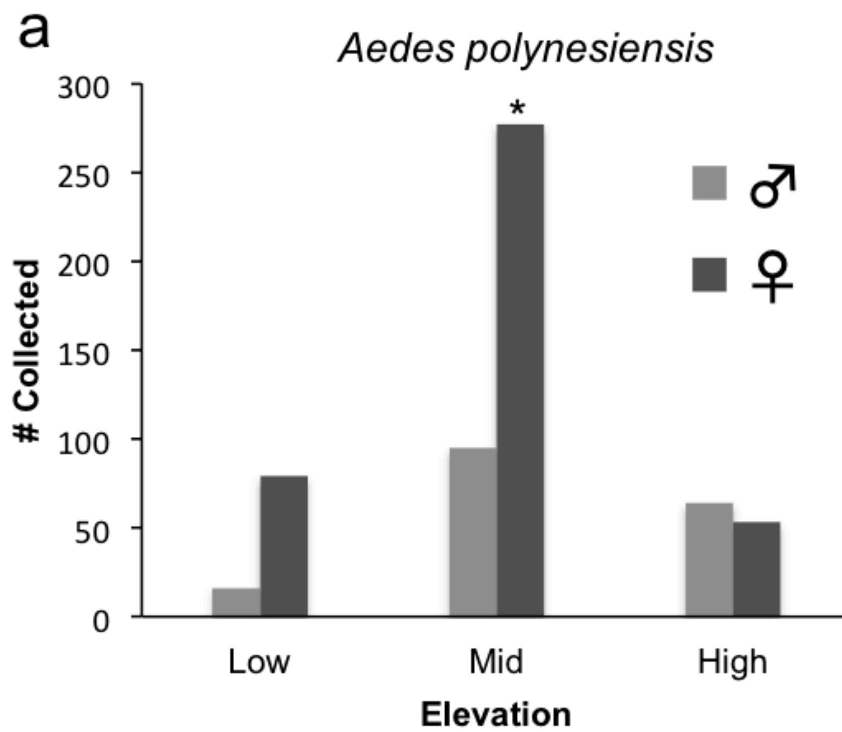
the strategy at a larger scale will ultimately rely on releases of males only. Development of genetic sexing for *Ae. polynesiensis* would significantly reduce cost for sorting. Irradiation could also be contemplated to sterilize potential female contaminants before release. Low level of radiation would render contaminant female sterile as female *Ae. polynesiensis* are more susceptible to radiation than males (Brelsfoard et al. 2009).

Finally, the proposed *Wolbachia* suppression strategy should be part of an integrated vector control program. In the case of Maupiti, community participation for the reduction of breeding sources in coconuts groves harvested for copra, and peridomestic containers coordinated by local leaders would greatly reduce the number of released males required for elimination. Though burrows of land crabs were not issues on Onetahi (crab absent from this motu), burrows found throughout the South Pacific islands can form significant breeding sites. They also are sources of harborage for *Ae. polynesiensis* eggs as they are governed by fluctuating sea levels thus less vulnerable to rainfall fluctuations. The use of insecticide impregnated crab baits could provide effective and specific control to mosquito larvae in crab burrows (Lardeux et al. 2002a). Furthermore, recent study on landing response of *Ae. polynesiensis* to different colors (Chambers et al. 2013) could be applied to insecticide treated targets to further reduce adult population. All together, the inclusion of a *Wolbachia* suppression strategy as part of an integrated vector control could overcome the shortcomings of MDA in selected endemic areas of the South Pacific. This would certainly break the LF transmission cycle initially in Maupiti thus providing a pertinent solution to LF in other South Pacific islands where *W. bancrofti* is vectored by the *Ae. polynesiensis* mosquito.

Appendix

Table 7.1 Summary of worm challenges of APk backcrosses to *B. pahangi* (Chapter 2). The location where filarial larvae were found 14 days after worm challenge, the number of mosquitoes and the average LF are provide. All larvae were infective (L3 stage).

Backcross	Location	N	Average LF#
BC1	Proboscis	6	2.5
	Proboscis & head	8	2.8
	Head	10	1.7
	Head & thorax	1	5
	Thorax	2	1
BC2	Proboscis	2	1.5
	Proboscis & head	4	3.2
	Head	11	1.7
	Thorax	1	2
	Thorax & abdomen	1	2
BC3	Proboscis	3	1
	Proboscis & head	7	2.7
	Proboscis & abdomen	3	3.3
	Proboscis, head, & thorax	1	5
	Proboscis, thorax, & abdomen	1	5
	Head	8	1.5
	Head & thorax	3	3
	Thorax	1	1
	Abdomen	2	1.5
BC4	Proboscis	9	2.9
	Proboscis & thorax	1	2



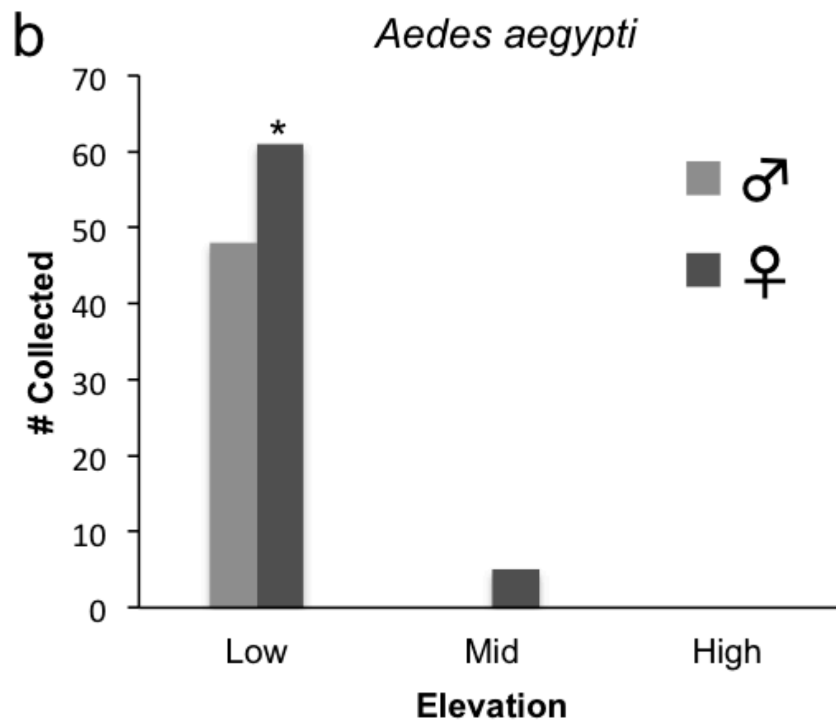


Figure 7.1. Total number of (a) *Ae. polynesiensis* and (b) *Ae. aegypti* male and female mosquitoes collected in Afareaitu (Chapter 3). * Significant difference (P<0.01) from other elevations.

Table 7.2. Summary of hatch rates of compatible brood of Tetiaroa before, during and after releases (Chapter 5). Lines shaded in gray show measurements (weeks 6-14) where fertility on Onetahi is significantly different from that on Tiaraunu (Fisher's Test).

Release	Measurement	Hatch rate of compatible brood (mean ± SEM; n compatible; n incompatible)	
		Tiaraunu	Onetahi
Before	1	85% ±3; 38; 0	88%±3; 15; 0
	2	80% ±3; 62; 0	90%±3; 36; 0
	3	78% ±5; 25; 0	88%±3; 24; 0
	4	81% ±4; 42; 0	94%±1; 39; 0
	5	57% ±6; 32; 0	86%±3; 32; 0
	6	60% ±6; 32; 1	74%±5; 37; 0
	7	82% ±4; 33; 0	88%±2; 42; 0
	8	87% ±3; 45; 0	78%±4; 42; 2
	Total Eggs	20,103	17,194
During	1	89% ±2; 48; 0	83%±3; 42; 1
	2	92% ±2; 19; 0	76%±6; 30; 0
	3	89%±4; 22; 1	90%±5; 22; 3
	4	91%±3; 39; 1	96%±2; 41; 3
	5	89%±4; 34; 0	95%±2; 32; 5
	6	98%±1; 35; 0	97%±3; 30; 6
	7	94%±3; 41; 1	87%±6; 26; 11
	8	92%±3; 34; 0	94%±3; 32; 6
	9	98%±1; 43; 0	98%±1; 31; 11
	10	96%±2; 40; 0	94%±4; 24; 14
	11	94%±3; 40; 0	95%±2; 31; 7
	12	91%±3; 42; 0	92%±4; 31; 6
	13	91%±3; 45; 0	87%±3; 39; 5
	14	78%±4; 36; 1	79%±4; 29; 11
	Total Eggs	25,900	33,255
After	1	94%±3; 29; 0	96%±2; 40; 0
	2	95%±2; 46; 0	91%±3; 45; 0
	3	97%±1; 41; 0	95%±3; 38;0
	Total Eggs	6,644	7,957

References

Agnew, P., C. Haussy, and Y. Michalakis. 2000. Effects of density and larval competition on selected life history traits of *Culex pipiens quinquefasciatus* (Diptera: Culicidae). Journal of Medical Entomology 37: 732-735.

Ali, S. R., and L. E. Rozeboom. 1971a. Cross-mating between *Aedes* (*S.*) *polynesiensis* Marks and *Aedes* (*S.*) *albopictus* Skuse in large cages. Mosquito News 31: 80-84.

Ali, S. R., and L. E. Rozeboom. 1971b. Cross-insemination frequencies between strains of *Aedes albopictus* and members of the *Aedes scutellaris* group. Journal of Medical Entomology 8: 263-265.

Ali, S. R., and L. E. Rozeboom. 1973. Comparative laboratory observations on selective mating of *Aedes* (*Stegomyia*) *albopictus* Skuse and *A.*(*S.*) *polynesiensis*. Marks. Mosquito News 33: 23-28.

Andrews, E. S., P. R. Crain, Y. Fu, D. K. Howe, and S. L. Dobson. 2012. Reactive Oxygen Species Production and Brugia pahangi Survivorship in Aedes polynesiensis with Artificial Wolbachia Infection Types. PLoS Pathogens 8: e1003075.

Backhouse, T. C., and A. R. Woodhill. 1954. Wuchereria bancrofti from New Caledonia in relation to certain Scutellaris group mosquitoes. South Pacific Commission. Mosquitoes-borne diseases. Filariasis: 8.

Bain, O. 1971. Transmission of filariasis. Limitation of passages of ingested microfilariae to the hemocoele of the vector; interpretation. Annales de Parasitologie Humaine et Comparée 46: 613-631.

Bain, O. 1976. Traversée de la paroi stomacale du vecteur par les microfilaires: techniques d'étude utilisées, importance épidémiologique. Bulletin of the World Health Organization 54: 397-401.

Ball, T. S., and S. R. Ritchie. 2010. Sampling biases of the BG-Sentinel trap with respect to physiology, age, and body size of adult *Aedes aegypti* (Diptera: Culicidae). Journal of Medical Entomology 47: 649-656.

Bayoh, M., and S. Lindsay. 2003. Effect of temperature on the development of the aquatic stages of *Anopheles gambiae* sensu stricto (Diptera: Culicidae). Bulletin of Entomological Research 93: 375-382.

Bedhomme, S., P. Agnew, C. Sidobre, and Y. Michalakis. 2003. Sex-specific reaction norms to intraspecific larval competition in the mosquito *Aedes aegypti*. Journal of Evolutionary Biology 16: 721-730.

Behbahani, A., T. Dutton, N. Davies, H. Townson, and S. Sinkins. 2005. Population differentiation and *Wolbachia* phylogeny in mosquitoes of the *Aedes scutellaris* group. Medical and Veterinary Entomology 19: 66-71.

Belkin, J. N. 1962. The mosquitoes of the South Pacific (Diptera, Culicidae), University of California Press, Berkeley.

Bellini, R., A. Medici, A. Puggioli, F. Balestrino, and M. Carrieri. 2013. Pilot Field Trials with *Aedes albopictus* Irradiated Sterile Males in Italian Urban Areas. Journal of Medical Entomology 50: 317-325.

Bellini, R., M. Calvitti, A. Medici, M. Carrieri, G. Celli, and S. Maini. 2007. Use of the sterile insect technique against *Aedes albopictus* in Italy: First results of a pilot trial, pp. 505-515. In M. J. B. Vreysen, A. Robinson and J. Hendrichs (eds.), Area-Wide Control of Insect Pests. Springer, Vienna, Austria.

Benedict, M. Q., and A. S. Robinson. 2003. The first releases of transgenic mosquitoes: An argument for the sterile insect technique. Trends in Parasitology 19: 349-355.

Benedict, M. Q., B. Knols, H. Bossin, P. Howell, E. Mialhe, C. Caceres, and A. Robinson. 2009. Colonisation and mass rearing: learning from others. Malaria Journal 8: S4.

Bhalala, H. V., J. D. Smith, B. A. O'Dea, and J. R. Arias. 2010. The Efficacy of the BG-Sentinel CO2 Nozzle in Collecting Host-Seeking Mosquitoes in Fairfax County, Virginia. Journal of the American Mosquito Control Association 26: 226-228.

Boakye, D. A., H. A. Baidoo, E. Glah, C. Brown, M. Appawu, and M. D. Wilson. 2007. Monitoring lymphatic filariasis interventions: Adult mosquito sampling, and improved PCR - based pool screening method for *Wuchereria bancrofti* infection in *Anopheles* mosquitoes. Filaria journal 6: 13.

Bock, M. E., W. K. Reisen, and M. M. Milby. 1983. Lifetime mating pattern of laboratory-adapted *Culex tarsalis* males. Mosquito News 43: 350-354.

Bonnet, D. D., and H. Chapman. 1958. The larval habitats of *Aedes polynesiensis* Marks in Tahiti and methods of control. The American Journal of Tropical Medicine and Hygiene 7: 512-518.

Bossin, H., and L. K. M. Hapairai. 2012. Hôtel LE TAHA'A Island Resort & Spa, Nuisances Entomologiques sur le motu TauTau, Taha'a, pp. 18. Institut Louis Malarde, Papeete.

Bourtzis, K., and A. Robinson. 2006. Insect pest control using Wolbachia and/or radiation, pp. 225-246. In K. Bourtzis and T. A. Miller (eds.), Insect Symbiosis, vol. 2. CRC Press, Boca Raton, Florida.

Boyer, S., J. Gilles, D. Merancienne, G. Lemperiere, and D. Fontenille. 2011. Sexual performance of male mosquito *Aedes albopictus*. Medical and Veterinary Entomology 25: 454-459.

Boyer, S., C. Toty, M. Jacquet, G. Lemperiere, and D. Fontenille. 2012. Evidence of multiple inseminations in the field in Aedes albopictus. PLoS One 7: e42040.

Brelsfoard, C. L., and S. L. Dobson. 2012. Population genetic structure of Aedes polynesiensis in the Society Islands of French Polynesia: implications for control using a Wolbachia-based autocidal strategy. Parasites and Vectors 5: 80.

Brelsfoard, C. L., Y. Séchan, and S. L. Dobson. 2008. Interspecific Hybridization Yields Strategy for South Pacific Filariasis Vector Elimination. PLoS Neglected Tropical Diseases 2: e129.

Brelsfoard, C. L., W. St Clair, and S. L. Dobson. 2009. Integration of irradiation with cytoplasmic incompatibility to facilitate a lymphatic filariasis vector elimination approach. Parasites and Vectors 2: 38.

Brengues, J., and O. Bain. 1972. Passage des microfilaires de l'estomac vers l'hémocèle du vecteur, dans les couples *Wuchereria barzcrofti-Anopheles gambiae* A, *W. bancrofti-Aedes aegypti* et *Setaria Iabiatopapillosa-A. aegypti.* Cahier ORSTOM, Série Entomologie Medicale Et Parasitologie 10: 235-250.

Brunhes, J., and P. Bousses. 2009. Description of *Culex* (Culex) *sechani n. sp*. from Tahiti (Society Islands)(Diptera, Culicidae). Bulletin de la Societe Entomologique de France 114: 21-28.

Burkot, T., and K. Ichimori. 2002. The PacELF programme: will mass drug administration be enough? Trends Parasitol 18: 109-115.

Burkot, T. R., D. N. Durrheim, W. D. Melrose, R. Speare, and K. Ichimori. 2006. The argument for integrating vector control with multiple drug administration campaigns to ensure elimination of lymphatic filariasis. Filaria Journal 5: 10.

Catteruccia, F., J. P. Benton, and A. Crisanti. 2005. An Anopheles transgenic sexing strain for vector control. Nature Biotechnology 23: 1414-1417.

Chambers, E. W., L. Hapairai, B. A. Peel, H. Bossin, and S. L. Dobson. 2011. Male mating competitiveness of a *Wolbachia*-introgressed *Aedes polynesiensis* strain under semi-field conditions. PLoS Negl Trop Dis 5: e1271.

Chambers, E. W., H. C. Bossin, S. A. Ritchie, R. C. Russell, and S. L. Dobson. 2013. Landing response of *Aedes* (*Stegomyia*) *polynesiensis* mosquitoes to coloured targets. Medical and Veterinary Entomology: DOI: 10.1111/j.1365-2915.2012.01065.x.

Chambers, E. W., S. K. McClintock, M. F. Avery, J. D. King, M. H. Bradley, M. A. Schmaedick, P. J. Lammie, and T. R. Burkot. 2009. Xenomonitoring of *Wuchereria bancrofti* and *Dirofilaria immitis* infections in mosquitoes from American Samoa: trapping considerations and a comparison of polymerase chain reaction assays with dissection. The American Journal of Tropical Medicine and Hygiene 80: 774-781.

Chanteau, S., and J. Roux. 2008. Bancroftian lymphatic filariasis: toward its elimination from the Pacific? Bulletin de la Société de Pathologie Exotique et de ses Filiales 101: 254-260.

Chu, B. K., P. J. Hooper, M. H. Bradley, D. A. McFarland, and E. A. Ottesen. 2010. The economic benefits resulting from the first 8 years of the Global Programme to Eliminate Lymphatic Filariasis (2000-2007). PLoS Neglected Tropical Diseases 4: e708.

Collins, F. H., M. A. Mendez, M. O. Rasmussen, P. C. Mehaffey, N. J. Besansky, and V. Finnery. 1987. A ribosomal RNA gene probe differentiates member species of the *Anopheles gambiae* complex. The American Journal of Tropical Medicine and Hygiene 37: 37-41.

Costantini, C., S. G. LI, A. D. Torre, N. F. Sagnon, M. Coluzzi, and C. E. Taylor. 1996. Density, survival and dispersal of *Anopheles gambiae* complex mosquitoes in a West African Sudan savanna village. Medical and Veterinary Entomology 10: 203-219.

Craig, D. A., D. C. Currie, and D. A. Joy. 2001. Geographical history of the central-western Pacific black fly subgenus *Inseliellum* (Diptera: Simuliidae: *Simulium*) based on a reconstructed phylogeny of the species, hot-spot archipelagoes and hydrological considerations. Journal of Biogeography 28: 1101-1127.

Curtis, C. 1985. Genetic control of insect pests: growth industry or lead balloon? Biological Journal of the Linnean Society 26: 359-374.

Curtis, C., J. Akiyama, and G. Davidson. 1976. A genetic sexing system in *Anopheles gambiae* species A. Mosquito News 36: 492-498.

Curtis, C., G. Brooks, M. Ansari, K. Grover, B. Krishnamurthy, P. Rajagopalan, L. Sharma, V. Sharma, D. Singh, and K. Singh. 1982. A field trial on control of *Culex quinquefasciatus* by release of males of a strain integrating cytoplasmic incompatibility and a translocation. Entomologia Experimentalis et Applicata 31: 181-190.

Davis, T. R. 1949. Filariasis control in the Cook Islands. The New Zealand Medical Journal 48: 362-370.

Dean, J. L., and S. L. Dobson. 2004. Characterization of *Wolbachia* infections and interspecific crosses of *Aedes* (*Stegomyia*) *polynesiensis* and *Ae*. (*Stegomyia*) *riversi* (Diptera: Culicidae). Journal of Medical Entomology 41: 894-899.

Delatte, H., G. Gimonneau, A. Triboire, and D. Fontenille. 2009. Influence of Temperature on Immature Development, Survival, Longevity, Fecundity, and Gonotrophic Cycles of Aedes albopictus, Vector of Chikungunya and Dengue in the Indian Ocean. Journal of Medical Entomology 46: 33-41.

Dev, V., and K. S. Rai. 1985. Genetic relationships among certain species of the *Aedes* (*Stegomyia*) *scutellaris* group (Diptera: Culicidae). Annals of Tropical Medicine and Parasitology 79: 325-331.

Dobson, S. L., C. W. Fox, and F. M. Jiggins. 2002. The effect of *Wolbachia*-induced cytoplasmic incompatibility on host population size in natural and manipulated systems. Proceedings of the Royal Society B: Biological Sciences 269: 437-445.

Dutton, T. J., and S. P. Sinkins. 2005. Filarial susceptibility and effects of *Wolbachia* in *Aedes pseudoscutellaris* mosquitoes. Medical and Veterinary Entomology 19: 60-65.

Dyck, V. A., J. Hendrichs, and A. S. Robinson. 2005. Sterile insect technique: principles and practice in area-wide integrated pest management, pp. 787. In V. A. Dyck, J. Hendrichs and A. S. Robinson (eds.). Springer Heidelberg, Germany.

Esterre, P., E. Vigneron, and J. Roux. 2005. Histoire de la lutte contre la filariose lymphatique en Polynésie Française: leçons de 50 années d'efforts. Bulletin de la Societe de Pathologie Exotique 98: 41-50.

Esterre, P., C. Plichart, Y. Séchan, and N. L. Nguyen. 2001. The impact of 34 years of massive DEC chemotherapy on *Wuchereria bancrofti* infection and transmission: The Maupiti cohort. Tropical Medicine and International Health 6: 190-195.

Eyraud, M., and G. Quelennec. 1976. *Aedes polynesiensis*, technique d'élevage, observations sur son comportement sexuel et sa reproduction, essai de chimiostérilisation au Thiotépa. Cahier ORSTOM, Série Entomologie Medicale Et Parasitologie 14: 3-11.

Failloux, A. B., A. Ung, M. Raymond, and N. Pasteur. 1994. Insecticide susceptibility in mosquitoes (Diptera: Culicidae) from French Polynesia. Journal of Medical Entomology 31: 639-644.

Failloux, A. B., M. Raymond, A. Ung, C. Chevillon, and N. Pasteur. 1997. Genetic differentiation associated with commercial traffic in the Polynesian mosquito, *Aedes polynesiensis* Marks 1951. Biological Journal of the Linnean Society 60: 107-118.

Failloux, A. B., M. Raymond, A. Ung, P. Glaziou, P. M. V. Martin, and N. Pasteur. 1995. Variation in the vector competence of *Aedes polynesiensis* for *Wuchereria bancrofti*. Parasitology 111: 19-29.

Farajollahi, A., B. Kesavaraju, D. C. Price, G. M. Williams, S. P. Healy, R. Gaugler, and M. P. Nelder. 2009. Field efficacy of BG-Sentinel and industry-standard traps for *Aedes albopictus* (Diptera: Culicidae) and West Nile virus surveillance. Journal of Medical Entomology 46: 919-925.

Farid, H. A., Z. S. Morsy, H. Helmy, R. M. Ramzy, M. El Setouhy, and G. J. Weil. 2007. A critical appraisal of molecular xenomonitoring as a tool for assessing progress toward elimination of Lymphatic Filariasis. The American Journal of Tropical Medicine and Hygiene 77: 593-600.

Ferguson, H. M., B. John, K. R. Ng'habi, and B. G. J. Knols. 2005. Addressing the sex imbalance in knowledge of vector biology. Trends in Evolution and Ecology 20: 202-209.

Ferguson, H. M., K. R. Ng'habi, T. Walder, D. Kadungula, S. J. Moore, I. Lyimo, T. Russell, H. Urassa, H. Mshinda, G. F. Killeen, and B. G. J. Knols. 2008. Establishment of a large semi-field system for experimental study of African malaria vector ecology and control in Tanzania. Malaria Journal 7: 158-173.

Focks, D. A. 1980. An improved separator for the developmental stages, sexes, and species of mosquitoes (Diptera: Culicidae). Journal of Medical Entomology 17: 567-568.

Gauzere, B., M. Bohrer, D. Drouet, P. Gasque, M. Jaffar-Bandjee, L. Filleul, and D. Vandroux. 2011. Infection a virus Chikungunya a La Reunion en 2005-ì2006: formes graves emergentes de l'adulte en reanimation. Reanimation 20: 211-222.

Gerberg, E. J. 1970. Manual for mosquito rearing and experimental techniques, Mosquito News, Selma, California.

Gillespie, R. G. 2002. Biogeography of spiders on remote oceanic islands of the Pacific: archipelagoes as stepping stones? Journal of Biogeography 29: 655-662.

Gimnig, J. E., M. Ombok, S. Otieno, M. G. Kaufman, J. M. Vulule, and E. D. Walker. 2002. Density-dependent development of Anopheles gambiae (Diptera: Culicidae) larvae in artificial habitats. Journal of Medical Entomology 39: 162-172.

Gomulski, L. M. 1985. Larval density, adult size and mating competitiveness in the mosquito *Anopheles gambiae*. Transactions of the Royal Society of Tropical Medicine and Hygiene 79: 276-277.

GraphPad 2010. GraphPad Prism computer program, version 5. By GraphPad, La Jolla, California.

Gratz, N. 2004. Critical review of the vector status of Aedes albopictus. Medical and Veterinary Entomology 18: 215-227.

Gubler, D. J. 1970a. Comparison of reproductive potentials of *Aedes* (*Stegomyia*) *albopictus* Skuse and *Aedes* (*Stegomyia*) *polynesiensis* Marks. Mosquito News 30: 201-209.

Gubler, D. J. 1970b. Induced sterility in *Aedes* (*Stegomyia*) *polynesiensis* Marks by cross-insemination with *Aedes* (Stegomyia) *albopictus* Skuse. Journal of Medical Entomology 7: 65-70.

Gubler, D. J. 1970c. Competitive displacement of *Aedes* (*Stegomyia*) *polynesiensis* Marks by *Aedes* (*Stegomyia*) *albopictus* Skuse in laboratory populations. Journal of Medical Entomology 7: 229-232.

Gubler, D. J. 1971. Studies on comparative oviposition behavior of *Aedes* (*Stegomyia*) *albopictus* and *Aedes* (*Stegomyia*) *polynesiensis* Marks. Journal of Medical Entomology 8: 675-682.

Gubler, D. J. 1981. Transmission of Ross River virus by *Aedes polynesiensis* and *Aedes aegypti*. The American Journal of Tropical Medicine and Hygiene 30: 1303-1306.

Hapairai, L. K., M. A. Cheong-Sang, S. Sinkins, and H. C. Bossin. 2013. Population studies of the filarial vector *Aedes polynesiensis* (Diptera: Culicidae) in two typical island settings of French Polynesia. Journal of Medical Entomology In press.

Harrington, L. C., T. W. Scott, K. Lerdthusnee, R. C. Coleman, A. Costero, G. G. Clark, J. J. Jones, S. Kitthawee, P. Kittayapong, and R. Sithiprasasna. 2005. Dispersal of the dengue vector *Aedes aegypti* within and between rural communities. The American Journal of Tropical Medicine and Hygiene 72: 209-220.

Harris, A. F., A. R. McKemey, D. Nimmo, Z. Curtis, I. Black, S. A. Morgan, M. N. Oviedo, R. Lacroix, N. Naish, N. I. Morrison, A. Collado, J. Stevenson, S. Scaife, T. Dafa'alla, G. Fu, C. Phillips, A. Miles, N. Raduan, N. Kelly, C. Beech, C. A. Donnelly, W. D. Petrie, and L. Alphey. 2012. Successful suppression of a field mosquito population by sustained release of engineered male mosquitoes. Nature Biotechnology 30: 828-830.

Hendrichs, J., M. J. B. Vreysen, W. R. Enkerlin, and J. P. Cayol. 2005. Strategic options in using sterile insects for area-wide integrated pest managment, pp. 563-600. In V. A. Dyck, J. Hendrichs and A. S. Robinson (eds.), Sterile insect technique: principles and practice in area-wide integrated pest management. Springer, Heidelberg, Germany.

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

Hoffmann, A. A., B. L. Montgomery, J. Popovici, I. Iturbe-Ormaetxe, P. H. Johnson, F. Muzzi, M. Greenfield, M. Durkan, Y. S. Leong, Y. Dong, P. E. Cook, J. Axford, A. G. Callahan, N. Kenny, C. Omodei, E. A. McGraw, P. A. Ryan, S. A. Ritchie, M. Turelli, and S. L. O'Neill. 2011. Successful establishment of Wolbachia in *Aedes* populations to suppress dengue transmission. Nature 476: 454-457.

Huang, Y. M. 1972. Contributions to the mosquito fauna of Southeast Asia. XIV. The subgenus Stegomyia of Aedes in Southeast Asia I-The Scutellaris Group of Species. Contributions of the American Entomological Institute 9.

Ichimori, K. 2001. Entomology of the filariasis control programme in Samoa, *Aedes polynesiensis* and *Ae. samoanus*. Medical Entomology and Zoology 52: 11-21.

Ingram, R. L. 1954. A study of the bionomics of *Aedes* (*Stegomyia*) *polynesiensis* Marks under laboratory conditions. American Journal of Epidemiology 60: 169-185.

Ito, Y., and K. Yamamura. 2005. Role of Population and Behavioural Ecology in the Sterile Insect Technic, pp. 177-208. In V. A. Dyck, J. Hendrichs and A. S. Robinson (eds.), Sterile insect technique: principles and practice in area-wide integrated pest management. Springer, Heidelberg, Germany.

Iyengar, M. O. 1955. Distribution of mosquitoes in the South Pacific region, pp. 57. South Pacific Commision, Noumea, New Caledonia.

Iyengar, M. O. 1960. A review of the mosquitoes fauna of the South Pacific (Diptera: Culicidae). South Pacific Commission, Noumea, New Caledonia.

Jachowski, L. A. 1954. Filariasis in American Samoa: V. Bionomics of the principal vector, *Aedes polynesiensis (*Marks). American Journal of Epidemiology 60: 186-203.

Jachowski, L. A., and G. F. Otto. 1952. Filariasis in American Samoa: II. Evidence of Transmission outside of Villages. The American Journal of Tropical Medicine and Hygiene 1: 662-671.

Joseph, H., A. Clough, A. Peteru, S. Crawley, T. Pulu, F. Maiava, and W. Melrose. 2010. Exploratory study investigating factors influencing mass drug administration (MDA) compliance for lymphatic filariasis in Samoa. Samoa Medical Journal 2: 12-25.

Kambris, Z., P. E. Cook, H. K. Phuc, and S. P. Sinkins. 2009. Immune Activation by Life-Shortening *Wolbachia* and Reduced Filarial Competence in Mosquitoes. Science 326: 134-136.

Kawada, H., S. Honda, and M. Takagi. 2007. Comparative laboratory study on the reaction of *Aedes aegypti* and *Aedes albopictus* to different attractive cues in a mosquito trap. Journal of Medical Entomology 44: 427-432.

Kessel, J. F. 1957. An effective programme for the control of filariasis in Tahiti. Bulletin of the World Health Organization 16: 633-664.

Kimura, E., L. Penaia, and G. Spears. 1985. Epidemiology of subperiodic bancroftian filariasis in Samoa 8 years after control by mass treatment with diethylcarbamazine. Bulletin of the World Health Organization 63: 869-880.

Knipling, E. F. 1955. Possibilities of insect control or eradication through the use of sexually sterile males. Journal of Economic Entomology 48: 459-462.

Knipling, E. F. 1979. Agriculture Handbook no. 512: The basic principles of insect population suppression and management. USDA, Washington, DC.

Knols, B. G. J., B. N. Njiru, W. R. Mukabana, E. M. Mathenge, and G. F. Killeen. 2003. Contained semi-field environment for ecological studies on transgenic African malaria vectors: benefits and constaints, pp. 91-108. In W. Takken and T. W. Scott (eds.), Ecological aspects for application of genetically modified mosquitoes. Kluwer Academic Publishers, Dordrecht, The Netherlands.

Krafsur, E. S. 1998. Sterile insect technique for suppressing and eradicating insect populations: 55 years and counting. Journal of Agricultural Entomology 15: 303-317.

Krockel, U., A. Rose, E. Eiras, and M. Geier. 2006. New tools for surveillance of adult yellow fever mosquitoes: comparison of trap catches with human landing rates in an urban environment. Journal of the American Mosquito Control Association 22: 229-238.

Lacroix, R., H. Delatte, T. Hue, and P. Reiter. 2009. Dispersal and survival of male and female *Aedes albopictus* (Diptera: Culicidae) on Reunion Island. Journal of Medical Entomology 46: 1117-1124.

Lacroix, R., A. R. McKemey, N. Raduan, L. K. Wee, W. H. Ming, T. G. Ney, S. R. AA, S. Salman, S. Subramaniam, O. Nordin, A. T. N. Hanum, C. Angamuthu, S. Marlina Mansor, R. S. Lees, N. Naish, S. Scaife, P. Gray, G. Labbe, C. Beech, D. Nimmo, L. Alphey, S. S. Vasan, L. Han Lim, A. N. Wasi, and S. Murad. 2012. Open Field Release of Genetically Engineered Sterile Male *Aedes aegypti* in Malaysia. PLoS One 7: e42771.

Lambdin, B. H., M. A. Schmaedick, S. McClintock, J. Roberts, N. E. Gurr, K. Marcos, L. Waller, and T. R. Burkot. 2009. Dry Season Production of Filariasis and Dengue Vectors in American Samoa and Comparison with Wet Season Production. The American Journal of Tropical Medicine and Hygiene 81: 1013-1019.

Lance, D. R., and D. O. McInnis. 2005. Biological Basis of the Sterile Insect Technic, pp. 69-98. In V. A. Dyck, J. Hendrichs and A. S. Robinson (eds.), Sterile insect technique: principles and practice in area-wide integrated pest management. Springer, Heidelberg, Germany.

Lardeux, F. 1991. Contrôle d'Aedes aegypti et d'Aedes polynesiensis, vecteurs de dengue et de filariose, avec le copépode prédateur Mesocyclops aspericornis : essais de terrain dans des petites îles du Pacifique (rapport sur la période : Janvier 1990 - Décembre 1990, 3ème et dernière période de financement, et rapport final) pp. 70. ITRMLM Papeete.

Lardeux, F. 1992. Biological control of Culicidae with the copepod *Mesocyclops aspericornis* and larvivorous fish (Poeciliidae) in a village of French Polynesia. Medical and Veterinary Entomology 6: 9-15.

Lardeux, F., L. Nguyen, and J.-L. Cartel. 1995. *Wuchereria bancrofti* (Filariidea: Dipetalonematidae) and its vector *Aedes polynesiensis* (Diptera: Culicidae) in a French Polynesian village. Journal of Medical Entomology 32: 346-352.

Lardeux, F., Y. Séchan, and M. Faaruia. 2002a. Evaluation of insecticide impregnated baits for control of mosquito larvae in land crab burrows on French Polynesian atolls. Journal of Medical Entomology 39: 658-661.

Lardeux, F., F. Riviere, Y. Séchan, and B. H. Kay. 1992. Release of *Mesocyclops aspericornis* copepoda for control of larval *Aedes polynesiensis* Diptera Culicidae in land crab burrows on an atoll of French Polynesia. Journal of Medical Entomology 29: 571-576.

Lardeux, F., F. Riviere, Y. Séchan, and S. Loncke. 2002b. Control of the Aedes vectors of the dengue viruses and *Wuchereria bancrofti*: The French Polynesian experience. Annals of Tropical Medicine and Parasitology 96: S105-S116.

Lardeux, F., Y. Séchan, S. Lonke, X. Deparis, J. Cheffort, and M. Faaruia. 2002c. Integrated Control of Peridomestic Larval Habitats of *Aedes* and *Culex* Mosquitoes (Diptera: Culicidae) in Atoll Villages of French Polynesia. Journal of Medical Entomology 39: 493-498.

Laven, H. 1951. Crossing experiments with *Culex* strains. Evolution 5: 370-375.

Laven, H. 1967. Eradication of Culex pipiens fatigans through Cytoplasmic Incompatibility. Nature 216: 383-384.

Lincoln, F. C. 1930. Calculating waterfowl abundance on the basis of banding returns. *In* U. D. o. Agriculture [ed.], Washington, DC.
Lowrie, R. C. 1973a. Displacement of *Aedes* (*S.*) *polynesiensis* Marks by *A.*(*S.*) *albopictus* Skuse through competition in the larval stage under laboratory conditions. Journal of Medical Entomology 10: 131-136.
Lowrie, R. C. 1973b. The effect of competition between larvae of *Aedes* (*S.*) *albopictus* Skuse and *A.* (*S.*) *polynesiensis* Marks. Journal of Medical Entomology 10: 23-30.
Lyimo, E. O., W. Takken, and J. C. Koella. 1992. Effect of rearing temperature and larval density on larval survival, age at pupation and adult size of *Anopheles gambiae*. Entomologia Experimentalis et Applicata 63: 265-271.
Macdonald, W. W. 1962. The genetic basis of susceptibility to infection with semi-periodic Brugia malayi in Aedes aegypti. Annals of Tropical Medicine and Parasitology 56: 373-382.
Macdonald, W. W. 1976. Mosquito genetics in relation to filarial infections. Symposia of the British Society for Parasitology 14: 1-24.
Macdonald, W. W., and C. P. Ramachandran. 1965. The influence of the gene fm (filarial susceptibility, *Brugia malayi*) on the susceptibility of *Aedes aegypti* to seven strains of *Brugia, Wuchereria and Dirofilaria.* Annals of Tropical Medicine and Parasitology 59: 64-73.
Macia, A. 2009. Effects of larval crowding on development time, survival and weight at metamorphosis in *Aedes aegypti* (Diptera: Culicidae). Revista de la Sociedad Entomologica Argentina 68: 107-114.
Maciel-de-Freitas, R., C. T. Codeco, and R. Lourenco-de-Olivera. 2007. Body size associated survival and dispersal rates of *Aedes aegypti* in Rio de Janeiro. Medical and Veterinary Entomology 21: 284-292.
Maguire, T., F. N. MacNamara, J. A. R. Miles, G. F. S. Spears, and J. U. Mataika. 1971. Mosquito-Borne Infections in Fiji: II. Arthropod-Borne Virus Infections. Journal of Hygiene 69: 287-296.
Mahmood, F., and W. K. Reisen. 1982. *Anopheles stephensi* (Diptera: Culicidae): changes in male mating competence and reproductive system morphology associated with aging and mating. Journal of Medical Entomology 19: 573-588.
Maiga, H., R. K. Dabire, T. Lehmann, F. Tripet, and A. Diabate. 2012. Variation in energy reserves and role of body size in the mating system of *Anopheles gambiae*. Journal of Vector Ecology 37: 289-297.
Marie, J., and H. Bossin. 2013. First Record of *Wyeomyia* (*Wyeomyia*) *mitchellii* (Diptera: Culicidae) in French Polynesia. Journal of Medical Entomology 50: 37-42.
Marini, F., B. Caputo, M. Pombi, G. Tarsitani, and A. Della Torre. 2010. Study of *Aedes albopictus* dispersal in Rome, Italy, using sticky traps in mark-release-recapture experiments. Medical and Veterinary Entomology 24: 361-368.
Medici, A., M. Carrieri, E.-J. Scholte, B. Maccagnani, M. L. Dindo, and R. Bellini. 2011. Studies on *Aedes albopictus* larval mass-rearing optimization. Journal of Economic Entomology 104: 266-273.
Meek, S. R. 1984. Occurrence of Rickettsia-like symbionts among species of the Aedes scutellaris group (Diptera: Culicidae). Annals of Tropical Medicine and Parasitology 78: 377-382.

Meek, S. R. 1988. Compatibility of members of the *Aedes* (*Stegomyia*) *scutellaris* subgroup of mosquitoes (Diptera: Culicidae) and its relevance to the control of filariasis, pp. 115-132. In M. W. Service (ed.), Systematics Association Special Volume no. 37. Clarendon Press, Oxford.

Meek, S. R., and W. W. Macdonald. 1982. Studies on the inheritance of susceptibility to infection *Brugia pahangi* and *Wuchereria bancrofti* in the *Aedes scutellaris* group of mosquitoes. Annals of Tropical Medicine and Parasitology 76: 347-354.

Melrose, W. D. 2002. Lymphatic filariasis: new insights into an old disease. International Journal for Parasitology 32: 947-960.

Mercer, D. R. 1999. Effects of larval density on the size of *Aedes polynesiensis* adults (Diptera: Culicidae). Journal of Medical Entomology 36: 702-708.

Mercer, D. R., G. R. Wettach, and J. L. Smith. 2005. Effects of larval density and predation by *Toxorhynchites amboinensis* on *Aedes polynesiensis* (Diptera : Culicidae) developing in coconuts. Journal of the American Mosquito Control Association 21: 425-431.

Mercer, D. R., H. Bossin, M. C. Sang, L. O'Connor, and S. L. Dobson. 2012a. Monitoring Temporal Abundance and Spatial Distribution of *Aedes polynesiensis* Using BG-Sentinel Traps in Neighboring Habitats on Raiatea, Society Archipelago, French Polynesia. Journal of Medical Entomology 49: 51-60.

Mercer, D. R., J. Marie, H. Bossin, M. Faaruia, A. Tetuanui, M. C. Sang, and S. L. Dobson. 2012b. Estimation of Population Size and Dispersal of Aedes polynesiensis on Toamaro motu, French Polynesia. Journal of Medical Entomology 49: 971-980.

Météo-France. 2010. La métérologie en Polynésie française. *In* Météo-France [ed.], Direction interrégionale de Météo-France en Polynésie française. Météo-France, Faa'a.

Michael, E., and D. A. P. Bundy. 1997. Global mapping of lymphatic filariasis. Parasitology Today 13: 472-476.

Mogi, M. 1976. Notes on the northern records of *Aedes* (Stegomyia) *riversi* Bohart and Ingram. Mosquito Systematics 8: 347-352.

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

Morrison, A. C., E. Zielinski-Gutierrez, T. W. Scott, and R. Rosenberg. 2008. Defining challenges and proposing solutions for control of the virus vector *Aedes aegypti*. PLoS Medicine 5: e68.

Mou, Y., C. Plichart, A.-M. Legrand, H.-P. Mallet, N. Cerf, and L. Nguyen. 2009. Évaluation de la prévalence de la filariose lymphatique en 2008 en Polynésie française. Bulletin Epidémiologique Hebdomadaire: 504-507.

Nayar, J. K. 1969. Effects of larval and pupal environmental factors on biological status of adults at emergence in *Aedes taeniorhynchus* (Wied.). Bulletin of Entomological Research 58: 811-827.

Ng'habi, K. R., B. John, G. Nkwengulila, B. G. J. Knols, G. F. Killeen, and H. M. Ferguson. 2005. Effect of larval crowding on mating competitiveness of *Anopheles gambiae* mosquitoes. Malaria Journal 4: 49.

O'Connor, F. W. 1923. Researches in the Western Pacific. Research Memoirs of the London School of Tropical Medicine and Hygiene 4: 1-57.

O'Connor, L., C. Plichart, A. C. Sang, C. L. Brelsfoard, H. C. Bossin, and S. L. Dobson. 2012. Open release of male mosquitoes infected with a *Wolbachia* biopesticide: field performance and infection containment. PLoS Negl Trop Dis 6: e1797.

O'Neill, S. L., A. A. Hoffmann, and J. H. Werren. 1997. Influential passengers: inherited microorganisms and arthropod reproduction, Oxford University Press, New York.

Otsuka, Y., and H. Takaoka. 1997. Elimination of *Wolbachia pipientis* from Aedes albopictus. Medical Entomology and Zoology 48: 257-260.

Ottesen, E. A. 2006. Lymphatic filariasis: Treatment, control and elimination. Advances in parasitology 61: 395-441.

Ottesen, E. A., B. O. Duke, M. Karam, and K. Behbehani. 1997. Strategies and tools for the control/elimination of lymphatic filariasis. Bulletin of the World Health Organization 75: 491-503.

Packer, M. J., and P. S. Corbet. 1989. Size variation and reproductive success of female Aedes punctor (Diptera: Culicidae). Ecological Entomology 14: 297-309.

Paine, R. W. 1943. An introduction to the mosquitoes of Fiji. Bulletin of the Fiji Department of Agriculture 22: 1-35.

Papathanos, P. A., H. C. Bossin, M. Q. Benedict, F. Catteruccia, C. A. Malcolm, L. Alphey, and A. Crisanti. 2009. Sex separation strategies: past experience and new approaches. Malaria Journal 8: S5.

Phuc, H. K. H., M. H. M. Andreasen, R. S. R. Burton, C. C. Vass, M. J. M. Epton, G. G. Pape, G. G. Fu, K. C. K. Condon, S. S. Scaife, C. A. C. Donnelly, P. G. P. Coleman, H. H. White-Cooper, and L. L. Alphey. 2007. Late-acting dominant lethal genetic systems and mosquito control. BMC Biology 5: 11.

Pichon, G. 1974. Relations mathématiques entre le nombre des microfilaires ingérées et le nombre des parasites chez différents vecteurs naturelles ou expérimentaux de filarioses. Cahier ORSTOM, Série Entomologie Médicale Et Parasitology 12: 199-216.

Pichon, G. 2002. Limitation and facilitation in the vectors and other aspects of the dynamics of filarial transmission: the need for vector control against *Anopheles*-transmitted filariasis. Annals of Tropical Medicine and Parasitology 96: S143-S152.

Plichart, C., and A. Legrand. 2005. Detection and characterization of *Wolbachia* infections in *Wuchereria bancrofti* (Spirurida: onchocercidae) Var. *pacifica* and *Aedes* (*Stegomyia*) *polynesiensis* (Diptera: Culicidae). The American Journal of Tropical Medicine and Hygiene 73: 354-358.

Plichart, C., Y. Séchan, N. Davies, and A. M. Legrand. 2006. PCR and dissection as tools to monitor filarial infection of *Aedes polynesiensis* mosquitoes in French Polynesia. Filaria Journal 5: 2.

Plichart, C., S. Laney, Y. Sechan, N. Davies, and A.-M. Legrand. 2007. Correction: PCR and dissection as tools to monitor filarial infection of *Aedes polynesiensis* mosquitoes in French Polynesia. Filaria Journal 6: 5.

Ponlawat, A., and L. C. Harrington. 2009. Factors associated with male mating success of the dengue vector mosquito, Aedes aegypti. The American Journal of Tropical Medicine and Hygiene 80: 395-400.

Rakai, I. M., J. D. Naserua, F. N. Macnamara, and J. S. Pillai. 1974. Mosquito-borne infections in Fiji. IV. Biting times for village mosquitoes and human filaria transmission potential of Aedes polynesiensis and Aedes pseudoscutellaris. Journal of Medical Entomology 11: 588-594.

Ramachandran, C. P., and M. A. Zaini. 1968. Studies on the transmission of sub-periodic *Brugia malayi* by *Aedes* (*Finlaya*) *togoi* in the laboratory. II. The development of the parasite to the infective form; the relationship between concentration of microfilariae in the vertebrate host and infection in the mosquitoes. The Medical Journal of Malaya 22: 198-203.

Ramalingam, S. 1968. The epidemiology of filarial transmission in Samoa and Tonga. Annals of Tropical Medicine and Parasitology 62: 305-323.

Ramzy, R. M., M. El Setouhy, H. Helmy, E. S. Ahmed, K. M. Abd Elaziz, H. A. Farid, W. D. Shannon, and G. J. Weil. 2006. Effect of yearly mass drug administration with diethylcarbamazine and albendazole on bancroftian filariasis in Egypt: a comprehensive assessment. Lancet 367: 992-999.

Rao, R. U., L. J. Atkinson, R. R. M., H. Helmy, H. A. Farid, M. J. Bockarie, M. Susapu, S. J. Laney, S. A. Williams, and G. J. Weil. 2006. A real-time PCR-based assay for detection of *Wuchereria bancrofti* DNA in blood and mosquitoes. American Journal of Tropical Medicine and Hygiene 74: 826-832.

Rasband, W. 2004. ImageJ computer program, version 1.44o. By Rasband, W.

Reisen, W., M. Milby, and M. Bock. 1984. The effects of immature stress on selected events in the life history of *Culex tarsalis*. Mosquito News 44: 385-395.

Riviere, F., B. H. Kay, J. M. Klein, and Y. Séchan. 1987. *Mesocyclops aspericornis* (Copepoda) and *Bacillus thuringiensis* var. *Israelensis* for the biological control of *Aedes* and *Culex* vectors Diptera Culicidae breeding in crab holes tree holes, and artificial containers. Journal of Medical Entomology 24: 425-430.

Riviere, F., J.-M. Klein, R. Thirel, and M. Chebret. 1998a. Ecology of *Aedes* (*Stegomyia*) *polynesiensis* Marks, 1951 (Diptera: Culicidae), vector of Bancroft filariasis. I. Studies in coconuts eaten by rats as breeding sites. Annales de la Societe Entomologique de France 34: 195-207.

Riviere, F., J.-M. Klein, J. Duval, Y. Séchan, R. Thirel, M. Faaruia, and L. Colombani. 1998b. Ecology of *Aedes* (*Stegomyia*) *polynesiensis* Marks, 1951 (Diptera: Culicidae), mosquito vector of Bancrofti filariasis: II. Studies in land crab holes of *Cardisoma carnifex* Herbst. Annales de la Societe Entomologique de France 34: 259-283.

Rivière, F., G. Pichon, and M. Chebert. 1979a. Ecologie D'*Aedes* (Stegomyia) *polynesiensis* Marks, 1951 (Diptera, Culicidae) en Polynesie Francaise. I. Lieux de repos des adults-applicaion dans la lutte antimoustique a Bora Bora. Cahier ORSTOM, Série Entomologie Medicale Et Parasitologie 17: 235-241.

Rivière, F., G. Pichon, J. Duval, R. Thirel, and A. Toudic. 1979b. Introduction of *Toxorhynchites* (*Toxorhynchites*) *amboinensis* (Doleschall, 1857) (Diptera: Culicidae) in French Polynesia. Cahier ORSTOM, Série Entomologie Medicale et Parasitology 17: 225-234.

Rosen, L. 1955. Observations on the epidemiology of human filariasis in French Oceania. American Journal of Hygiene 6: 219-248.

Rosen, L., and L. E. Rozeboom. 1954. Morphologic variations of larvae of the scutellaris group of *Aedes* (Diptera, Culicidae) in Polynesia. The American Journal of Tropical Medicine and Hygiene 3: 529-538.

Rosen, L., L. E. Roseboom, B. H. Sweet, and A. B. Sabin. 1954. The transmission of dengue by *Aedes polynesiensis* Marks. The American Journal of Tropical Medicine and Hygiene 3: 878-882.

Rosen, L., L. E. Rozeboom, W. C. Reeves, J. Saugain, and D. J. Gubler. 1976. A field trial of competitive displacement of *Aedes polynesiensis* by *Aedes albopictus* on a Pacific atoll. The American Journal of Tropical Medicine and Hygiene 25: 906-913.

Rozeboom, L. 1971. Relative densities of freely breeding populations of *Aedes* (*S.*) *polynesiensis* Marks and *A.*(*S.*) *albopictus* Skuse: a large cage experiment. The American Journal of Tropical Medicine and Hygiene 20: 356-362.

Rozeboom, L., and B. N. Gilford. 1954. The Genetic relationship of *Aedes pseudoscutellaris* Theobald and *A. polynesiensis* Marks (Diptera: Culicidae) American Journal of Epidemiology 60: 117-133.

Rozeboom, L., and D. McLean. 1956. Transmission of the virus of Murray valley encephalitis by *Culex tarsalis* Coquillet*, Aedes poynesiensis* Marks and *A. pseudoscutellaris* Theobald. American Journal of Hygiene 63: 136-139.

Rozeboom, L., B. H. Sweet, and A. B. Sabin. 1954. The transmission of dengue by *Aedes polynesiensis* Marks. The American Journal of Tropical Medicine and Hygiene 3: 878-882.

Russell, J., L. Faulquier, and M. Tonione. 2011. Rat invasion of Tetiaroa Atoll, French Polynesia, pp. 118-123. In C. R. Veitch, M. N. Clout and D. R. Towns (eds.), Island invasives: eradication and management. International Union for Conservation of Nature, Gland, Switzerland.

Russell, R. C. 2004. The relative attractiveness of carbon dioxide and octenol in CDC- and EVS-type light traps for sampling the mosquitoes *Aedes aegypti* (L.), Aedes *polynesiensis* Marks, and *Culex quinquefasciatus* Say in Moorea, French Polynesia. Journal of Vector Ecology 29: 309-314.

Russell, R. C., C. E. Webb, and N. Davies. 2005a. *Aedes aegypti* (L.) and *Aedes polynesiensis* Marks (Diptera : culicidae) in Moorea, French Polynesia: A study of adult population structures and pathogen (*Wuchereria bancrofti* and *Dirofilaria immitis*) infection rates to indicate regional and seasonal epidemiological risk for dengue and filariasis. Journal of Medical Entomology 42: 1045-1056.

Russell, R. C., C. E. Webb, C. R. Williams, and S. A. Ritchie. 2005b. Mark-release-recapture study to measure dispersal of the mosquito *Aedes aegypt*i in Cairns, Queensland, Australia. Medical and Veterinary Entomology 19: 451-457.

Samarawickrema, W., F. Sone, and R. Cummings. 1987a. Natural infections of Wuchereria bancrofti in *Aedes* (*Stegomyia*) *polynesiensis* and *Aedes* (*Finlaya*) *samoanus* in Samoa. Transactions of the Royal Society of Tropical Medicine and Hygiene 81: 124-128.

Samarawickrema, W., F. Sone, L. Self, R. Cummings, and G. Paulson. 1992a. Distribution of breeding and control of the filariasis vector *Aedes samoanus* in leaf axils of Pandanus in Samoa. Medical and Veterinary Entomology 6: 367-370.

Samarawickrema, W. A., F. Sone, and R. F. Cummings. 1987b. Seasonal abundance, diel biting activity and parity of *Aedes polynesiensis* marks and *A. samoanus* (Grünberg)(Diptera: Culicidae) in Samoa. Bulletin of Entomological Research 77: 191-200.

Samarawickrema, W. A., E. Kimura, F. Sones, G. S. Paulson, and R. F. Cummings. 1992b. Natural infections of *Dirofilaria immitis* in *Aedes* (*Stegomyia*) *polynesiensis* and *Aedes* (*Finlaya*) *samoanus* and their implication in human health in Samoa. Transactions of the Royal Society of Tropical Medicine and Hygiene 86: 187-188.

Samarawickrema, W. A., F. Sone, E. Kimura, L. S. Self, R. F. Cummings, and G. S. Paulson. 1993. The relative importance and distribution of *Aedes polynesiensis* and *Ae.aegypti* larval habitats in Samoa. Medical and Veterinary Entomology 7: 27-36.

Samarawickrema, W. A., E. Kimura, G. F. S. Spears, L. Penaia, F. Sone, G. S. Paulson, and R. F. Cummings. 1987c. Distribution of vectors, transmission indices and microfilaria rates of subperiodic *Wuchereria bancrofti* in relation to village ecotypes in Samoa. Transactions of the Royal Society of Tropical Medicine and Hygiene 81: 129-135.

Schmaedick, M. A., T. S. Ball, T. R. Burkot, and N. E. Gurr. 2008. Evaluation of three traps for sampling *Aedes polynesiensis* and other mosquito species in American Samoa. Journal of the American Mosquito Control Association 24: 319-322.

Schoeler, G. B., S. S. Schleich, S. A. Manweiler, and V. L. Sifuentes. 2004. Evaluation of surveillance devices for monitoring *Aedes aegypti* in an urban area of northeastern Peru. Journal of the American Mosquito Control Association 20: 6-11.

Service, M. 1997. Mosquito (Diptera: Culicidae) dispersal: The long and short of it. Journal of Medical Entomology 34: 579-588.

Sherron, D. A., and K. S. Rai. 1983. Genetics of speciation in the *Aedes* (*Stegomyia*) *scutellaris* group (Diptera: Culicidae) 2. Crossing relationships of *Aedes cooki* with six sibling species. Journal of Medical Entomology 20: 520-525.

Shiu, S., D. R. Mercer, P. M. V. Martin, F. Rodhain, M. Raymond, and A.-B. Failloux. 1997. *Aedes polynesiensis* in the Society Islands: Environmental correlates of isoenzyme differentiation. Medical and Veterinary Entomology 11: 349-354.

Silver, J. B. 2008. Mosquito Ecology: Field Sampling Methods, Third Edition ed. Springer New York.

Sinkins, S. P. 2004. *Wolbachia* and cytoplasmic incompatibility in mosquitoes. Insect Biochemistry and Molecular Biology 34: 723-729.

Sinkins, S. P., and S. L. O'Neill. 2000. Wolbachia as a vehicle to modify insect populations, pp. 271-287. In A. M. Handler and A. A. James (eds.), Insect transgenesis: Methods and applications. CRC Press, Boca Raton, Florida.

Sinkins, S. P., and H. C. J. Godfray. 2004. Use of *Wolbachia* to drive nuclear transgenes through insect populations. Proceedings of the Royal Society B: Biological Sciences 271: 1421-1426.

Sinkins, S. P., and F. Gould. 2006. Gene drive systems for insect disease vectors. Nature Reviews Genetics 7: 427-435.

Smallegange, R., W. Schmied, K. van Roey, N. Verhulst, J. Spitzen, W. Mukabana, and W. Takken. 2010. Sugar-fermenting yeast as an organic source of carbon dioxide to attract the malaria mosquito Anopheles gambiae. Malaria Journal 9: 292.

Smith-White, S., and A. R. Woodhill. 1954. The nature and significance of non-reciprocal fertility in *Aedes scutellaris* and other mosquitoes. Proceedings of the Linnean Society of New South Wales 79: 163-176.

Snow, L. C., M. J. Bockarie, and E. Michael. 2006. Transmission dynamics of lymphatic filariasis: vector specific density dependence in the development of *Wuchereria bancrofti* infective larvae in mosquitoes. Medical and Veterinary Entomology 20: 261-272.

Southgate, B. 1992. The significance of low density microfilaraemia in the transmission of lymphatic filarial parasites. Journal of Tropical Medicine and Hygiene 95: 79-86.

Suzuki, T. 1975. Filarial infection in vector mosquitoes after mass drug administration in Western Samoa. Tropical Medicine 16: 147-156.

Suzuki, T., and F. Sone. 1974. The bionomics of filariasis vectors in Western Samoa. Japanese Journal of Sanitation Zoology 25: 251-257.

Tesfa-Yohannes, T., and L. Rozeboom. 1974. Experimental crossing of *Aedes polynesiensis* and *Aedes scutellaris malayensis* Diptera Culicidae. Journal of Medical Entomology 11: 323-331.

Tripet, F., Y. T. Toure, G. Dolo, and G. C. Lanzaro. 2003. Frequency of multiple inseminations in field-collected *Anopheles gambiae* females revealed by DNA analysis of transferred sperm. The American Journal of Tropical Medicine and Hygiene 68: 1-5.

Trpis, M., W. Hausermann, and G. B. Craig. 1995. Estimates of Population Size, Dispersal, and Longevity of Domestic A*edes aegypti* (Diptera: Culicidae) by Mark Release Recapture in the Village of Shauri Moyo in Eastern Kenya. Journal of Medical Entomology 32: 27-33.

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

Turner, S. L., N. Li, T. Guda, J. Githure, R. T. Carde, and A. Ray. 2011. Ultra-prolonged activation of CO2-sensing neurons disorients mosquitoes. Nature 474: 87-91.

Weil, G. J., and R. M. Ramzy. 2007. Diagnostic tools for filariasis elimination programs. Trends in Parasitology 23: 78-82.

Werren, J. H. 1997. Biology of *Wolbachia*. Annual Review of Entomology 42: 587-609.

WHO. Year. Published. Neglected Tropical diseases, pp. 1-39. *In* W. H. O.-R. o. f. t. W. Pacific (ed.), Sixty-third session, 2012, Hanoi, Viet Nam. World Health Organisation.

Williams, C. R., S. A. Long, R. C. Russell, and S. A. Ritchie. 2006. Field efficacy of the BG-Sentinel compared with CDC Backpack Aspirators and CO2-baited EVS traps for collection of adult *Aedes aegypti* in Cairns, Queensland, Australia. Journal of the American Mosquito Control Association 22: 296-300.

Williams, S. A., S. J. Laney, L. A. Bierwert, L. J. Saunders, D. A. Boakye, P. Fischer, D. Goodman, H. Helmy, S. L. Hoti, V. Vasuki, P. J. Lammie, C. Plichart, R. M. Ramzy, and E. A. Ottesen. 2002. Development and standardization of a rapid, PCR-based method for the detection of *Wuchereria bancrofti* in mosquitoes, for xenomonitoring the human prevalence of bancroftian filariasis. Annals of Tropical Medicine and Parasitology 96 Suppl 2: S41-46.

Woodhill, A. R. 1949. A note on experimental crossing of *Aedes* (*Stegomyia*) *scutellaris scutellaris* Walker and *Aedes* (*Stegomyia*) *scutellaris katherinensis* Woodhill (Diptera Culicidae). Proceedings of the Linnean Society of New South Wales 74: 224-226.

Woodhill, A. R. 1950. Further notes on experimental crossing within the *Aedes scutellaris* group of species (Diptera, Culicidae). Proceedings of the Linnean Society of New South Wales 75: 251-253.

Wright, J. D., and A. R. Barr. 1981. *Wolbachia* and the normal and incompatible eggs of *Aedes polynesiensis* (Diptera: Culicidae). Journal of Invertebrate Pathology 38: 409-418.

Young, E. C. 2007. Mosquitoes of Rarotonga, Cook Islands: a survey of breeding sites. New Zealand Journal of Zoology 34: 57-61.

Zhou, W., F. Rousset, and S. O'Neill. 1998. Phylogeny and PCR-based classification of *Wolbachia* strains using wsp gene sequences. Proceedings of the Royal Society B: Biological Sciences 265: 509-515.

Zielke, E. 1976. Studies on quantitative aspects of the transmission of *Wuchereria bancrofti*. Tropical Medicine and Parasitology 27: 160-164.