UNIVERSITY OF CALIFORNIA,
IRVINE

Insert Brilliant Insightful Title Here

DISSERTATION

submitted in partial satisfaction of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

in Biological Sciences

by

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2013

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DEDICATION

(Optional dedication page)
To ...

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ACKNOWLEDGMENTS

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Conference name

Another awesome paper **Aug 2012**
Conference name

SOFTWARE

Magical tool http://your.url.here/
C++ algorithm that solves TSP in polynomial time.

ABSTRACT OF THE DISSERTATION

Insert Brilliant Insightful Title Here

By

William Augustine Dunn

Doctor of Philosophy in Biological Sciences

University of California, Irvine, 2013

Anthony A. James, Chair

The abstract of your contribution goes here.

Chapter 1

Introduction

1.1 Dengue fever and malaria are mosquito borne diseases

There are many mosquito borne diseases that afflict the world's population. Dengue fever and malaria are two important such diseases resulting in major health and productivity deficits in many developing nations worldwide CITEME. The causative agents of these two diseases are a suite of four species of RNA viruses (Dengue Virus 1-4) and a group of five species of protist from the genus *Plasmodium* (*Plasmodium falciparum*, *P. vivax*, *P. ovale*, *P. malariae*, and *P. knowlesi*). However, in any **single** geographic location each disease is primarily transmitted by a single species of mosquito CITEME(Table 1.1).

Disease	Region	Mosquito Vector
Dengue Viruses	Wolrdwide	*Aedes aegypti*
Human Malaria	Africa	*Anopheles gambiae*
	Asia	*Anopheles stephensi*

Table 1.1: Primary geographic vectors of dengue fever and malaria

The fact that both of these diseases are caused by multiple pathogen species complicates efforts to prevent human disease: particularly when addressed from the perspective of providing therapeutic or prophylactic care directly to human patients.

TONY: MORE HERE ABOUT WHY THIS COMPLICATES THINGS?

1.2 Hematophagy (bloodfeeding) is an essential part of mosquito-borne disease

Another very important aspect of both of these diseases is that people do not become infected through interaction with other people, and mosquitoes do not become infected through interaction with other mosquitoes. The transmission cycles transmission of both diseases require that the pathogen be passed from human to mosquito or from mosquito to human. This pattern has important consequences that can be exploited to reduce the number of people who will become infected with dengue fever or malaria.

1.2.1 Transmission cycle

In Brief:

1. A female mosquito bites an infected person.
2. The pathogen is taken into the mosquito's midgut along with bloodmeal.
3. The pathogen must escape the midgut and gain access to the mosquito's circulatory system (the animal is now **infected**).

4. The pathogen must gain access to the mosquito's salivary glands (the animal is now **infectious**).

5. The infectious female mosquito bites an uninfected human and the pathogens in her saliva are introduced to the person's body as she feeds.

Figure 1.1: Cross section of a female mosquito with tissues important to the transmission cycle highlighted.
CITEME

While the specifics of how viruses and protists live and reproduce in mosquitoes and humans are quite different, the transmission cycles of dengue and malaria are very similar in their critical events. The transmission cycle of either pathogen type can be encapsulated into five fundamental events. First, a female mosquito feeds on an infected human's blood taking up the pathogen as well (Figure 1.1). The bloodmeal is digested in the mosquito's midgut, and escape from the midgut is the first crucial step for the survival of the pathogen inside the mosquito. If it is trapped in the midgut, it will eventually be passed as waste after the bloodmeal is digested. If it manages to escape to the hemolymph, the pathogen must survive long enough to gain access to the mosquito's salivary glands. If it is unable to infiltrate the salivary glands, then the mosquito, while itself **infected**, is not **infectious** to other humans. For this, the pathogen must be injected into the bloodstream of the next human along with the contents of the mosquito's saliva.

1.3 Public health: *multiple targets*

Because the arrows of transmission essentially[1] points from mosquito to human or from human to mosquito, the cycle will collapse and a local area would eventually be cleared of the pathogen if either of those arrows are broken, provided that the intervention is maintained. This model suggests two primary targets for public health interventions aiming to reduce the incidence (followed by prevalence) of infection in a population:

1. Prevent mosquitoes from infecting humans.
2. Prevent humans from infecting mosquitoes.

However, many interventions can not be clearly divided into addressing purely the human or vector side of the cycle. For example, efforts to reduce the number of bites that humans receive from mosquitoes affects both the probabilities of the vector *and* the human becoming infected. This is one reason that vector control is almost always part of prevention strategies CITEME. For the purposes of this document, I will define human-based interventions to be those that are directly administered to a human body. Essentially: medical interventions.

In this respect, the long term goals of public health are more focused on prevention than the treatment of acute cases. Of course sick people need to be treated. Additionally, identifying and curing people who are infected also prevents mosquitoes from acquiring the pathogen as well. However, in the long term prevention is preferred.

[1] While some mosquito to non-human vertebrate transmission may occur, it is not thought to be sufficient to maintain the pathogen if the mosquito:human loop is broken (CITEME).

1.3.1 Addressing the human side of the cycle

Because of the definition of human-based interventions above, there are relatively few effective options in this category for malaria and dengue fever. Normally, this section would include vaccines and swift, effective patient identification and treatment to clear the infection. However, at this time these types of interventions simply do not exist on the market or in a cost-effective form applicable to the isolated and impoverished areas that are most affected CITEME.

1.3.2 Non-transgenic options for addressing the vector side of the cycle

Medical options are scarce generally in the regions of the world where these diseases are most common. The alternative is to focus attention toward controlling access of the vector populations to human contact. This can include removal of nearby mosquito breeding sites (usually various forms of standing water), spraying of insecticides, introduction of biological predators, and/or bed nets, etc. One fairly recently developed approach that has shown great promise is Sterile Insect Technique (SIT).

Sterile insect technique

SIT exploits a peculiar aspect of some insects' reproductive behavior. In many insects, the first mating event provides the only genetic material that the female will use to fertilize her eggs for the rest of her life CITEME. Subsequent mating events contribute little to no genetic material to the females progeny, **even if the first event involves a sterile male**. This means that inundative numbers of sterilized males introduced into a native population will effectively sterilize significant proportions of wild females. This has dramatic effects on the local population. Negative population growth results from the inability of each generation to fully replace itself, and the effect is compounded for each subsequent generation as long as the program is maintained.

Perhaps most famously, SIT was used successfully to eradicate the screwworm from the southern United States in the 1950s [6].

1.3.3 Transgenic techniques provide a new strategy for controlling disease transmission

Most conventional vector control strategies involve what might be termed vector population reduction. In addition to population reduction, transgenic techniques provide a novel vector control strategy that has considerable implications to the sustainability of the vector control aspect of public health interventions for dengue and malaria. In contrast to population reduction, this novel strategy could be conceptualized as vector population conversion. It's goal is not to reduce or eliminate contact between humans and vectors but to drive a transmission-refractory trait through the vector population that renders the vector unable to become infectious at all, converting it into a non-vector.

1.4 Elements of a successful transgenic vector control product

1. the ability to introduce transgenes into the vector genome in a heritable way
2. the discovery or construction of effector genes
3. the ability to control when and where the effector genes are expressed
4. the ability of your transgenic vectors to have swift, dramatic, and clinically meaningful effects on the native vector population (gene drive)

1.4.1 Introducing heritable transgenes into mosquito genomes

Before one can employ transgenic methods for vector control, one must have the capacity to make transgenic vectors. For mosquitoes, this is more labor intensive than for other fly species like *Drosophila melanogaster*. However, it has been accomplished, and protocols to reliably introduce transgenes into many mosquito species now exist CITEME. Unfortunately, the relative difficulty is high. It takes roughly 2-3 months to establish a stable line. Thus many experimental approaches that are straightforward or even routine in *D. melanogaster*or other species remain impractical for mosquitoes.

The method of introducing a transgene into a mosquito genome generally relies on re-purposing the activities of transposons (transposable elements). These nucleotide elements have the ability to cut themselves out of one part of the DNA and re-insert themselves into another. It is possible to engineer them to insert selected transgenes into the mosquito genome instead of the genes that they usually contain.

Until recently, most if not all transposons used for transgenesis in mosquitoes inserted the transgenes into more-or-less random locations. The number of locations and number of copies at any **single** genomic location was unpredictable [1, 14]. This makes characterization of the effects of a transgene complicated. However, with the successful implementation of the ϕC31 site-specific integration system in *Ae. aegypti* and *An. stephensi* [16, 13, 9], we can now reliably target specific, pre-characterized regions of these genomes for transgene integration of a single copy.

Heritability is achieved through specifically targeting the germline cells of mosquito embryos for transgenesis. In flies like mosquitoes, the cells that will become the germline always position themselves at one end (or pole) of the embryo (Figure 1.2). The transgenesis cocktail containing the transgene construct is injected into the embryos where the pole cells are located. This

means that the first generation of mosquitoes that may contain the transgene in every cell of their body is **not** the injected generation but the progeny of the injected mosquitoes.

Figure 1.2: ***oskar*** **gene expression in the vector mosquitoes,** ***Anopheles gambiae*** **and** ***Aedes aegypti*****:**
Hybridizations *in situ* of *Anga osk* and *Aeae osk* RNA probes to whole-mount *An. gambiae* and *Ae. aegypti* embryos. Embryos are orientated with anterior on the left. *An. gambiae* embryos (A,B) shown have reached cellular blastoderm with pole cells indicated by an arrow. *Ae. aegypti* embryos (C,D) shown are in the preblastoderm stage. Strong staining is exclusive to the posterior pole cells (A) and posterior end (C) (arrows), when compared with embryos of the same stage, determined by DAPI staining (not shown), probed with *Anga* and *Aeae osk* sense RNA probes (B,D). Bar = 50 μm.

Excerpted from [10]

1.4.2 Effector genes

In addition to the ability to integrate transgene constructs into the vectors' genomes, it is necessary to design constructs that contain transgenes that will effect a useful response in the vector. Two broad strategies are commonly applied when designing an anti-transmission phenotype: population reduction and population conversion.

Vector population reduction:

The goal of vector population reduction is the same as conventional vector control modalities. *Reduce the local population of a disease vector with the goal of reducing the probability that an infectious individual will have an opportunity to participate in a transmission event.*

A **transgenic tactic** for the population reduction strategy is illustrated by Oxitec's *Ae. aegypti*strain OX3604C developed with support from the Bill and Melinda Gates Foundation's Grand Challenges for Global Health Initiative. Strain OX3604C represents a female-specific Release of Insects carrying a Dominant Lethal phenotype (RIDL) approach which uses a poison transgene that kills any female adult expressing the transgene unless the female is provided an "antidote" through its water supply [17, 2, 7].

Releasing enough of these mosquitoes will affect the local mosquito species population in a way that is analogous to SIT.

Vector population conversion:

Vector population conversion is a novel strategy for vector control that primarily exists within the context of genetically altered vectors (CITEMEwolbacia). It promises to be the most long-lasting vector-focused intervention. Unlike **all** vector population reduction tactics, there is a practical point in a conversion intervention (the anti-transmission trait works and is present in local populations at near 100%) when the human management of the intervention can be ceased, yet it continues to function. For population reduction to approach this result, the vector species must not simply be eliminated from the local area, but approach elimination on a continental scale, or more realistically achieve global eradication.

The reason is that these mosquitoes (especially *Ae. aegypti*) can and do travel long distances, as eggs or larvae, in the backs of trucks (between villages) or in the pools of water collected

in super-tankers (transcontinentally). So a local village is only free of the vectors until more migrate into the area. But in a conversion scenario, those migrants mate with the local vector population, and their offspring are assimilated into transmission-deficient mosquitoes. The protection of the local village can be preserved *even if some surrounding villages fail to maintain control of their mosquitoes.*

An example of a **transgenic tactic** for population conversion of *Ae. aegypti* into a transmission-deficient phenotype involves an effector gene that produces a double stranded RNA molecule that contains a portion of the dengue virus genome [8, 11]. Because most animal cells have a system that detects and degrades double stranded RNA[2] in a sequence specific manner, this primes the mosquito cells' antiviral response to specifically attack the dengue virus if the effector gene is expressed in the cell before it gets infected.

1.4.3 Controlling when and where the effector genes are expressed

Even with an effector gene that clears 100% of the pathogen 100% of the time, the intervention will not be successful in limiting transmission if the transgene is not turned on at the right time and place. If the transgene is most effective when the pathogen is in the midgut, it is not useful if the gene is only expressed in the antennae.

The region of DNA directly before the sequence of the gene is a major determinant of the pattern of expression and is referred to as the promoter. It influences when the gene will be turned on (e.g., directly following the ingestion of a bloodmeal), and in which tissue type (e.g., the midgut). Promoters influence the transcription of genes through the binding of special proteins called transcription factors that recognize specific DNA sequences called *cis*-regulatory elements (CREs). CREs are sometimes organized into modules that represent groups of binding sites for a specific combination of transcription factors. These modules are *cis*-regulatory

[2] Double stranded RNA generally signals that a virus is active in the cell.

modules (CRMs) CITEME(Figure 1.3a). Once a set of transcription factors are bound to the promoter they recruit the special machinery needed for the gene to be turned on or silenced depending on the context of the cellular environment (Figure 1.3b).

(a) CREs make up CRMs **(b)** One model of transcriptional regulation CITEME

Figure 1.3: Elements of transcriptional regulation

A typical strategy to obtain regulatory sequences suited to drive a transgene following the expression characteristics used as an example above (expressed directly following ,a bloodmeal, in the midgut) would be to identify genes in the mosquito that already have a similar expression pattern to the ideal. The promoter from that gene would then be copied and inserted in front of our transgene in the final integration construct.

This is a very common process used to engineer the expression patterns of real transgenes in mosquitoes. In [12], the promoter sequences of a gene called carboxypeptidase (normally expressed in the midgut after a meal) from *Ae. aegypti* and *An. gambiae* were inserted in front of a reporter gene.

From the abstract of [12]:

> Six independent transgenic lines were obtained with the AeCP construct and one with the AgCP construct. Luciferase mRNA and protein were abundantly expressed

in the guts of transgenic mosquitoes in four of the six AeCP lines and in the AgCP line. Expression of the reporter gene was gut-specific and reached peak levels at about 24 h post-blood ingestion.

1.4.4 Achieving swift, dramatic, and clinically meaningful effects on the native vector population

In many ways, this is the most difficult part of the system. In order for the transgene to have its self-sustaining properties as well as achieve effective anti-transmission results, it must spread through the native mosquito population to the point that the percent of individuals possessing the gene approaches 100%.

Making the optimistic assumption that the transgene carries a negligible fitness cost [3], or even a slight fitness advantage, achieving near 100% conversion could take decades. Funding terms for efforts like this in poor nations can be closer to 5 years or less. To enable the population conversion strategies to work, genetic "tricks" must be exploited that cause the gene to spread through a mosquito population **much** faster than could happen naturally. Efforts to discover or design these gene drive systems are an on going and active area in this field.

An example of such a method is the Medea system successfully engineered in *D. melanogaster* (CITEME).

In Brief:

1. The construct consists of a **death gene** under control of a maternal effect promoter and a **rescue gene** under control of an early embryonic promoter.

[3] By fitness cost/advantage here, I mean that the transgene causes the mosquitoes that inherit it to be either less or more successful at producing offspring, respectively.

2. The **death gene** is produced by the mother and deposited into 100% of her developing embryos: *even those that did not inherit the transgene due to mating with a wild type male.*

3. However, since the **rescue gene** is produced by only those embryos that inherited the transgene construct, only those embryos will survive.

4. The female has on average half as many progeny survive as usual if she mated with a wild type male, but 100% of those that survive inherit the transgene.

5. Over time this results in the fixation or near fixation of the transgene in the population.

1.5 Conservation of bloodmeal-regulated transcription

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1.5.1 Evolutionary relationship between mosquitoes and hematophagy

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1.5.2 Implications for design of transgenic vector control systems?

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Chapter 2

Development of Analytical Approach

2.1 Purpose

Review a selection of my contributions to the literature that have informed the approach used in this project throughout my tenure as a member of the lab

1. Comparative genomics allows the discovery of cis-regulatory elements in mosquitoes
    - Use of comparative genomics to discover putative CREs from orthologous promoter regions in mosquitoes that were able to be corelated with bloodmeal associated transcription control
2. RNA-seq analyses of blood-induced changes in gene expression in the mosquito vector species, Aedes aegypti
    - relevance
3. Strain Variation in the Transcriptome of the Dengue Fever Vector, Aedes aegypti
    - relevance

4. Comparative Transcriptome Analyses of Deltamethrin-Resistant and-Susceptible Anopheles gambiae Mosquitoes from Kenya by RNA-Seq

    - relevance

5. Complex Modulation of the Aedes aegypti Transcriptome in Response to Dengue Virus Infection

    - relevance

2.2 Comparative genomics allows the discovery of cis-regulatory elements in mosquitoes

Figure 2.2

Figure 2.1

2.3 RNA-seq analyses of blood-induced changes in gene expression in the mosquito vector species, Aedes aegypti

Figure 2.3

Figure 2.4

Figure 2.1: Associations of mosquito motifs with gene expression profiles in *An. gambiae*:
Motif enrichment within (A) 5′-end flanking regions of genes in clusters responsive to blood meal ingestion, and in (B) 5′-end flanking regions of genes in clusters enriched in selected tissues. The significance of motif enrichment is indicated by pseudocolor of -log10 (P-value) determined through hypergeometric statistics, and the median expression profile of each gene cluster is shown below each respective column. Red and green colors represent higher and lower relative mRNA accumulation, respectively. Asterisks (*) indicate a match to a previously described mosquito TFBS. Heatmaps were created with Matrix2png (CITEME:local-58). FB, fat body; hPBM, hours post blood meal; MG, midgut; OV, ovaries; TC, time course clusters.
Adapted from [15]

Modified from: Sieglaff et al. PNAS. 2009 Mar 3;106(9):3053-8

Figure 2.2: Phylogenetic relationships between four vector mosquitoes compared with representative *Drosophila* species:
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Adapted from [15]

2.4 Strain Variation in the Transcriptome of the Dengue Fever Vector, Aedes aegypti

Figure 2.5

2.5 Complex modulation of the Aedes aegypti transcriptome in response to dengue virus infection

Figure 2.6

2.6 $\text{like}_{\text{this}}$

Figure 2.3: Examples of amendments to the *Ae. aegypti* annotation supported by HMMSplicer results: Black bars in the top tracks represent the current gene annotations. Blue histograms in the second track represent the non-normalized coverage of RNA-seq reads at each position. The range of the histogram values shown in each view is depicted on the labeled y-axis of each RNA-seq track. Black boxes in the lower track represent splice-site predictions based on the RNA-seq reads using HMMSplicer determined in this study. Each function has a unique identifier listed below and its HMMSplicer score is listed in red. If multiple reads support a single junction, "junc = x" lists the number of supporting reads. This information provides evidence to link two islands of transcription as a single transcription event, therefore, exons of a common mRNA. All predicted junctions shown here also are supported by EST alignments. Genes are (A) AAEL006259; (B) AAEL010818; and (C) AAEL001774 and AAEL001759.
Excerpted from [3]

Figure 2.4: Motif map of putative CREs discovered by SCOPE using transcripts detected significantly only in bloodfed female *Ae. aegypti*: Locations of representative SCOPE-derived CRE motifs in the 2000 bp upstream of the annotated translational start site in the 40 transcripts detected significantly only in bloodfed females. Transcript names on the left are ordered from most (top) to least (bottom) abundant. Candidate CRMs are highlighted in like-colored rectangles.
Adapted from [3]

Figure 2.5: Fatty acid biosynthesis and elongation in mitochondria: Proteins corresponding to transcripts accumulated in a strain-specific manner are circled in a color corresponding to the strain and condition defined in the legend (panels A and B).
Excerpted from [5]

`convert figures/figs/bonizzoni2012complex-cre.tif `dirname figures/figs/bonizzoni20

Figure 2.6: MEME analysis of nine genes with $FPKM_{DENVI} \geq 100$ in carcasses and salivary glands at 14 dpi These genes also were identified with transcripts exhibiting significant differential accumulation in analyses of salivary gland samples of the Liverpool strain infected with DEV2 Thailand 16881 [26]. Colored boxes represent individual putative CREs and their locations in promoters of each gene. Red and blue arrows adjacent to Ensembl Gene ID indicate those genes whose transcripts were detected previously as more or less abundant following DENV infection [26]. Distances in base-pairs are provided below the schematic of each gene. doi:10.1371/journal.pone.0050512.g004
Excerpted from [4]

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Glossary

gene drive molecular genetic tactics that cause a linked trait or gene to spread through a population at faster rates than expected based on fitness alone; generally operating independently of natural selection and genetic drift. 12

germline the cells responsible for eventually forming the sperm or egg cells in a mature sexual organism. 7

hemolymph A fluid analogous to blood that constitutes the circulatory system of most invertebrates. 3

incidence a measure of the frequency with which new cases of illness, injury, or other health condition occurs among a population during a specified period. 4

ϕC31 a site-specific bacteriophage recombinase that performs precise, unidirectional recombination between two sites called attB and attP. 7

population conversion a novel vector control strategy that aims to drive a transmission-refractory trait into a local vector population with the goal of preventing individuals from every becoming infectious. 6, 8

population reduction a vector control strategy that focuses on reducing the local population of a disease vector with the goal of reducing the probability that an infectious individual will have an opportunity to participate in a transmission event. 6, 8

prevalence the number or proportion of cases or events or attributes among a given population. 4

transgene a gene that has been transfered from one organism to another. 7

transposable element relatively short regions of DNA in an organism's genome that have the ability to cut (or copy) themselves from the genome and insert themselves into a new location in the genome. 7

transposon . 7, *see* transposable element

Acronyms

CRE *cis*-regulatory element. 10

CRM *cis*-regulatory module. 10

RIDL Release of Insects carrying a Dominant Lethal phenotype. 9

SIT Sterile Insect Technique. 5, 6, 9

Appendix A

Methods

Appendix B

Code