ABSTRACT

PAA, SANDRA ALEXIS. Creation of a Strain of *Drosophila melanogaster* with a Killer-Rescue Gene-Drive Mechanism and Observation of Wild-Type Frequencies in Population Cages over Multiple Generations. (Under the direction of Dr. Fred Gould, Dr. Jim Mahaffey, and Dr. Nick Haddad.)

A strain of *Drosophila melanogaster* was created, such that the flies were homozygous for two alleles in a gene-drive mechanism called "Killer-Rescue". In this system, the transgenes borrowed from *Saccharomyces cerevisiae*, called Gal4 and Gal80, acted as Killer and Rescue, respectively. In order for the Gal4-Gal80 system to function as a killer and rescue system, all flies contained a wild-type gene, called UAS-DiscoM, which, when activated by inheritance of the Killer allele alone, would kill the individual embryonic *Drosophila*. If the individual also inherited a copy of Rescue, the Killer was blocked from activating the UAS gene. All non-transgenic flies ("wild-type") carried the UAS-DiscoM gene. The first objective of the researcher was to test if such flies could, indeed be created and be maintained in a stable stock, and it was indeed discovered to be true.

The second, and perhaps more applicable objective of this creation of a transgenic strain was to examine its function in population-cage level experiments, to determine frequencies of the Killer and Rescue alleles (indirectly, through observation of the frequency of wild-type flies relative to the frequency transgenic flies, and directly, through molecular techniques) over a period of fifteen generations. These trials would show that, at a release ratio of 50% transgenic to wild-type, and with seemingly zero fitness cost to the transgenic flies carrying Killer and Rescue, that the transgenic alleles would go approximately to fixation at a given level, and that the frequency of wild-type flies would plateau at around 7%--a number which would persist over the majority of the generations (F3-F15). In a separate experiment, transgenic flies were introduced to population cages at a much lower

release ratio: 10% transgenic alleles to 90% wild-type. At the current time, eight generations have passed, and the four most recent generations (F5-F8), have continuously increased in the amount wild-type phenotype (the greatest increase being 6.99%, starting at 14.12% in the F5 generation and increasing to 21.11% by F8). This indicates the possibility for a very slow wild-type recovery in cases where the release ratio of transgenics to wild-type is small enough, even when the cost to carrying the gene-drive alleles is minimal.

Creation of a Strain of *Drosophila melanogaster* with a Killer-Rescue Gene-Drive Mechanism and Observation of Wild-Type Frequencies in Population Cages over Multiple Generations

by
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DEDICATION

I would like to dedicate my Thesis to my family members, who have given me the most wonderful, enduring support and are always ready to be at my side.

To my Grandfather, Ralph Boers, and my Grandmother, Betty Boers, for teaching me through mastery of their respective crafts, that hard work and patience beget beauty,
To my Grandfather, Joseph Paa, who taught me that music is the best kind of release,
To my Grandmother, Leni Paa, who taught me to love all creatures (even spiders!)
To my Tante Jutta and Onkel Gerhard, who taught me to have fun
To my Father, Helfried Paa, who taught me to persist and work hard, especially when times are rough,
To my Mother, Mary Elizabeth Paa, who taught me to be creative at an early age,
To my Sister, Ashley Lance, who taught me to always be happy.

Thanks, and Love, to all.

BIOGRAPHY

Sandra Alexis Paa was born September 3, 1985, to Helfried and Mary Elizabeth Paa of Transylvania County, North Carolina. In the mountainous terrain of Western N. C., she enjoyed all manner of outdoor activities, not limited to lifting all kinds of stones to see what creatures lay beneath. Sandra attended Brevard High School, where she was best known for leading the drumline of the high school marching band. In the fall of 2004, she attended N. C. State University in Raleigh, where she continued her role as a percussionist but also evolved a taste for the Biological Sciences. After taking a general entomology course, Sandra discovered she had quite a liking for all manner of arthropods, and pursued an internship at the N.C. Museum of Natural Sciences, in its Arthropod Zoo. After a positive experience taking care of arthropods at the museum, Sandra sought and acquired a summer job raising *Heliothis* moths in the lab of Dr. Fred Gould.

Sandra Graduated in December 2007, with a Bachelor's Degree in Biological Sciences with minors in History and Entomology, and decided to begin her Master's Degree work as early as possible, and was fortunate to have been brought into the Entomology Department in Dr. Fred Gould's lab.

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I would also like to thank all the post-doctoral research associates in both the Gould and Mahaffey labs—their help has been unbelievably valuable in my experiments, from running PCR reactions to babysitting flies over the weekend. I have made some great friends and I hope to keep strong connections with them in the futures.

I also thank the NCSU EGSA, for providing me with opportunities to extend the reach of our entomology program into the community. I've had a lot of fun with outreaches, and have enjoyed meeting Graduate students and again, hope to keep many of them as friends throughout my career.

Many thanks to the Entomology Department at NCSU, and to the staff—without them I would not have had the great teaching opportunity that I was granted.

Finally, I thank the National Institutes of Health for helping fund my experiments. I greatly appreciate the financial support, and hope that I have contributed worthwhile information to the overall cause.

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CHAPTER ONE: INTRODUCTION

For my Master's Degree thesis project, I conducted population cage-level experiments with the fruit fly, *Drosophila melanogaster*, testing a synthetic gene drive system, called *Killer-Rescue* (Gould et al. 2008). First, I developed a strain of flies that contain transgenes called *Killer* and *Rescue*. Ultimately, I wanted to observe how the frequencies of the genes varied over several generations, and how this gene-drive system would function when released into lab-controlled populations of "wild-type" flies. In the following chapters, I discuss my reasoning for embarking on a project of this nature, citing the importance of fly-borne disease and the usefulness of *Drosophila* as a model in simulating populations of vector-carrying Dipterans, like mosquitoes. I also detail how the flies were created, through a series of crosses, and then discuss the results after several generations when the flies were released at frequencies of 0.50 and 0.10 in populations of wild-type flies. I begin by discussing the importance of insect-vectored disease, with some emphasis on three globally important fly-borne diseases.

I. Insect-Vectored Diseases

A. A Brief Overview of Insect-Vectored Diseases.

Arthropod-vectored diseases have played a substantial role in the quality of life of humans across the span of the globe, and across the span of history (Hill 1987). One of the earliest, and most devastating examples of insect-vectored disease affecting human society was the Bubonic Plague of Europe in the 1300s (Gunn 1916)—which resulted in

approximately one-fourth of the population of Europe dying due to fleas carrying the bacterium *Yersinia pestis* (Walker 2004).

Arthropod-vectored pathogens that cause human disease are members of diverse taxa, including species of bacteria, protozoans, and viruses. In many cases, these pathogens multiply within the arthropod before being transmitted to a vertebrate host (Mitchell 2002, [CDC 7], [CDC 8]). The Centers for Disease Control and Prevention list a worldwide count of 537 arboviruses alone that affect humans ([CDC 1]), including West Nile virus and Yellow Fever virus. Arthropod-vectored bacteria and protozoans, such as those causing Chagas' Disease and Malaria, have also proven problematic to humans (Gubler 1998, Schneider 2000). The arthropods that vector these pathogens range from Diptera (e.g. mosquitoes) to Hemiptera (e.g. kissing bugs), to Acari (e.g. ticks). .

Control of arthropod-borne disease is clearly important for enhancing the worldwide quality of life in the face of a growing human population (Cohen 2003, Cohen 1995) and globalization of the world economy that fosters the transport of diseases and their vectors. To avoid future disease outbreaks, there is a need to understand and manipulate vector organisms and the pathogens they transmit. The order Diptera includes some of the most important disease-vectoring taxa, and they are the focus of much recent research aimed at developing novel tools for disease control. In the next sections I will present three examples of diseases vectored by dipterans, and will provide the real-world justification for my thesis research. I will then discuss how an understanding of the biology of the dipteran midgut and salivary glands where pathogens multiply can help us develop novel approaches for managing pathogen transmission.

B. Human Pathogens Vectored by Dipterans

Of the 537 Arboviruses listed from around the world by the ([CDC 1]), many utilize mosquitoes as their main vector. In cases where little is known about the epidemiology of a specific arbovirus, the CDC list often references mosquitoes as suspected in transmission ([CDC 1]).

Several strategies for combating fly-borne diseases are being investigated in the different areas of pest management. These range from the most basic strategies, like sprays applied aerially to control adults (Mount et al. 1996), or chemicals applied in ponds where mosquito larvae may develop (Jondiko 1986), to area-wide programs like the sterile insect technique—all of which are limited in their success. The sterile insect technique has proven helpful in other families in Diptera, such as Tephritidae and Calliphoridae (McInnis 1994 and McGraw 2001, respectively) in which males are irradiated *en masse* to cause sterility and are released into the wild, (Knipling 1959). This increases the likelihood several-fold that a female will mate with a male who is unable to produce viable sperm, ultimately wasting her energy and leaving her without fertilized eggs. The vector for Dengue Fever, *Aedes aegypti*, has been referenced as a likely useful candidate for this technique, due to success in manipulation of this species in laboratory mass-rearing settings—however, there are no current programs for using this strategy in Dengue-troubled areas (Fu et al 2010). One complication is that irradiated males have been shown to suffer a fitness disadvantage when trying to compete with their wild-type counterparts, which can lead to problems in efficacy in a sterile-male release program (Collins et al. 2008).

As mentioned above, mosquitoes are also vectors of protozoan diseases, such as Malaria. To give some perspective on the biology and control of dipteran-vectored diseases, I will provide an overview of two protozoan and one viral disease that have and are having major impacts on humans.

1. Malaria

Background

The protozoan *Plasmodium sp*. (most commonly, *P. falciparum*) that causes malaria is mostly vectored by the mosquito *Anopheles gambiae (sensu latu)*. Eighty-nine percent of the world's cases of this disease occur in Africa. Malaria is the continent's second most-deadly disease (behind HIV-AIDS), killing an estimated 863,000 people in 2008 alone ([CDC 4]).

As is the case with most protozoan parasites, the life cycle of *P. falciparum* is a complex one. During its life cycle, it undergoes a five-stage metamorphosis, and invades three physical barriers in the mosquito's body (Ghosh et al. 2000). An infected, *Plasmodium*-bearing mosquito takes a blood meal from a human host, releasing the motile sporozoite stage of the protozoan (which had multiplied in the infected mosquito's midgut and salivary glands). When the sporozoites are injected via the insect's saliva, they enter the victim's bloodstream and travel to the liver, where they infect those cells and multiply as merozoites (a motile stage). Eventually, merozoites become too numerous for the liver cells, causing those cells to rupture and then the merozoites re-enter the bloodstream, where they infect red blood cells. Here, they become trophozoites (a feeding stage of this protozoan's life cycle), and these feed on the hemoglobin of the red blood cells. Once gorged, these protozoans

undergo schizony, during which the trophozoites reproduce themselves as merozoites ("daughter cells") in the remains of the red blood cell, then burst out and re-infect more blood cells. Reproductive versions of the protozoan are also created within the borders of the red blood cell, and these gametocytes are the stage taken up by a mosquito as she feeds and then becomes infected. In the mosquito, the gametocytes morph into reproductive cells called macrogametocytes, which give rise to a motile, ookinete stage. The ookinetes attach to the mosquito's midgut and develop into oocysts, which form sporozoites within. When the sporozoites develop, the oocyst bursts and the sporozoites emerge and head to the mosquito's salivary glands. It is in this stage that they are injected into their next human host by the mosquito ([CDC 7]).

Vector Biology

Anopheles gambiae, the most well-known vector of Malaria in Sub-Saharan Africa, is a nocturnal blood-feeding mosquito which, like many mosquitoes, uses olfactory cues in host detection (Carey et al. 2010). Blood meals are taken in order to provide nutrients to developing eggs in the female (male mosquitoes feed only on sugar sources, such as nectar, for sustenance). Females lay eggs singly on the surface of still water, often in containers where water has collected. The eggs hatch into larvae, which align themselves with the surface of the water, since they lack the underwater breathing tubes of *Aedes* larvae. Larvae feed on algae and microorganisms under the water's surface, and go through four larval instars before pupation. Only a few days later, the adult mosquito emerges from the pupa, and if the female takes a blood meal from a human host infected with Malaria, the cycle begins again ([CDC 5]).

Control measures, such as insecticide-treated bed nets, and indoor residual spraying, help dissuade mosquitoes to a certain extent. Occasionally, there can be insecticide treatment of still water areas to control larvae. Making conscious efforts to decrease standing water containers can also help to some degree ([CDC 6]).

Epidemiology and Treatment

The CDC lists that approximately half the world's population lives in areas that are at risk for malaria transmission—especially Sub-Saharan Africa and parts of Asia ([CDC 4]). Malaria can take two forms of severity. One is considered "uncomplicated," during which victims may experience a variety of unpleasant symptoms, including fever, headaches, muscle pains, sweats, chills, and occasionally nausea and vomiting. These symptoms are difficult to distinguish from many other illnesses, but can be diagnosed when parasites are found in a blood sample. The other, more severe form affecting humans is called "severe malaria", in which victims may experience a high level of confusion or may go into a coma. They have trouble breathing and focusing, and they experience severe anemia, along with the regular signs of uncomplicated malaria ([CDC 8]).

Treatment comes in the form of several different drugs (depending on severity of symptoms, other illnesses, or whether the victim is pregnant)—and some of these drugs are very expensive. That can be problematic in areas where population is high and quality of patient care may suffer ([CDC 10]). As is always the case, however, the goal is to stop the vector before it takes a blood meal, and ultimately stop the spread of *P. falciparum* as best as humanly possible.

2. Dengue Fever

Background

Dengue Fever is a viral disease caused by flaviviruses of any of four serotypes (Dengue 1, 2, 3, or 4), occurring most frequently within South America, Central America ([CDC 9]), and tropical Asia ([WHO 1999]) and Australia (McKenzie et al. 1996). The insect vectors for the virus are mosquitoes in the genus *Aedes*, most commonly *Aedes aegypti*, but also *A. albopictus*. Two-fifths of the human population lives in areas that are habitable by *A. aegypti*, and the World Health Organization approximates 50 million people are infected annually ([WHO, 2010]). Regarding the more severe form of the disease, the CDC lists several hundred thousand cases of Dengue Hemorrhagic Fever (DHF) annually, with outbreak case fatality rates as high as 10% ([CDC 9]).

Vector Biology

Just as in the case with *Anopheles*, the female mosquito can take a blood meal from a human infected with any of the Dengue strains, and then the virus travels to her midgut, where it multiplies (Gomez-Machorro 2004), but does not exhibit such a diverse and complex life cycle as the Plasmodium protozoan (CDC 8). The virus then travels to the salivary glands, where it continues to multiply. The parasites are then injected into the next human host via saliva of the mosquito (Gomez-Machorro 2004), thus infecting the human and beginning the cycle anew.

Aedes aegypti is a diurnal mosquito, so bed-nets are not helpful. Rather, people are encouraged to patrol and clean out any water-bearing containers near their homes, which could give rise to mosquito larvae if a female finds suitable habitat there ([CDC 9]).

Community-wide education programs and surveillance measures are being championed by the World Health Organization ([WHO 1999]).

The female mosquito does prefer standing water that is fairly fresh and nutrient-rich; she will lay her eggs on the side of the container near the water line. The life cycle from a laid egg to a reproductive adult takes as little as eight days, so vigilance is important when eliminating mosquito larvae and pupae from containers ([CDC 9]).

Epidemiology and Treatment

As the name might imply, the main symptoms experienced by victims of Dengue include high fever, as well as intense headache and pain behind the eyes. Other symptoms include joint, muscle, and bone pain, delicate skin prone to bruising, and occasionally rashes or mild bleeding. The CDC reports that children and first-time victims of Dengue have a less severe form of the disease than older people, or those previously exposed to the virus ([CDC 9]).

Victims of the more severe form of the disease, Dengue Hemorrhagic Fever, experience many of the same symptoms as DF, but they experience a fever that can run to around 7 days. However, when the fever declines in severity, other symptoms take its place, including persistent vomiting, severe abdominal pain, and difficulty inflating the lungs, in order to breathe. At this time, capillaries can become “leaky,” causing hemorrhages under the skin, which may give rise to circulatory system failure and shock, after which the patient may die if their circulatory failure remains untreated ([CDC 8]).

There is no vaccine for Dengue Fever or DHF (Scott et al. 2002); suspected victims should alert their physicians, and make sure to get plenty of rest and fluids. Pain relief

medication is said to help; if a patient's health severely deteriorates within a day after the initial fever wanes, it is highly recommended they seek medical assistance at a hospital ([CDC 8]).

Once again, the best defense is offense—the most highly emphasized method of control has proven to be the emptying and rinsing of any types of containers that may hold mosquito larvae or pupae—including children's swimming pools, animal water dishes, discarded tires, and outdoor garbage cans ([CDC 9]).

3. African Trypanosomiasis

Background

Also called African sleeping sickness, this disease is caused by the protozoans *Trypanosoma brucei rhodesiense* in Eastern Africa, and *Trypanosoma brucei gambiense* in Western Africa. These forms of trypanosomiasis are vectored by the Tsetse flies of the genus *Glossina* ([CDC 2]).

Vector Biology

The Tsetse flies, *Glossina sp.*, feed on the blood of vertebrates, and exhibit a unique strategy for taking care of their young. Once she has mated, the female tsetse fly produces a single egg that hatches and grows within her body, nourished indirectly by the blood meals that she takes, as they are converted into a nutritious fluid by the mother's accessory glands (Buxton, 1955). Once the larva is large enough and is ready to pupate, the female births the maggot on top of soil which is typically under a shrub, where there are less drastic temperature fluctuations. The maggot burrows underground and pupates near where its

mother deposited it (Buxton 1955). Since they are viviparous, Tsetse flies have relatively few offspring compared to other Diptera—birthing no more than twenty offspring per female (Gooding and Krafsur, 2005).

The cycle of African Sleeping Sickness can be seen as starting with a Tsetse fly ingesting a blood meal from a person infected with the protozoan, *Trypanosoma brucei*. Once inside the fly, the protozoan exhibits unique strategy for multiplying. Unlike the cases above, where the infectious agent migrates through the body cavity of the insect to travel from the midgut to the salivary glands, in the case of the Tsetse fly the journey is linear and exclusively relegated to the digestive system. Upon intake of the blood meal, the trypanosomes spend a short time in the insects' crop before being emptied into its midgut, where the meal starts to become digested. Here, the trypanosomes (in their flagellated, motile form called a trypomastigote) undergo active and rapid division, after which they penetrate the rigid membrane lining the interior midgut (called the peritrophic membrane) with little difficulty. They migrate anteriorally, along the ectoperitrophic space of the esophagus and hypopharynx, and then into the salivary glands. As they venture to glands, they elongate and become epimastigotes, the infectious stage of this trypanosome's life cycle. Once the infected fly bites an uninfected vertebrate host, the trypanosomes are released in the fly's saliva, and the victim then becomes infected (Vickerman et al. 1988). The trypanosomes multiply by binary fission within the infected human [(CDC 2)].

Epidemiology and Treatment

Both forms (Eastern and Western) are fatal if no treatment is undertaken—within three months for *T.b. rhodesiense*, or up to a year or more for *T.b.gambiense*. Symptoms include the development of a chancre around the site of the original bite, which swells painfully, as do lymph nodes, the face, and the hands. Headaches and fever become persistent, as do joint pain and fatigue, as the course of the infection continues. The CDC also reports that neurologic problems occur, such as confusion and personality changes, after the disease has infected the victim's nervous system. And, as the name might imply, fatigue leads to the victim's need to sleep more often, especially during the day, as the body tries to fight off the infection ([CDC 2]).

C. Spread of Disease: The Dipteran Midgut and Salivary Glands

The midgut and salivary glands are targeted by agents of disease as sites of multiplication and transmission to subsequent vertebrate hosts. The midgut of any insect is an active place, in which digestive enzymes are excreted for the digestion of food, and where nutrients from those food sources can be absorbed into the body. This organ carries out ion transport and the absorption of amino acids, lipids and sugars (Popova-Butler and Dean 2008). Cells lining the luminal portion of the midgut form structures called microvilli (Chapman 1998), and share characteristics of form and function with the villi of the vertebrate small intestine (Palay and Karlin 1959), in that these structures greatly enhance the surface area for which absorption and excretion may take place (Chapman 1998).

In most insects, the interior surface of the midgut is lined with extracellular secretions, secreted by cells at one end of the midgut epithelium. These secretions harden in laminar fashion, and form a membrane that is constantly regenerated every few hours (Chapman 1998). This layer of laminar, chitinous excretion is commonly referred to as the peritrophic membrane (henceforth referred to as the PM). In Diptera, the PM is formed in a belt-like manner at the anterior of the midgut and moves towards the posterior (Richards and Richards 1977), separating food from the epithelial layer of cells that make up the midgut (Chapman 1998). In adult Diptera, the PM is not formed until the insect takes a meal, and in the midgut of Culicidae, the creation of the membrane is only induced when the mosquito takes a blood meal (Chapman 1998). Parasites typically traverse the PM through their own secretion of chitinase, locally disrupting it so they may escape the midgut and move onward to the midgut epithelium, and then to the salivary glands (Ghosh et al. 2000).

The salivary glands also play a significant role in disease transmission—as seen in all three of the diseases described above. The infectious agent is injected into a vertebrate host with the saliva as the Diptaran takes a blood meal. Salivary glands are an integral component of the insect digestive system. Flanking either side of the esophagus, these glands are present within the thorax of the insect and are connected by tubes called salivary ducts to the head. These ultimately fuse into a common salivary duct toward the proboscis, which opens at the base of the hypopharynx (Dhar and Kumar 2003). In hematophagous Diptera, these glands are responsible for several tasks having to do with the uptake and digestion of a blood meal (Chapman 1998). Saliva facilitates the uptake of the blood meal, by releasing anti-

coagulants and anti-histamine compounds into the vertebrate host at the locality of the bite, ultimately suppressing the host's immune response.

In those Dipterans that transmit disease, the glands not only carry out transmission of the pathogen to the vertebrate host, but they also contain proteins (such as nitric oxide synthases and bactreriolytic lysozymes) that act in the immune response of the mosquito to minimize adverse effects to the mosquito after the first blood meal has been taken (Dhar and Kumar 2003). Before getting to the salivary glands, the pathogen must first traverse the barrier between the insect's hemocoel and layers of tissue that comprise the glands themselves. A study has shown (Sidjanski 1997) that pathogens, such as a sporozoite form of *Plasmodium falciparum*, recognize and bind specifically to proteins on the outer surface of the glands in *Anopheles stephensi* by means of the protozoans' own circumsporozoite proteins (1997), so the current thinking is that the process of traversing the barrier may be receptor-mediated. However, much still remains unknown about the process for recognition of the salivary gland epithelium by the protozoan (Sidjanski 1997, Dhar and Kumar 2003).

Traversing the barrier of the salivary gland may be the easier of the two barriers a disease agent must cross, in order to venture from the midgut to the salivary glands—considering the glands are not protected with the rigid lining of the PM. The PM in various members of Diptera are relatively robust, permeable only to molecules with a radius less than 4.5 nanometers—while in grasshoppers and larval Lepidoptera, molecules may be as big as 35 nm (Chapman 1998). To put this into perspective, the typical human red blood cells measure 6-8 micrometers in diameter, and are 2-3 micrometers thick ("Red Blood Cell" 2010). Meanwhile, arthropod-borne flaviviruses typically range from 40-60 nanometers in

diameter (“Flaviridae” 2006). According to one group of researchers, the PM has been found to not be the primary determinant of susceptibility to midgut traversing by the pathogen, though it likely impacts the number of individual pathogens that do the traversing and that ultimately get to the salivary glands (Shahabuddin et al, 1998).

Though there is still debate about the exact methods that pathogens use to traverse the major barrier of the midgut, a current hypothesis is that the pathogens are small enough to get around the PM and then embed themselves into midgut epithelial cells. Infected PM cells are eliminated by shedding, by the mosquito’s response to parasite invasion (Baton 2005). Those shed cells are then broken down by enzymes in the hemocoel, and the parasite escapes into that body cavity--where it is somewhat vulnerable to the immune response of the insect, but can indeed make its way to the salivary glands.

It is understood (Pimenta et al 1994 ; CDC 8, CDC 9) the salivary glands are the main site of introduction of parasites, be they protozoan, bacteria, or virus, from the vector into the human host, and as mentioned above, the arthropod midgut is a site for pathogen replication. Therefore, it may make sense to try combating transmission of disease in a genetic sense, through physical or physiological modification of these organs.

D. Making use of the Mosquito Midgut as a means of combatting disease transmission

At the present time, genetic strategies to manage vector organisms are being heavily researched. There exists a great interest within this field, especially given that vectors like mosquitoes are becoming increasingly resistant to various pesticides, and that vaccines still do not exist for dengue (Scott et al. 2002) or malaria ([NIAID 2009]).

As indicated above, in order to complete the necessary components of their life cycle in the mosquito, *Plasmodium sp*. and dengue virus must cross the epithelial layers of the mosquito midgut and salivary glands (Ghosh et al 2001 and Gomez-Machorro et al. 2004, respectively). If we were to consider driving an effector gene (that is, a gene interfering with pathogen transmission between the mosquito's organs) into a population, we would need to know the method of action of that gene, and its overall efficacy, as shown in laboratory trials.

Natural barriers associated with the insect's internal digestive system would be a target for exploitation by researchers in preventing pathogen transmission. The less than perfect action of these barriers give rise to the insect's competency as a vector, also called "vector competence" or "VC". VC is a measure of the ability of the virus to make use of the insect's digestive system—successfully infecting it, replicating within it, and escaping it through transmission to the next host (Bennett et al. 2005). Physical barriers, again, would include those barriers to pathogen movement out of the midgut, and barriers to movement into the salivary glands once the disease agent has traversed the midgut lining. Populations of *A. aegypti* (the vector for Dengue) have demonstrated genetic diversity for the permeability of these barriers, and in turn, have genetic diversity in their vector competence, exhibiting high or low resistance to infection, and exhibiting high or low dissemination of the virus (Bennett et al. 2002).

Before those physical barriers are encountered by pathogens, the pathogens must deal with naturally-occurring physiological barriers in the midgut. In one mosquito study, for example, a polymorphism in the expression of a serine protease was found to limit the ability of the mosquito to harbor protozoan parasites (Han et al. 1999). Proteases in the midgut of

the mosquito serve many roles—they can help destroy developing Plasmodium ookinetes, however, they also break down blood proteins from the vertebrate that would naturally attack the pathogen (Shahabuddin 1998).

Once pathogens like Plasmodium invade the epithelial layer of the midgut, they trigger the mosquito's antimicrobial response, during which a large number of antibacterial peptides, called Cecropins, are induced (Shahabuddin 1998). Specific genes are induced in *Anopheles gambiae* by a *Plasmodium berghei* infection 20 to 30 hours after an infectious blood meal, when the ookinetes invade the midgut epithelium. These genes included those coding for bacteria-binding proteins, protease homologs, and an anti-microbial peptide called defensin (Dimopolous et al. 1997, Riehle et al. 2003).

Much work along the anti-malarial front has involved genes that exploit the physiology of the midgut and salivary glands. Marelli and colleagues found that anopheline mosquitoes expressing a peptide named SM1 in the lumen of the midgut had difficulty transmitting *Plasmodium berghei,* and that expression of the peptide conferred an actual fitness advantage (Marelli et al 2007). The peptide, named SM1 for "salivary gland and midgut binding protein 1" (Ito et al. 2002) strongly inhibited the traversing of both the midgut and salivary gland epithelia by the Plasmodium parasites (Ghosh et al. 2001), suggesting strong evidence that Plasmodium development could be blocked if the SM1 peptide is produced and expressed in the lumen of the mosquito gut (Ito et al. 2002).

In the study by Marelli, et al., one group of transgenic *A. stephensi* mosquitoes expressing this protein was allowed to feed on uninfected mice, and another group of transgenic mosquitoes fed on mice infected with *P. berghi*. In the study, both transgenic and

wild-type mosquitoes had equal fitness when feeding on uninfected mice, but when feeding on infected mice, transgenic mosquitoes had significant fitness advantages over the wild-type in fecundity and viability and by nine generations, 70% of the mosquitoes in the population contained the transgenes (Marelli et al. 2007). There may be great worth in the future, in finding peptides to strengthen the epithelial layers of organs needed by parasites in order to complete their development.

Discovery and manipulation of genes that express peptides in the midgut, or finding genes that alter the physiology of the mosquito's digestive system, takes a large amount of effort. Once they have been discovered and delivered into a mosquito stock, researchers must confirm that the genes are being expressed, and they need to run several tests to understand how inheritance of the genes affects the organism's behavior, phenotype, and progeny (among other effects). However, without a proper mechanism for driving genes into a population, the construction of those transgenes may be of limited use. In the following section, I examine some potential strategies for pushing genes into populations.

II. GENE DRIVE MECHANISMS AND THEIR USE IN MODEL SYSTEMS

Gene drive, as the name implies, is a mechanism for pushing genes into a population where those genes previously did not exist. For example, if we wish to drive an anti-malarial ("effector") gene like the one that codes for SM1 into a population so that all anopheline mosquitoes have difficulty transmitting malaria, we would need to be able to get it to spread to a great majority of mosquitoes in a given population. Whereas my experiments address the Killer-Rescue strategy, which is discussed in further detail in section C below, there are other strategies that researchers may choose to employ. Most of these use "selfish" genetic elements that replicate themselves within the germline of the organism or have super Mendelian inheritance.

A. Selfish Genes

The predictable inheritance of genetic information, often based on observable phenotypes of an organism and its progeny, is usually defined as basic Mendelian inheritance (Campbell and Reece 2002). However, many genes exist that are not inherited in predictable, Mendelian fashion, and instead are able to be passed on to the next generation at a higher frequency than expected and can be said to be inherited in a "biased manner" (Burt and Trivers 2006). Most often, these genes are able to drive themselves to high frequency in populations through one of three tactics: self-overreplication, disruption of the transmission of other alleles, or preferential movement of genes toward the germline and away from somatic cells (Burt and Trivers 2006, p.4-5). In cases where researchers want to drive an anti-pathogen gene into a population, a mechanism must be developed to drive that gene into

almost every individual. "Selfish genes," as described by Burt and Trivers (2006) exhibit mechanisms needed for gene drive, and some are briefly discussed below.

B. A Brief Introduction to four Gene Drive Mechanisms

There exist several strategies for driving genes through populations; below I will describe four of the most straightforward techniques.

Transposons

Transposable elements (also called transposons or mobile genetic elements), are "the most widespread selfish element, the best studied, the most complex to master, and the element showing the most unusual form of drive" (Burt and Trivers 2006). Approximately half of the human genome is composed of remnants of mobile DNA (Craig 2002). Transposons compose approximately 15% of the genome of the Malaria mosquito, *Anopheles gambiae* (Holt 2002). Therefore, understanding the structure and biochemistry of these segments of DNA is of great importance. Among some of the best-studied transposons are the P-elements of *Drosophila melanogaster* (Burt and Trivers 2006), and the flies I used in my experiments had their transgenes inserted with the aid of such P-elements.

Transposons take part in "recombination reactions in which discrete segments of DNA are moved between nonhomologous sites" in the genome of an organism (Craig 2002). Due to their often random insertions within the genome, transposition of a transposon is expected to be associated with a fitness cost to individuals—especially in cases where transposons disrupt genes that impact fecundity or egg viability (Riehle et al. 2003). Some models, however, have predicted that transposons would be able to drive effector genes, even

in the presence of a fitness cost, provided the benefits of carrying the genes (such as decreasing the probability of infection or effectively decreasing parasite numbers in the mosquito) are high enough (Boete and Koella 2003).

Drive Towards Sex-Bias

Some selfish elements operate by hijacking sex chromosomes, such that a sex-skew occurs in the progeny of the individual possessing an affected chromosome (Burt and Trivers 2006). For instance, experiments with *Drosophila quinaria* and *D. recens* have shown that males that act as carriers for "Killer X" chromosomes sire completely female progeny (Jaenike 1996). It follows that, if enough generations were to take place with males inheriting this type of sex-skew, that entire species could be wiped out if no counter-balancing selection took place (Jaenike 1996). Fortunately for the organism, strategies such as polyandry (mating with several males such that sperm can compete) exist to counter-balance the sweep towards fixation of a population towards a single sex (Price et al. 2010).

A tool that is seeing greater usage to skew sex-ratio in experimental populations of a variety of insects is a group of endobacteria from the genus *Wolbachia* (Pfarr and Hoerauff 2006). This bacteria can alter sex-ratio of a population in several ways, including specific male-killing (bacteria kill embryonic males so that the bacterium has a better chance of being passed to the next generation), or feminization (causing genetic males to develop as females) (Burt and Trivers 2006). The bacteria manipulate the gametogenesis of its host, and therefore "selfishly" increase the occurrence of their own reproduction (Heath et al. 1999).

Cytoplasmic Incompatability

Wolbachia is best known for causing cytoplasmic incompatibility between the gametes of males infected with the bacteria, and of females that are uninfected (or infected with a different strain of *Wolbachia*) (Burt and Trivers 2006). Cytoplasmic incompatibility involves the "modification" of the male's sperm, and the "*rescue*" of female eggs when both male and female contain the same *Wolbachia* strain. In the cases where male and female have non-compatible strains, embryogenesis is disrupted (Werren 1997) and the fertilization event becomes effectively null. Some have estimated that anywhere between 20-80% of insect species are infected with some species of the bacteria (Pfarr and Hoerauff 2006), though other, more conserved estimates hover between 10 and 20% (Heath 1997, Werren 1997).

Wolbachia is maintained in a population of organisms via vertical transmission, (from mother to offspring), and can also be transmitted horizontally (from an infected individual to one uninfected, or, from one species of arthropod to another) (Heath et al. 1999). Population dynamics can be influenced by fecundity and viability of infected females relative to uninfected females, and by the proportion of infected eggs lain by infected females, indicative of the amount of vertical transmission in the population (Werren 1997). Ultimately, *Wolbachia* decrease the fitness of uninfected females, and this increases the relative fitness of infected females (Burt and Trivers 2006), and thereby infected males, given the degree of cytoplasmic incompatibility (and "cytotype") conferred by the bacteria (Werren 1997).

Maternal Effect and *Medea* elements

Another method for driving a gene through a population is to use selfish genetic elements called "*Medea*" elements ("*Medea*" being an acronym for Maternal Effect Dominant Embryonic Arrest). These elements cause the death of any progeny from a mother carrying the element, that do not receive a copy of the element themselves (Beeman 1992, Beeman and Friesen, 1999). For example, a female carrying a *Medea* element in homozygous condition that mates with a male that does not carry the gene at all, will have completely heterozygous offspring—all will inherit a copy of the selfish genetic element to survive. Meanwhile, if a heterozygous female carrying the element mates with male not carrying the element, half of their progeny will inherit a copy of the element, while the other half will not inherit it, and die as embryos.

Medea can quickly sweep through a population from low frequency to fixation—within less than 100 generations (Burt and Trivers 2006). However, despite any positive, population-wide benefits the *Medea* strategy may confer (such as higher resistance to infection by a pathogen), the strategy has the potential to backfire, since pathogens have a tendency to replicate and adapt quickly in potentially hostile cellular environments, wherein the components of the immune system attempt to attack the invading pathogen. The population of mosquitoes may succumb to a virus strain that successfully becomes resistant to the anti-pathogen effector gene once the *Medea* genes have swept to a high frequency within a population (Gould et al. 2008). If pathogens mutate and become resistant to the influences of the effector gene, the Medea strategy would lose its potential for driving an effector gene through a population.

C. The Killer-Rescue Gene Drive System

The gene drive strategy, called "Killer-Rescue," has recently been proposed (Gould et al. 2008) but has only been explored theoretically. In this system, two genes are used, in which one gene (the "Killer") would theoretically produce a fatal toxin, while the other (the "Rescue") would provide a life-sustaining antitoxin. Inherited alone, *Killer* would cause embryonic lethality, or termination of life at an early stage, depending on the specific type of *Killer* gene. When inherited with *Rescue*, the effect of the *Killer* would be negated, and the organism would live and continue to grow into a healthy, fertile adult. The goal of my graduate research has been to develop and test this Killer-Rescue system in a laboratory setting.

Ultimately, for driving an effector gene through a population, one would link the effector gene to the *Rescue* gene, such that it is inherited along with the gene that confers the fitness advantage of survival.

D. Expectations of Killer -*Rescue* frequencies

For researchers who want to release genetically-modified organisms into a wild population and increase the frequency of an anti-pathogen gene, the ability to predict the change in allele frequencies prior to release is of great importance. My experiments tested what actually happens as a result of insects with the Killer-Rescue system being released into "wild" populations.

However, before I started my experiments, models were created to simulate activity of these alleles under several different circumstances. The following is a brief synopsis of

what we expected going into the experiments and is largely derived from Gould et al., 2008.

The special utility of the Killer-Rescue system over a gene drive system like Medea is that the Killer-Rescue system should enable researchers to push an effector gene into a discrete population for a limited period of time, so that the stability of the effector gene could be tested in a real population. Ultimately, the goal is to engineer a strain of flies that exhibits a 5%-20% fitness cost to carrying the *Rescue* gene, such that, once the *Rescue* gene is no longer beneficial to the organism carrying it (i.e., when the killer gene removes itself from the population), the cost of keeping the *Rescue* gene outweighs the benefit, and eventually the gene falls out of the population. In some cases it could also be useful to have a cost associated with the *Killer* gene.

The chromosomes of a hypothetical insect with *Killer* and *Rescue* genes are depicted in Figure 1. In the diagrams, pairs of homologous chromosomes are represented, with colored blocks indicating the specific genes.

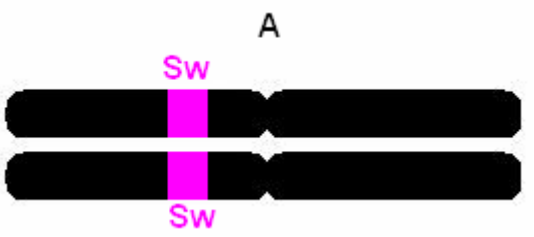

Figure 1: The first component of a successful Killer-Rescue system. All flies, both in the wild population, and those created with transgenes, contain a switch gene (Sw), which triggers lethality of the organism if activated. Under normal circumstances, this gene remains latent and unused, but has the potential to be activated—only then will it cause lethality. I will designate this as chromosome "A".

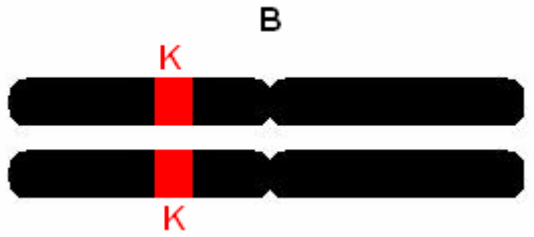

Figure 2: The second component of the system. We will designate this as our Killer (K) gene. It is the activator for the trigger gene—that is, without inhibition, inheriting Killer will activate the switch gene, causing death of the organism. I designate this pair of homologous chromosomes as "B."

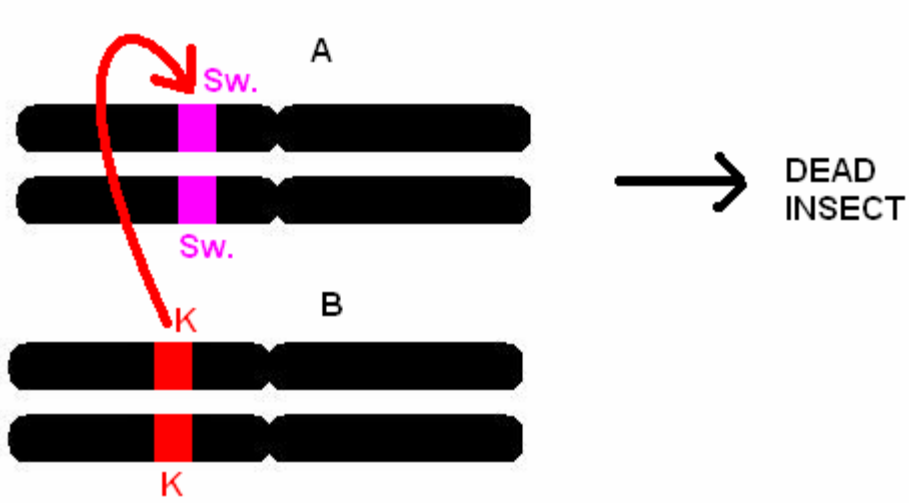

Figure 3: *Killer* gene activates the Switch gene without any inhibition. Lethality is triggered, and the insect is killed.

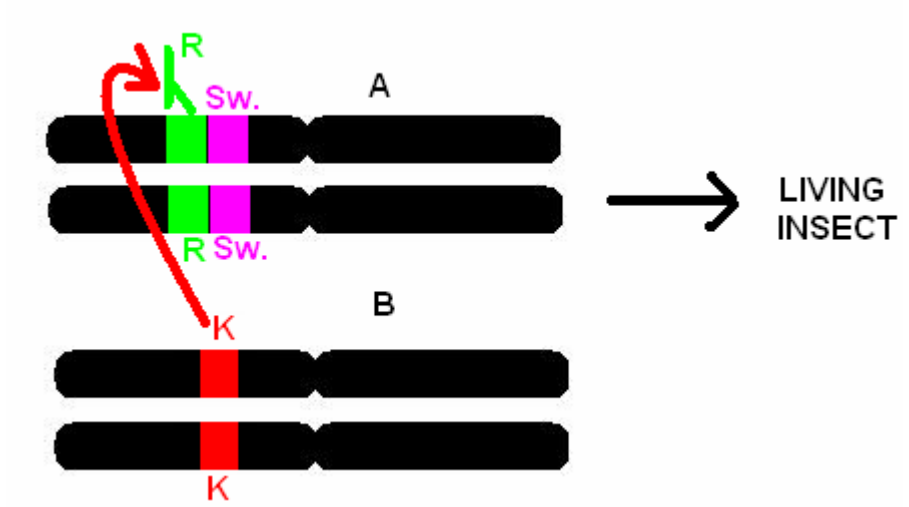

Figure 4: In the presence of the *Rescue* gene, *Killer* is blocked from activating the Switch gene. The organism continues to develop normally into a living insect. The rescue gene in our case is placed on the same chromosome as the Switch gene.

As shown in figures 1-4, the *Rescue* gene lies on a chromosome separate from that of the *Killer* gene. We denote the inheritance of *Killer* with an upper case K, and the inheritance of *Rescue* with an upper case R. Lower case k and r respectively indicate that the

Killer and *Rescue* genes are absent from a specific chromosome. Inheriting one copy of *Rescue* (R) is sufficient to overcome any lethal effects coded for by *Killer* (K), either in homozygous or heterozygous condition. However, if an individual was to inherit one or two copies of *Killer* without a single copy of *Rescue*, the individual would die. Possible genotypes of flies that survive are KKRR, KKRr, KkRR, KkRr, kkRR, kkRr and kkrr (the genotypes of flies expected to die are KKrr and Kkrr).

In order to appreciate the dynamics of the *Killer* and *Rescue* genes in a population, it is helpful to examine a series of Punnett squares. Here, assume that transgenic flies are released on a 1:1 ratio with wild-type flies.

In Figure 5, we see the first cross as an introduction of transgenic (KKRR) to wild-type (kkrr). Notice all progeny survive (as indicated in light green), since they all have a copy of the dominant *Rescue* allele. Figure 6, however, shows what results from a cross of those heterozygous progeny (KkRr x KkRr). Notice that three of every sixteen offspring die (as indicated in red), since they inherit no copy of the *Rescue* allele. In this cross, the wild-type genotype is produced at low frequency (indicated in light blue). Figures 7, 8, and 9 demonstrate different potential scenarios in which progeny of the heterozygous cross may mate with each other: Figure 7: KkRR x KkRr, Figure 8: KkRr x kkRr, Figure 9: kkRR x kkRr. (For practical purposes, all possible combinations are not given.)

	KR	KR	KR	KR
kr	KkRr	KkRr	KkRr	KkRr
kr	KkRr	KkRr	KkRr	KkRr
kr	KkRr	KkRr	KkRr	KkRr
kr	KkRr	KkRr	KkRr	KkRr

Figure 5: KKRR x kkrr. This represents the homozygous cross, when transgenic individuals are first released into the population of wild-type individuals. Assume a 1:1 release ratio. Living progeny are represented on a green field.

	KR	Kr	kR	kr
KR	KKRR	KKRr	KkRR	KkRr
Kr	KKRr	KKrr	KkRr	Kkrr
kR	KkRR	KkRr	kkRR	kkRr
kr	KkRr	Kkrr	kkRr	kkrr

Figure 6: KkRr x KkRr. This represents the heterozygous cross, when progeny from the cross above mate with each other. Living individuals are represented in green. Dead individuals are represented in red, wild-type in blue.

	KR	Kr	kR	kr
KR	KKRR	KKRr	KkRR	KkRr
KR	KKRR	KKRr	KkRR	KkRr
kR	KkRR	KkRr	kkRR	kkRR
kR	KkRR	KkRr	kkRR	kkRr

Figure 7: Sample scenario: KkRr x KkRR. All progeny inherit a copy of *rescue*, hence, all progeny live and are able to contribute to the next generation.

	KR	Kr	kR	kr
kR	KkRR	KkRr	kkRR	kkRr
kR	KkRR	KkRr	kkRR	kkRr
kr	KkRr	Kkrr	kkRr	kkrr
kr	KkRr	Kkrr	kkRr	kkrr

Figure 8: Sample scenario: KkRr x kkRr. Here is a scenario in which some individuals do not receive a copy of the *Rescue* allele, and therefore, they die.

	kR	kR	kR	kR
kR	kkRR	kkRR	kkRR	kkRR
kr	kkRr	kkRr	kkRr	kkRr
kR	kkRR	kkRR	kkRR	kkRR
kr	kkRr	kkRr	kkRr	kkRr

Figure 9: Sample scenario: kkRR x kkRr. Once again, all individuals receive a copy of the *Rescue* allele, and remain living.

A population genetics model developed by Gould et al. (2008) examined a variety of scenarios where transgenic flies homozygous for these *Killer* and *Rescue* genes are released into wild-type fly populations. On the next pages are some of those scenarios, reprinted with permission from the Royal Society, from Gould et al. 2008 (Material Copyright © 2008, Copied with permission from The Royal Society).

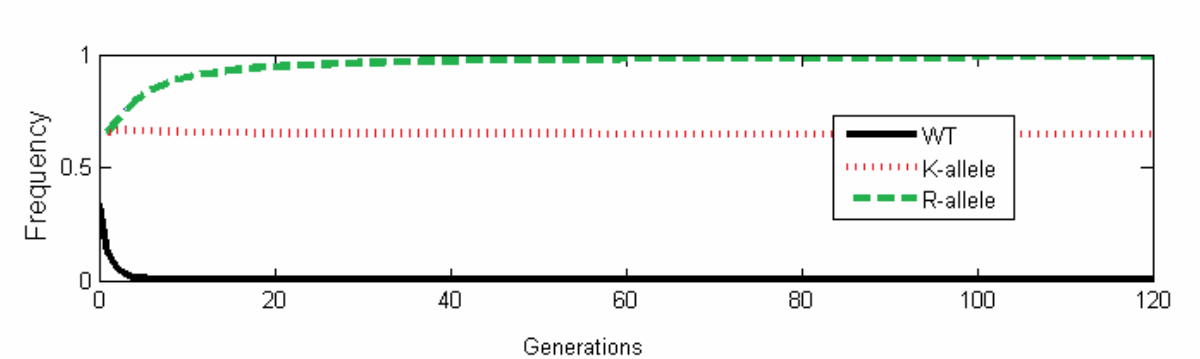

Figure 10A: One initial release of transgenic flies, at a ratio of 66.6% Transgenic to 33.3% Wild-Type. No fitness cost associated with carrying *Killer* or *Rescue*. (With permission from the Royal Society. From Gould et al. 2008. Copyright © 2008, The Royal Society).

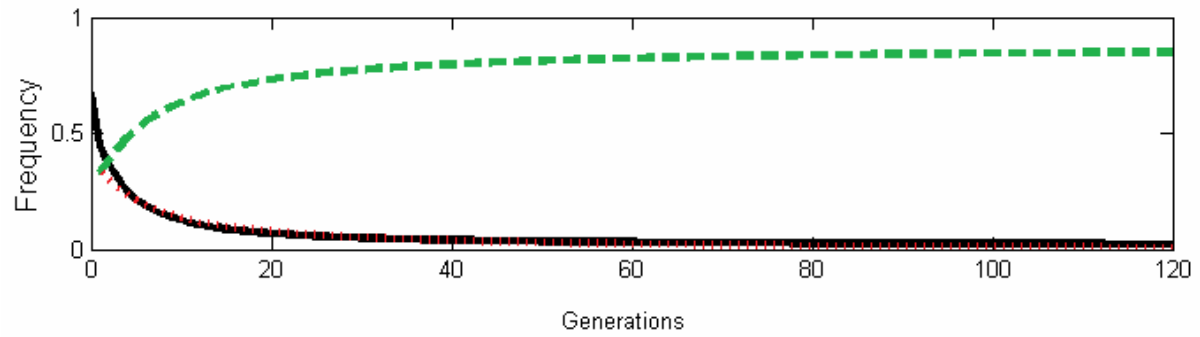

Figure 10B: One initial release of transgenic flies, at a ratio of 33.3% Transgenic to 66.6% Wild-Type. No fitness cost associated with carrying *Killer* or *Rescue*. (With permission from the Royal Society. From Gould et al. 2008. Copyright © 2008, The Royal Society).

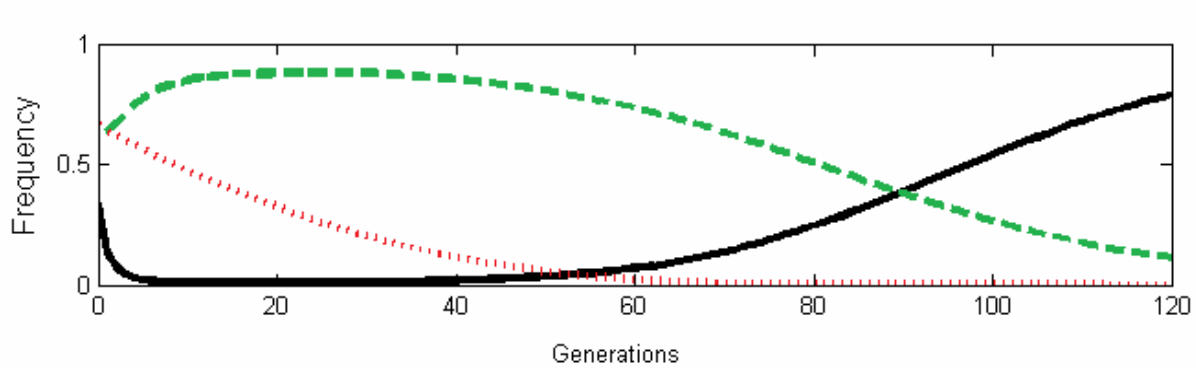

Figure 11A: One initial release of transgenic flies, at a ratio of 66.6% Transgenic to 33.3% Wild-Type. A 10% fitness cost is associated with carrying *Killer* and with carrying *Rescue*. (With permission from the Royal Society. From Gould et al. 2008. Copyright © 2008, The Royal Society).

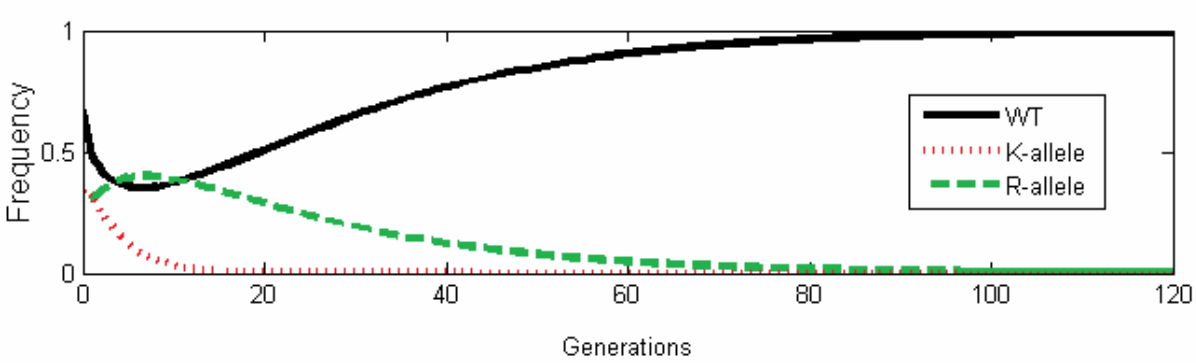

Figure 11B: One initial release of transgenic flies, at a ratio of 33.3% Transgenic to 66.6% Wild-Type. A 10% fitness cost is associated with carrying *Killer* and with carrying *Rescue*. (With permission from the Royal Society. From Gould et al. 2008. Copyright © 2008, The Royal Society).

In Figures 10A and 10B, scenarios are presented in which *Killer* and *Rescue* genes cause no decrease in the fitness of the insects carrying them (i.e. no fitness cost). In Figure 10A, transgenic individuals are released at a 2:1 ratio to wild-type individuals. The frequency of *Rescue* increases rapidly over the first twenty generations, and continues to increase in frequency until it reaches near fixation. Because there is no fitness cost associated with the *Rescue* gene, there is no selection to eliminate it from the population. While *Killer* is still present in the population, *Rescue* does confer a fitness benefit—in that the organism is not killed by the *Killer* allele if it has been inherited. When the *Rescue* gene (R) is at high frequency, the likelihood of inheriting an "r" decreases. This, along with the lack of a fitness cost to individuals due to the inactive *Killer* gene, results in the frequency of *Killer* very slowly decreasing.

Figure 10B indicates the expected response if transgenic individuals are released at a 1:2 ratio to wild-type flies. Again *Rescue* keeps insects alive that have inherited copies of *Killer* in the early generations after release, and this is sufficient that *Rescue* surges to a high frequency, though not to fixation within 120 generations. *Killer* is not able to maintain itself in the population, as a compounded effect of a release ratio that is one-fourth of the ratio presented in Figure 10A (meaning, far fewer flies inherit it), along with its inherent fitness disadvantage--killing itself out of the population—whereas flies with *Rescue* still have an advantage when *Killer* is present. The *Rescue* gene remains in the population at a stable frequency after *Killer* has been eliminated, because *Rescue* does not have a fitness cost in this scenario. Note that the wild-type takes a much longer time to reach a frequency near zero than it did in the scenario of Figure 10A.

As described briefly above, Gould and colleagues suggest the strategy for bringing the wild-type back, and for avoiding the most-feared outcome: the event that "a strong gene-drive mechanism spreads an anti-pathogen gene to high frequency over a wide area and then the pathogen rapidly adapted to the gene product" (2008). The idea: engineer the transgenic insects such that carrying *Killer* and *Rescue* have associated fitness costs. The fitness costs could come from many different scenarios. Perhaps the fertility of either males or females, or viability of eggs, is adversely affected, so the fitness of the wild-type is comparatively greater than that of transgenics. Thus, the wild-type flies can regain their original status at 100% frequency. The two scenarios presented in figures 10A and 10B, then, would not be ideal when releasing real strains of transgenic flies into a wild population for the first time—at least, not ideal in the sense that the wild-type cannot regain a frequency of 100%. In the following two scenarios, however, applying a cost to carrying the genes (via reduced fertility or viability, for example) is examined.

In Figure 11A, the model shows a release ratio at 2:1, transgenic to wild-type, but in this scenario, both *Killer* and *Rescue* have an associated fitness cost of 10%. Once again, *Rescue* proves beneficial at first, but here we see the *Rescue* gene frequency ultimately falls, due to a higher fitness cost than benefit once *Killer* has reached a frequency nearing zero. Notice, too, that the frequency of the *Killer* allele falls almost instantly, due to the compounding costs of 1) killing off individuals that inherit it without a copy of *Rescue*, and 2) the fitness cost of carrying the *Killer* gene.

Finally, in Figure 11B, the release ratio is low so that not as many wild-type individuals mate with transgenic individuals (as in the scenario of Figure 10B), and those that

inherit a copy of *Rescue* experience an immediate fitness advantage over the wild-type, in that they are being "rescued" from death if they also inherited one or two copies of *Killer*. However, when the transgenes are released at such a low frequency (compounded with the 10% cost associated with carrying both alleles), they fail to establish themselves at a high enough frequency in the population. This results in a much quicker return of the wild-type to 100% frequency in the population.

It should be noted that molecular geneticists expect that most insect strains with an added transgene will have a fitness cost, so this is not a difficult outcome to achieve. Whereas a fitness disadvantage among released individuals would have been harmful to a sterile male release program, a fitness disadvantage to carrying transgenes in a Killer-Rescue gene drive system could be helpful in the long run, providing that it could enable wild-type individuals (those lacking transgenes) to have better fitness thereby returning the native population to a high frequency (Gould et al. 2008), should scenarios adversely circumnavigating the gene-drive system (such as pathogen resistance through mutation) occur. Indeed, it may be ideal for the population to re-establish itself at 100% wild-type.

Dynamics of the *Killer* and *Rescue* genes as represented in time series graphs from Gould et al. (2008) are important to consider before releasing transgenic mosquitoes into an area. However, these models have very simple assumptions about the biology of the insect, so it is also important to test the dynamics of the *Killer* and *Rescue* genes in a real insect system. Here we turn to *Drosophila melanogaster*, to test whether frequencies of *Killer* and *Rescue* in a transgenic fly laboratory system reflect predictions made by the models.

CHAPTER TWO: BUILDING A *DROSOPHILA* STRAIN WITH KILLER-RESCUE PROPERTIES.

The major goal of my research was to determine if a Killer-Rescue system composed of GAL4, GAL80, and disco-M would have dynamics similar to those predicted by mathematical models (Gould et al. 2008). However, before this could be done, I needed to construct strains containing all three of these genes in homozygous form. In this chapter, I describe the creation of such a transgenic *Drosophila melanogaster* strain.

A. Background Information

A variety of strategies exist for driving certain desired genes through a population of organisms, as mentioned in Chapter One. In my population-level experiment (Chapter Three), the goal was to test the efficacy of the gene drive system, called "The Killer-Rescue Gene Drive System." As mentioned in Chapter One, our system works such that, if an individual inherits a *Killer* (K) gene without a *Rescue* (R) gene, that individual will experience embryonic lethality. However, if the individual inherits a *Rescue* gene, that *Rescue* gene will block the activation of the embryonic lethality triggered by the *Killer* gene, and the individual will survive.

This strategy has great potential for use in the future for two main reasons. First and most importantly, it is believed that this system can be employed against fly-borne diseases, such as those described in the preceding chapter (Sinkins and Gould 2006). An anti-pathogen ("effector") gene construct may be created, and ideally linked to the Rescue construct. In that situation, flies (mosquitoes) not inheriting the Rescue /anti-pathogen construct will die; flies that inherit *Rescue* will not only live, but would also inherit a copy of

the anti-pathogen gene. This anti-pathogen gene may, for example, interfere with the physical transmission of the virus from the midgut of the insect to its salivary glands as described in Chapter One. If the salivary glands do not receive the virus, the mosquito's next human host would not become infected.

Secondly, we hypothesized this strategy would likely be useful in that the *Killer* and *Rescue* alleles will likely bestow some fitness cost to the fly strain carrying them (Gould et al. 2008). That way, the frequency of the transgenes would be expected to fall to very low levels in the population within a predictable number of generations. Ultimately, if there is a fitness cost associated with carrying *Rescue* (but death occurs when inheriting only *Killer*), only flies carrying R survive—until the fitness cost of carrying R is greater than the fitness benefit of suppressing K with R—at which point R is lost from the population, and the population returns its original status, in which all individuals contain the wild-type genotype. This is important, not only experimentally (if a genetic mistake occurs, the population can recover) but also politically, since mosquitoes do not respect arbitrary country borders, and asking several bordering countries to agree to host trial releases with transgenic organisms may prove difficult (Gould et al. 2008).

As all scientific procedures begin with preliminary testing, I focused my efforts upon the fruit fly—*Drosophila melanogaster*. Though *Drosophila* does not carry any human pathogens, I could still create a strain of flies that contained the basic Killer -Rescue System (lacking any anti-pathogen construct). Once I had obtained these flies, my next goal was to observe the frequencies of the wild-type phenotype for 15 generations, as the transgenes

moved through the population. I then compared these frequencies to those predicted (Gould et al. 2008).

B. GAL4 and GAL80 in the *Drosophila melanogaster* System

Drosophila melanogaster is a key model organism for understanding gene mechanics and functions, both in insects and, due to homology of many genes, also in vertebrates. It exhibits all the characteristics of good model organisms, such as large numbers of offspring, short generation time, and relative ease of rearing in mass quantity (Rubin and Lewis 2000, Schneider 2000).

To simulate a killer/rescue system in Drosophila, I took advantage of the GAL4/UAS system (Brand and Perrimon 1993, Duffy 2002). All flies that were used contained the UAS-discoM construct (Robertson et al 2002), which has the open reading frame of the *Drosophila disconnected* (*disco*) gene downstream of the upstream activation sequence. GAL4 is a transcription factor that binds to this UAS sequence to activate genes (Duffy 2002). In my case, this would lead to ectopic expression of *disco*, which is known to cause embryonic lethality by interfering with normal development (Robertson et al. 2002). I used the armGAL4 transgene to produce ubiquitous GAL4 expression. This transgene has a portion of the *armadillo* (*arm*) regulatory region driving expression of yeast GAL4 in a fairly ubiquitous manner throughout the fly embryo. Activation of *disco* by this driver has previously been shown to cause embryonic lethality (Robertson et al. 2002). In this respect, armGAL4 represents the "Killer" gene and UAS-*disco* the Switch gene. The final component of the system is tub-GAL80, a GAL80 open reading frame driven by the *Drosophila tubulin* promoter. In yeast, GAL80 is a repressor that keeps the galactose metabolic genes inactive

when other sugars are available. The GAL80 protein also binds to the UAS, blocking binding of GAL4 and thereby activation of galactose metabolizing genes. Therefore, in the Killer/Rescue model, GAL80 prevents activation of the UAS-*disco* construct, so functions as the Rescue component.

Origin of GAL4 and GAL80

GAL4 and GAL80 are genes from the galactose metabolism pathway of the yeast, *Saccharomyces cerevesiae* (Melcher and Xu 2001). In the yeast, GAL4 regulates the genes GAL10 and GAL1 (responsible for transporting galactose into the cell and beginning to break it down into simpler sugars) (Lohr et al. 1995). It does so by directly binding to an Upstream Activation Sequence (UAS) that activates the transcription of GAL1 and GAL10, (Giniger et al 1985). Meanwhile, GAL80 functions as a negative regulator, binding to the same UAS sequence when the yeast can only uptake glycerol or glucose as its food source. GAL80 inhibition of GAL4 is relaxed when the yeast takes up galactose once again as its source of sugar nutrition (Lohr et al. 1995). These genes have been introduced into *Drosophila* as a mechanism of controlling spatial and temporal expression of transgenes (Brand and Perrimon 1993, Lee and Lou 2001) through P-element transformation (Spradling 1986).

C. Materials

Initial Fly Stocks used to develop the Killer-Rescue strain are described below in Table 1. In this table, I indicate first the official name of the stock, then the notation given by Flybase at the Bloomington Stock Center At Indiana University, or the reference from which the flies were obtained. Next, I give a brief description of both genotype and phenotype, as

they pertain to my experiments. Finally, I describe how I will denote them, in a simple form, henceforth in this paper.

Table 1: Initial fly stocks used in creation of the Killer-Rescue Flies

Stock Name, Role in Construction	Flybase at Indiana University number, or Reference	Phenotype	Notation Henceforth (II ; III)
▪ UAS-disco-M (III) (Homozygous, Wild-Type Stock)	(Robertson et al. 2004)	Mottled ommatidia, white background in eyes. Yellow body.	$\frac{+}{+}$; $\frac{UAS\ +}{UAS\ +}$
▪ GAL80 (III) (Homozygous, Rescue Stock)	Flybase ID= FBst0005135 y[1] w[*]; P{ry[+t7.2]=neoFRT}82 B P{w[+mC]=tubP-GAL80}LL3	Brick-Red eye color. Yellow body.	$\frac{+}{+}$; $\frac{+\ Gal80}{+\ Gal80}$
▪ arm-GAL4 (II) (Homozygous, Killer stock)	W. McGinnis, University of California at San Diego (Robertson et al. 2002)	Orange eye color. Yellow body.	$\frac{armGal4}{armGal4}$; $\frac{+\ +}{+\ +}$
▪ TM3/TM6B (III) (Balancer Stock)	Flybase ID= FBst0003720 y[1] w[*]; TM3, Sb[1]/TM6B, Tb[+]	White eye color. Ebony body, Tubby, stubble bristles on dorsum, extra humeral bristles.	$\frac{+}{+}$; $\frac{TM3}{TM6B}$

That final notation represents Chromosomes II and III of the fruit fly, with a "+" indicating the absence of a gene. Genes on separate chromosomes are separated both with a semicolon, and are not underlined together. Two genes on the same chromosome are separated by a long space, but they share an underline.

For example, a fly with the notation $\frac{A}{A} ; \frac{B\ C}{+\ +}$ is homozygous for gene A on Chromosome II, but is heterozygous for both genes B and C on Chromosome III. GAL4 is always on Chromosome II, while UAS and GAL80 are always on Chromosome III. I will continue to use this notation when describing the order of the crosses I used, to produce my line of transgenic flies.

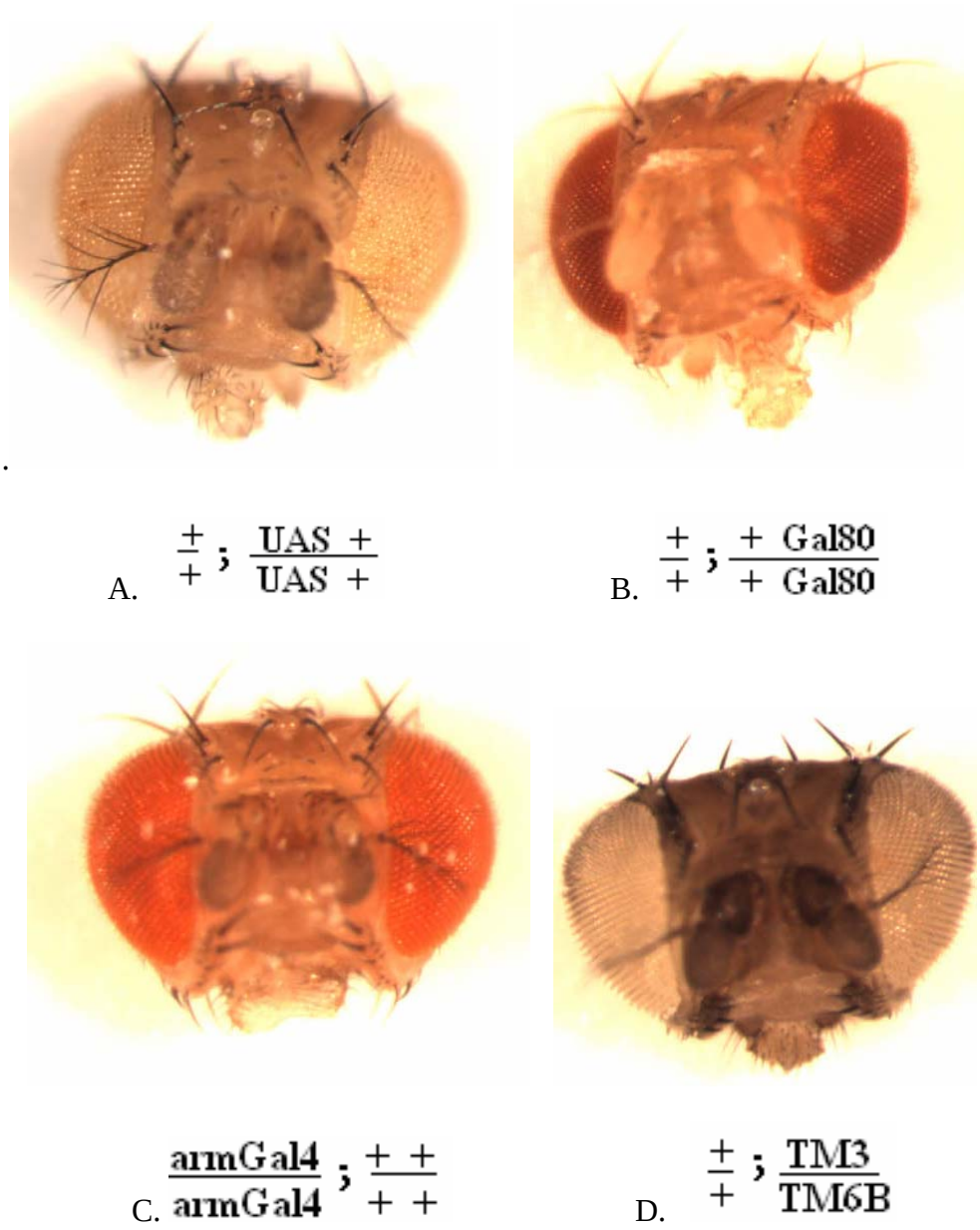

Figure 12: Eye phenotypes in *Drosophila melanogaster* stocks used to create the Killer-Rescue flies. A) UAS Disco-M displaying white background with mottling of ommatidia pigmentation. B) GAL80 fly, with brick red eye color. C) armGAL4 fly, with orange eye color. D) TM3/TM6B fly, with white eye color and an ebony cuticle.

Photos made using Qcapture MicroPublisher 5.0 and Qcapture software (© 2006 QImaging) using a Leica CamZ 16F Stereoscope at 12.5x Magnification.

Marker genes called “mini-whites” were present in the P-elements that had been transformed into the Drosophila lines upon creation of the stocks listed above. These markers are often used to help researchers distinguish phenotypically between flies based on amount of pigmentation in the eyes, with a positive relationship between the number of mini-whites and the number of transgenes. Sun and colleagues describe mini-whites as reporter genes that express uniform pigmentation in the developing *Drosophila* eye, and note that mini-whites are helpful in identifying genes involved in forming imaginal patterns (Sun et al 1995).

The eye colors of GAL4 (*Killer*) and GAL80 (*Rescue*) flies were chosen in our in *D. melanogaster* gene drive system, such that eye color could be used to score individuals possessing the Killer and *Rescue* genes in generations beyond F_1.

UAS disco-M served as our “wild-type” fly stock, (only called such because of its lack of transgenes linked to an eye-pigment coding “miniwhite” gene—real wild-type Drosophilds are red-eyed). This wild-type fly possessed a white-eyed phenotype, wherein relatively few, and random, ommatidia contain red pigment cells (Figure 12A). This gives the eyes a “mottled” appearance. The name of this stock, UAS disco-M, was named such as it is an Upstream Activation Sequence of the *disconnected* gene, which is a gene responsible for ventral appendage development (Patel et al. 2007), including maxillae and legs (Dey et al. 2009). Ubiquitous, ectopic expression of Disco is lethal (Patel et al. 2007)—so when Disco is activated in regions of the developing fly where it is not typically needed in development, lethality is induced in the fly’s embryonic stage. We tested and confirmed this hypothesis in crosses to arm-GAL4, making use of the UAS/GAL4 system (Brand and Perrimon 1993).

D. Methods: Creation of the Killer-Rescue Flies

The following presents a series of crosses used to obtain the ultimate goal: flies possessing both *Killer* and *Rescue* in homozygous condition. For each mating, all females used were virgin female flies. Before creating the Killer-Rescue flies, we conducted a test to make sure the *Killer* actually functioned as a "Killer," when mating armGAL4 stock flies to Disco-M stock flies. As noted in further detail below, *Killer* did work as expected, and killed flies in their embryonic stage of life.

In order to create the flies, we divided the overall process into three main goals:

Goal 1: Create a stock of flies homozygous for UAS and for Gal-80 on Chromosome III.

Goal 2: Create a stock of flies homozygous for Gal-4 on Chromosome II, and get two balancers in place on the third chromosome (TM3 and TM6B).

Goal 3/Ultimate Goal: Conduct crosses between flies from Goal 1 and Goal 2 in a manner that results in flies homozygous for all three genes: Gal-4, Gal-80, and the UAS.

Procedure for Goal 1 flies

In Step 1, we combined two homozygous starting lines of flies to create progeny which were all heterozygous for GAL80 and for the UAS. All possible progeny would be heterozygous.

Step 1. Mate: $\frac{+}{+}$; $\frac{UAS \quad +}{+ \quad UAS \quad +}$ x $\frac{+}{+}$; $\frac{+ \quad GAL80}{+ \quad GAL80}$

Progeny: $\frac{\mathbf{+}}{\mathbf{+}}$ **;** $\frac{\mathbf{UAS \quad +}}{\mathbf{+ \quad GAL80}}$

In Step 2, we began the process toward keeping disco-M and GAL80 on the chromosomes they were already on, but preventing recombination events with the addition of a balancer. Balancers prevent recombination due to their nested, inverted repetitions of DNA within the chromosome ("Balancer" 2010). The possible progeny here could be either white-eyed or red-eyed. If individuals had white eyes, they were discarded because it indicated the absence of GAL80. Darker red eyes indicated the greater likelihood that GAL80 would be present with UAS, lighter red eyes would indicate that Gal80 was not present with the UAS—so those with lighter red eyes were discarded. All flies collected would be those exhibiting the stubble phenotype, indicating that TM3 was the balancer that Gal80 and disco-M were paired with—and is easier to distinguish at a quick glance than humeral bristles (the visual marker for TM6B) are.

Step 2: Mate: + ; UAS discoM + (females) x + ; TM3 (males)
+ + GAL80 + TM6B

Progeny: **+ ; UAS GAL80 (Collect these! Darker red eye color.)**
+ TM3 (Stubble-bristles. Discard long bristles.)

+ ; + + (white eyes—discard)
+ TM3 or TM6

+ ; + GAL80 or + ; UASdiscoM + (lighter red
+ TM3 or TM6B + TM3 or TM6B eyes—discard)

For Step 3, flies collected from step 2 were backcrossed to TM3/TM6B flies, to make sure that progeny produced conveyed the phenotypes we had predicted (the possibilities are shown below). Indeed, the predicted phenotypes occurred, so we could continue to select

flies heterozygous for UAS and GAL80 that were paired with a TM3 balancer, via the stubble bristles.

Step 3: Mate: + ; UAS GAL80 / + TM3 x + ; TM3 / + TM6B

Progeny:

+ ; UAS GAL80 / + TM3 → Retain (stubble bristles—males and females)

+ ; UAS GAL80 / + TM6B → Discard (long bristles)

+ ; TM3 / + TM6B → Discard (White eyes, Two balancers)

In step 4, We mated the selected progeny together. This would allow us to create only three possible offspring types (indicated below), one of which would automatically die, due to the lethality of having two of the same balancer on one chromosome. The flies with stubble bristles obviously still retained the TM3 balancer. However, flies exhibiting long bristles were likely homozygous for both GAL80 and UAS, and flies of that genotype were the ones we sought for Goal 1. From these, we created a stock, such that we knew they would continue to breed with each other and produce fertile, viable offspring.

Step 4: Mate: + ; UAS GAL80 / + TM3 x + ; UAS GAL80 / + TM3

Progeny:

+ ; UAS GAL80 / + UAS GAL80 →Retain! This is Goal 1. Mate these together and Goal 1 Stock is finished. (Long bristles!)

+ ; UAS GAL80 / + TM3 →Discard (stubble bristles)

+ ; TM3 / + TM3 → Automatically dies.

Steps 5-6: Testcross and PCR

At this point, it was important to check to see if we truly had both genes accounted for. In order to make sure GAL80 is working, I mated pairs of Goal 1 individuals (once the stock was doing well and producing lots of progeny) to some arm-GAL4 individuals (making sure to carry out reciprocal male: female and female: male crosses). If GAL80 was not present, GAL4 would have activated the UAS gene (assuming it was there) and killed off all the new embryos in their eggs. This did not happen—the flies lived.

Since they lived, that meant one of two things—Gal 80 was working, or the UAS was not present. To test for the presence of the UAS, we performed PCR. The pUAST primers we used were as follows: 5'-CACCACAGAAGTAAGGTTCCT-3'for the Forward Primer, and for the Reverse Primer, 5'-CTGCAACTACTGAAATCTGCC-3'. The PCR protocol used was in accordance with the instructions provided by QIAGEN's DNeasy® Blood & Tissue handbook, p. 1-19 (reagents) and p. 28-30: Spin Column Protocol for Animal Tissues.

PCR confirmed that the UAS disco gene was present, and Goal 1 had been achieved.

Procedure for Goal 2 flies

Step 7 begins much like Step 1 did in creating Goal 1. Here we started with a stock homozygous for GAL4 and mated it with the balancer stock, creating flies heterozygous for Gal4 and containing one of the two possible balancer genes. In this case, however, we retained both phenotypes so we could mate them together and produce our desired Goal 2 progeny: flies with both balancers and homozygous for GAL4.

Step 7: Mate: GAL4 ; + x +; TM3
GAL4 + + TM6B

Progeny: **GAL4 ; TM3**
+ +

GAL4 ; TM6B
+ +

In Step 8, the two phenotypes from above were mated to each other. This would produce twelve genotypically different progeny types, but only one progeny type was truly sought—and that was the Goal 2 genotype, containing both balancers and homozygosity for GAL4. The flies we wanted would have orange eyes, and since both balancers were present, the flies would have ebony bodies, stubble dorsal bristles, and extra humeral bristles—so we could eliminate progeny types based on their lack of those desired phenotypes. However, our desired progeny type was indistinguishable phenotypically from one other progeny type, so both types were collected.

Step 8: Mate: GAL4 ; + x + TM6B
+ TM3 GAL4 ; +

Progeny Possibilities: (12 total):

GAL4 ; TM3 → **Desired! This is the Goal 2 Genotype. (Collect.)**
GAL4 TM6B

GAL4 ; TM3 →Indistinguishable from above (Collect also.)
+ TM6B

Progeny Possibilities (cont'd)—and the traits that distinguish them;

all but one of the following are yellow-bodied flies.:

Genotype	Trait 1	Trait 2
GAL4; + GAL4 +	Long Bristles	Normal Humeral
GAL4; + + +	Long Bristles	Normal Humeral
+; + + +	Long Bristles	White Eyes
+; TM3 +; TM6B	Ebony Body	White Eyes
GAL4; TM3 GAL4; +	Stubble Bristles	Normal Humeral
GAL4; TM3 + +	Stubble Bristles	Normal Humeral
GAL4; TM6B + +	Long Bristles	Extra Humeral
GAL4; TM6B GAL4 +	Long Bristles	Extra Humeral
+ ; + + TM3	Stubble Bristles	White Eyes
+ ; + + TM6B	White Eyes	Normal Humeral

Step 9: Once the two indistinguishable progeny genotypes described in the above paragraph have been collected, 70 single-pair matings were carried out between males and females from the same vials. I wanted a large number because it is predicted from simple population

genetics that approximately ¼ of my randomly-paired flies would prove to be crosses between flies that were homozygous for GAL4 (see below).

In order to confirm homozygosity of flies from a specific cross, the 70 single-pair lines were each allowed to continuously breed in their own vials for three generations. If any white-eyed flies emerged in succeeding generations, we would know that at least one of the pair-mated flies was a heterozygote, as follows:

1. Pair-mates are both homozygotes: Only red-eyed, homozygous GAL4 flies will ever be produced. (This is what we wanted. Their progeny was collected and a stock vial was started.)
2. One mate is a heterozygote: The F_1 Generation will not reveal any white-eyed flies, but the F_2 Generation and succeeding generations should. These were discarded.
3. Both flies within the pair are heterozygotes: White-eyed flies will be visible in the F_1 Generation. These were discarded.

After two generations, there remained 21 vials from the original 70 that had survived to the F_2 generation and had not given rise to any white-eyed progeny. After three generations, there remained only three strains that reached the F_3 adult stage and did not have any white-eyed individuals. These were the true-breeding homozygous lines—and only one of those three had the armGAL4 in its lineage, while the other two were from a GAL4 lineage with a darker eye color, which we decided not to pursue, with the anticipation that armGAL4 would be better for examining eye color in later generations in the population cages.

At this point, these true-breeding strains become stocks, and **Goal 2 had been achieved**.

Step 9: Mate:

GAL4 ; TM3	x	GAL4; TM3
? TM6B		GAL4; TM6B

→Select vials of flies that do not produce white-eyed flies over three generations

Goal 2: **GAL4 ; TM3**
GAL4 TM6B

Procedure for Goal 3 Flies

Once again, we begin the process for obtaining the Goal 3 "ultimate goal" flies by crossing two established stocks in Step 10—but this time, I had created both of those stocks. Here again I select for stubble-bristled flies, as they are easy to quickly score to use in the next crosses.

Step 10: Mate:

GAL4 ; TM3	x	+; UAS GAL80
GAL4 TM6B		+ UAS GAL80

Progeny: **GAL4 ; UAS GAL80 → Stubble bristles: Retain.**
+ TM3

GAL4 ; UAS GAL80 → Long bristles: discard.
\+ TM6B

For Step 11, I back-crossed the stubble-bristled progeny to the Goal 2 stock, which would produce five possible genotypes—two would have long dorsal bristles and would be discarded due to our need for the stubble visual marker in upcoming crosses—two more genotypes, both having stubble bristles, would be phenotypically indistinguishable from each other (so we kept them both), and the final type was an ebony fly, which we would not need anymore.

Step 11: Mate: GAL4 ; UAS GAL80 / + TM3 x GAL4; TM3 / GAL4 TM6B

Progeny:

GAL4; UAS GAL80 / GAL4 TM3 (or TM6B) → **select for stubble (discard TM6Bs)**

GAL4; UAS GAL80 / + TM3 (or TM6B) → **select for stubble (discard TM6Bs)**

GAL4; TM3 / GAL4; TM6B → discard (ebony)

Here again, I faced a scenario where it was impossible to distinguish the two retained genotypes, because eye color of GAL4 heterozygotes and homozygotes was similar. Of the selected progeny in the above step, males were mated twice, as follows: (females cannot be mated twice since they retain sperm from each male they mate with; once we confirmed the identity of the male, we could mate him to a female with an understanding of what his progeny should look like.)

We first mated the males to TM3/TM6 white-eyed flies. If any of his progeny were white-eyed, we'd know he was a heterozygote, and was not useful in contributing offspring toward Goal 3. He and all his progeny would be discarded. Since the flies do not have a long life span, the male was extracted for his second mating when his offspring had hatched and started grazing.

The second mating was to a Goal 2 female (homozygous for GAL4 and having both TM3 and TM6B), so we would know her phenotype for sure and create predictable progeny without wasting females that arose from Step 11—all progeny would be homozygous for GAL4, while being heterozygous for both GAL80 and UAS.

Step 12A: Mate Male GAL4; UAS GAL80 x (female) +; TM3
(?) TM3 + TM6B

Step 12 B: Mate Male GAL4; UAS GAL80 x (female) GAL4; TM3
(?) TM3 GAL4 TM6B

Progeny (if male is homozygote): **GAL4; UAS GAL80**
GAL4 TM3 (or TM6B) →retain stubble flies, discard long-bristled flies

Finally, in step 13, the stubble-bristled progeny from step 12 were crossed together. If the offspring had inherited two sets of TM3, they would have died; any stubble progeny was discarded. Progeny arising with long dorsal bristles were those of the Goal 3 genotype, exhibiting homozygosity for each of the three genes we desired.

Step 13: Mate: GAL4; UAS GAL80 x GAL4; UAS GAL80
GAL4 TM3 GAL4 TM3

Progeny: **GAL4 ; UAS GAL80 → GOAL 3/ULTIMATE GOAL**
GAL4 UAS GAL80 Make a stock of these.

(These had long thoracic bristles. I mated them together to create a stock population of transgenic flies.) These are the flies that may be referred to henceforth as the "transgenic strain" in my analysis of the population dynamics in my laboratory-created Killer -*Rescue* System.

Once again, the "Wild-type" strain was a regular strain only containing the UAS, such that, if the *Killer* gene was inherited without *Rescue*, UAS was activated and the organism died as an embryo.

Fertility and Viability Assays

As important, precautionary measures, before beginning the large-scale population cages, I wanted to make certain that my Killer-Rescue transgenic flies were indeed homozygous for both *Killer* and *Rescue*—and also I wanted to make sure they were fertile and would produce viable offspring on their own before trying to mate them with flies of the wild-type genotype. We conducted a variety of trials to comprehend the qualities of the transgenic flies.

Methods: Testing the transgenic flies

First, I conducted some simple, qualitative "sight" tests—in which I examined the wild-type stock vials for approximate number of adults and amount of larval activity, and then examined the stock vials for my transgenic lines. Through basic surveying of the stocks, I concluded that the transgenics had qualitatively the same large number of adults (around 50 individuals) and the same type of larval activity (approximately equal numbers of larvae, of similar proportions of each instar, eating the substrate).

Second, I set up some viability tests to count the approximate number of larvae that hatched from eggs produced by both the wild-type females and the transgenic females. For three consecutive days, we set up cups for egg collection. These are plastic, see-through cups with the bottoms cut off and replaced with Petri dishes containing grape agar; the tops are specially-constructed heavy-weight paper and create a tight seal so the flies cannot escape. I selected twenty non-virgin, transgenic females to lay eggs in one cup, and twenty non-virgin, wild-type females to lay eggs in a separate cup, and allowed them to lay eggs for approximately six to eight hours. I then collected 200 eggs from each cup and placed those

eggs on new grape plates, in piles of ten, for ease of counting, and allowed them 24 hours to hatch. I then counted the number hatched and the number that did not hatch. Handling eggs can be a delicate and sometimes difficult procedure, and that some error may occur from damage incurred by the egg if handled incorrectly. On average, 94.6% of transgenics hatched from their eggs, and 92.6% of wild-types hatched. In a Chi-squared test, using the number of wild-type flies as the expected number, it was determined that egg hatch rates in this test were not significantly different.

I conducted a fertility test to examine how many eggs females were laying in both the wild-type and transgenic stocks. Female disco-M flies were mated with male disco-M flies for three days, then put into cups with grape agar to deposit eggs. Female flies from Goal 3 were mated with Goal 3 males for three days as well, and also put into cups with grape agar for laying eggs. Over the course of 24 hours, females from the disco-M cup laid 134 eggs (88.1% had hatched within 48 hours), while females from the Goal 3 cup laid 142 eggs (88.73% hatched within 48 hours). One Goal 3 female died in the next day, but I allowed all remaining females to lay eggs for a total of four days. After that female had died, the amount of eggs laid by Goal 3 flies was approximately 2/3 that of what the disco-M flies had lain. At 48 hours, the three disco-M females laid 128 eggs, while the two Goal 3 females laid 81 eggs, and at 72 hours, the disco-M females laid 139 eggs and the Goal 3 females laid 76 eggs. Indeed, it appears egg-laying rates are very similar. A Chi-squared test was also used here to determine if egg-laying rates were significantly different. Again, the number of eggs laid by wild-type females was used as the expected number. Since one Goal 3 female had died, the expected number for 48 hours and 72 hours was 2/3 that of the average number of

Disco-M eggs over the course of three days. Again, it was determined that the number of eggs laid by transgenics was not significantly different from those laid by the wild-type.

Over the course of those four days, average hatch rates were 89.545% for disco-M flies, and 95.035% for Goal 3 flies.

Killer -Rescue Assays

Finally, and perhaps most importantly, we to conducted tests to demonstrate that our Killer could carry out its job, and that our transgenic flies did not immediately kill the wild-type flies when they mated to each other.

To ensure that our stock homozygous only for the *Killer* allele would truly kill flies of the wild-type stock, individual virgin female wild-type (UAS disco-M) flies were mated in single-pair fashion to Killer (armGAL4) flies, on three different trial occasions. In each of these trials, there were at least three vials, each of which contained a single mating pair. Approximately 30-50 eggs were laid by each female, and in all but one case, all the eggs turned brown and did not develop. In only one vial, three larvae emerged but none lived long enough to pupate.

I also set up four vials in which I pair-mated Goal 3 male transgenics to virgin female UAS-DiscoM flies. We expected their offspring to be completely heterozygotes, both for GAL4 and GAL80. We expected living flies to emerge, and that the ubiquitous expression of arm-GAL4 did not take control and kill the embryos. A large number of these pupated and eclosed (this was only observed in a qualitative manner; flies were not counted), so we deduced that indeed, the *Rescue* alleles in our flies were working.

CHAPTER THREE: TESTING THE DYNAMICS OF A KILLER-RESCUE GENE DRIVE SYSTEM IN *DROSOPHILA*

A. Introduction

As mentioned above in greater detail (Chapter 1), gene drive involves pushing a desired gene (or in this case, a pair of desired genes), into a population of organisms. It was our expectation that if a group of the Killer-Rescue flies were released into a population of UAS flies, the frequency of the R genes would increase over time, at least initially. Once the K-R containing flies had been created, and we had ascertained that we had a viable and stable stock population of them—and once the preliminary, qualitative tests for fertility had been conducted and the transgenic females seemed to produce just as many viable eggs as the wild-type females—we could finally begin population-level experiments. In these experiments, we introduced "transgenic" individuals (containing Killer and *Rescue* alleles) to a cage of "wild-type" individuals (not containing K and R alleles) at a frequency of either 0.50 or 0.10. Here again, "wild-type" individuals have mottled eyes and contain the UAS disco-M transgene only, while "K-R" flies have eyes varying in shades of red. All of these contain UAS disco-M, along with either GAL4 (the Killer), GAL80 (the *Rescue*), or both.

B. Methods

Replicate population cages were made from Stouffers ® Brand Animal Crackers containers, dimensions 8" x 8" x 12". Lids were removed and replaced by light-colored cloth sleeves. The cloth was taped to the container mouth around this circumference, so that items could be easily added or removed from the cage. The sleeves could be tied off with a

slipknot in order to keep flies from escaping. Each cage was given a 250mL-beaker for water, into which Kimwipes® were added for wicking water. Each cage was also provided with a 60x15mm Petri dish, filled halfway with grape agar, and sprinkled with Fleischmann's® dry active yeast.

Rearing Conditions

Flies were grown at 25°C with a 12 hour light/dark cycle on standard Drosophila diet, comprised of a cornmeal and molasses agar blend (see Supplementary Materials for diet recipes).

In this incubator, flies took approximately 10-12 days to complete development, from egg to eclosed adult (depending on the stock). Virgin females were collected within 8 hours of eclosion, and males were collected as needed.

During the creation of transgenic lines, flies were reared in vials, filled approximately 1" high with larval diet, enhanced with a slight sprinkling of dry active yeast for the adults. The vials were plugged with standard-sized cotton balls to prevent flies from escaping.

For the replicate cages in which transgenic flies were released at a 50:50 ratio to wild-type flies, the parental generation for each cage consisted of 50 virgin female wild type flies and 50 male transgenic flies. The flies were four days or less past adult eclosion, virgin females having been collected *en masse* from large rearing bottles within eight hours of emergence. These flies were allowed to breed for five days, and approximately 340 eggs were collected on the 3rd, 4th, and 5th days from the grape agar plates in each cage. This enabled us to have approximately 1000 F_1 offspring in each replicate cage. The agar

containing the appropriate number of eggs was excised from the Petri dish and placed into a bottle containing regular fly food.

For each of the generations, F_1-F_{14}, the first day of adult eclosion was noted and recorded as "Day 1" for that generation. At the end of Day 5, rearing bottles (from which adult flies eclosed) were removed from the cage, while water and food were still provided daily (via grape agar). Approximately 340 Eggs from each of these generations were collected on subsequent Days 6 through 8, in the same fashion as noted above; eggs on grape diet were removed from the Petri dish and placed into bottles as mentioned above. When adults were no longer needed (Day 8), they were frozen in a -80°C freezer in Eppendorf tubes until they could be counted and scored for eye color. After scoring, the flies were returned to the tubes and placed back into the -80°C freezer.

Flies were counted by emptying around 150 individuals at a time from their Eppendorf storage tubes into weighing boats so that they could be counted and scored easily. Flies not dumped out were kept on ice, with the intent of preserving their genetic material, while flies being counted were tallied as quickly as possible and then returned to a new Eppendorf tube on ice. When counting, males were separated from females, Disco-M eye color was separated from "Red" eye color. "Red" included any flies with any dark background of pigment, from dark red to light orange—while disco-M flies' eyes had a mottled white appearance. Tallies were added to achieve the final count.

During the approximate time of the seventh generation of the cages that started at a 0.50 frequency, I noticed the plateau in the percentages of disco-M flies, and it was clear that those flies were maintaining frequencies around 7% disco-M phenotype from one generation

to the next, starting at the third generation—which was quite different than our expectations. We assumed there would exist a fitness cost to carrying the transgenes, and in turn our expectations were that the amount of wild-type in the population would increase, instead of plateau. It was decided that a new experiment should be started, with a skewed release ratio to see if this ratio brought about drastically different results. We decided to use a ratio of 90% wild-type to 10% transgenic alleles, and observe the resulting gene frequencies. To initialize 90:10 cages, 81 wild-type females were mated to wild-type males, 18 wild-type females were mated to transgenic males, and 1 transgenic female was mated to a transgenic male. Virgin females were collected first, then matings were allowed to take place for a period of five days. Two replicate cages were constructed as described above. Egg collection and adult counting were also carried out using the methods described above: egg collection from the Parental generation took place on Days 3-5 after the 5-day mating period; Days 6-8 would continue to be the egg collection dates in each subsequent generation.

Preparation of flies to be used in PCR

Individual flies were examined at the molecular level to detect the presence or absence of Killer (GAL4) and *Rescue* (GAL80) genes because it was not possible to accurately relate fly eye color with genotype. Flies were ground in "Squish Buffer" (Nairz, et al. 2007):

10X Squish Buffer (SB): 100mM Tris-HCl pH 8.2; 10mM EDTA pH 8.0; 2% Triton X-100 (Sigma-Aldrich), 250mM NaCl. This was diluted before use--for each individual fly, 50uL 1X SB will be used. In this diluted SB, there should be 5uL 10X SB in 44.5uL water. Proteinase K is added to each individual reaction in 0.5uL quantity.

A single fly is placed in a 0.7mL Eppendorf tube, and a drop of the 1X SB is dispensed near the body of the fly. Using a separate pipette tip, the fly's body is ground up as homogeneously as possible, before the rest of the SB is dispensed into the Eppendorf tube. The ground fly and SB are pippetted up and down, in order to ensure a homogenous mixture.

The squished flies were incubated at room temperature for thirty minutes, before they were spun down and the liquid is collected from the tubes. Chitinous cuticle pellets were discarded. The liquid was collected in PCR tubes, which are then taken to the thermocycler for a two-minute, 95ºC deactivation of Proteinase K. After this, the DNA is diluted 1:4, and was used in PCR reactions.

PCR Protocol

I used the DreamTaq PCR Kit from Fermentas™ (Glen Burnie, Maryland) as follows. The recipe below is given for a one-sample reaction, though can be multiplied proportionally to satisfy sample number requirements (multiply this recipe by ten for a 10-sample reaction).

Recipe

-10X DreamTaq Buffer (includes MgCl2):	2.5	uL
-dNTPs	0.25	uL
-forward primer	0.5	uL
-reverse primer	0.5	uL
-Taq polymerase	0.125	uL
-H2O	21.25	uL
-DNA	5.00	uL

DNA samples were placed in the thermocycler, centered in the middle rows and columns. The block was heated to 94ºC for two minutes in an initialization step, after which the denaturing, annealing, and elongation took place respectively over 40 cycles. In each cycle, the denaturing temperature was 94ºC held for 30 seconds. The annealing temperature varied

based on the primers I used; for the GAL4 primers I used 60ºC and for the GAL80 primers I used a 50 ºC annealing temperature. The annealing step for each cycle was 45 seconds. Elongation in each cycle was at 72ºC for 2 minutes. The final elongation step was held at 72ºC for ten minutes, and finally, the reactions were held at 4ºC until removed from the thermocycler.

Initially, we used primers that primed from both directions within the GAL4 and GAL80 genes. However, it was discovered that, since the flies eat the same yeast from which their genes are derived, we were actually getting false positives on gels, simply from the amount of yeast present on the bodies of the individuals that were ground up and used for PCR samples. We solved this problem by creating a forward primer that was found in the mini-white gene of the fly, and used it with both reverse primers.

For Priming GAL4: Forward: 5'-GACATTGACGCTAGGTAACGC-3'
Reverse: 5'-TGCTGCTATCACTGAAGGACG-3'

For Priming GAL80: Forward: 5'-GACATTGACGCTAGGTAACGC-3'
Reverse: 5'-CTATAATGCGAGATATTG-3'

PCR products were analyzed by agarose gel electrophoresis. 50mL TAE Buffer was added to 0.5grams of agarose, to make a 1.0% gel. In instances where small DNA fragments needed to be resolved, a 1.5% gel was used.

C. Results

In the following section, I present results first from the cages in which transgenic flies were released at a 50:50 ratio to the wild-type flies in 15 generations. Second, I show the results obtained in the cages where the release ratio was 10% transgenic to 90% wild-type. From the 10:90 cages, we conducted PCR experiments on 30-50 randomly-selected male flies from generations F_1-F_7 and F_{10} to understand how prevalent the frequency of GAL4 was in each population (see Table 2). In generation F_5 we also used PCR to test for the presence of GAL80 in the population, which also confirmed that any flies exhibiting the mottled eye disco-M phenotype lacked both transgenes.

Finally, I pair composite images of the flies from generation F_2 with their respective GAL4 PCR results, and did the same with generation F_6 (Figures 3 through 6). For example, in the picture of the F_2 flies (Figure 3), some flies have an obvious red eye color, while others are not so obvious and appear orange under the microscope. Those exhibiting the disco-M phenotype are easiest to distinguish. When taking the image for the F_6 generation (Figure 5), I rotated suspected orange flies so that they faced left, while flies I suspected were red-eyed face to the right. Flies exhibiting the disco-M phenotype are at the bottom of this image. Notice, however, flies #7, #12 and #13, and 21 do not show up as positive for GAL4 on the corresponding PCR image (Figure 6). All orange-eyed flies do not show presence for GAL4, and we believe that those with orange eyes in any cage lack GAL4 but still contain GAL80, as evidenced by the relatively low presence of the disco-M phenotype in the 10:90 cages.

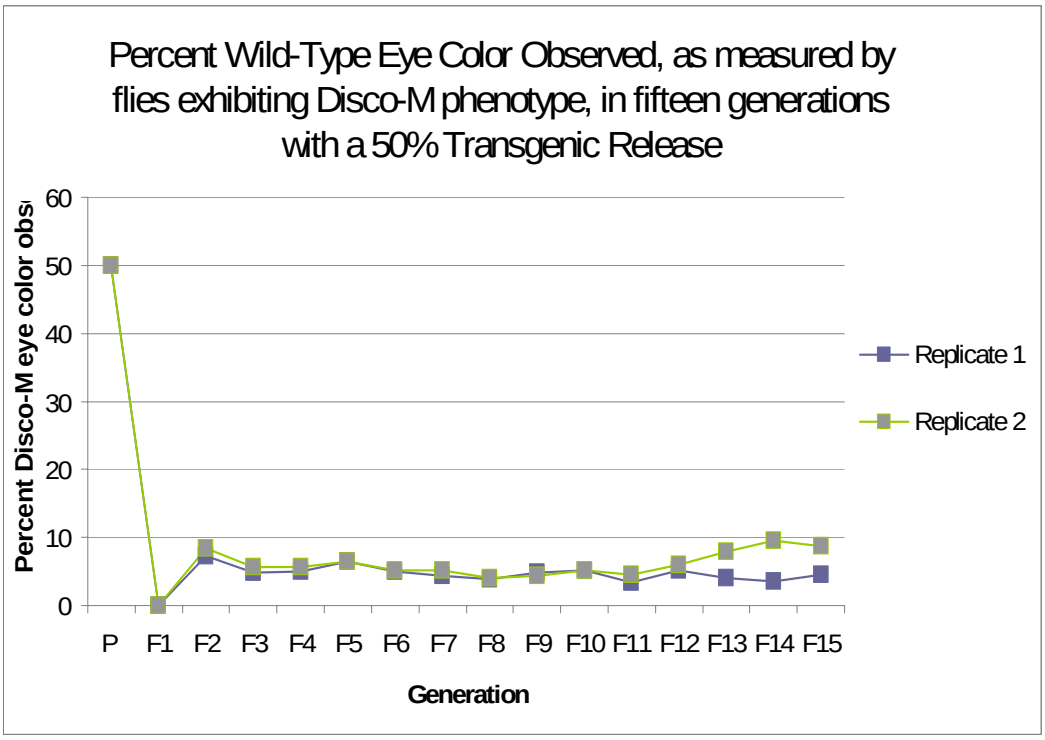

Figure 1. Graph of Percent wild-type over 15 generations, in population cages with 0.5 release frequency of transgenic flies.

From Figure 1, it appears evident that, at a 50% release ratio of transgenic flies into a wild-type population, *Killer* and *Rescue* have very little cost at all. Contrary to the predictions presented in Gould, et al. 2008 displaying a cost to carrying the alleles (Figures 11A and 11B in Chapter 1), this graph seems to indicate that very little cost, if any, is actually present. My graph actually looks much more like Figure 10A in Chapter 1—exhibiting a high transgenic release ratio, in which there is no cost to carrying the alleles. The wild-type appears to have a plateau; generations 3-15 all have approximately 7% wild-type, in Replicates 1 and 2. It may have been the case that the wild-type had lower fitness than the transgenics, in traits other than fecundity and viability.

During the approximate time of the seventh generation of the cages that started at a 0.50 frequency, I noticed the plateau in the disco-M flies, maintaining their frequencies around 7% from one generation to the next, starting at the third generation. It was decided that a new experiment should be started, with a new ratio of transgenic flies to wild-type flies. We then conducted a release resulting in an initial K and R frequency of 0.10, to determine if the changes in K and R frequencies would be more informative than in the 0.50 frequency release. In this manner, the population would be skewed towards the wild-type, so if there was indeed a cost to carrying *Killer* and *Rescue*, it would likely be more noticeable and perhaps the genes would fall out of the population more quickly. The objective here was to investigate whether the wild-type would be able to re-establish a frequency of 90% or higher after 15 generations.

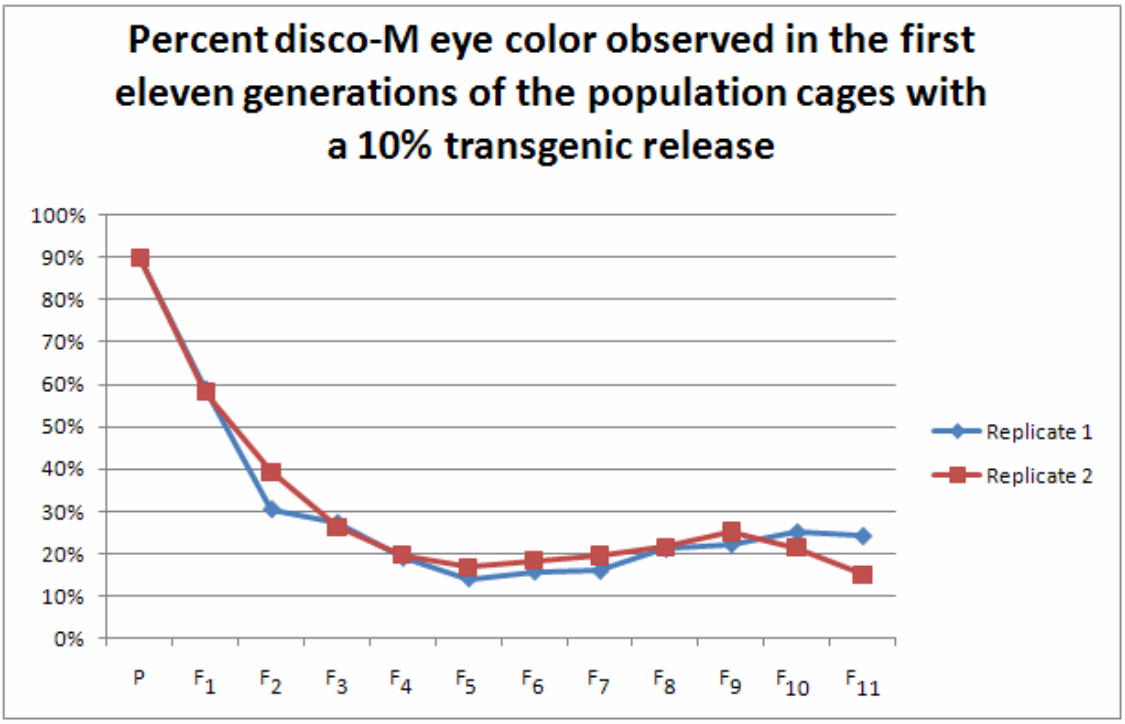

Figure 2. Graph of 90:10 population cages, percent wild-type over 11 generations.

The wild-type frequency in the cages with the 10% release ratio of transgenics to wild-type decreases to about 15% within the first five generations—but begins to rise again—and by generation 8, about 21% of individuals have the disco-M phenotype. The disco-M phenotype increases in frequency again in the ninth generation, but then begins to fall once more in the tenth and eleventh generations. By F_{11}, the first replicate had a disco-M frequency of 24.13%, and the second replicate had gone decreased to 15.11%, for an average during that generation of 19.62% of the wild-type phenotype.

Table 2: Population cages with 10% Transgenic Release Ratio. PCR was conducted with GAL4 primers to perceive a snapshot of the genotypes in the population, collected at random, in the first seven generations, from Replicate 1.

All flies with mottled white eye color were assumed to have neither GAL4 nor GAL80, and this was ascertained via PCR of 9 white-eyed flies in generation F_2 and 3 white-eyed flies in generation F_5, and was also confirmed in several other miscellaneous tests from other cages and generations.

	Sample Size	% Pos, GAL4	% Pos, GAL80*	%Wild-type
Generation F_1	**30**	12/30 =40.00%	12/30=40.00%*	18/30=60.00%
Generation F_2	**30**	18/30=60.00%	21/30=70.00%*	9/30=30.00%
Generation F_3	**30**	9/30=30.00%	21/30=70.00%*	9/30=30.00%
Generation F_4	**30**	9/30=30.00%	23/30=76.67%*	7/30=23.33%
Generation F_5	**37**	**19/37=51.35%**	**34/37=91.89%**	**3/37=8.11%**
Generation F_6	**30**	11/30=36.67%	21/30=70%*	9/30=30.00%
Generation F_7	**50**	17/50=34.00%	39/50=78%*	11/50=22.00%
Generation F_{10}	**30**	13/30=43.33%	22/30=73.33%*	8/30=26.67%

*For purposes of practicality, all flies with "red" (not disco-M colored) eyes are assumed to have GAL80. Generation F_5 was used to ascertain that all red-eyed flies indeed had GAL80, proving also that every fly with GAL4, the *Killer* allele, contained GAL80, the *Rescue* allele, and this generation is shown in bold above.

The data presented above in Table 2 reflect molecular data collected from eight of the eleven generations in the cages with a 10% transgenic release ratio. All were tested for the presence of GAL4 with the PCR primers listed in the methods section of this chapter, only generation F_5 was tested for the presence of GAL80. Indeed, any flies that had solid pigmentation in the eyes (typically a red or orange color) showed positive for GAL80. Flies displaying the disco-M phenotype showed negative for GAL80 and for GAL4.

Table 2 reflects the amount of disco-M shown in Figure 2. In Figure 2, the percent disco-M phenotype decreases dramatically until the F_5 generation, and that is also represented in the above chart (frequencies of disco-M decrease from 60% to 8.11% in these random samples). In Figure 2, the frequency of the phenotype goes on to increase and then stabilize in subsequent generations. This fact is not reflected well in Table 2, though it may be representative of the comparatively less drastic change in percent of disco-M phenotype frequency from generations F_6-F_{11}. Also, the sample sizes here are very small, so variation about the GAL4 estimate may be very large. Importantly, variation about the disco-M phenotype can be seen when superimposing the percentages of disco-M in Table 2 onto the graph of Figure 2. For many of the generations, the variation between the actual results and the PCR results is small, but in F2, F6, and F7, the variation can be as far as ten percentage points away from the true number within the population. In order to get a more accurate representation of the frequency of GAL4 in the population, larger sample sizes should be examined.

With respect to the frequencies of GAL4 in the randomly-selected samples, the F_1 generation shows that 40% of flies had GAL4, and that reflects the still relatively high

frequency of the wild-type phenotype. In F_2, more individuals inherited the transgenes, and this is reflected in Table 2, where the GAL4 frequency is at 60% in the random sample. Generations F_3-F_7 have GAL4 frequencies ranging from 30% in F_3 and F_4 to 51. 35% in F_5, and the average for those five generations is around 37.5% GAL4 in the random samples. Later, the sixth generation gave a random drawing in which 34% of males chosen contained GAL4, and in the seventh generation, we see a similar amount of GAL4 at 39.53% (it should be noted that the sample sizes between these two generations differed by 20 individuals). In the F_{10} generation the frequency of GAL4 among 30 randomly selected males was 43.33%. Ever-present, though, is the effect of the small sample sizes on the numbers given here.

In the next two pages, I present generations F2 and F6 from Table 2, in pictorial form. F2 flies, including disco-M flies, were used in PCR to determine presence of GAL4 (Figures 4 and 6). Images of the flies (Figures 3 and 5) were taken in order to determine if researchers would be able to visually distinguish flies containing GAL4 from those lacking the transgene.

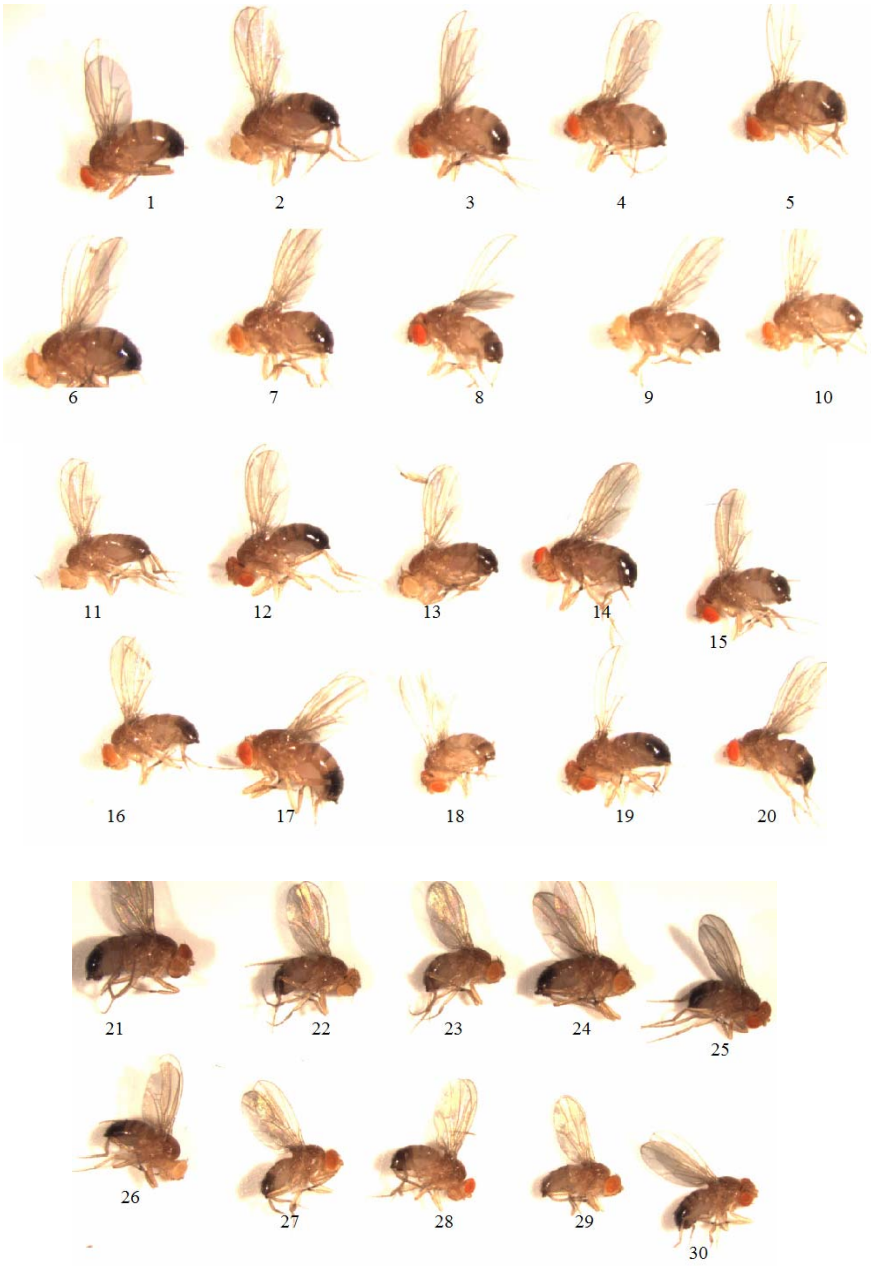

Figure 3: Image of individuals from generation F_2 represented in Table 2.

Photos made using Qcapture MicroPublisher 5.0 and Qcapture software (© 2006 QImaging) using a Leica CamZ 16F Stereoscope at 7.1x Magnification.

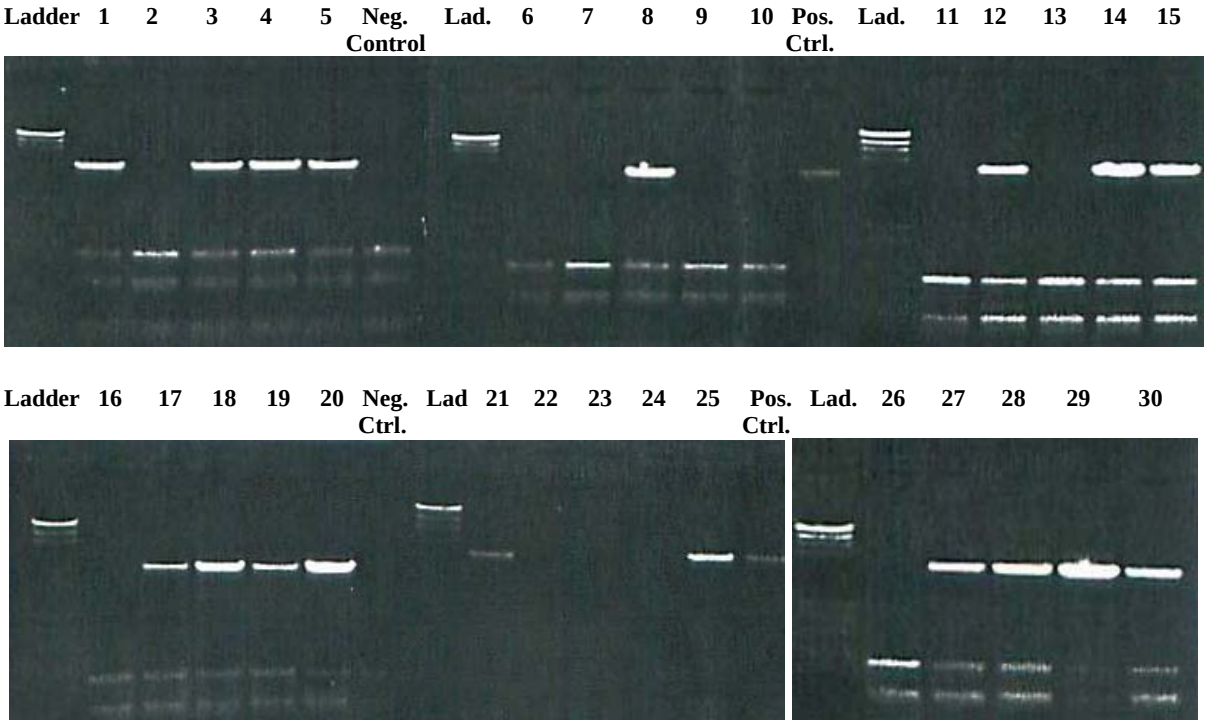

Figure 4: PCR Results, using primers for Gal 4, of red-eyed and white-eyed F2 individuals represented in Table 2 andcorresponding to the individuals in Figure 3

When comparing Figure 3 with the corresponding molecular data in Figure 4, it is important to notice that there are flies lacking GAL4, that have thorough pigmentation in their eyes. Flies 7, 10, and 16 have orange eyes, and do not show presence of GAL4 in the PCR. (Flies exhibiting the disco-M phenotype are flies 2, 6, 9, 11, 13, 22, 23, 24, and 26 and also show negative for GAL4).

The case is slightly different by the F6 generation, however, in that many flies have red eye pigmentation but lack GAL4.

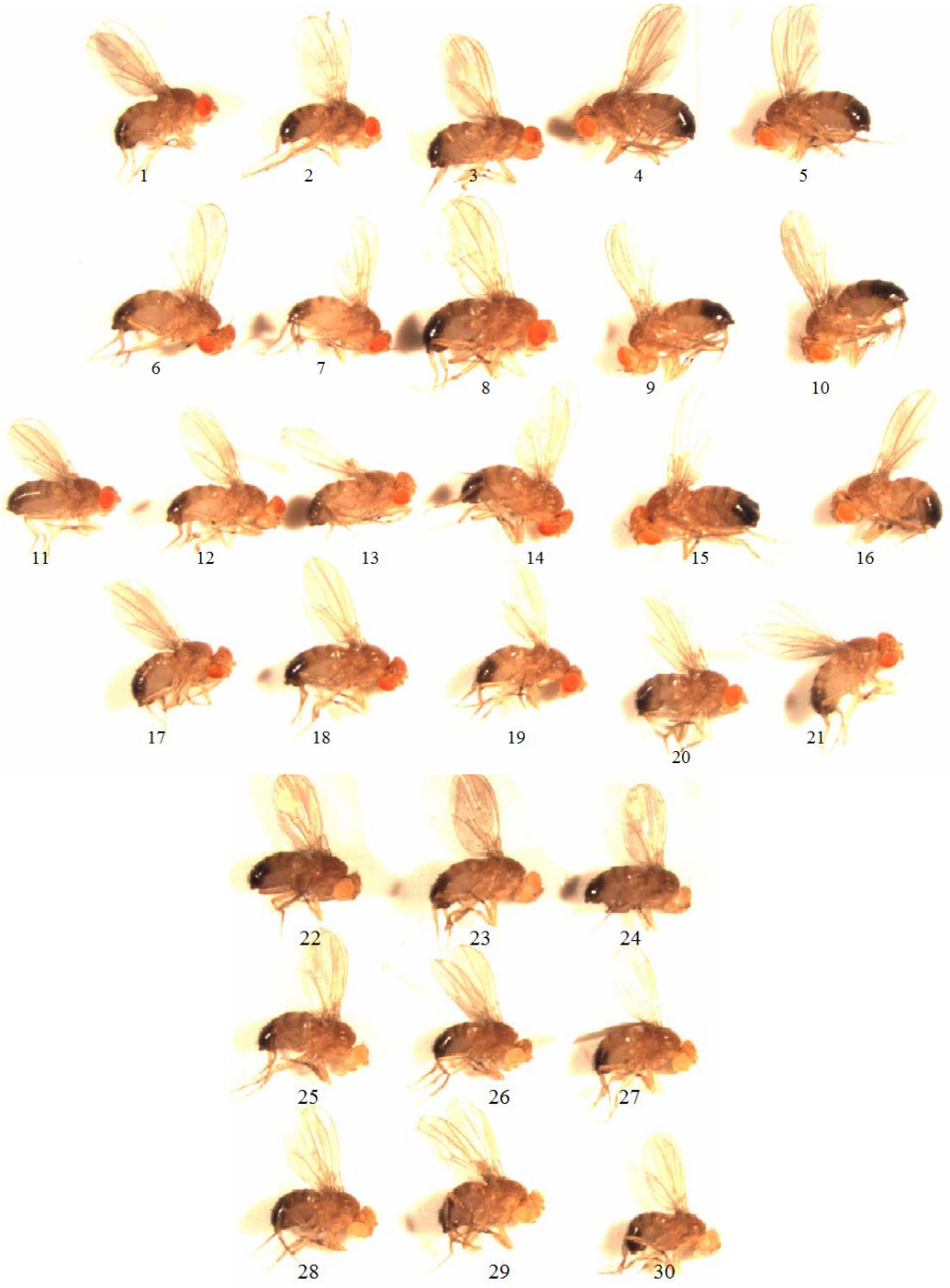

Figure 5: Image of individuals from generation F_6 represented in Table 2

Photos made using Qcapture MicroPublisher 5.0 and Qcapture software (© 2006 QImaging) using a Leica CamZ 16F Stereoscope at 7.1x Magnification.

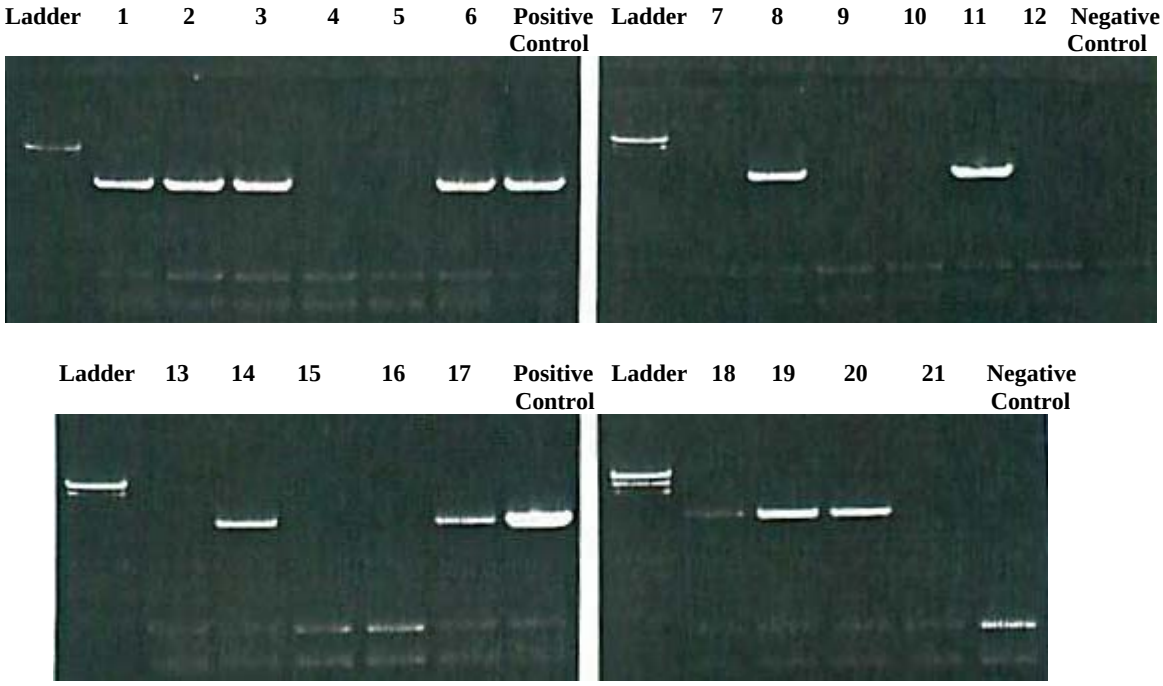

Figure 6: PCR Results, using primers for Gal 4, of the red-eyed F6 individuals represented in Table 2

Once again, there are flies represented in Figure 5 that did not show presence of GAL4 in figure 6. I tried to predict those that I thought lacked GAL4, and turned them so that they faced left in the picture (those are flies 4, 5, 9, 10, 15, and 16). However, there still remained some flies with red eyes (flies 7, 12, 13, and 21) that show an absence of the *Killer* gene in the PCR results. Flies 22-30 were diagnosed as having the wild-type phenotype and were subsequently not used in PCR, since at that point, we had collected enough data showing that flies exhibiting the disco-M phenotype lacked both transgenes, GAL4 and GAL80.

D. DISCUSSION AND CONCLUSIONS

I. Discussion

The general results shown in Figures 1 and 2 of this chapter indicate that, after the transgenic flies are released, the wild type frequency tended to decrease and then sustain itself at a certain frequency, seeming to plateau. When we released transgenic flies at a 50% ratio, the wild-type would plateau around 7%, and when we released them at a 10% release ratio, the wild-type phenotype seemed to plateau around 20%. In both scenarios, there is little justification for assuming any return to initial frequency by the wild-type. Indeed, there appears to be a fitness advantage to carrying the transgenes, instead of any fitness disadvantage.

Clearly, from my set of population cages with a 50% transgenic release, it can be seen in Figure 1 that a plateau was reached with respect to the percentage of wild-type existing in the population at a rate that is more or less constant, wavering only between five percentage

points, over twelve generations.

There appears to be no overall positive or negative trend, in the amount of disco-M after ten generations in either release scenario. That may be indicative of two possible situations. First, it is possible that GAL80 is approaching a fixed frequency in the population and GAL4 is slowly declining. Second, it is possible to assume GAL80 and GAL4 are both approaching fixed frequencies in the population. This assumption is supported by the results for GAL4 positives in randomly-selected males that went through PCR (Table 2) and also by noting the trend that currently appears in the cages with 10% transgenic release (Figure 2). The overall trend is neither positive nor negative, so we assume that a plateau is being formed in the disco-M phenotype frequency. Therefore, it is likely that the other genes are probably functioning similarly to how they functioned in the 50% release cages.

Time will allow us to better understand what we are seeing in the 10:90 cages. We may find that, regardless of the introduction rate of transgenics to wild-type, a plateau of the wild-type frequency should be expected (though different transgenic release ratios may be predicted to beget different plateau frequencies of the wild-type). The fate of these cages remains to be seen in the coming months. There are plans to monitor the 10:90 cages until at least their fifteenth generation, and perhaps beyond. The discovery of a true upswing and return of the disco-M phenotype frequency would negate the above plateau assumption.

After we had witnessed the trend towards a plateau in both treatments, we wanted to find out if we could manipulate our models to reflect the actual plateaus and frequencies we observed. Software similar to that described in Jongsma et al. 2009 was used to predict a the following circumstances to try and understand why my system deviated from the predicted

scenarios in the Gould, et al. 2008 paper. Scenarios similar to those presented in Figures 10 and 11 in Chapter 1 were investigated, but we used my release ratios (.5 and .1) instead of those presented in the Gould, et al. 2008 paper (.66 and .33). Strangely, with both 50% and 10% release ratios, and with a variety of costs imposed on Killer or Rescue or Both, no predictive model showed what happened in the true populations of fruit flies.

These models are presented on the next several pages in Figures 7 and 8. Figure 7A shows the 50% transgenic release, with zero cost to carrying *Killer* or *Rescue*, and Figure 7B shows the 50% transgenic release with a 10% fitness cost to carrying *Killer* and a 10% fitness cost to carrying *Rescue*. Notice that neither indicate a plateau formation.

Figures 8A and 8B demonstrate models generated with a 10% transgenic release, 8A giving no fitness cost to carrying either gene, and Figure 8B representing a 10% fitness cost to carrying *Killer* and a 10% fitness cost to carrying *Rescue*. Again, the models are not indicative of the frequency of wild-type I observed in my population cages.

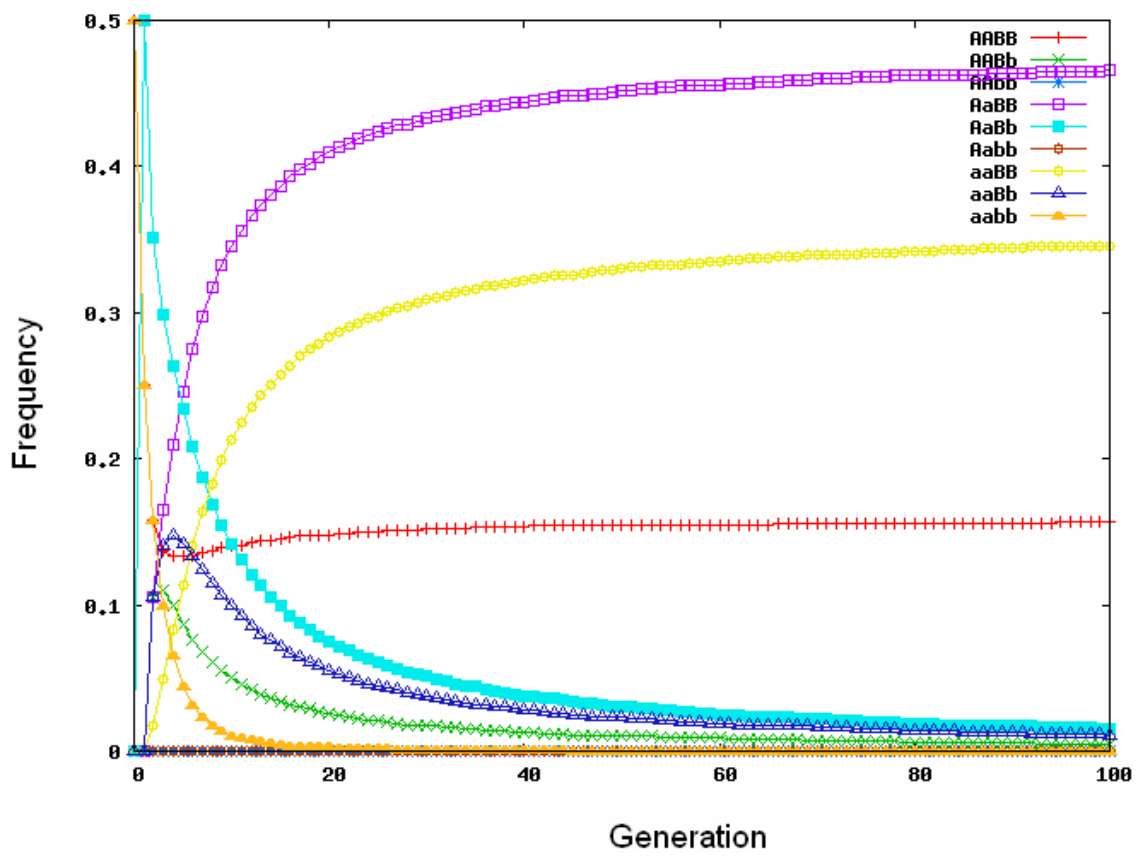

Figure 7A: Model of a release of transgenic flies at a 50:50 ratio to wild-type flies, no fitness cost to either *Killer* (A) or *Rescue* (B). The gold line with triangular blocks indicates the frequency of the wild-type (aabb). Notice, it decreases dramatically within the first 15 generations, and continues to decline over the course of 100 generations, asymptotically approaching zero.

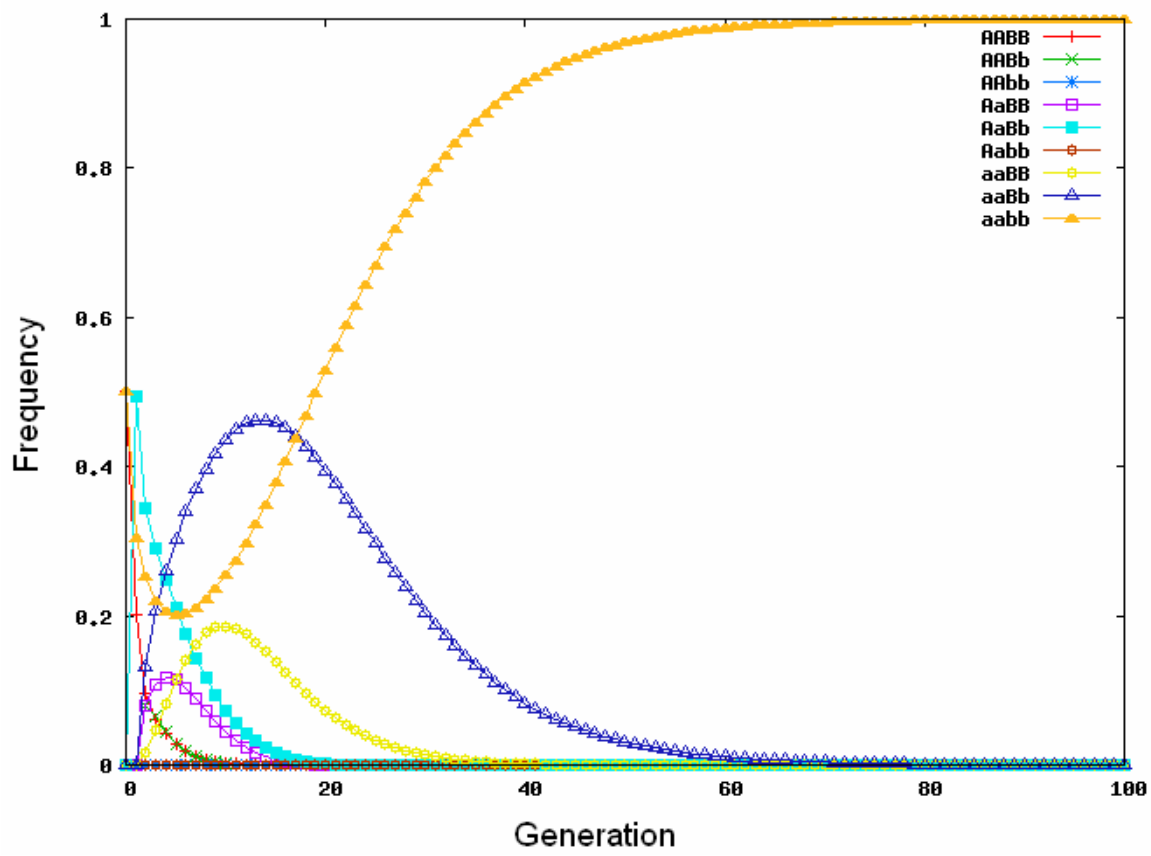

Figure 7B: Model of a release of transgenic flies at a 50:50 ratio to wild-type flies, with a 10% cost to each *Killer* and *Rescue*. The gold line with triangular blocks indicates the frequency of the wild-type (aabb). Notice in this scenario, the wild-type decreases to a frequency of 20% before the cost of Rescue eliminates that gene from the population, and the wild-type returns to 100% frequency around 60 generations.

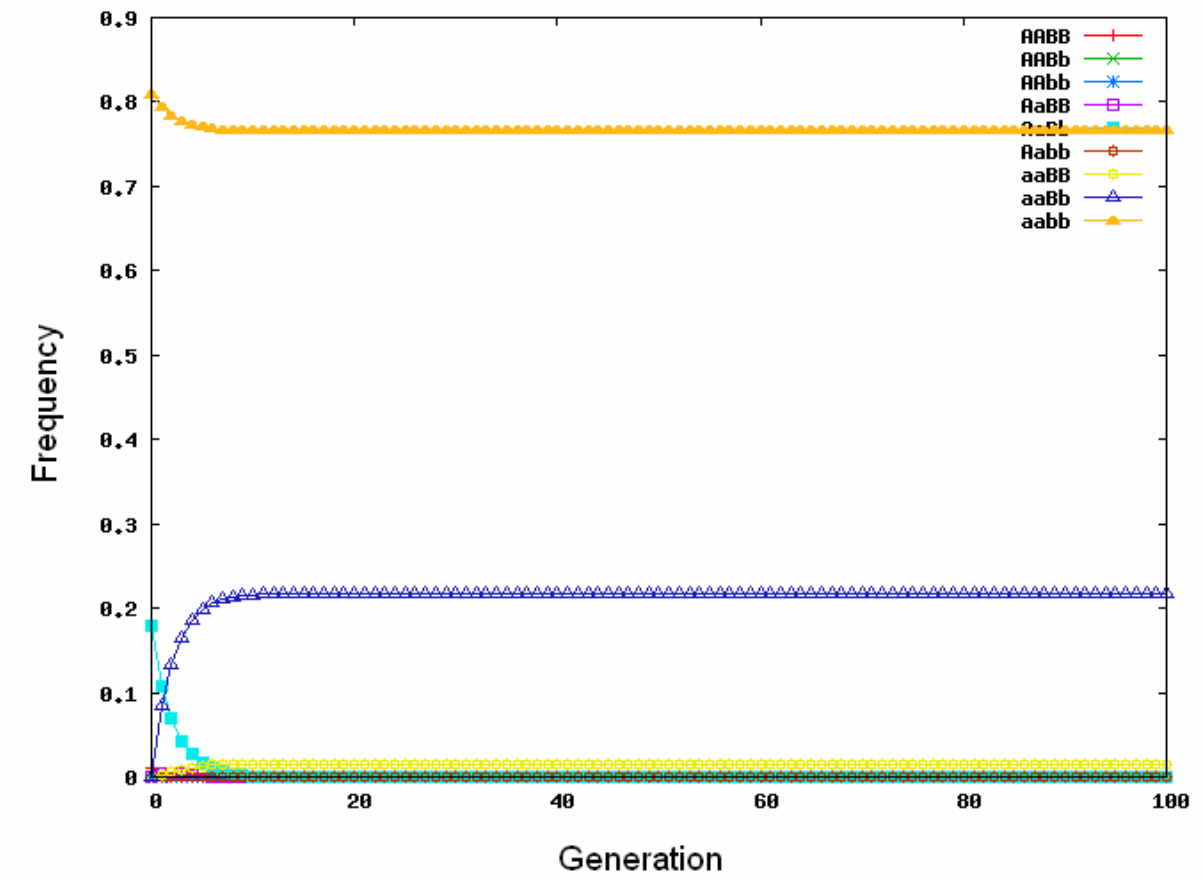

Figure 8A: Model of a release of transgenic flies at a 10:90 ratio to wild-type flies, no fitness cost to either *Killer* or *Rescue*. The gold line with triangular blocks indicates the frequency of the wild-type (aabb). The wild-type in this scenario maintains a high frequency, which was not seen in any of my cages,

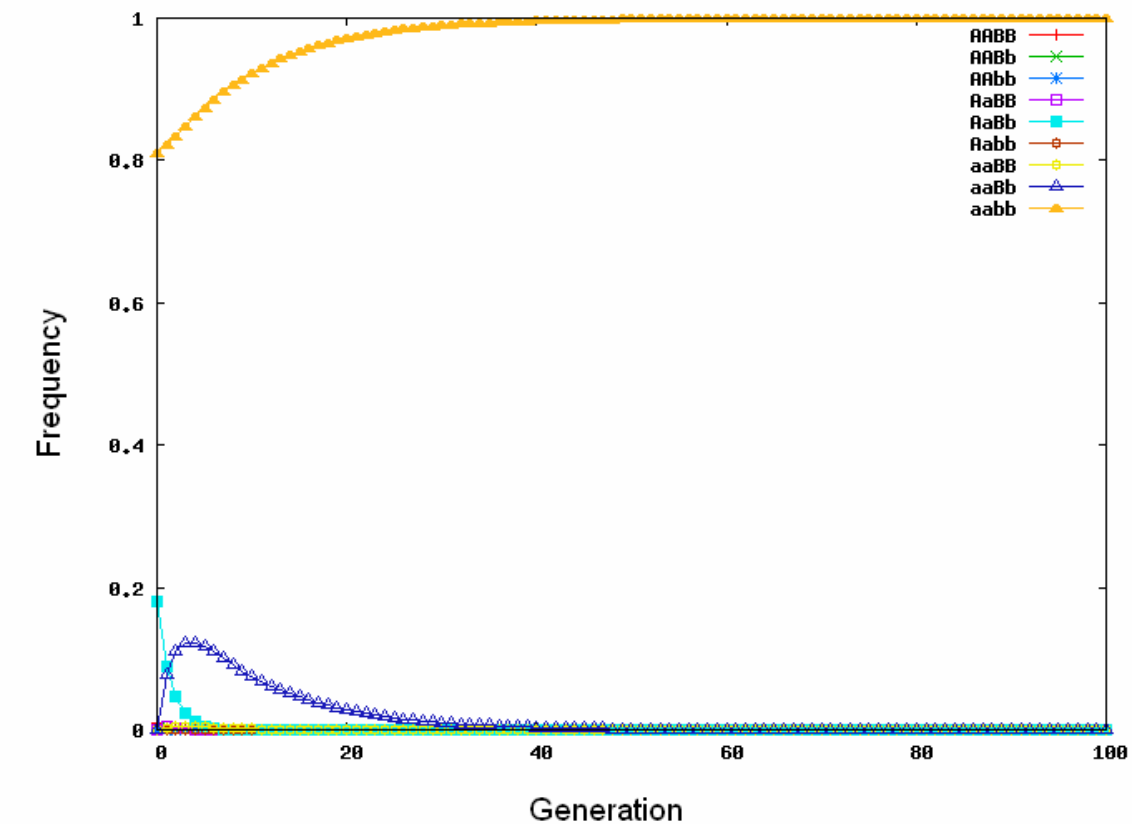

Figure 8B: Model of a release of transgenic flies at a 10:90 ratio to wild-type flies, with a 10% cost to each *Killer* and *Rescue*. The gold line with triangular blocks indicates the frequency of the wild-type (aabb). Notice in this scenario, the wild-type decreases to a frequency of 80% by the second generation, but ultimately increases back to 100% frequency after 40 generations. Again, this scenario does not represent what happened in my cages whatsoever.

The question remains—why are we seeing these plateaus, when the models do not predict them? It appears certain that the genes are not behaving as we predict, and at the current time it is difficult to understand what exactly is happening, genetically, with the flies in the cages. It may be possible that linkage between GAL80 and the UAS was interrupted, or that GAL4 isn't 100% lethal. Perhaps the population of wild-type individuals is experiencing inbreeding depression, adversely impacting the number of viable eggs laid in the cages by the females, and this is overcome by some advantage conferred by inheritance of the transgenes. Another possibility, still, is that of a heterozygote advantage in the population that we do not currently understand.

When the experiments began, we assumed fitness costs would exist, for carrying *Killer* and *Rescue,* and these costs would be revealed in our transgenic flies. Upon simple observation and a few experiments (Chapter 2), it certainly appeared that the transgenic lines were just as healthy, fertile, and viable as their wild-type counterparts.

II. Improvements

Clearly, the best improvement that can be made to the data I have collected would be to increase the sample sizes of flies analyzed by PCR (Table 2). Using 96-well plate PCR would be a viable option for obtaining data from a large number of individuals per generation, so that we could get a clearer picture of the true frequency of both GAL4 and GAL80 in the population. We had tried this, but too many human errors led to results that were difficult to interpret. Therefore, we used PCR of 10-20 individuals at a time, in an effort to not only get clear, valid data, but also to conserve resources and lessen mistakes. It

should be noted that individuals with more PCR experience would likely make less mistakes and perhaps get better results. More flies can be ground up for their DNA and can be tested for the presence or absence of both GAL4 and GAL80.

Since the flies from all generations were saved in the -80°C incubator, this is still a very feasible project that can be conducted in the near future.

If this experiment could have been done differently, in retrospect I would suggest having two replicates of each of several transgenic release ratios, perhaps at 90%, 75%, 50%, 25%, 10%, and perhaps even 1%—keeping the number at 2 replicates for feasibility with the cages in the incubators—release percentages that may be significantly different from, but easily comparable to those release ratios already used (50% and 10%). If unlimited space were available, indeed, three or four replicates would be ideal. I also feel that even though they may be cumbersome to count, 1000 flies per cage is the necessary number to get a really accurate picture of the percent wild-type in the population. There were several instances where my adviser and I would look at the percentages of disco-M in counts after I had only counted about 200 flies, and they differed notably from the actual percentages when I had completed the counts. For example, when counting F_8 flies from one of the 50:50 cages, the first two dumpings of male flies give 1.705% disco-M, but the overall percentage of male flies with disco-M phenotype was 3.081%, differing by nearly two percentage points.

One of the methods used, that may have a greater degree of influence than we are able to measure, is the procedure for the collection of eggs from grape agar. Currently, a section of approximately 350-400 eggs is sliced out from the grape agar. I count the eggs in groups of ten, though I do miss some eggs, especially where females laid eggs on the sides

where the agar meets the Petri dish, so true egg numbers can often vary. Also, this may be a biased means of collecting eggs, as I have seen females often deposit several eggs around one general area. If, for some reason, disco-M females liked to lay their eggs away from the yeast on the grape plates, while red-eyed flies laid theirs closer to the yeast, I may have inadvertently caused a bottleneck by randomly selecting a place to cut the agar. So there is indeed a high potential for human error here. It would be useful to look into a method that gently washes all the eggs from the plate, and the researcher could perhaps mix the eggs around and fill an Eppendorf tube to a certain level delineating 350 eggs per collection. Certainly there is a better way to collect eggs than the method used here; I feel the method I used was likely very biased towards females that layed many eggs in one spot. The population of any of the genotypes could have been misrepresented in the population excised from the grape agar.

With regard to the use of enhanced green fluorescent protein (EGFP), this was a strategy we had only briefly discussed in the beginning, planning-phases of the experiment. It may be helpful in future projects to link genes coding for the protein, to our genes of interest, if possible—and in this way screen for the presence or absence of the *Killer* and/or *Rescue* genes by the presence or absence of fluorescence under UV light.

III. Moving Forward

The cages with the 10% transgenic allele release will be continued, perhaps for another few months, and wild-type frequencies will be monitored in each generation. It will be worthwhile to see whether the frequency of the wild type does, in fact, recover to a certain percentage, or if it even appears to surge back to initial frequency—as that result would truly

indicate that a fitness cost to carrying K and R is present. However, it seems from the current trend that an upswing is unlikely. Plans are in place to monitor at least the next three generations, so data comparing the two sets of fifteen generations can exist side by side.

With respect to the real-world implications of this experiment, the results seem to indicate that a plateau of wild-type individuals will be maintained in a population regardless of the release ratio, though more testing would be necessary to determine if multiple different release ratios lead to similar wild-type plateaus. Perhaps GAL4 and GAL80 are too beneficial in *Drosophila* to reflect a real fitness cost to carrying them. Certainly, other constructs should be examined that confer a fitness disadvantage to *Drosophila*.

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Supplementary Materials

Grape Agar Recipe (makes 500mL) Protocol of Dr. Jim Mahaffey's Lab.

-In a 600mL beaker, add 171 mL dH_2O, put this on a hot plate turned to full heat, with stir bar on.

-Add 57mL Welch's ® Concentrated Grape Juice, 29grams dextrose (brand?), 14.5grams sucrose (brand?) and 9 grams Brewer's Yeast (brand?). Add 10-15 extra milliliters dH2O, then cover beaker with aluminum foil.

-Meanwhile, in a separate beaker, add 272mL dH2O to 11grams Bacto® Agar. Microwave and stir until agar has completely dissolved.

-Add agar mix to grape juice mix on hot plate.

-Once all agar has visibly dissolved into grape mix (as discernable by lack of agar flecks in the mixture), turn heat off but keep stir bar on. Once substantially cooler, add 6.5mL Tegosept ®, and continue to stir for 5 minutes.

-At this point, the grape agar mixture may be poured into Petri dishes. This recipe can make around 60 Petri dishes' worth of grape plates.

-Let cool for two minutes, then place lids on Petri dishes, and continue to let cool for 30 minutes until agar has solidified and is no longer warm to the touch. Store in 4°C refrigerator.

Regular Agar Diet (used in vials and bottles): Protocol of Dr. Trudy McKay's Lab. Makes 3000 Vials.

-Stir 200 grams agar into 23 Liters of water, and bring to a boil until all the agar has dissolved.

-Meanwhile, dissolve 390 grams of yeast in 5L cold water, then stir in 1955g Yellow Cornmeal until all lumps have been dissolved.

-Once Agar has dissolved, add 1360mL molasses into the agar mix, and then stir the cornmeal/water mix into the batch as well.

-Allow the mixture to boil again, and then let simmer for ten minutes.

-Turn off heat source, then allow the mixture to cool in the large volume for around five minutes. Add 355mL Tegosept and 160mL Proprionic Acid and stir in well. Allow to cool for another five minutes, then dispense into vials or bottles.

-Allow to cool before plugging if time allows, or cover tops of vials and/or bottles with Cling Wrap® and refrigerate.