HORIZONTAL AND VERTICAL TRANSMISSION OF A PANTOEA SP. IN CULEX SP.

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By

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Abstract

Mosquitoes serve as vectors for several life-threatening pathogens such as *Plasmodium* spp. that cause malaria and Dengue viruses that cause dengue hemorrhagic fever. Control of mosquito populations through insecticide use, human-mosquito barriers such as the use of bed nets, and control of standing water, such as areas where rainwater has collected, collectively work to decrease transmission of pathogens. None, however, continue to work to keep disease incidence at acceptable levels. Novel approaches, such as paratransgenesis are needed that work specifically to interrupt pathogen transmission. Paratransgenesis employs symbionts of insect vectors to work against the pathogens they carry. In order to take this approach a candidate symbiont must reside in the insect where the pathogen also resides, the symbiont has to be safe for use, and amenable to genetic transformation. For mosquito species, *Pantoea agglomerans* is being considered for use because it satisfies all of these criteria. What isn't known about *P. agglomerans* is how mosquitoes specifically acquire this bacterium, although given that this bacterium is a typical inhabitant of the environment it is likely they acquire it horizontally through feeding and/or exposure to natural waters. It is possible that they pass the bacteria to their offspring directly by vertical transmission routes. The goal of my research is to determine means of symbiont acquisition in *Culex pipiens*, the Northern House Mosquito. I chose *C. pipiens* as a model organism due to its ease of rearing in a laboratory setting.

My research involved monitoring the fate of *P. agglomerans* that contained a fluorescent marker that was ingested by laboratory-reared adult male and female *C. pipiens* to verify horizontal transmission and determine if vertical transmission occurs. I

used a combination of standard microbiological techniques and tests in conjunction with fluorescent microscopy. I found that both male and female *C. pipiens* acquire *P. agglomerans* horizontally and that females pass the bacteria to their offspring. To my knowledge, this is the first report of vertical transmission in *C. pipiens*. I also found that identification of *P. agglomerans* using standard biochemical tests and databases can be unreliable which can impact its use in the field raising risk assessment and environmental impact questions.

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Chapter 1

Malaria

History of Malaria

According to the Centers for Disease Control and Prevention, malaria or a disease resembling malaria has been around for over 4,000 years. The name "malaria" comes from the Italian word "mal-aria" which means "bad-air," and reflected the belief that this disease was contracted through foul smelling air[1]. Foul smelling air was associated with swamps and marshes which are the perfect breeding grounds for mosquitoes, the actual vectors of the pathogens that cause the disease.

In 1880, Charles Laveran, a French army surgeon stationed in Algeria was the first to notice parasites in the blood of a patient suffering from malaria[1]. Subsequently in 1890, two Italian scientists, Giovanni Grassi and Raimondo Filetti first introduced the names *Plasmodium vivax* and *Plasmodium malariae* for two malarial parasites[1, 2]. In 1897, Ronald Ross, a British officer in the Indian Medical Service, demonstrated that the malarial parasite could be transmitted from an infected patient to a mosquito. He also studied malaria in birds and showed that a mosquito could transmit malarial parasites from bird to bird[1, 2]. Thus, both the pathogens that cause the malaria and their mode of transmission were now identified.

Malaria has a history of devastation. For example, between 1905 and 1910, malaria was a major cause of death and disease among the workers constructing the Panama Canal. In 1906, there were 26,000 employees working on the Canal and of those, 21,000 were hospitalized for malaria[1]. Once the mosquito population was under control,

the number of hospitalized workers decreased by tenfold[1]. During World War I (April 6, 1917 – November 11, 1918), malaria accounted for 4,746 hospital admissions and seven deaths, and most of these cases occurred in the Americas and Caribbean region[8]. World War II was affected by malaria more than WW I. In WW II (December 7, 1941 – August 14, 1945), there were 113,256 new cases of malaria and ninety deaths. *Plasmodium vivax* was reported to be responsible for 30% of the deaths, P. *falciparum* infection accounted for 54% of deaths, mixed infections for 4%, and unspecified malarial parasites for the other 11%[8]. The unspecified malarial parasites may have been *P. vivax,* and was misdiagnosed as *P. falciparum,* or it was an undiagnosed mixed infection. During the Korean War (June 25, 1959 – July 27, 1953), there were 4,542 new diagnoses of malaria, but no deaths occurred in the naval forces. P. *vivax* was the main parasite to that caused malaria during this war. During the Vietnam War (August 4, 1964 – January 27, 1973), there were 24,606 cases of malaria, and 46 deaths resulted from the disease[8].

According to the World Malaria Report, in 2013 there were an estimated 198 million malaria cases worldwide with 584,000 cases that resulted in death[18]. Moreover, seventy-eight percent of those deaths were children 5 years old and younger. In 2014, 97 countries and territories had ongoing malaria transmission. An estimated 3.3 billion people are at risk for getting malaria, of which 1.2 billion are at high risk. The highest risk of transmission is found in Africa, South of the Sahara and in parts of Oceana such as New Guinea[1]. Roughly 1,000 cases of malaria are introduced into the United States each year. Recent outbreaks in California were in 1988, 1989, and 1990[2]. These incidents were due to introductions of infected humans into areas with competent *Anopheles* vectors[3];

two of the three cases occurred in San Diego County and one case occurred in the city Colton[1] located in San Bernardino County.

Symptoms of malaria can range from flu-like and cold symptoms to seizures, and may include physiological dysfunction such as kidney failure[18]. As mentioned earlier, *Plasmodium* spp. that cause malaria are transmitted by mosquitoes and thus, malaria is considered a vector-borne disease. *Anopheles* spp. are the vectors for *Plasmodium* spp[1, 13]. Malaria in humans is caused by five different *Plasmodium* species: *Plasmodium falciparum, P. vivax, P. ovale P. malariae,* and *P. knowlesi*[2]. There are other *Plasmodium* species which infect monkeys, birds, and reptiles, but these have not been reported to infect humans[13]. A female *Anopheles* mosquito acquires the parasite when she takes a blood meal from an infected host. She can further transmit the pathogen to another host during another blood meal. While this cycle of infection can be simply stated, in actuality, the cycle involves complex interactions between the mosquito and its host, and a complex parasite life cycle.

Plasmodium **Life Cycle**

The *Plasmodium* life cycle involves forms of sexual and asexual reproduction and includes three life-cycle stages: gametocytes, sporozoites, and merozoites[4, 5] For the purposes of my research, I will describe reproduction of the protozoan parasite in terms of human infection, *Anopheles,* and the *Plasmodium* spp, life cycle (Figure 1). The sexual phase, gametogony, begins in the blood of the human host and is completed within the lumen of the midgut of the mosquito. The first phase of asexual reproduction, sporogony, occurs on the outer wall of the midgut of the mosquito. The second phase of asexual

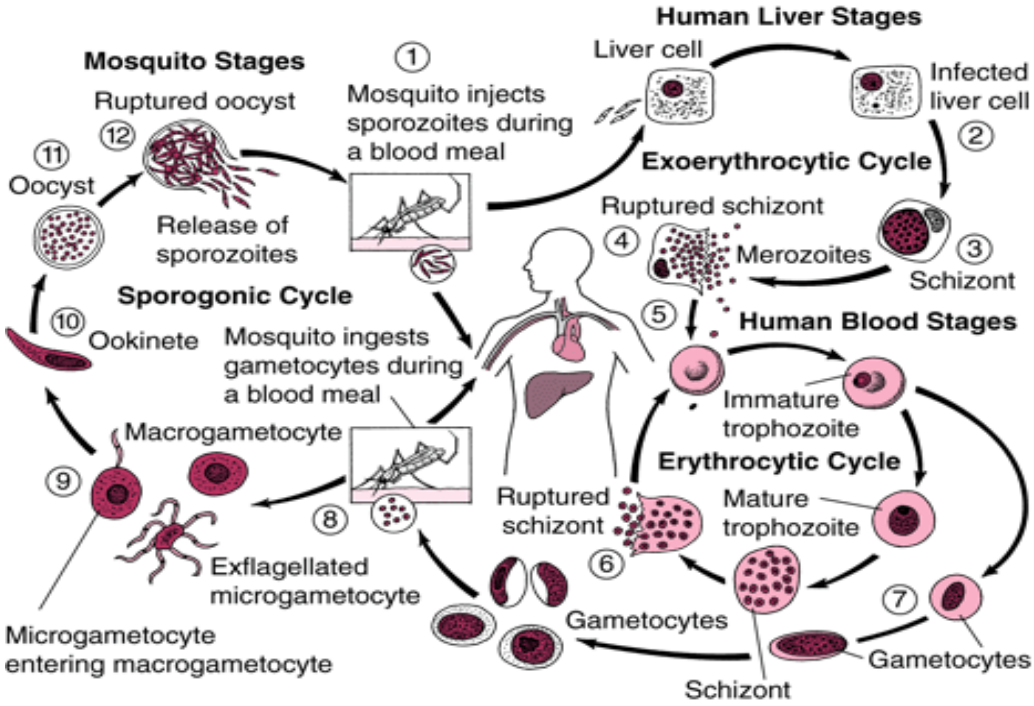
reproduction occurs first in the human liver, known as the exoerythrocytic stage, and then later in the blood of the human host (erythrocytic stage). Sporogony is often referred to as the exogenous phase of malarial parasite development because it occurs outside of the human host. Merogony is the term used to describe the endogenous phase of development within the human host.

A gametocyte is the sexual stage of the malarial parasite. A male gamete is called a microgametocyte and a female gamete is called a macrogametocyte. This stage takes place in the gut of the mosquito and will produce gametes inside the mosquito host. The male and female gametes fuse to form diploid zygotes. The zygotes become motile ookinetes that burrow into the mosquito midgut wall and form oocysts. The growth and the division of the oocyst produce thousands of sporozoites. The oocyst bursts and releases the sporozoites into the body cavity of the mosquito, which then travel to the mosquito's salivary glands. The sporozoites are injected into the blood stream of a human host when the female mosquito takes a blood meal. A female mosquito is able to locate a blood meal via carbon dioxide emitted by the human host through the use of receptors on her antennae[9]. The sporozoites travel to the human's liver and invade the liver cells. During the exoerythrocytic cycle, the sporozoites infect liver cells and then mature into schizonts. The schizonts rupture and release merozoites. The merozoites proceed to infect red blood cells. This stage is when the clinical manifestations of the disease occur. The merozoite has a bristly surface of filaments which are used to capture and invade red blood cells [10]. While inside the red blood cell, the merozoite feeds on it through a small, dense ring where small portions of the erythrocyte's cytosol are pulled into the parasite's

vacuole membrane and is then digested [10]. The merozoites feed, grow, and replicate asexually for 1-3 days and some develop into gametocytes which circulate through the human's bloodstream. The gametocytes are then ingested by a female *Anopheles* mosquito when she takes a blood meal.

Plasmodium multiply in the mosquito's midgut known as the sporogonic cycle. Zygotes are generated and in turn become motile and elongated to become ookinetes. The ookinetes develop into oocysts. The oocysts grow, rupture, and release sporozoites, which make their way to the mosquito's salivary glands. Upon feeding, the female mosquito introduces the sporozoites into the human host. Once the female mosquito ingests the gametocytes, the cycle starts all over again.

Figure 1. *Plasmodium* Life Cycle



The Merck Manual for Healthcare Professionals. Merck Sharp and Dohme, Jan. 2010. Web. 20 Jan. 2014. <http://www.merckmanuals.com/professional/infectious_diseases/extraintestinal_protozoa/malaria.html>.

There are more than 200 species of *Plasmodium* that can infect the blood of birds, reptiles, various mammals, and humans; the most common forms of human malaria are caused by *Plasmodium falciparum, P. vivax, P. malariae, P. ovale* and *P. knowlesi*[1]. All members in the genus *Plasmodium* are parasitic[41] and suggests evidence that they are remotely related to each other, and that their evolutionary divergence predates the origin of the hominids.

Almost every malarial death is caused by *P. falciparum.* Around the world, malaria is the most significant parasitic disease of humans, and claims the lives of more children worldwide than any other infectious disease[1, 5, 6]. Since 1900, the area of the world exposed to malaria has been halved, yet two billion more people are presently exposed[5]. Infection rates in children in endemic areas, such as Africa, are on the order of 50% [5]. Chronic infection has been shown to reduce school scores by up to 15%. Reduction in the incidence of malaria coincides with increased economic output[5]. *P. falciparum* causes the most dangerous form of malaria in humans almost every malarial death is caused by *P. falciparum*. An estimated 1 million people are killed by *P. falciparum*, especially in Africa where the species dominates[5].

Plasmodium vivax is mainly found in the United States, Asia, Latin America, and in Sub-Saharan Africa. *P. vivax* is the most prevalent malarial parasite, but is not as deadly as *P. falciparum*. *P. vivax* can still lead to severe disease and causes splenomegaly, and can go dormant in the liver, with relapse several months or years after the initial infecting bite[1, 5].

Plasmodium malariae is found worldwide, but is known as "benign malaria" and is not as severe as *P. falciparum* and *P. vivax*[1]. This species has a three-day infection cycle, known as a quartan pattern, where every 72 hours, the patient's temperature spikes [11]. *P. malariae* is the least studied because it has a lower prevalence and a milder clinical appearance.

Plasmodium ovale is rare compared to *P. falciparum* and *P. vivax* and is less dangerous than these two parasites. The clinical course with *P. ovale* is similar to that of *P. vivax*[1]. In established infections, temperature spikes occur at 48-hour intervals, a two day cycle known as tertian pattern[11].

Plasmodium knowlesi is found in Southeast Asia and is a natural pathogen to macaques. It can also infect humans but is known as "primate malaria." *P. knowlesi* has a 24-hour replication cycle and can rapidly progress from an uncomplicated to severe infection. The symptoms of acute knowlesi infection are of a nonspecific infectious illness similar to those seen in *P. falciparum* and *P. vivax* malaria[13]. A severe infection can lead to severe thrombocytopenia, renal failure, hypotension, jaundice, and deranged liver enzymes[13].

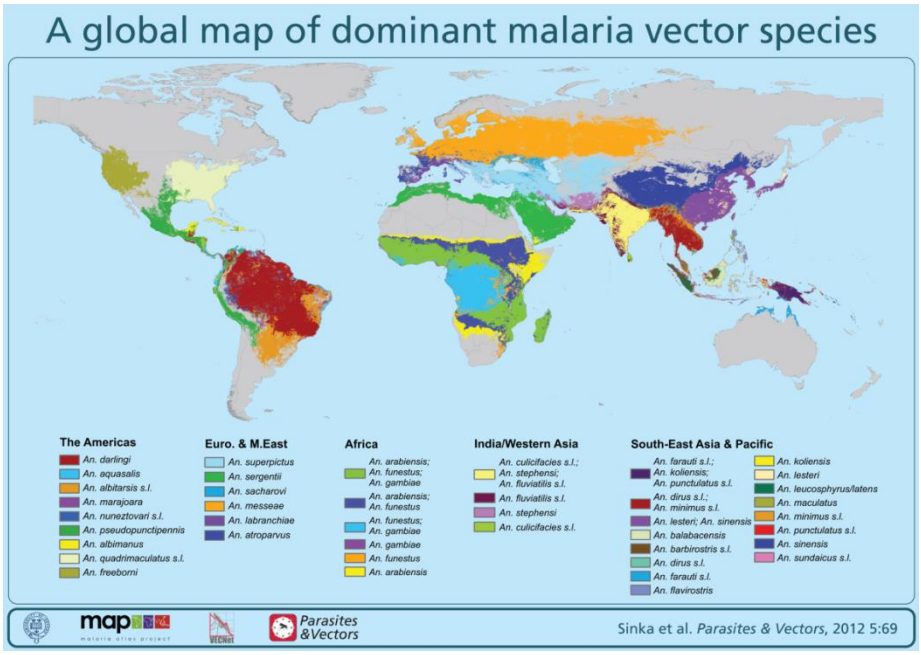
Mosquito Vectors

Control of mosquito populations in areas where *Plasmodium* species prevail is a daunting task for several reasons, including economic, climatic, and host availability. In general, mosquitoes are the most important arthropods affecting human health [14]. While mosquitoes are known vectors of malaria, they also transmit parasites that cause other devastating diseases, such as filariasis, encephalitis, yellow fever, and dengue. These

diseases are especially severe in developing regions of the tropics where malaria is endemic. Therefore, control of mosquito populations is meaningful in terms of decreasing disease in general.

Mosquitoes occur in practically every region of every continent in the world except for Antarctica[2]. The greatest species diversity occurs in tropical forests, but extremely high densities of mosquitoes are common even in the species-poor biomes, such as the tundra[3] (Figure 2). Even in extreme areas, mosquitoes can flourish and serve as reservoirs for pathogens. For example, Wagner et al. (1975) found California encephalitis virus from tundra mosquitoes of the *Aedes hexodontus-punctor* group collected in the Keewatin District, Northwest Territories.

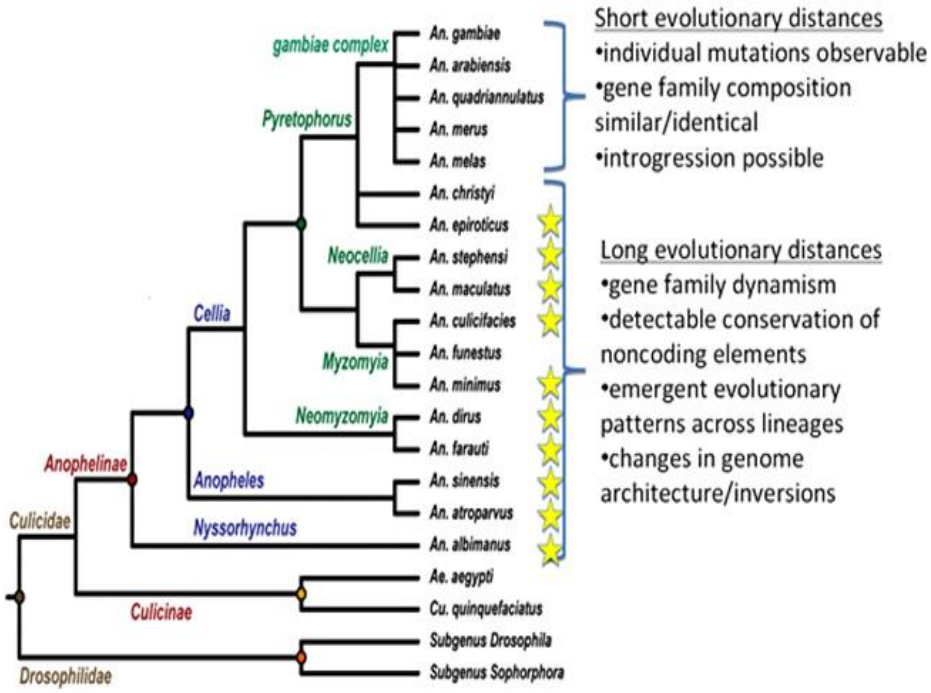
Figure 2. Global Distribution of *Anopheles* spp.



Marianne E. Sinka (2013). Global Distribution of the Dominant Vector Species of Malaria, Anopheles mosquitoes - New insights into malaria vectors, Prof. Sylvie Manguin (Ed.), ISBN: 978-953-51-1188-7, InTech, DOI: 10.5772/54163. Available from: http://www.intechopen.com/books/anopheles-mosquitoes-new-insights-into-malaria-vectors/global-distribution-of-the-dominant-vector-species-of-malaria

My research involved mosquitoes in the family Culicidae subfamily Culicinae because of their availability in California. Culicidae consists of 3,200 species[3]. Current classification recognizes two subfamilies: Anophelinae and Culicinae (Figure 3). Anophelinae is considered the primitive group and consist of 3 genera. Culicinae includes 40 genera. There are 15 genera in North America north of Mexico[3]. Of the 15 genera, there are three important groups of mosquitoes which are the *Anopheles gambiae* complex, *Culex pipiens* complex, and *Aedes* species.

Figure 3. Evolutionary Distance of Mosquitoes



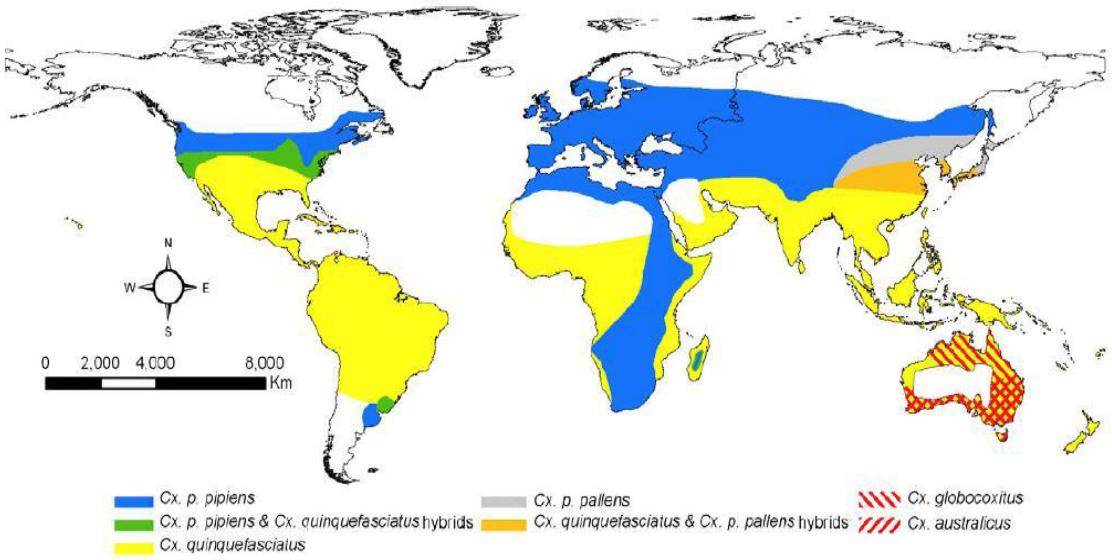
Carlton, Jane M., Daniel E. Neafsey, and Rhoel R. Dinglasan. "'Omics Approach to P. vivax Biology." The New York Academy of Sciences. New York. Speech http://www.nyas.org/Publications/Ebriefings/Detail.aspx?cid=d73bddd8-a497-417b-8673-2cb0607e82b4

Anopheles gambiae is an important vector of *Plasmodium* because it is more anthropophilic, that is: these mosquitoes prefer biting humans over other animals, is

endophilic, prefers to be in inside dwellings, and endophagic and thus feeds indoors more than the other species in its complex[3]. Interestingly, typical models used to estimate malaria risk in regions and inform control and prevention strategies utilize measures of mean outdoor temperature. Paaijmans and Thomas (2011) provide evidence, however, that some malaria vectors spend large parts of their adult life resting indoors.

Culex pipiens is a group of closely related domestic and peridomestic species (Figure 4). *Culex pipiens*, the Northern House Mosquito, and *Culex quinquefasciatus*, the Southern House Mosquito, are the most medically important taxa worldwide[3]. The two species overlap and commonly interbreed creating a hybrid species in the United States[3].

Figure 4. Global Distribution of *Culex* spp.



Global distribution of *Cx. pipiens* complex mosquitoes. Geographic range for *Cx. p. pipiens* includes both forms (pipiens and molestus). *Cx. australicus* and *Cx. globocoxitus* are restricted to Australia.

Aedes species are medically important and are found worldwide in the tropics and subtropics. They are the primary vector of both dengue and yellow fever viruses. One interesting aspect of *Aedes* behavior is that most do not disperse readily. Harrington et al. (2005) found that adult *Ae. Aegypti* disperse relatively short distances, suggesting that

people rather than mosquitoes are the primary mode of dengue virus dissemination within and among communities[44].

The mosquito's life cycle is completed in two different environments, one aquatic and one terrestrial[2]. Eggs are laid in the aquatic habitat, either in standing water or areas that will flood. In water larvae undergo four instar molts or stages, and then pupate. After the pupal stage, the adult mosquitoes emerge and exist in terrestrial environments. Typically during the first 3-5 days of adulthood, mosquitoes obtain sugar from plant nectar or honeydew, become sexually mature, and mate[7]. The sugar provides energy for flight, mating, and dispersal[2, 7].

Male and female mosquitoes exhibit different ecological behaviors. The males' only source of nutrition is from feeding on nectar and plant saps[2, 7]. Nectar and plant saps mainly contain sugar; this substance is the male's primary energy source. Female mosquitoes, on the other hand, are hematophagous. This means that while females do feed on nectar and plant sap, additionally they typically need a blood meal. The ingestion and digestion of blood initiate egg development. The large amounts of protein in the hemoglobin provide the building blocks for the egg yolk and serves as a substrate for building glycogen and lipids which also contributes to the female's energy reserves for flight and survival[2, 7].

Most female mosquitoes are anautogenous, meaning their egg follicles remain in a resting stage until a blood meal is taken. An autogenous female of *C. pipiens* is usually able to only lay a single egg raft without blood feeding[7]. Some other female mosquitoes are autogenous, and can develop eggs without a blood meal. Autogeny is genetically

determined and is an inherited partially dominant trait[7].

Antimalarial medication can be linked back to second century China where sweet wormwood was used to fight fevers and the indigenous Indian tribes of the New World used Peruvian bark to help fight fevers[1]. The medicines found within the wormwood and the bark is now known as artemisinin and quinine[5]. Both are the most effective antimalarial drugs available today. Artemisinin targets *Plasmodium* organisms in the schizont stage of development. When the schizonts mature from sporozoites, they contain insoluble iron called hemozoin[15]. Hemozoin is formed within schizonts as they feed on hemoglobin in the cytoplasm of human red blood cells. Artemisinin contains a peroxide group that reacts with hemozoin, by producing radicals that attack the parasite proteins, thereby killing the organisms[15]. Quinine interferes with the growth and reproduction of the malarial parasites only during the erythrocytic cycle[16]. When quinine treatment is terminated many recovered patients experience another attack of malaria several weeks later. This recurrence stems from the failure of quinine to kill the malarial parasites in cells of the body other than the red blood cells[16]. These parasites persist and, after a time, reinvade the red blood cells and precipitate the second malarial attack, or relapse[16]. Because quinine fails to produce a complete cure of malaria, better antimalarial drugs have been developed such as chloroquine[16]. Chloroquine is more effective than quinine in suppressing the growth of *Plasmodium* during the erythrocytic cycle. Primaquine is also a synthetic drug and acts upon both the blood and tissue stages of the parasite, thus producing complete cures and preventing relapses[16].

The insecticide known as DDT was used at the end of World War II as a form of malarial control[5, 8, 16]. With the success of DDT and the production of more effective antimalarial medications, the World Health Organization submitted a proposal in 1955 for the eradication of malaria worldwide[8, 16]. Success was limited, because malaria was eliminated in nations with temperate climates and seasonal malaria. Some countries had vast reductions in malaria cases, but then cases substantially increased when eradication efforts stopped[8]. Completion of the eradication campaign was eventually abandoned, due to the emergence of drug resistance, resistance to insecticides, wars and population movements, and lack of long-term funding from donor countries[8]. The current goal is to reduce the number of malaria-related cases and deaths using a variety of approaches aimed to circumvent factors that caused the eradication campaign to fail.

Malaria has been a difficult disease to eradicate. Mosquitoes have consistently become resistant to the insecticides that are being used and there is a lack of an effective malarial vaccine. Wearing personal protective clothing and placing netting over beds helps to decrease mosquito contact with human skin, however in some areas and conditions, their use is not feasible or is under-utilized. New methods or revisitation and improvement of older methods need to be considered in order to fight this disease. Recently, genetic modification of male mosquitoes such as the use of the Sterile Insect Technique has shown promise (described in more detail below). Sterile Insect Technique is a species-specific method that relies on releasing large numbers of sterile male mosquitoes into the environment to mate with native female mosquitoes[17].

The aims of mosquito control include preventing mosquito bites, keeping mosquito populations at acceptable densities, minimizing mosquito-vertebrate contact, and reducing the longevity of the female mosquitoes[19]. Eradication of mosquito species or there associated pathogens is no longer viewed as a viable option because getting entirely rid of a living entity might result in trophic issues. A more realistic option is the combination of pest management, personal protection, and reducing disease prevalence.

Personal protection is the most direct and simple approach to preventing mosquito bites. Mesh suits with hoods can be worn over clothing. Head nets reduce bites along the face and neck. Chemical repellants are applied to the skin or clothing which prevents mosquitoes from landing on a person or causes the mosquitoes to leave before "biting" a person. Avoiding the outdoors during peak mosquito activity and having screens on windows and doors can prevent mosquitoes from getting inside human dwellings. Bed nets are infused with synthetic pyrethroid and are strung over beds at night to repel mosquitoes and kill the ones that land on the nets[20]. Other insecticidal devices create a vapor or smoke that reduces mosquito attacks within the vicinity[20].

The use of insecticides as a mosquito control measure is the principle way to kill mosquitoes that bite indoors; however, after prolonged exposure to an insecticide, mosquitoes begin to develop resistance over several generations[20]. Levels of resistance increase quickly due to the multiple generations mosquitoes have per year. In a comprehensive study by Liu (2015), it appears that increased metabolic detoxification of insecticides and decreased sensitivity of the target proteins or genes responsible for such proteins are likely responsible for insecticide resistance.

Altering a mosquito's habitat is also a reliable tool in mosquito management. Harborage alteration renders the adult resting sites unsuitable[21]. Changing the larval habitat which prevents oviposition, hatching, or larval development is known as source reduction. For example, water can be altered or eliminated. Water alteration includes adding plastic foam beds on top of the water which provides a barrier over latrine water, underground sewage lines, waste tire shredding, natural container elimination, lids for water-storage barrels, vegetation changes in a pond, and altered flow in tidal marshes[21].

Biological control of mosquitoes has been studied extensively. Most efforts are directed at the larval stage because aerial predators such as dragonflies and bats do not specialize in adult mosquitoes and have little effect on their population densities[20]. Aquatic predators affect the larval stages. These predators include the Mosquito Fish (*Gambusia affinis*) and the Killifish (*Fundulus* spp.) [20]. Other fish feed on the vegetation that provides harborage for larvae. There have been attempts to develop the use of parasites and pathogens of mosquito larvae as control agents, but they have limited effectiveness and are not routinely used. One exception is the bacterium *Bacillus thuringiensis israelensis*, or *Bti*, which kill the larvae upon ingestion by producing proteinacious toxins[20].

Some *Anopheles* species are poor vectors of malaria. For these species, the *Plasmodium* parasite does not develop well within the mosquito. For example, select strains of *Anopheles gambiae* that are refractory to parasite infection[22]. In these species, the oocysts fail to develop in the refractory mosquitoes and result in ookinete death[22]. There are studies looking into the genetic traits of these mosquitoes and hopes of

releasing genetically modified mosquitoes that are refractory to malaria into the wild mosquito population[22].

Genetic control is a subcategory of biological control. Genetic control has been successful against some pests; however, its use against mosquitoes still remains experimental. There are two main approaches: release of sterilized males, and the introduction of incompatible strains of mosquitoes[21]. The latter approach creates a decrease in the wild mosquito population, and replaces that natural population with strains that are not susceptible to infectious agents. The sterile male approach releases males carrying a dominant lethal gene that allows the males only to have male offspring[21].

Chemical control includes using insecticides that kill larvae or adults. Larvicides are applied to the water where the larvae develop or where water can accumulate providing a larval habitat. Adulticides are applied to surfaces where adults will rest or in the air where they fly. This approach is still widely used on the inner walls of human dwellings. Residual adulticides can also be used outdoors on buildings or vegetation, but have short-term effects because the wind, sun, and rain causes the insecticide to degrade[22].

Today, malaria control depends on epidemiological surveillance, investigations of outbreaks of locally transmitted malaria, the spraying of insecticides, and investigation into novel approaches of pathogen transmission. One such novel approach involves a genetically modified microorganism to help inhibit the spread of the *Plasmodium* parasite. This approach is known as Paratransgenesis.

Paratransgenesis

Despite great advances in public health, insect-transmitted infectious diseases remain a leading cause of morbidity and mortality worldwide [23]. Insects have developed an array of resistance mechanisms by which they can inactivate toxic substances, such as insecticides. Even biological methods, such as the Sterile Insect Technique, have been rendered less effective by changes in insect behavior[24, 30]. From an ecological standpoint, it is not desirable to completely eliminate insect pests because they may and often have an important role in the food chain. This is why development of new methods to reduce the competence of insect vectors and their disease-causing microorganisms is necessary.

Among these new methods is paratransgenesis. Paratransgenesis employs the interactions between pathogen-transmitting vectors, bacterial symbionts of the vectors, and the pathogen[25]. Symbiotic bacteria are isolated from the insect vector and genetically transformed *in vitro* to export molecules that interfere with pathogen transmission. The genetically altered symbionts are then introduced into the host vector, where expression of engineered molecules affects the host's ability to transmit the pathogen, i.e., its vector competence[23]. This approach attempts to decrease pathogen transmission without adverse effects on the vectors themselves.

General requirements must be in order for paratransgenesis to be considered as a potential vector control application. Requirements include: 1) an appropriate symbiotic association must exist within a given pathogen-transmitting vector; 2) bacterial symbionts should be amenable to culture and genetic manipulation; 3) genetically altered symbionts should remain stable; 4) fitness of the genetically altered symbionts should not be

compromised. Furthermore, normal symbiotic functions should not be altered; 5) transgene products released from the genetically altered symbionts should interact effectively with the target pathogen(s); and 6) a method must exist for dispersal of the genetically altered symbionts amongst naturally occurring populations of vectors.

Several projects aim to develop a paratransgenic approach for control of pathogen transmission, particularly in mosquitoes[28, 29]. Significant progress has been made in the area of Chagas disease vectors. Chagas disease is known as American trypanosomiasis, and is caused by the protozoan *Trypanosoma cruzi. T. cruzi* is transmitted by the insects in the family Reduviidae, subfamily Triatominae, and are often referred to as Kissing Bugs[23, 26]. The first demonstration of paratransgenesis was through the expression of Cecropin A by *Rhodococcus rhodnii* within the midgut of the reduviid, *Rhodnius prolixus*[24]. The *T. cruzi* was eliminated almost completely within the insect and the transformed bacteria appeared to maintain a stable relationship within the insect host [24]. Additionally, transformed symbionts were shown to be horizontally transmitted to *R. prolixus* carrying non-transformed symbionts via reduviid coprophagic habits[27].

Paratransgenesis also appears to be a promising approach to reducing African trypanosomes transmission via *Glossina* spp. Machado, the tsetse fly. Genetically transformed *Sodalis*, a bacterial symbiont of the tsetse flies was found to be transmitted vertically through the female milk glands[27]. Vertical transmission was demonstrated 75% of the P_1 offspring and 68% of the P_2 descendants[27]. This indicated that the symbiont was able to spread through the tsetse population. Also, the *Sodalis* was able to be transformed and colonized in a non-native tsetse host species without reducing the hosts' fitness[27, 30].

Current studies include the development of ways to increase the stability of trypanocidal transgene expression over time, and identification of novel and effective effector molecules for use in the system[31].

Paratransgenesis can also be applied to the management of plant diseases. Research findings (i.e. Bextine et al. 2004) indicate a potential use of paratransgenesis to control Pierces disease, an incurable disease of grapevine caused by the plant pathogen, *Xylella fastidiosa*. *Homalodisca coagulata,* the Glassy-Winged Sharpshooter is a xylem-feeding hemipteran and a major vector of *X. fastidiosa.* A symbiont of *H. coagulata* mouthparts, *Alcaligenes xylosoxidans denitrificans* is safe (Biosafety Level 1), easily transformed, and genetically modified. *A. x. denitrificans* persists in the xylem of many host plants of *X. fastidiosa*[31]. Interestingly, it is in the same insect order as *R. prolixus*, Reduviidae, the vector of Chagas disease.

The release and use of a genetically modified organism into the environment raises scientific, social, regulatory, and environmental issues. There are no regulatory guidelines currently in place that fit paratransgenesis. As a result, the science is in a "holding pattern" in terms of field application. Regardless, the science of paratransgenesis is moving forward.

Identification and characterization of the mosquito midgut microbiota is likely to contribute towards better understanding of mosquito biology including longevity, reproduction and mosquito-pathogen interactions that are important to create strategies for vector control mechanisms[32]. Insects can be considered as holobionts, units in which the insect host and its microbiota are often involved in complex reciprocal multipartite

interactions[33]. The bacterial microbiota contribute to an array of biological functions with mosquitoes such as supplying nutrients, inducing resistance to pathogens and parasitoids, and conferring tolerance of temperature stress[33]. While some bacterial symbionts within the mosquito gut may be viable but non-culturable, those that can be cultured offer insight toward the diverse roles bacteria may play within the mosquito.

An interesting phenomenom is observed in mosquito gut microbiota in the metamorphosis of the larval stage to an adult mosquito. During metamorphic transition from the larval form to the adult form, the microbiota is 'cleared' and adult mosquitoes acquire a new set of microorganisms. This process of microbial cleansing and acquisition of new microorganisms is called gut-sterilization[32]. During gut sterilization a mechanism is operating during mosquito metamorphosis and adult emergence. This mechanism appears to involve the sequestration of the remaining larval gut bacteria within the confines of the meconial peritrophic membrane one or two and the possible bactericidal effect of the exuvial (molting) fluid, which is ingested during the process of adult emergence[36].

Moro, et al. (2013) studied the gut microbiota of 104 *Aedes albopictus* in Madagascar. The three major phyla cultured were: Actinobacteria, Firmicutes, and Proteobacteria. *Pantoea,* in the phylum Proteobacteria, was the most abundant genera isolated from both male and female mosquitoes, representing 25.8% of the total isolates compared to the genera *Klebsiella*, *Enterobacter, Acinetobacter,* and *Staphylococcus*, just to name a few[33]. The abundance of *Pantoea* in these mosquitoes suggest that it is a good candidate for paratransgenic management of *Aedes*-borne diseases.

In another study, *Anopheles stephensi* caught in Southern Iran and surveyed for their midgut bacteria yielded: *Pseudomonas*, *Alcaligenes*, *Bordetella*, *Myroides*, and *Aeromonas*[34]. The findings in this study contrasted previous findings (Straif et. al., 1998, Rani et. al., 2009, and Pumpuni et. al., 1996) and reflect the possible effect of biogeographical factors on adaptation of bacterial species based on region of capture[34]. For example, in 1996, a study of wild *Aedes triseriatus*, *Cx. pipiens*, and *Psorophora columbiae* using routine laboratory bacteriologic techniques indicated the presence of *Serratia marcescens*, *Klebsiella ozonae*, *Pseudomonas aeruginosa*, and *Enterobacter (Pantoea) agglomerans*[35] dominant inhabitants in these species.

Pantoea agglomerans is a Gram negative bacterium formerly known as *Enterobacter agglomerans* and belongs to the *Enterobacteriaceae* family. *Pantoea* is a diverse genus and as such, *Pantoea* strains and their possible association with animal hosts and disease remain unclear[37]. In addition, the identification of *Pantoea* species can often be difficult[37]. Despite this, *Pantoea* spp. are currently classified as Biosafety Level 1 bacteria that typically can be found in association with plants, soil, water and insects, including mosquitoes[31].

While assessing the phylogenetic diversity of culturable bacteria in adult mosquitoes, Valiente Moro et al. (2013) found that *Pantoea* was the most abundant genera isolated from both male and female *Aedes* mosquitoes. Given this finding, it is possible that *Pantoea* may be an important partner to the mosquitoes.

Insect hosts and their microbiota can be involved in complex partnerships[31] and in line, a bacterial symbiont, such as *Pantoea* spp. can contribute to different biological

functions within the mosquito, such as breaking down complex macronutrients[39], inducing resistance to pathogens[31], and helping the insect host tolerate environmental stressors[31].

It remains unknown how mosquitoes acquire *Pantoea* spp.; however, most symbionts are acquired either vertically or horizontally. In some cases, symbionts can be acquired through both routes[40]. One of the objectives of my thesis research is to determine if *Culex* mosquitoes can pass *Pantoea agglomerans* vertically.

The aim of my research is to provide information relevant to the goal of using *P. agglomerans* in a paratransgenic strategy for control of pathogen transmission in mosquitoes.

My thesis research involves the following objections:

1. To verify that *P. agglomerans* is horizontally acquired through feeding and establishes in the gut of *Culex pipiens.*

2. To determine if *P. agglomerans* is passed vertically to *C. pipiens*.

Chapter 2

Materials and Methods

Mosquito rearing:

Mosquito pupae and adults used in this research experiment were provided in part by The Alameda County Mosquito Abatement District where an active colony of *Culex pipiens* mosquitoes is maintained. The colony was collected in 1999-2000 from a sewer plant in Oakland, CA. Pupae were culled from larval trays using a 3 ml pipette and transferred to a pupal chamber. A water sample containing larvae was placed in the

bottom portion of the pupal chamber. A plastic lid between the two sections contained a vinyl funnel through which the emerging adults could fly into the upper portion. Aluminum screening on the top of the chamber allowed for ventilation. The pupal chamber used was 8-3/8" high x 4-7/8" diameter (21 x 12 cm), and consisted of two clear, quart-sized styrene containers (BioQuip, Rancho Dominguez, CA). Once pupae were collected and put within the chamber, the chamber was placed inside an 8 cubic feet mosquito cage. The cage's sides consist of insect screening on the sides and top, a ¼ inch thick Plexiglas bottom, and a top half-screened front. The screens were nailed into a 5/16 inch x ¾ in aluminum screen frame. The front bottom half of the cage is split in half vertically. Each quarter is fitted with insect netting that can be tied shut. The mosquito cage also contained an 8 oz. cup of tap water used as a water source for oviposition, and a Petri dish containing 1-3 C&H (Crockett, CA) sugar cubes as a food source for emergent adults. Once the egg rafts were laid, they were transferred to a small larval tray 220 mm X 170 mm with approximately 300 ml of tap water. After 6-7 days, six hundred to eight hundred milliliters of water were added to the larval trays because of evaporation. Once first instar larvae emerged, they were provided with 0.01 g mixture of liver powder and Brewer's yeast hydrolysate (BioServ, Fleming, NJ). Twice a week the powder mixture was mixed with approximately 6-8 oz. of water to create a slurry. Approximately 2 mL of the slurry were then added to the larval trays using a 3 mL disposable plastic transfer pipet by submerging the tip of the pipette beneath the surface of the water. Once the first instars grew into second and third instars, the trays were divided into larger trays measuring 300 mm X 220 mm. The larger trays required 1 to 1.5 liters of water

depending on the larval size. Additional water was added to the trays as needed. The second, third, and fourth instars required 0.1 g Brewer's Yeast hydrolysate twice a week.

Mosquito Treatment:

Once the *Culex pipiens* adults emerged from pupae, they were provisioned with 1-3 C&H sugar cubes on a Petri dish. The sugar cubes were inoculated with a 24 hour old culture of *Pantoea agglomerans* previously modified to express the fluorescent protein, Ds red or a strain of *Pantoea agglomerans* (ATCC) in Tryptic Soya Broth (TSB) (Difco Laboratories, Detroit, MI). Approximately 1 mL of the culture was taken from the culture tube using a 1 mL disposable plastic transfer pipette. Three to five applications (approximately 200 uL) of a fresh (approximately 18 h old) culture of *P. agglomerans* culture were dropped onto the tops of each C&H sugar cube. Male and female *Culex pipiens* adults were given approximately 24-48 hours of exposure to the C&H sugar cubes for verification of horizontal transmission and determination of vertical transmission experiments To insure that mosquitoes would actually feed on bacteria deposited on the sugar cubes, a subset of adult mosquitoes were provisioned with sugar cubes containing combination of *P. agglomerans*, growth medium (TSB) and blue food coloring (Safeway, Pleasanton, CA) and others were provisioned with TSB on sugar cubes. If adults fed on the sugar cubes, the blue dye would be evident in their guts and throughout their abdomens.

At the end of the 24 hours, the adult mosquitoes were collected with a mechanical aspirator which pulls in mosquitoes using a vacuum and into a detachable collection tube. The detachable collection tube was removed from the aspirator once the desired number

of mosquitoes are collected, between 5 and 15 mosquitoes were normally collected at a time. Mosquitoes were killed by freezing and were then transported in the collection tube on ice from the Alameda County Mosquito Abatement District to California State University East Bay campus in Hayward, CA.

Mosquito Dissection

Individual chilled mosquitoes were extracted from the collection chamber using sterilized dissecting forceps. The remaining mosquitoes were left in the collection chamber and on ice during each dissection. The mosquitoes were sexed and then were dissected aseptically under a dissecting microscope. The mosquito's legs and wings were removed until only the proboscis, head, thorax, and abdomen remained. On a Petri dish, the mosquito was held by the thorax using sterile dissecting forceps with the left hand, and with the right hand, the end of the mosquito's abdomen was grasped and pulled using sterile dissecting forceps with the right hand. The mosquito's midgut became exposed. Using a disposable glass Pasteur pipette, 0.5 mL of sterile Phosphate Buffered Saline (PBS) solution was poured onto the midgut and then sucked up into the Pasteur pipette. The sterile PBS and midgut were transferred to a labeled 10 x 100 mL test tube containing 5 mL of sterile nutrient broth (Tryptic Soya Broth). This process was repeated for each mosquito. The test tubes were placed in a 26°C incubator for one week.

Unless the presence of *Pantoea agglomerans* was observed using microscopy directly, all cultures from the mosquitoes were 1) determined pure, 2) characterized by Gram staining and cellular morphology, and 3) colonial morphologies were recorded. Once pure, catalase and oxidase tests were performed.

Verifying *P. agglomerans* Uptake (horizontal transmission)

The dissected midgut was allowed to incubate in TSB 26°C until turbid. Often, the physiological state of bacteria from medium to insect gut back to medium causes a slowed growth in the latter environment (Lauzon, pers. commun.). Each culture was streaked for isolation on to Tryptic Soya Agar plates. The plates were placed into the incubator and were checked approximately 24 hours later. The colonies were viewed and described. Tentative verification of *P. agglomerans* containing the DsRed marker is pink pigmentation to the colonies. Further verification was done using staining, biochemical tests, and API 20e strips (BioMerieux, Cambridge, MA). The tests performed on the isolated midgut bacteria included: Gram staining, Triple Sugar Iron, MacConkey agar plate, Urease Broth, Citrate, Methyl-Red Vogues Proskauer, and Motility Indole Ornithine tests. The results from the biochemical tests were corroborated with the API 20e strip results and data from Phenol Red Broth containing glucose, lactose and sucrose individually. The tests were also corroborated with arginine decarboxylase, lysine decarboxylase, and ornithine decarboxylase tests (Sigma, St. Louis, MO).

As a note, the mosquito-derived symbiont previously identified as *Pantoea agglomerans* at times is biochemically similar to a *Serratia* sp. I found this to be true of the ATCC culture of *P. agglomerans. P. agglomerans* is known to be biochemically plastic and thus, I adhered to the earlier identification of *P. agglomerans* in my analyses. The use of the genetically-modified *P. agglomerans* was important in this regard.

Determination of vertical transmission of *Pantoea* sp. in adult mosquitoes

Egg rafts laid in plastic 8 oz. water cups were acquired from the treated *C. pipiens* adults and distributed into larval trays. Once the eggs hatched, the larvae were reared as stated above. When the P1 larvae metamorphosed into pupae, the pupae were transferred into the bottom part of the pupal chamber using a 1 mL disposable plastic transfer pipette. All of the P1 pupae were then placed into the adult mosquito cage and allowed to emerge and feed on one non-inoculated sugar cube.

Adult P1 mosquitoes were collected, dissected, and cultured as described above. The dissected midgut was allowed to incubate in TSB at 26°C. Each culture was streaked for isolation on to Tryptic Soya Agar plates. The plates were placed into the incubator and were checked approximately 24 h later. The colonies were viewed and described. Identification of *P. agglomerans* was achieved as described earlier.

A few P1 adult mosquitoes were dissected with their midguts and eggs placed on clean glass microscope lenses with phosphate buffered saline. They were individually covered with a clear glass cover slip and the corners were painted with clear nail polish. The midguts were viewed using a Leica TCS SPE-II Confocal Laser Scanning Microscope or an Olympus Fluorescent BX51 Microscope.

Chapter 3

Results

Two limitations existed in my research: 1) The capture of mosquitoes from cages at the Alameda laboratory often involved uneven distribution of male and female adults, and 2) Ages of emergent mosquitoes may have differed enough to affect feeding

behavior. Data revealed (presented below) that neither of these limitations affected the overall aim of my work in determining if *Culex pipiens* adult mosquitoes acquire symbionts solely through feeding and/or contact (horizontal transmission) solely or through a mix of horizontal and vertical transmission.

Before experiments began, I first made certain that mosquitoes would feed on a sugar diet containing bacteria. After 72 h of exposure to control and *Pantoea agglomerans* diets containing blue food coloring, mean feeding rates for adults provisioned with the bacterial diet were 57% and 35% for adults on a sugar diet (data not shown).

Experiment 1: Verification of horizontal transmission of a *Pantoea* sp. in adult mosquitoes.

Seventy one percent (5/7) of mosquitoes yielded bacterial cultures. Eight isolates were retrieved from these mosquitoes that fed on a mosquito-derived genetically-modified symbiont. Forty percent of the isolates were identified as the *Pantoea* sp. and these isolates were obtained from three of the five mosquitoes (Table 1). This strain of *Pantoea* was not isolated from control mosquitoes.

In a second test, a strain of *Pantoea agglomerans* (ATCC 49010) was used. In this case, fifty nine percent of mosquitoes yielded 22 bacterial cultures. *P. agglomerans* was isolated from 68 % percent of the mosquitoes (15/22) (Table 1).

Table 1. Verification of horizontal transmission of *Pantoea* sp. in adult *Culex pipiens*.

	Number of *Culex pipiens* Dissected	% *Culex* yielding bacterial cultures	% of cultures identified as DsRed *Pantoea*	% *Culex* containing DsRed *Pantoea*
Treatment 1	7	71	40	60
Treatment 2	22	50	68	58
Control	5	67	58*	22*

*Naturally occurring *Pantoea* sp. not introduced

In addition, confocal laser scanning and fluorescent microscopy was used to verify the presence of *Pantoea* containing the fluorescent marker (Fig. 5). *Pantoea* was found in 100% of the treatment mosquitoes (n=4) and not observed in control mosquitoes (n=5) (Figure 6 and 7). The *Pantoea* containing Ds red was found on eggs within females (Figure 8).

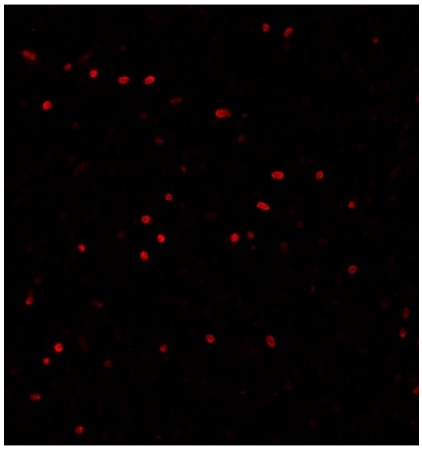


Figure 5. Confocal laser scanning image of *P. agglomerans* expressing DsRed fluorescent protein, 100X excitation 558 nm.

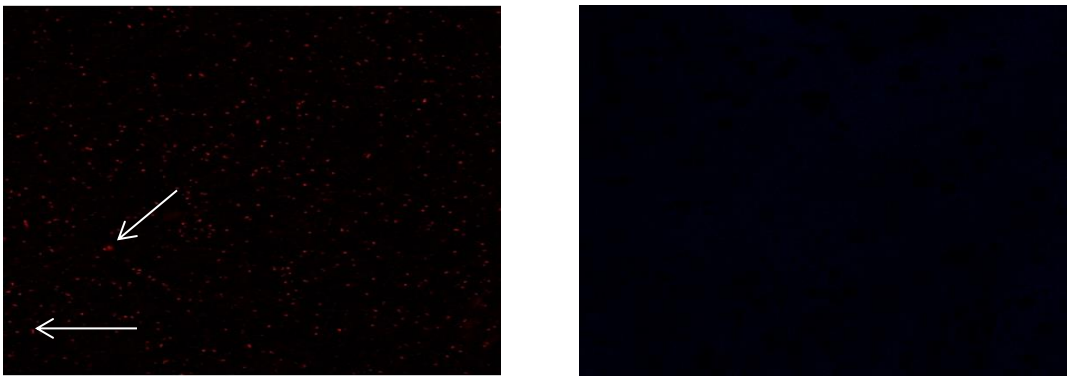


Figure 6. Confocal laser scanning image of *P. agglomerans* expressing DsRed fluorescent protein within the midgut of an adult female *C. pipiens* (left) and a *C. pipiens* midgut from a control adult (right). Magnification =10X

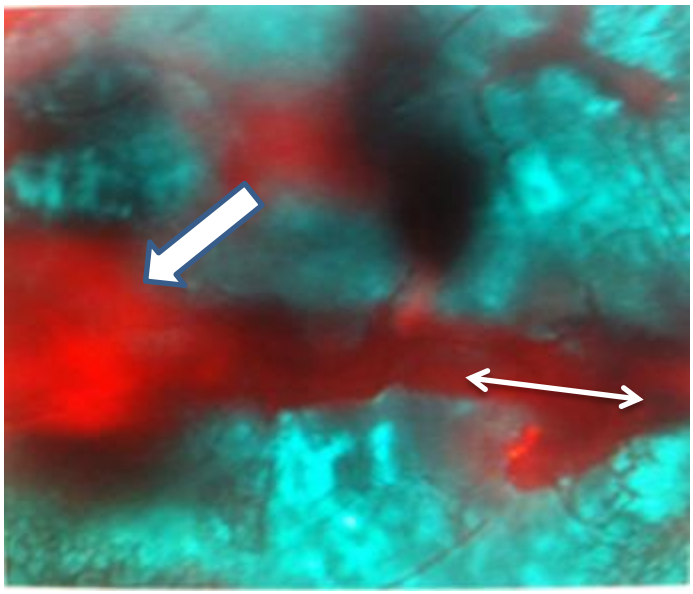


Figure 7. Fluorescent image of the crop (single arrow) and midgut (double arrow), transected, of an adult female *C. pipiens* containing *P. agglomerans* with Ds red (thick arrow). Magn. = 10x

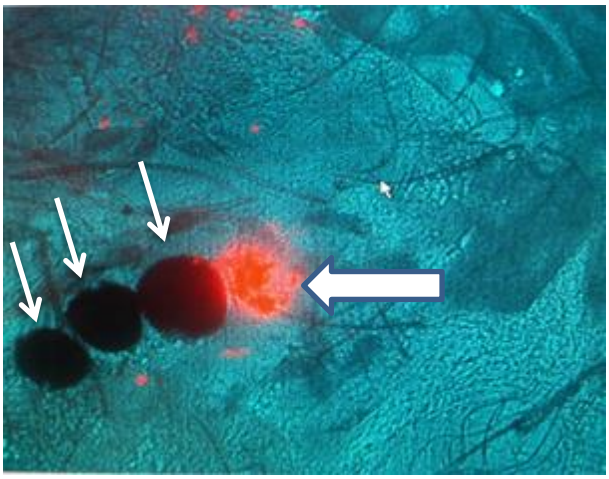


Figure 8. Fluorescent image of eggs (thin arrows) containing *P. agglomerans* containing Ds red dissected from within a female *C. pipiens*. Notice the presence of *P. agglomerans* in surrounding reproductive tissue (thick arrows). Magnification= 10x.

Experiment 2: Determination of vertical transmission of a *Pantoea* sp. in mosquito adults.

Mosquitoes that fed on *Pantoea* were allowed to mate. Eggs collected from females were reared to adulthood (P1). In replicate one, emergent adults (n=8) yielded 8 isolates and none were identified as *Pantoea*. In replicate two, fifty percent of P1 adults yielded twelve isolates. Thirty percent of the isolates were identified as *Pantoea.* In replicate three, three adults yielded three isolates, all of which (100%) were identified as *Pantoea* (Table 2). Sixty seven percent of control mosquitoes yielded 19 isolates and 58% were identified as *Pantoea* sp. but not *Pantoea* containing the fluorescent marker.

Table 2. Percent vertical passage of *Pantoea* sp. in P1 generation *C. pipiens*

	Number of *Culex pipiens* Dissected	% *Culex* yielding bacterial cultures	% of cultures identified as DsRed *Pantoea*
Replicate 1	8	100	0
Replicate 2	7	50	30
Replicate 3	3	100	100
Control	5	60	58*

*Naturally occurring *Pantoea* spp. not introduced

Chapter 4

Discussion

I found that upon ingestion *Pantoea agglomerans* could later be recovered from the gut of adult *C. pipiens. P. agglomerans* has been reported to be isolated from many different insect species such as *Schistocera gregaria*[47], *Rhagoletis pomonella*[48] and from mosquitoes[28, 48]. *P. agglomerans* is a typical inhabitant of soil, plants, and natural waters[49] and likely insects pick up this bacterium while feeding on natural food sources such as bird feces and/or contact with sources[50]. *P. agglomerans* is a free-living bacterium in these sources and thus can easily serve as an inoculum[51]. Other insects acquire important or necessary symbionts through coprophagy. For example, *Rhodnius prolixus*, the kissing bug, demonstrate symbiont dispersal and acquisition when hatching nymphs feed on adult *R. prolixus* frass. Microbiota rapidly populate inside their guts and provide supplementary dietary needs[23]. *Rhodococcus rhodnii* is one of the bacterial symbionts *R. prolixus* nymphs acquire from their mother's feces. *R. rhodnii* plays a vital role in the growth and development of *R. prolixs* and without these bacteria, most nymphs die after the second developmental molt[25]. The roles of bacterial symbionts have also been evaluated in other insect disease vectors. The *Glossina* spp. (tsetse) vectors of African sleeping sickness (trypanosomiasis) harbor as many as three distinct populations of bacterial species. Like *R. prolixus*, tsetse flies are obligate blood feeders, and at least one population of bacteria is presumed to be nutritional mutualist symbionts[25].

To my knowledge and for the first time, I found that *P. agglomerans* is vertically passed from female *Culex* adults to next generation adults. It remains unclear what

factors are involved that select for vertical transmission in insects but it is likely that pressures arose from mixed transmission, horizontal and vertical, with selection favoring vertical acquisition[52]. Mixed patterns of symbiont transmission behavior in insects have been observed to be common[53] and likely reflect how a host adapts to various environmental niches. Lauzon et al. (2009) found that the Mediterranean fruit fly, *Ceratitis capitate* engages in symbiont dispersal both horizontally and vertically and suggested that multiple acquisitions and replacements of symbionts these tephritids suggest that symbionts play vital roles in overcoming biological constraints encountered by these insects in nature.

Bacterial symbionts that are transmitted to offspring via vertical transmission could be important for the health and growth of the offspring into adulthood. For example, the cereal weevil, *Sitophilus oryzae*, harbors intracellular symbiotic bacteria which supply the weevil with several vitamins such as pantothenic acid, riboflavin, and interact with mitochondrial oxidative phosphorylation by increasing the mitochondrial respiratory control ratio and mitochondrial enzymatic activities[46]. *Sitophilus oryzae* principle endocytobiotes also have physiological and behavioral consequences on their hosts: they increase the host fertility, decrease the larval development time, and increase the flying ability of adults[46].

Symbiont transfer from parent to progeny in some cases has been well defined such as in the case of *Wolbachia* spp. that infect insects[51]. *Wolbachia* is a genus of intracellular bacteria found in many species of arthropods[25]. *Wolbachia* are maternally transmitted from parent to offspring and are often involved in a variety of reproductive

anomalies, such as cytoplasmic incompatibility (reproductive incompatibility due to maternal, nongenetic factors), sex ratio determination and distortions, and parthenogenesis[25].

Asaia is a bacterium found in the gut of *Anopheles* spp. and *Aedes* spp. *Asaia* has been found as an extracellular bacterium in the gut, the salivary glands, and reproductive organs, from which it is transmitted vertically to the progeny, venereally from males to females, and then to the offspring[38]. *Asaia* is also transmitted horizontally, through the co-feeding of mosquitoes on the same food source[38]. The diverse mode of transmission of *Asaia* spp. among mosquitoes indicates that these insects are potentially exposed to diverse strains, suggesting that differently from symbionts transmitted only vertically, several strains could coexist in different populations and even within single insect individuals[38].

I found that *P. agglomerans* can successfully be transmitted vertically and it offers great potential for use in impacting mosquito-borne pathogen transmission. Stable passage of *P. agglomerans* through subsequent mosquito populations through mixed acquisition would more rapidly control pathogen spread. In addition, *P. agglomerans* offers a way to decrease mosquito populations through direct contact with eggs. It is possible that *P. agglomerans* could be modified to express an ovicide.

Paratransgenesis appears to be a promising strategy to reduce African trypanosomes transmission by tsetse flies using a genetically transformed *Sodalis*, a symbiont of the tsetse fly. *Sodalis* was transformed with GFP, and colonized in non-native tsetse host species at a density similar to a native colonization and without

reducing host fitness[27]. A recombinant bacterium with minimal fitness cost to mosquitoes is also an important factor for the success of future field applications. According to Wang et al. (2012), no significant difference in mosquito lifespan was detected among any of the mosquito groups when they tested the impact of a recombinant *P. agglomerans* expressing anti-*Plasmodium* molecules on the mosquitoes. This suggests that these recombinant anti-Plasmodium products pose no obvious negative impact on mosquito fitness in laboratory conditions[54]. In my work, it appeared that *C. pipiens* did not show any obvious fitness costs post-consumption and establishment of *P. agglomerans* yet this area needs further research.

The release of any genetically modified bacterium (GMB) into nature poses several issues. Horizontal transfer of the transgene of interest between mosquito sibling species and other microorganisms in nature are of primary concern. Technology to prevent the potential horizontal transposon transfer by viruses and to inhibit transposition activity mediated by endogenous transposases still needs to be developed[27]. Researchers have countered concern with GMB release, however, through the development of GMB that can only exist in the presence of synthetic compounds not found naturally in the environment[55, 56]. In this case, known as biocontainment, a GMB cannot metabolically bypass their biocontainment machinery using natural compounds, and they demonstrate resistance to horizontal gene transfer and mutagenesis[55].

Pantoea agglomerans has been described as "The *Pantoea agglomerans* complex" and was previously designated *Erwinia herbicola* and *Enterobacter agglomerans*[57]. Deletoile et al. (2009) stated that "most strains initially identified as *P.*

agglomerans by use of API 20E strips belonged to diverse phylogenetic branches corresponding to other species of *Pantoea* or *Enterobacteriaceae* and to the probable novel species". The strain of *Pantoea agglomerans* that I used in this work as well as the reference strain acquired from the American Type Culture Collection often provided fluctuating biochemical results. As such, monitoring this bacterium in any applied or release setting mandates the use of a marker to insure originality. This would be a key criterion for risk assessment and environmental impact studies. At the very least, a multilocus gene sequencing method that has been determined to be a sound tool for *Pantoea* species delineation and identification might assist for strain tracking.

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