Title

Behavioral and physiological ecology of mosquito disease vectors (Diptera: Culicidae) as a function of aquatic macrophyte invasions

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by

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University of California, Berkeley

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Abstract

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Doctor of Philosophy in Environmental Science, Policy, and Management

University of California, Berkeley

Professor George K. Roderick, Chair

To investigate the impact of invasive aquatic weeds on mosquito populations in the Sacramento-San Joaquin River Delta, field and laboratory experiments were conducted to test the impact of invasive aquatic plants (water hyacinth, water primrose, and Brazilian waterweed) on the behavioral ecology of *Culex pipiens*, a primary mosquito vector for West Nile Virus (WNV). In an outdoor caged experiment containing larval mosquitoes and predatory Mosquitofish, mosquito survival was significantly higher among high densities of the three plant species than vegetation-free water. In intermediate plant densities, mosquito survival was higher among water hyacinth than both Brazilian waterweed and water primrose. In low plant densities, mosquito survival was higher among water hyacinth than Brazilian waterweed and vegetation-free water. In another caged experiment containing mesocosms, mosquito larval development time was completed more rapidly in the presence of intermediate densities of water hyacinth than all other treatments. In an outdoor caged choice experiment, mosquitoes laid more eggs in mesocosms containing intermediate densities of water hyacinth than all other treatments. Laboratory choice tests and an olfactometer experiment revealed that mosquitoes were more attracted to water that contained plants or plant infusions than water alone. These results suggest that water hyacinth provides both physical and chemical cues to some species of mosquitoes. Effective management of invasive water hyacinth in waterways may thus reduce mosquito populations and reduce human health risk.

Dedication

This dissertation is dedicated to my wife, Kenyetta, who, after living in Florida all her life, graduating from college and marrying me, booked a one-way ticket to rural Ithaca, NY where I was pursuing my M.S. degree at Cornell University. After having established herself and making friends in NY over the course of that following year she showed me her support once again when I decided to relocate us to Berkeley, CA so that I could pursue my Ph.D. at the University of California, Berkeley. I am thankful to her for providing to us our beautiful children who were born during this academic journey.

This is also dedicated to my mother and the rest of my family and friends who supported me along the way.

Quote

“If we knew what we were doing it would not be called research, would it?”

-- Albert Einstein

Table of Contents

Chapter 5. Flight and oviposition responses in mosquito disease vectors, *Culex pipiens*, *Aedes aegypti*, and *Anopheles quadrimaculatus* (Diptera: Culicidae): Impacts by invasive aquatic macrophytes and their infusions……………….…57

Appendices

List of Figures

List of Tables

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Chapter 1

Introduction

Literature Review

Environmental heterogeneity is known to shift species interactions in space and time (Thompson 1994, Leathwick and Austin 2001, Warren et al. 2010, Wiescher et al. 2011), and can significantly impact the distribution of organisms (Orr 1991). Patches of vegetation of varying sizes that occur in a defined geographic area is one way in which an environment may be considered heterogeneous and complex (Wiens 1977). Such an environment is often observed in freshwater habitats where aquatic macroinvertebrates are differentially influenced by aquatic macrophytes of different structural characteristics (Heck and Crowder 1991). These different plant characteristics may influence the quantity and quality of habitat available to aquatic macrophyte-associated organisms including insects (Dudley et al. 1986) and fish (Rozas and Odum 1988), and affect interactions between these groups. For instance, complexities within an environment can differentially influence aquatic organisms through the provision of predator-free refuge (Heck and Crowder 1991; Gotceitas and Colgan 1989), protection from turbidity (Orr and Resh 1989), egg-laying, resting and emergence sites (Orr and Resh 1992; Rooke 1984), surfaces on which epiphytes may attach (Diehl 1988), conducive microclimates (Lodge et al. 1989), and enhanced food resources (Soszka 1975). These types of complex, heterogeneous environments could also impact food webs (Power 1992), especially habitat structure created by invasive plants, which possess numerous attributes explaining their invasiveness (Hussner 2010) and often disrupt tropic interactions in native communities (Harvey and Fortuna 2012) for example by outcompeting native plant species (Dutarte 2004).

Additionally, invasive aquatic macrophyte species can impact not only aquatic macroinvertebrate communities but also humans. Some invasive aquatic macrophyte species can create unique habitat structure that indirectly increases risks to human health by facilitating populations of virulent disease vectors (Mack and Smith 2011), such as mosquitoes (Orr and Resh 1991) whose immature stages undergo aquatic development. Water hyacinth, *Eichhornia crassipes*, a notorious invasive aquatic macrophyte species, has been linked to malaria incidence for decades by its ability to enhance habitat availability to *Anopheles* mosquitoes, the primary vectors of malaria (Gopal 1987). Through its growth proliferation and dense concentrations of rametes the invader forms large mats on the surface of the water. These large mats make water stagnant by slowing down currents, a condition necessary for *Anopheles* oviposition (Merritt et al. 1992), and thus influencing the fitness, abundance and distribution of adult mosquitoes (Rejmankova et al. 2013). Water hyacinth has also been shown to indirectly increase disease risk to humans by supporting all developmental stages of *Mansonia* (Chandra et al. 2006; Burton 1960), a mosquito genus that infects humans with a nematode that causes lymphatic filariasis (Roberts and Janovy 2009). Orr and Resh (1992) demonstrated that habitat heterogeneity produced by the invasive parrotfeather, *Myriophyllum aquaticum*, also strongly influences the local distribution and abundance of *Anopheles* mosquitoes.

Invasive big sage, *Lantana camara*, cultivated in human settlements for its decorative aesthetic value can indirectly increase disease risk by enhancing the availability of resting sites for the tsetse fly, *Glossina spp.*, a vector of trypanosomiasis (sleeping sickness) (Mack 2001; Okoth 1986; Willett 1965). The deadly fly is provided habitat by the invader's impenetrable thicket of sprawling, intertwined (and often spiny) stems on otherwise open sites (Mack and Smith 2011). Syed and Guerin (2004) also demonstrated that the tsetse fly is attracted to *Lantana* leaf phytochemicals through wind tunnel experiments. In addition to habitat structure created by invasive plants, human host proximity to these plants also increases disease risk. The concentration of people living alongside fresh water bodies can exacerbate disease incidence by providing a large group of susceptible hosts (Morse 1995).

While an ecological context of disease transmission at broad levels has not been ignored, evidenced by studies and reviews on the biology of human parasites, their vectors, and other modes of dispersal and transmission (Rothman et al. 2008; Gregg 2002; Sousa and Grosholz 1991), identifying and preventing new categories and examples of disease transmission and risk is necessary to help protect human health. Such a new category involves the link between invasive plants and human disease risk. Identifying particular plant functional groups or life forms that facilitate disease vector populations deserve more attention (Mack and Smith 2011). One geographic area in California's Central Valley that has been unexplored in this research context is the Sacramento-San Joaquin River Delta ("the Delta"), formed by the confluence of California's two primary waterways, the Sacramento and San Joaquin Rivers. High levels of invasive aquatic vegetation occurring across a labyrinth of sloughs characterize the Delta, and West Nile Virus incidences have been steadily increasing in the Central Valley region. Thus, this setting presents a unique opportunity to investigate how habitat structure of different species of invasive aquatic macrophytes influences the fitness, behavior, and population dynamics of mosquitoes, particularly *Culex pipiens*, a primary vector of West Nile Virus.

Overview of Mosquito Species and their Associated Diseases

The transmission of mosquito-borne arboviruses of public health concern is greatly influenced by mosquito biology, behavior, and ecology, which in turn is influenced by a variety of factors including the environmental complexity (Farajollahi et al. 2011). The biology, behavior, and ecology of mosquitoes varies across genera and species, as does specific diseases with which they are associated.

The northern house mosquito, *Culex pipiens* L., and the southern house mosquito, *C. quinquefasciatus* Say, are common bridge vectors of West Nile virus in humans (Hamer et al. 2008). The latter species is also associated with lymphatic filariasis which impacts over 120 million people per year (Rinker, Pitts, and Zwiebel 2016).The yellow fever mosquito, *Aedes aegypti* L. *in* Hasselquist, is the primary vector of dengue, chikungunya, and yellow fever (Rinker, Pitts, and Zwiebel 2016). This mosquito has also been implicated in the recent outbreaks of Zika virus. The common malaria mosquito, *Anopheles quadrimaculatus* Say, is the primary vector of malaria, which impacts over 198 million people per year (Rinker, Pitts, and Zwiebel 2016).

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Chapter 2

Oviposition behavior in a mosquito disease vector, *Culex pipiens* (Diptera: Culicidae): Impacts by different invasive aquatic macrophytes

Abstract

Invasive aquatic plants tend to alter community dynamics within ecosystems, which in turn may impact the oviposition behavior of pest groups such as mosquitoes, in which the immature stages undergo aquatic development. Mosquitoes discriminate among potential oviposition sites based on factors such as temperature, light, and turbidity, and different species and types of aquatic vegetation may differentially impact these factors. Here we examine the impact of aquatic macrophytes on the oviposition preference of *Culex pipiens*, a primary West Nile virus mosquito vector in the Sacramento-San Joaquin Delta of California. The species of plants examined included *Eichhornia crassipes* (floating water hyacinth - invasive), *Ludwigia hexapetala* (emergent water primrose – invasive), *Myriophyllum aquaticum* (emergent parrotfeather – invasive), *Hydrocotyle umbellata* (floating pennywort – native), and *Azolla filiculoides* (floating mosquitofern – native). In a greenhouse cage choice bioassay, the highest proportions (36-40%) of egg rafts were laid among water hyacinth and water primrose, followed by pennywort (16%) and then parrotfeather (5%), while mosquitofern and the control (open water) did not differ (< 1%). A higher (by 19%) proportion of egg rafts was laid among invasive than native plants. In an outdoor caged choice experiment involving water hyacinth, water primrose, and a submersed species, *Egeria densa* (Brazilian waterweed)), water hyacinth and water primrose received 36% and 25% of egg rafts, respectively, which was four to six-fold higher than the proportions of egg rafts among Brazilian waterweed regardless of its density. Water among plants at intermediate plant densities received over two-fold higher proportions of egg rafts than among plants at high density and over 10-fold more than amon6 those at low density, regardless of plant species. Both water hyacinth and water primrose at intermediate density attracted almost 3-fold higher proportions of egg rafts than did these same weeds at high density, and 4 to 17-higher proportions than at low density.

Introduction

Selection of a suitable oviposition site within a microhabitat is critical for optimizing female reproductive success, particularly in aquatic environments (Millar et al. 1994). Various biotic and abiotic factors can influence the conduciveness of a site for aquatic immature stage survival and growth, creating selection pressure for gravid females to show preferences among potential oviposition sites (Petranka and Fakhourry 1991). Such selectivity is particularly noticed within groups whose immature stages are highly sensitive to environmental stresses and biotic mortality factors such as predators (Kifilawi, Blaustein, and Mangel 2003) and competitors (Blaustein et al. 2004) due to an inability to travel far from initial sites of oviposition (Onyabe and Roitberg 1997; Spencer et al. 2002). Vegetation is a biotic factor that can influence the suitability of local habitats for various animal groups (Neuman 1971; Tian et al. 1993; Downie 1995). For example, Liu et al. (2016) demonstrated that an invasive bullfrog species preferred waters with a high proportion of emergent plant coverage for oviposition. However, another study by Frouz (1997) revealed that terrestrial chironomids preferred to oviposit at sites with open and low levels of vegetation.

One important group of organisms that show discrimination during oviposition site selection is the mosquitoes (Takken and Knoll 1999; Blackwell and Johnson 2000; McCall 2002), whose immature stages develop in aquatic environments. Physical, chemical, and physiological factors including temperature, exposure to light, and water chemistry influence oviposition site preference in mosquitoes (Bentley and Day 1989; Lee 1991), which in turn may impact the hatch and larval survival rate and development time of mosquito eggs and larvae, respectively. Aquatic vegetation may be among the physical and chemical cues that play an important role in mosquito oviposition site selection. Different plant species may, to different degrees, alter air and water microhabitat temperature (Dale and Gillespie 1976, 1977, and 1978), light penetration through the water column, water velocity, and chemistry of an aquatic environment (Chambers 1999). These are all factors that contribute to the decision by females to select any given site for egg laying. However, there have not been adequate studies in the literature assessing the impact of aquatic vegetation on mosquito oviposition behavior.

Floating water hyacinth, *Eichhornia crassipes* (Mart.) Solms. (Pontederiaceae), is one of the world’s worst invasive weeds, reducing water availability and conveyance for human consumption and agriculture, impeding navigation, altering water quality, and degrading aquatic ecosystems (Villamagna

& Murphy 2010; Schoelhammer et al. 2012; Getsinger et al. 2014). Floating, rooted water primrose (*Ludwigia* spp.) (Onagraceae), consisting of a mix of invasive species and hybrids in the western U.S. (Hoch and Grewell 2012), spreads from shoreline habitats and disperses as floating fragments, choking rivers and canals (Okada et al. 2009). Submersed, rooted Brazilian waterweed (*Egeria densa* Planch.) (Hydrocharitaceae) has invaded rivers, sloughs and canals in the western U.S. (Pennington and Sytsma 2009), reducing water flow, hindering navigation and altering water quality (Yarrow et al. 2009). All three of these weeds are widespread non-native, invasive aquatic weeds in the Sacramento-San Joaquin Delta of northern California (Santos et al 2009, 2011). Other plants, such as non-native parrotfeather *Myriophyllum aquaticum* (Vell.) Verdc.) (Haloragaceae), as well as native floating mosquitofern (*Azolla filiculoides* Lam) (Azollaceae) and pennywort *Hydrocotyle umbellata* L.) (Araliaceae) can be locally invasive (Richerson and Grigarick 1967; Santos et al. 2009; Sytsma and Anderson 1993). Any or all of these aquatic plants may provide habitat superior to open water for larval mosquito development (Ofulla et al. 2010) but suitability is likely to vary due to variation in plant stature.

Approximately 20% of all infectious diseases in humans are caused by pathogens transmitted by vectors (Rinker, Pitts, and Zwiebel 2016), including mosquitoes in the *Culex*, *Aedes*, and *Anopheles* genera. As in many regions, control of *C. pipiens* and other mosquitos in California, including the Sacramento-San Joaquin Delta, is assessed in large part in terms of abatement of WNV transmission risk through monitoring of +WNV mosquito pools, and reduction of mosquito populations in and around aquatic habitats near human population centers (California Department of Public Health 2014). Invasive aquatic plants may affect the WNV exposure risk profile through their potential impacts on mosquito adult oviposition and larval survival. Elucidation of these interactions is thus likely to provide critical information for integrated mosquito population management.

This study was initially run as a greenhouse bioassay to examine the impacts of five floating and (except for mosquitofern) emergent aquatic plants on oviposition of egg rafts by *C. pipiens*. .I compared the relative oviposition responses of *C. pipiens* to three invasive aquatic macrophyte species – water hyacinth, yellow water primrose, and parrotfeather, and two native species – pennywort and mosquitofern. Based on the outcome of this study, a second outdoor caged experiment was conducted in which I compared the relative oviposition responses of *C. pipiens* to three invasive aquatic macrophyte species, including water hyacinth and water primrose, as well as submersed Brazilian waterweed, across three density levels. I hypothesized that the proportion of egg rafts laid by C. *pipiens* would be dependent on both plant species and density, and would differ between non-native and native aquatic plant species.

Materials and Methods

Biological materials. Adults of *C. pipiens* were obtained from laboratory colonies at the San Joaquin Mosquito and Vector Control District in Stockton, CA, and were reared as described in (Gerberg, Barnard, and Ward 1994). Floating non-native, invasive water hyacinth, *Eichhornia crassipes* (Mart.) Solms. (Pontederiaceae) was obtained from a greenhouse colony at the USDA-Western Regional Research Center, Albany, CA, and were maintained as in Moran et al. (2016). Emergent, rooted invasive water-yellow primrose (*Ludwigia hexapetala* (Hook. & Arn) Zardini et al, *Ludwigia grandiflora* (Michx.) Greuter & Burdet), or *Ludwigia peploides* subsp. *montevidensis* (Spreng.) P.H. Raven/*Ludiwigia peploides* subsp. *peploides* (Hoch and Grewell 2012) (Onagraceae), invasive Brazilian waterweed (*Egeria densa* Planch.) (Hydrocharitaceae), invasive parrotfeather (*Myriophyllum aquaticum* (Vell.) Verdc.) (Haloragaceae), native floating pennywort (*Hydrocotyle umbellata* L.) (Araliaceae), and native floating mosquitofern (*Azolla filiculoides* Lam) (Azollaceae) were collected from three field site in the Sacramento-San Joaquin Delta and maintained under water nutrient conditions similar to those used for water hyacinth.

Bioassay experimental design. A greenhouse cage-enclosure choice study was performed to investigate the effect of plant species (water hyacinth, water primrose, parrotfeather, pennywort, and mosquito fern) and plant status (invasive and native) on mosquito oviposition, defined as the mean proportion of egg rafts laid. The study was conducted during March and April 2015 in a greenhouse facility at the USDA-ARS Western Regional Research Center, Exotic and Invasive Weeds Research Unit, in Albany, CA. Temperature was maintained at 18°-30° C and natural light (daylength 14 hours). Within one mesh lumite cage (3 m long x 2.0 m wide x 2.2 m tall, 32-mesh, < 0.5 mm) (Bioquip, Rancho Dominquez, CA), a total of six clear plastic tanks (100 L volume; 85.7 cm long x 49.2 cm wide x 33.9 cm deep) were filled with 2/3rd of a bag (12 kg) of sand (KolorScape, Atlanta, GA) and 2/3rd of a bag (12 kg) of rock pellets (Vigoro, Lake Forest, IL). Dechlorinated water was added to each container to a height of 7 cm from the top. An aeration pump was added to each tank to add oxygen; movement of the water in each tank due to pumping was minimal. To standardize measurement of plant abundance across species, plant material for each species was added in each tank such that estimated 70-80% water surface coverage was achieved. Plants were allowed one month before the first replicate to root (water primrose and parrotfeather) and acclimate to container conditions, and 1-2 wk before subsequent replicates to recover from culling-related disturbance. Plant densities were maintained by removing plants (water hyacinth, pennywort, and mosquitofern) or trimming (water primrose and parrotfeather).

At the start of each experimental replicate, 80 *C. pipiens* adult females were introduced into the cage. Each tank was initially observed for 15 minutes to ensure that adult mosquitoes remained in good condition following release. Thereafter each container was observed once every 24 hours over five days. At each observation time the number of egg rafts in each of the six tanks was determined, and all egg rafts were removed. The next 24-hour period was thus considered a separate experimental replicate. The 24-hour bioassay was thus conducted a total of 20 times with 4 cohorts of adult mosquitos (i.e, five 24-hour bioassays per cohort). The six tanks were moved haphazardly within the cage between cohorts.

Outdoor Caged Experiment

A 3x3 factorial design outdoor cage-enclosure study investigating the effect of plant species (water hyacinth, water primrose, and Brazilian waterweed) and plant density (high, intermediate, and low) on mosquito oviposition behavior was conducted during June and July 2015 at the Aquatic Weed Research Laboratory of the USDA-Agricultural Research Service, Exotic and Invasive Weeds Research Unit, in Davis, CA where daily average outdoor temperatures averaged 29.9 °C (high), (12.6 °C (low). Within one of the same type of mesh lumite cage as was used in the greenhouse study, a total of 10 similar clear plastic tanks were filled with sand and gravel as above Dechlorinated water was added to each container to a height of 7 cm from the top. To standardize measurement of plant abundance across species, an estimation of percent area coverage was used as follows: 80-100% tank cover = high density, 50-80% tank cover = intermediate density and 10-50% tank cover = low density. The following treatment combinations were established: 1) water hyacinth – high density; 2) water hyacinth – intermediate density; 3) water hyacinth – low density; 4) water primrose – high density; 5) water primrose – intermediate density; 6) water primrose – low density; 7) Brazilian waterweed – high density; 8) Brazilian waterweed – intermediate density; 9) Brazilian waterweed – low density; or 10) control – no plant added.

Plants were allowed one month before the first replicate to root (water primrose and Brazilian waterweed) and acclimate to container conditions, and 1-2 wk before subsequent replicates to acclimate to recover from culling-related disturbance. Plant densities were maintained by removing plants (water hyacinth) or trimming (water primrose and Brazilian waterweed).

At the start of each experimental replicate, 100 *C. pipiens* adult females were introduced into the cage. Each tank was initially observed for 15 minutes to ensure that adult mosquitoes remained in good condition following release. Thereafter each container was observed once every 24 hours over 72 hours. At each observation time the number of egg rafts in each of the 10 tanks was determined. The experiment was conducted nine times (completely randomized block design with start dates as blocks), and containers were moved haphazardly within the cage between replicates (N adults per cage per replicate = 100 ; N total adults across replicates = 900).

Data analysis. Data were analyzed using generalized linear modeling (GLM) in SAS (Version 9.4), SAS Institute, Cary, NC, PROC GLIMMIX) with a binomial distribution assumption and random residual effect. For the greenhouse bioassay experiment, the analysis examined the main effect of plant species (water hyacinth, water primrose, parrotfeather, pennywort, and mosquitofern, or control-open water) across the 20 replicate tests. A subsequent analysis grouped the aquatic plants according to invasive status (two native species vs. three non-native and invasive species, control tanks omitted) on the mean proportion of egg rafts laid by *Culex pipiens* mosquitoes. In both analyses, Tukey's post-hoc mean adjustment and multiple comparisons tests were used to compare differences between specific groups. For the outdoor caged experiment, similar GLMs were run to assess the effects on mean proportion of egg rafts laid of the two independent variables (plant species and plant density), first grouped across density and species, respectively, and then as a two-factor analysis with interaction.

Results

Greenhouse Choice Bioassay

The effects of mosquito cohort, and of 24-hr replicate number (first to fifth day after adding adults) within each cohort, as well as their interactions with the plant species factor, were not significant, so those factors were removed from analysis. The results were thus analyzed using all 20 24-hour tests as replicates. Proportion egg rafts laid differed significantly across the three aquatic plant species ($F_{5, 114}$ = 46.09, $p < 0.0001$). In Tukey least-square mean comparisons (adjusted $p < 0.05$), the proportions of egg rafts laid among water hyacinth (mean ± SE) (0.404 ± 0.0186) and water primrose (0.364 ± 0.0214) were significantly higher than all other groups but not different from each other (Figure 2.1). The proportion of egg rafts laid among pennywort (0.164 ± 0.0181) was significantly higher than among parrotfeather (0.053 ± 0.0138), mosquitofern (0.006 ± 0.0211), and the open water control (0.008 ± 0.004) (Fig. 1). The proportion of egg rafts laid among parrotfeather was significantly higher than mosquitofern and the control (Fig 2.2). The proportion of egg rafts laid among mosquitofern and the control did not differ. Tanks containing invasive aquatic plants received ca. 2.5-fold higher proportions of egg rafts than did tanks with native aquatic plants ($F_{1, 98}$ = 33.19, $p < 0.0001$) . The average proportion of egg rafts laid in tanks containing any of the invasive plants (n = 60) was significantly higher (0.274 ± 0.0228) than in tanks (n =40) containing either of native plants (0.085±0.0156) (Figure 2).

Outdoor Caged Choice Bioassay

Plant species significantly affected oviposition ($F_{2,78}$ = 9.99, $p < 0.0001$). Averaged across all three densities, the proportions of egg rafts laid among water hyacinth (0.18 ± 0.029) and water primrose (0.12 ± 0.022) were significantly higher than among Brazilian waterweed (0.035±0.008) in Tukey mean comparisons, while water hyacinth and water primrose did not differ from each other (Figure 2.3). Plant density also significantly affected oviposition regardless of species ($F_{2,78}$ = 22.0, $p < 0.0001$). Averaged across all three plant species, the proportions of egg rafts laid in tanks containing the intermediate plant density (0.21±0.029) were significantly higher than in high plant density tanks (0.09±0.013), which were in turn higher than oviposition in low density tanks (0.02±0.007). (Figure 2.4).

In the two-factor analysis, which included the control open-water tanks (which received no egg rafts and are not shown in Figure 5), the main effects of plant species ($F_{2,80}$ = 11.78, $p <0.0001$) and density ($F_{2,80}$ = 17.62, $p< 0.0001$) were significant, as was their interaction ($F_{4,80}$ = 3.41, $p =0.0126$). The proportion of egg rafts laid among intermediate densities of water hyacinth (0.35 ± 0.031) and water primrose (0.25 ± 0.237) were significantly higher than all other treatments, and did not differ from each other (Figure 2.5). Water hyacinth attracted a 12.5-fold higher proportion of egg rafts at intermediate density and water primrose a 7-fold higher proportion than did Brazilian waterweed at this density (0.035 ± 0.017). Water hyacinth at high density (0.126 ± 0.028) and water primrose at high density (0.097

± 0.026) were the two next closest treatments, but even these tanks were both ca. 3-fold less attractive for egg raft deposition than those two weeds at intermediate density, and low density tanks of water hyacinth (0.043 ± 0.017) and water primrose (0.02 ± 0.01) were 8- and 12.5-fold less attractive than intermediate density, respectively. Low density tanks attracted few egg rafts in general (0.005 to 0.04 proportions), showing no difference among plant species, or from the open water control.

Discussion

This study investigated first the influence of five species of invasive and native aquatic plants on mosquito oviposition behavior. My hypotheses that differences in plant species and invasive status would lead to differences in oviposition microhabitat site selection were supported. The mean proportion of egg rafts laid among water hyacinth and water primrose was over 2-fold higher than on pennywort, the next-nearest plant in terms of egg raft oviposition. Water hyacinth and water primrose were 8-fold higher or more than parrotfeather or mosquitofern. Interestingly, the proportion of egg rafts laid on average on each of the three invasive plants was 2.5 higher than on either of the native plants. Given these results, invasive water hyacinth and water primrose were used in a subsequent outdoor caged experiment with the addition of another invasive weed, Brazilian waterweed. These plant species were thus chosen such that each of three main categories of aquatic macrophyte types was represented: floating (water hyacinth), emergent and rooted (water primrose) and submersed (Brazilian waterweed). In this study, my hypothesis that differences in plant type/species and density would lead to differences in oviposition microhabitat site selection was also supported. Specifically, water hyacinth and water primrose attracted four to six-fold higher proportions of eggs than did Brazilian waterweed, and, across all three macrophytes, intermediate (50-80% water surface coverage) plant densities attracted 2-fold higher proportions of egg rafts than did high densities and 10-fold more than low densities. In the two-factor analysis, water hyacinth and water primrose at intermediate density were at least 7-fold more attractive than Brazilian waterweed at any density, and at least 3-fold more attractive than water hyacinth or water primrose at high or low density.

These findings are peripherally consistent with other studies that found that *Culex pipiens, Culex restuans, Culex quinqefasciatus* show oviposition preference for water sources containing vegetation (e.g., grasses, tree leaves) (Kramer and Mulla 1979; Prasad and Daniel 1988; Bentley and Day 1989; Brust 1990; Reisen and Meyer 1990; Steinly and Novak 1990; Lampman and Novak

1996). Similarly, Orr and Resh (1992) found that selection of oviposition sites (measured as eggs m^{-2} d^{-1}) by adult female *Anopheles* mosquitoes was highest in intermediate densities of an invasive aquatic macrophyte species (*Myriophyllum aquaticum*). This finding is consistent with the observations in the above study in which it was found that intermediate densities of plants as main and interactive effects received the highest proportion of egg rafts laid. In addition to density effects, the impact of plant species on mosquito oviposition has also been demonstrated. For example, Reisking, Greene, and Lounibos (2009) demonstrated a difference in oviposition preference among two leaf species by an *Aedes* mosquito. This finding is consistent with the preference for water surfaces around water hyacinth and water primrose as oviposition sites for a *Culex* mosquito over water located among other available aquatic macrophyte species.

The selection of water hyacinth and water primrose as preferred plant species may be due to structural or chemical effects. Water hyacinth has large, broad, relatively rigid leaves and thick stems relative to the other species used. Water primrose has much smaller but rigid leaves and shares the characteristic of having thick rigid stems/stolons running parallel to the water surface. Leaf and stem rigidity may have played a role in the attractiveness of these two aquatic macrophytes to *C. pipiens* for oviposition. This logic is only partially supported by Overgaard (2007) in which it was demonstrated that plant structure impacted oviposition behavior of *Anopheles minimus*. Specifically, the study found that small-leaved plants were more attractive for oviposition than large-leaved plants, which does not support my findings as water hyacinth has the largest leaf size compared to all plants in the study. However, in the same study Overgaard (2007) found that large leaved plants were more attractive than grasses and soil. If grasses were less attractive due to low rigidity it may be the case that parrotfeather and pennywort, both of low rigidity compared to water hyacinth and water primrose, were less attractive for the same reasons. Additionally, invasive plants tend to exhibit higher rates of stomatal conductance than natives, increasing the rate of water vapor exiting the plant (Cavaleri and Sack 2010). Water vapor has been shown to be a pre-oviposition attractant for the malaria vector *Anopheles gambiae sensu stricto* (Okal et al. 2013). The observed preference for invasive plants observed in my study may reflect this trait, or may simply be a byproduct of the fact that structurally-superior water hyacinth and water primrose fell into the invasive category. The results strongly suggest that emergent, rigid plant structure above the water line is necessary for attraction of ovipositing mosquitos, even though eggs are deposited on the water, not the plant. Floating mosquitofern and submersed Brazilian water weed provided abundant plant cover at or below the surface, but were not attractive.

The results demonstrate the importance of investigating the impact of both plant species and density among other factors in order to support or refute generalizations on this topic. The preference for intermediate densities of aquatic macrophytes over high, as well as low density, suggests that mosquito females require multiple physical signals from both plants and open water in selecting oviposition sites. Additional investigations are needed to determine if plant species-specific chemical signals, in either the air or the water, are involved.

Mosquitoes are primary vectors of many human diseases and elucidating mechanisms that drive their populations is essential for informing management. Particular species of invasive aquatic vegetation may particularly be of concern due to their ability to outcompete native plants and impact community structure (Olden and Poff 2003; Sax and Gaines 2003; Winter et al 2009), and ultimately human well-being (Pejchar and Mooney 2009; Pysek and Richardson 2010). The results suggest that management of water hyacinth and water primrose to low densities will contribute to abatement of transmission risk of WNV and other vectored pathogens in areas containing both aquatic ecosystems, such as the Sacramento-San Joaquin Delta, that are vital for environmental health and human-well-being, and adjacent large human populations.

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Figure 2.1. *C. pipiens* mosquito oviposition preferences (mean proportion of egg rafts laid, across 20 tests) in the presence of five aquatic macrophyte species in tanks in a greenhouse cage choice bioassay. Tanks contained mosquitofern (native), water hyacinth (invasive), pennywort (native), water primrose (invasive), or parrotfeather (invasive), and control tanks contained no vegetation. Bars topped with a common letter do not differ significantly at the 0.05 level (Tukey's HSD test). Error bars =SE.

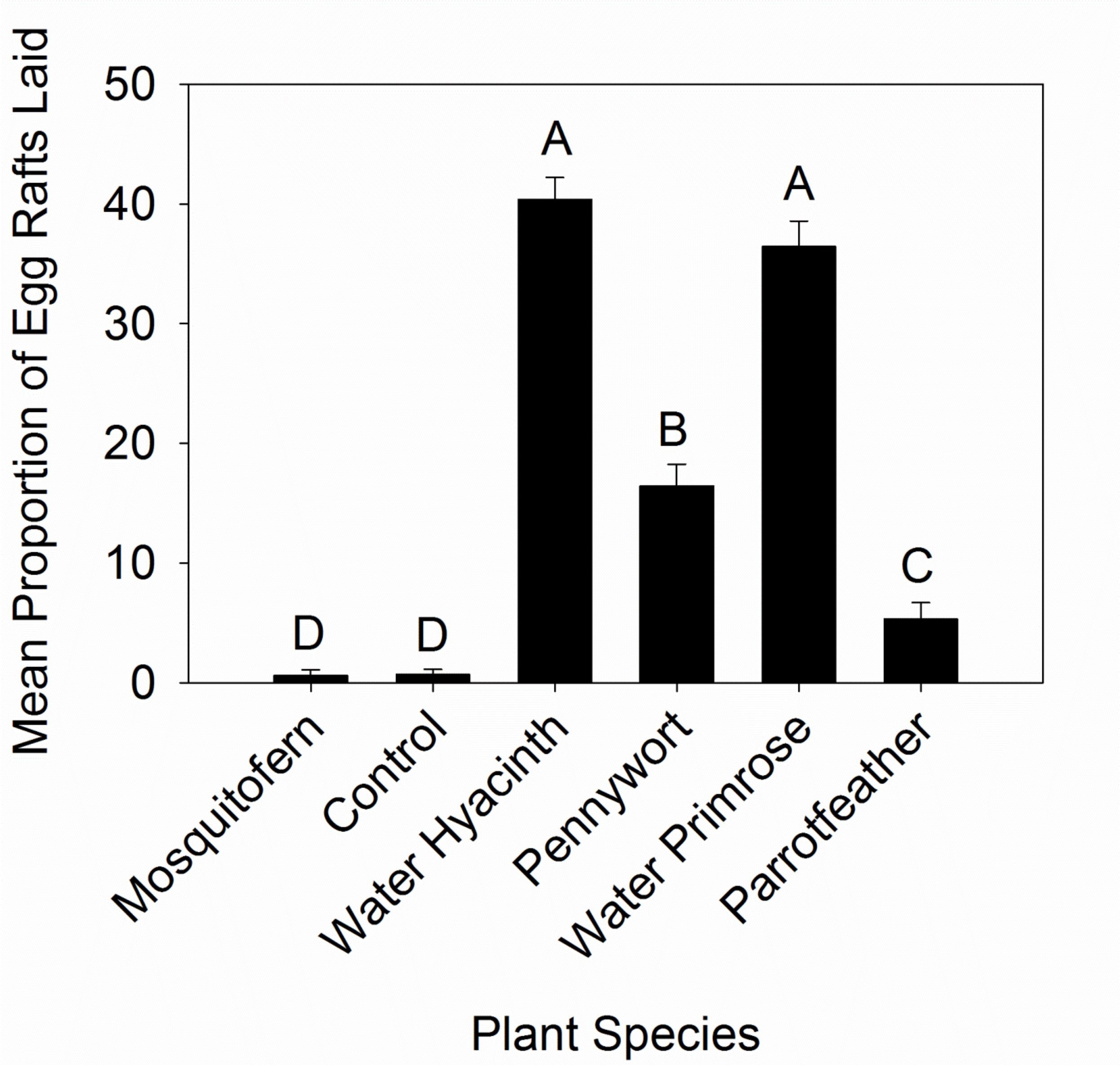


Figure 2.2. *C. pipiens* mosquito oviposition preferences (mean proportion of egg rafts laid) in the presence of one of three invasive aquatic plants or one of two native aquatic plants in the greenhouse cage choice bioassay (averages across 20 tests and three invasive or two native plants). Bars topped with a common letter do not differ significantly at the 0.05 level (Tukey's HSD test). Error bars=SE.

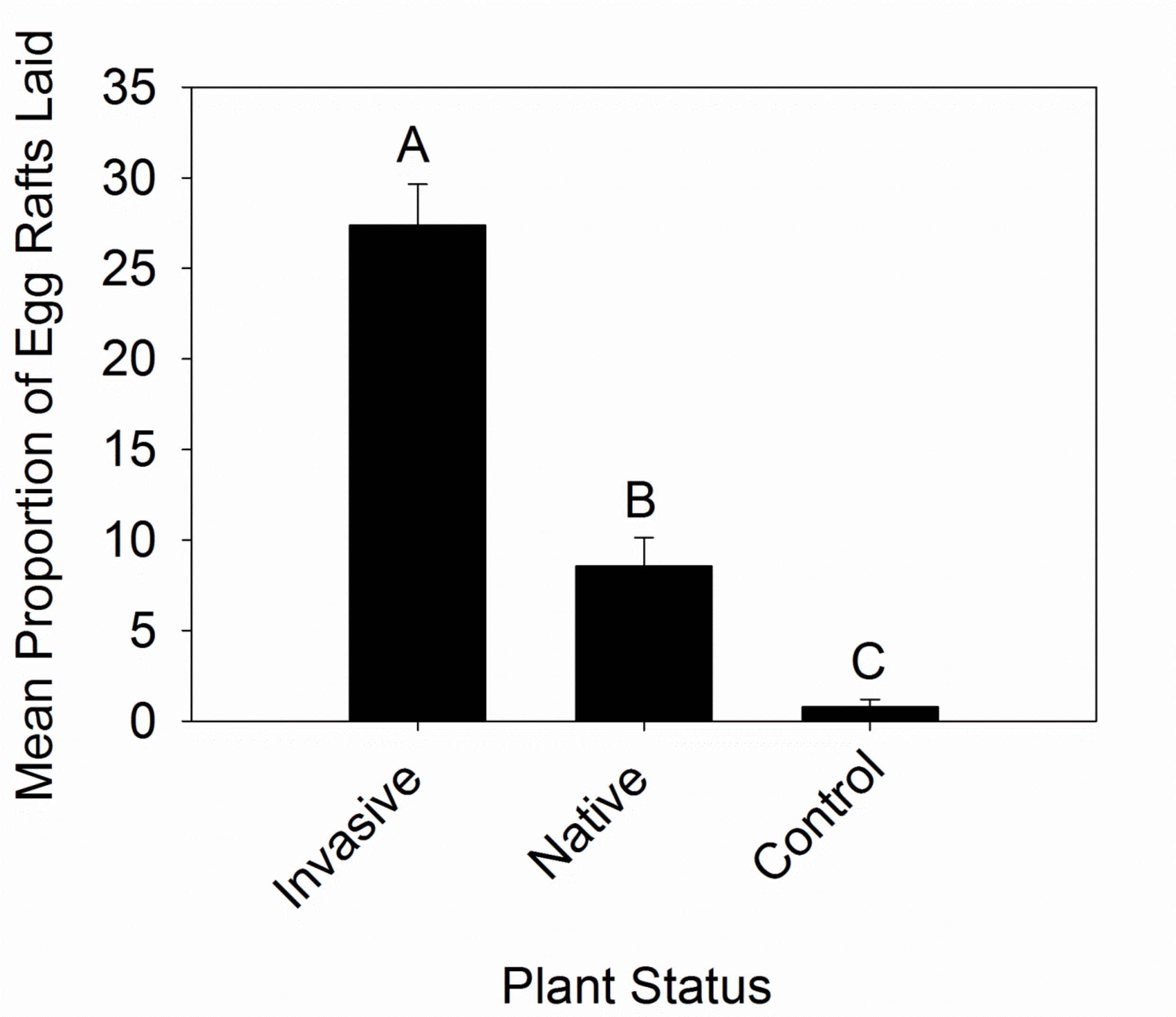


Figure 2.3. *C. pipiens* mosquito oviposition preferences in the presence of three invasive aquatic macrophytes, Brazilian waterweed, water hyacinth, and water primrose. Graph shows mean proportion of egg rafts deposited on each plant species across three densities per species (low, intermediate and high) in separate tanks, all within a cage enclosure (total of 27 tanks in each mean). Bars topped with a common letter do not differ significantly at the 0.05 level (Tukey's HSD test). Error bars = SE

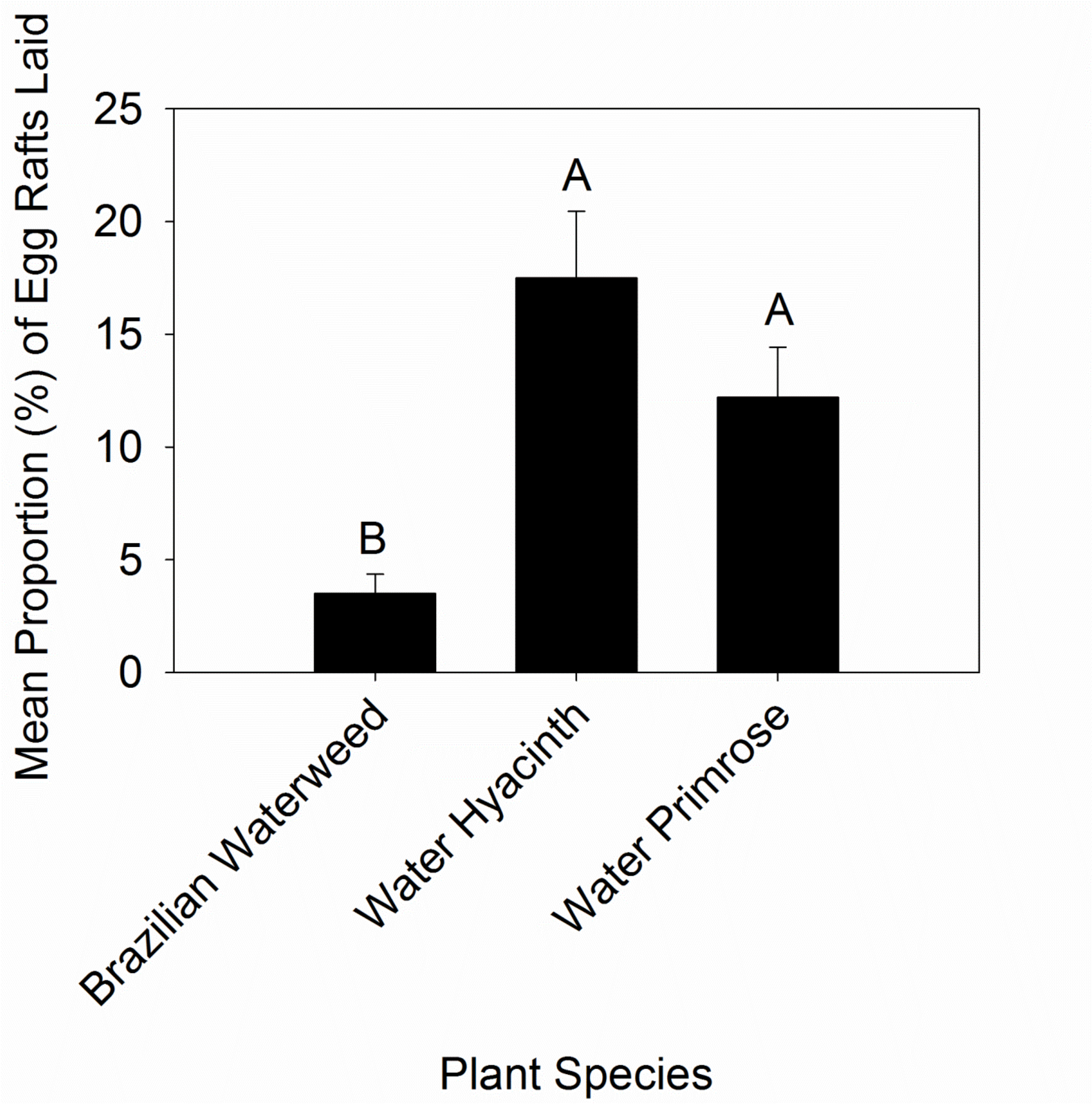


Figure 2.4. *C. pipiens* mosquito oviposition preferences in the presence of three aquatic plant densities, high (80-100% coverage), intermediate (50-80%), and low (10-50%). Graph shows the mean proportion of egg rafts laid at each plant density across the three plant species (water hyacinth, water primrose and Brazilian waterweed within a cage enclosure (total of 27 tanks in each mean). Bars topped with a common letter do not differ significantly at the 0.05 level (Tukey's HSD test). Error bars = S. E. M.

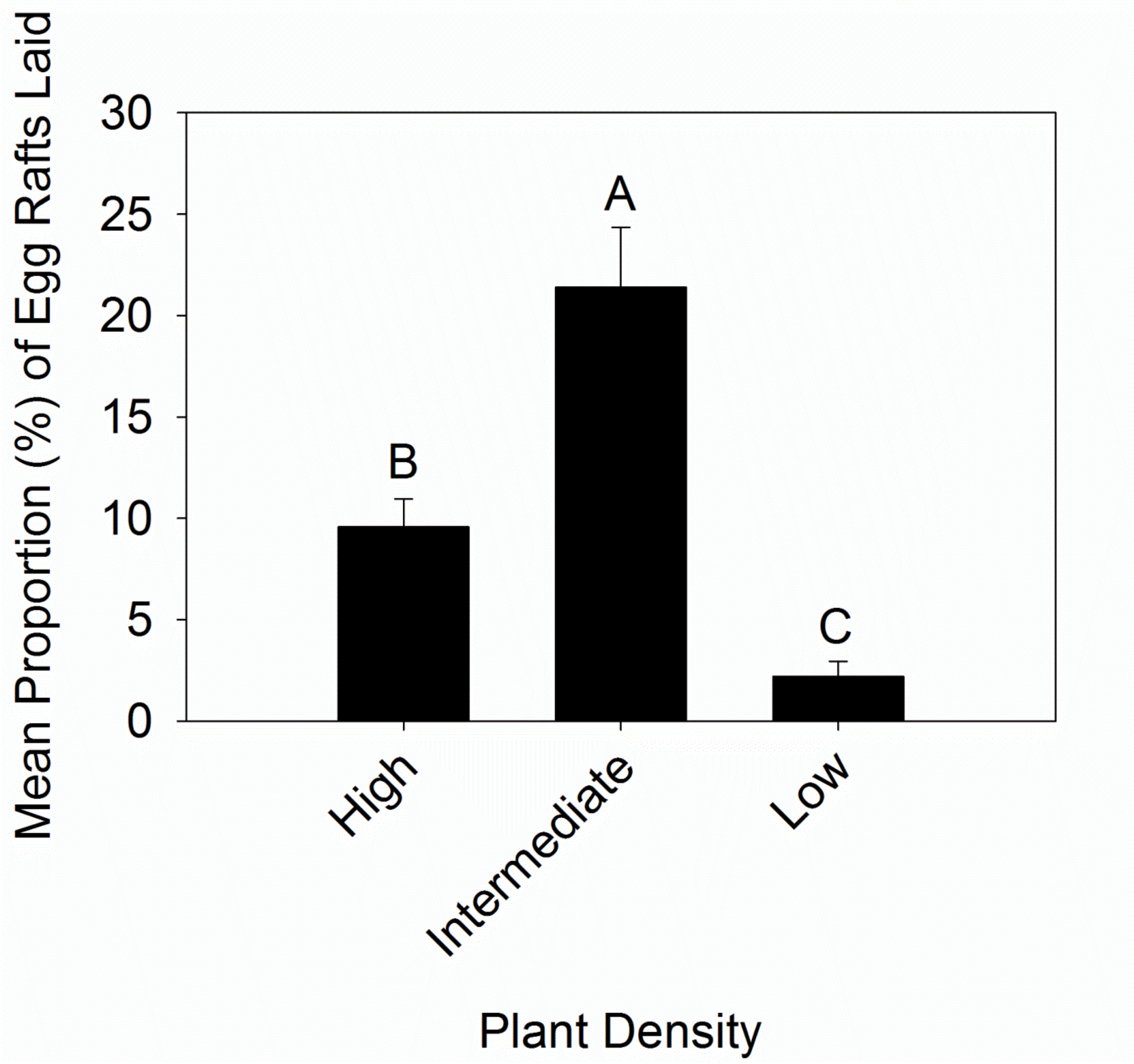
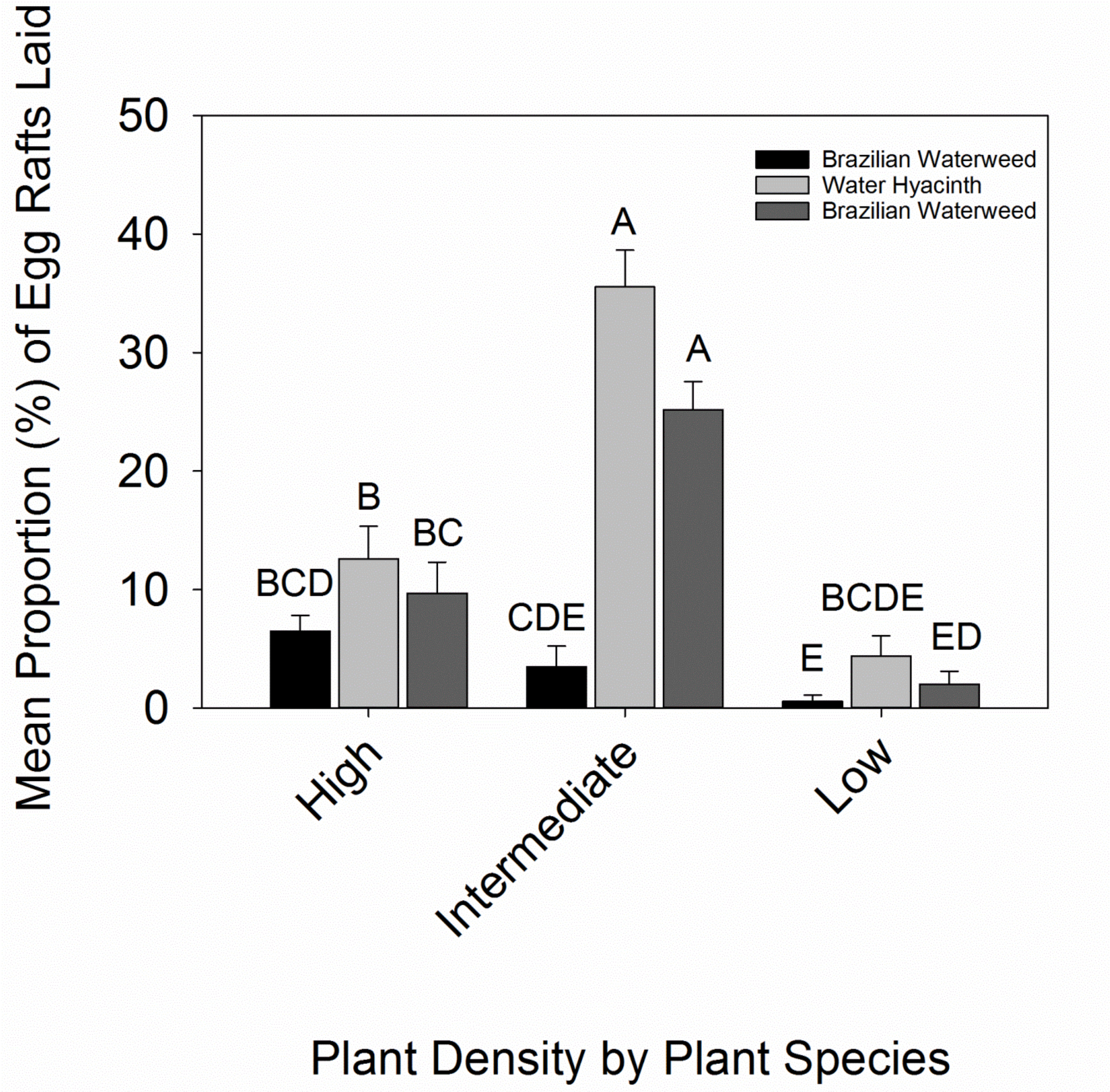


Figure 2.5. *C. pipiens* mosquito oviposition preferences in the presence of three aquatic plant species and three plant densities. Graph shows the mean proportion of egg rafts laid in each tank within a cage enclosure (total of 9 tanks in each mean). Bars topped with common letters do not differ significantly at the 0.05 level (Tukey's HSD test). Error bars = SE



Chapter 3

Predator-prey dynamics between a mosquito disease vector, *Culex pipiens* (Diptera: Culicidae) and Mosquitofish: Impacts by invasive aquatic macrophytes

Abstract

Non-native aquatic macrophytes often alter the ecological structure of habitats by providing refuge for prey against predators. Here we test a predator-refuge hypothesis to predict a potential impact of exotic aquatic macrophytes on larval mosquito survival in the presence of a predatory fish. Three species of weeds, *Eichhornia crassipes* (floating water hyacinth), *Ludwigia hexapetala* (emergent water primrose), and *Egeria densa* (submergent Brazilian waterweed), were compared at three densities for their relative impacts on the survival of larval *Culex pipiens*, a primary West Nile Virus mosquito vector, in the presence of predatory mosquitofish, *Gambusia affinis*. The study revealed that at intermediate (50 to 80% cover) plant densities, the survival curves for larvae after 72 h among water hyacinth was significantly higher (53% after 48 h) than for larvae among water primrose or Brazilian waterweed (42%) or in tanks without vegetation (34%). In contrast, the survival curves among the three plant species did not differ from each other at the high (80 to 100% cover) or low (10 to 50% cover) plant densities. Across all three plant densities, larval risk of mortality was significantly lower among water hyacinth than among the other two plant species, and risk was lower in the presence high or intermediate densities, across all three of the plant species, than at low densities or with no vegetation. These results suggest that water hyacinth in particular, and invasive aquatic weeds more generally, are likely to facilitate mosquito population survival in the presence of predatory fish, and should thus be managed in water bodies that could harbor disease-vectoring mosquitos.

Introduction

Invasive aquatic macrophytes often alter the landscape of invaded habitats by forming dense mats of vegetation that increase environmental heterogeneity (Sheley et al. 1998; Mack et al. 2000; Forseth and Innis 2004; Levine et al. 2003; Williams et al. 2009; Mattos and Orrock 2010; Orrock et al. 2010a). These invasive weed mats in turn may influence the quality and quantity of habitat available to aquatic macroinvertebrates, which often utilize aquatic vegetation as physical sources of shelter and refuge from predators (Valinoti et al. 2011; Chaplin and Valentine 2009; Martin and Valentine 2011). In addition to providing predator-free refuge (Rantala et al. 2004; Finke and Denno 2006), aquatic vegetation can reduce encounter rates between predator and prey by reducing the visibility of susceptible prey species (Hughes and Grabowski 2006), thus increasing survival of prey.

The level of refuge provided to prey by aquatic plants is largely dependent on the complexity of habitat structure that they create (Savino and Stein 1982). Complexity in this context is often divided into two categories, plant density and plant type (form or species) (Stoner and Lewis 1985; McCoy and Bell 1991). While the density of aquatic plants has often been shown to be proportional to aquatic macroinvertebrate abundance (Crowder and Cooper 1982; Stoner and Lewis 1985), such a relationship between plant type and the abundance of aquatic organisms is more difficult to generalize as different types of plants often support different epiphytic groups of organisms (Rooke 1986; Chilton 1990; Humphries 1996). However, Leber (1985) and Persson and Eklov (1995) demonstrated this type of relationship by showing that plant type rather than density has an influence on refuge. Additionally, because plant density and type need not be correlated, it is necessary to treat these two categories of complexity as separate influences on the level of refuge provided to prey (Stoner and Lewis 1985; McCoy and Bell 1991). Most studies, however, have not distinguished plant type from density, making it difficult to fully elucidate mechanisms that impact prey success within vegetative refuge.

Mosquitoes, whose immature stages occur in aquatic habitats, are a group of organisms thought to utilize some species of aquatic vegetation as habitat and refuge from predators (Orr 1991; Heck and Crowder 1991; Gotceitas and Colgan 1989). One of the most widespread and voracious predators of mosquitoes is the introduced mosquitofish, *Gambusia affinis.* Habitat complexity has been shown to reduce the effectiveness of fish predators in numerous studies (Gotceitas and Colgan 1989; Nelson and Bonsdorff 1990; Swisher et al. 1998) by creating complex structures that enhance protection for prey (Heck and Crowder 1991). However, in these studies habitat complexity was characterized by plant density, or simply presence/absence, rather than also taking plant type into account (Warfe and Barmuta 2006). It has been shown in laboratory settings that plant type can impact predator performance when plant density has no impact, making it necessary to assess the plant density and type categories of habitat complexity separately (Warfe and Barmuta 2004).

In this experimental study, we compare the refuge provided to a primary West Nile virus mosquito vector, *Culex pipiens*, created by different densities and types of invasive aquatic macrophytes in the presence of the predatory mosquitofish, *G. affinis*. Three species of plants were used, water hyacinth (*Eicchornia crassipes*), yellow water primrose (*Ludwigia hexapetala*), and Brazilian waterweed (*Egeria densa*), representing three distinct type of aquatic vegetation: floating, emergent, and submergent, respectively. We hypothesize that *C. pipiens* survival in the presence of *G. affinis* changes across plant type and density.

Materials and Methods

Biological materials. Larvae of *C. pipiens* were obtained from laboratory colonies at the San Joaquin Mosquito and Vector Control District in Stockton, CA, and were reared as described in (Gerberg, Barnard, and Ward 1994). Mosquitofish adults were obtained from the Contra Costa Mosquito and Vector Control District (CC MVCD) in Concord, CA, and were reared as described in (Hoy 1985). Studies on mosquitofish were conducted under an IAUC Protocol reviewed by the University of California-Berkeley (provide certification number). Floating water hyacinth, *Eichhornia crassipes* (Mart.) Solms. (Pontederiaceae) was obtained from a greenhouse colony at the USDA-Western Regional Research Center, Albany, CA, and were maintained as in Moran et al. (2016). Emergent, rooted water-yellow primrose (*Ludwigia hexapetala* (Hook. & Arn) Zardini et al, *Ludwigia grandiflora* (Michx.) Greuter & Burdet), or *Ludwigia peploides* subsp. *montevidensis* (Spreng.) P.H. Raven/*Ludiwigia peploides* subsp. *peploides* (Hoch and Grewell 2012) (Onagraceae) and Brazilian waterweed (*Egeria densa* Planch. (Hydrocharitaceae) were collected from one field site in the Sacramento-San Joaquin Delta and maintained under water nutrient conditions similar to those used for water hyacinth.

Experimental design. A 3x3 factorial design outdoor cage-enclosure study investigating the effect of plant species (water hyacinth, water primrose, and

Brazilian waterweed) and plant density (high, intermediate, and low) on mosquito survival was conducted. The study was conducted during June and July 2015 at the Aquatic Weed Laboratory of the USDA-Agricultural Research Service, Exotic and Invasive Weeds Research Unit, in Davis, CA where average outdoor temperatures averaged 29.9 °C (high), (12.6 °C (low). Within one mesh lumite cage (3 m long x 2.0 m wide x 2.2 m tall) (Bioquip, Rancho Dominquez, CA), a total of 10 clear plastic containers (100 L vlume; 85.7 cm long x 49.2 cm wide x 33.9 cm deep) were filled with 2/3rd of a bag (12 kg) of sand (KolorScape, Atlanta, GA) and 2/3rd of a bag (12 kg) of rock pellets (Vigoro, Lake Forest, IL). Dechlorinated water was added to each container to a height of 7 cm from the top. To standardize measurement of plant abundance across species, an estimation of percent area coverage was used as follows: 80-100% tank cover = high density, 50-80% tank cover = intermediate density and 10-50% tank cover = low density. The following treatment combinations were established: 1) water hyacinth – high density; 2) water hyacinth – intermediate density; 3) water hyacinth – low density; 4) water primrose – high density; 5) water primrose – intermediate density; 6) water primrose – low density; 7) Brazilian waterweed – high density; 8) Brazilian waterweed – intermediate density; 9) Brazilian waterweed – low density; or 10) control – no plant added. Plants were allowed one month before the first replicate to root (water primrose and Brazilian waterweed) and acclimate to container conditions, and 1-2 wk before subsequent replicates to acclimate to recover from culling-related disturbance. Plant densities were maintained by removing plants (water hyacinth) or trimming (water primrose and Brazilian waterweed).

At the start of each experimental replicate, 50 late-(third and fourth) instar *C. pipiens* larvae were introduced into each container. Two 24 hour-starved (in colony-derived water) *G. affinis* adults were then immediately added to each tank. The number of predators and prey were held constant across plant density as in previous studies (Cooper and Crowder 1979; Nelson 1979; Coen et al. 1981; Heck and Thoman 1981; Savino and Stein 1982; Main 1987; Ryer 1988; Nelson and Bonsdorff 1990; Jordan et al. 1997; Marcia et al. 2003). Each tank was initially observed for 15 minutes to ensure that mosquitofish mortality did not occur from exposure to new water. Thereafter each container was observed once every 24 hours for a total of 72 hours, or until all larvae in the container had been consumed. At each observation time the number of *C. pipiens* larvae and mosquitofish surviving was determined. No mosquitofish died during any of the replicates, and new mosquitofish from the CC MVCD colony were used for each replicate. The experiment was conducted nine times (completely randomized block design with start dates as blocks), and containers were moved haphazardly within the cage between replicates, resulting in a total of nine replicates for each treatment combination (N larvae per treatment per replicate = 50 ; N total larvae per treatment = 450).

Data analysis. Data were analyzed using nonparametric survival analysis (Kaplan-Meier method) in JMP Pro (Version 13, SAS Institute, Cary, NC) using log-rank estimation of χ^2 tests of significance. The adjustment for multiple comparisons for the log-rank test was performed using the Bonferroni method to control the familywise error rate. The family-wise Bonferroni threshold of 0.008 used for individual comparisons between treatments was calculated by dividing the significance level of 0.05 by K=6, which represents the number of comparisons. Hazard risk ratios for Kaplan-Meier survival curves were also calculated using plant species and plant density as effects, and separately for plant species across all densities in relation to control, to compare the relative risk of mortality across treatments.

Results

Mosquitofish consumed all mosquito larvae in all 10 of the treatments by 72 h. However, there was a significant effect of plant species (including control lacking plants) on survival over time at high plant density ($\chi^2_{df = 3} = 150.54$; P<0.0001), intermediate plant density ($\chi^2_{df = 3} = 108.19$; P<0.0001), and low plant density ($\chi^2_{df = 3} = 14.81$; P<0.002). At high plant density, the survival curves for mosquitoes among each of the three plant species were higher than the survival curve calculated for mosquito larvae in the absence of plants (P = or < 0.0001 for all three comparisons). After 48 h, survival probability in the presence of plants (any species) at high density averaged 50 %, while in the control it was 34% (Fig. 1). At intermediate plant density, the survival curve for mosquitoes among water hyacinth was significantly higher than the survival curves among Brazilian waterweed (P=0.0003) and water primrose (P=0.0005). After 48 h, the probability of mosquito larval survival was 53% on water hyacinth compared to 42% on Brazilian waterweed and 43% on water primrose (Fig. 3.1). Also at intermediate density, survival curves for mosquitoes among each of the three plant species were higher than the survival curve for the control lacking plants (P<0.0001 for all three comparisons). The average survival probability across all three plant species after 48 h was (46%). At low plant density, the survival curve for mosquito larvae among water hyacinth, but not among Brazilian waterweed or water primrose, was significantly higher than for mosquito larvae in the control container (P=0.001).

After 48 h, survival probability in water hyacinth at low density was 40%, and the average across all three plant species was 37% (Fig. 3.1).

The estimated relative risk of mortality among water primrose, Brazilian waterweed, and the control were significantly higher by 1.08%, 1.08%, and 1.24%, respectively, than among water hyacinth ($P<0.0026$, $P<0.0014$, and $P<0.0001$), respectively. The estimated relative risk of mortality in the control container was 1.14% higher than in container with water primrose ($P<0.0001$) and Brazilian waterweed ($P<0.0001$) (Table 3.1). The estimated relative risk of mortality in tanks of low weed density were 1.14% and 1.10% higher than tanks of high and intermediate plant densities, respectively [($P<0.0001$), ($P<0.0001$)] (Table 3.2).

Discussion

This study investigated the influence of both invasive aquatic plant density and type (species) on mosquito larval survival in the presence of a predator. Our hypothesis that differences in plant density and type would lead to differences in larval *C. pipiens* survival in the presence of *G. affinis* was supported. This effect was transient in these small containers, as mosquitofish found and consumed all of the larvae in all treatments by 72 h. We found that exposure of *C. pipiens* to *G. affinis* in the presence of floating water hyacinth at an intermediate (50 to 80% surface coverage) density resulted in the highest level of survival, ca. 10% higher after 48 h than in the presence of Brazilian waterweed or water primrose at this density, and 19% higher than in the absence of aquatic vegetation. At high (80 to 100% coverage), the presence of any of the three species, but not water hyacinth specifically, generated a transient but significant survival probability increase (of 16%) for *C. pipiens* after 48 h compared to containers lacking plants. At low (10 to 50% coverage) plant density, only water hyacinth transiently improved mosquito larval survival compared to open water, by % after 48 h. Across all three plant species, survival curves and average % survival probabilities after 48 h declined significantly as plant density declined. Thus, at sufficient densities, the presence of any of these three species provided higher levels of survival for *C. pipiens* in the presence of *G. affinis* than did the control; however, water hyacinth allowed transiently superior mosquito larval survival compared to both the other weeds and the control in the presence of up to 50% open water, and was unique in providing a survival benefit over the control even in the presence of up to 90% open water. These findings are consistent with other studies that found that specific types (Grutters et al. 2015; Valinoti, Ho and Armitage 2011; Martin and Valentine 2011; Rantala et al. 2004) or densities (Savino and Stein 1989) of aquatic vegetation resulted in higher survival for macroinvertebrate prey in aquatic systems. The specific effects of water hyacinth or any of these other plants on the level of protection provided to mosquitoes from predators has not been ascertained until now.

In contrast to this study in which prey survival was highest at intermediate plant densities of water hyacinth, other studies report that predator foraging success rather than prey survival is highest at intermediate plant densities of macrophyte species (Valley and Bremigan 2002; Wiley et al. 1984; Savino and Stein 1982; Crowder and Cooper 1982). Some studies found that prey survival increases only at high vegetation densities, i.e lacking open water (Coen et al. 1981; Stoner 1982; Orth et al. 1984; Nelson and Bonsdorff 1990 ; Orr and Resh 1991), whereas others found that higher plant densities does not necessarily result in the same effect (Canion and Heck 2009). Thus, more studies are needed to make generalizations about the effect of plant density on prey survival across multiple systems.

The maximization of survival among intermediate densities, particularly among water hyacinth, may be explained by similar effects of aquatic macrophytes on mosquito larvae and mosquitofish in the experiment. The intermediate plant density was likely high enough to disguise *C. pipiens* but low enough to make *G. affinis* remain conspicuous. It is possible that at high densities submerged plant shoots (Brazilian water weed), shoots and roots in combination (water primrose) or roots (water hyacinth) had a disguising effect on *G. affinis* that cancelled out the same disguising effect on *C. pipiens* reducing the time windows for prey to avoid the predator. It was expected, as observed, that mosquito survival would be higher across all vegetative treatments at sufficient density compared to the control treatment containing no vegetation, as a lack of refuge resulted in prey being more conspicuous. However, the observation of a protective effect of water hyacinth, even at low levels of refuge, illustrate the dependence of predator-prey interactions on plant species (Grutters et al. 2015) as some prey may be more successful at avoiding predators in low refuge settings if the prey moves relatively fast, and benefits from aquatic plant species-specific habitat structure. Thus, the impact of habitat complexity on prey survival may depend on predator and prey microhabitat use (Klecka and Boukal 2014; Power 1992). Detailed behavioral studies would be required to determine the specific benefit of water hyacinth to either reduce *C. pipiens* apparency or increase *G. affinis* apparency, and to determine resulting effects on predator search time. The results demonstrate the importance of testing various plant species within prey survival experiments in order to generalize or refute conclusions about the benefits of invasive aquatic macrophytes or other aquatic plants for mosquito larval survival. (Grutters et al. 2015; van Kleunen et al. 2014).

These results suggest that water hyacinth plays a beneficial role in mosquito development and thus integrated mosquito management activities should target this invasive weed species in addition to mosquitoes. The displacement of native aquatic macrophytes by invasive aquatic macrophytes like water hyacinth could impact predator-prey dynamics by providing more structurally suitable refuge to prey such as mosquitoes (Grutters et al. 2015). *Culex* mosquitoes are readily found breeding in the Sacramento-San Joaquin River Delta where water hyacinth invades waterways with dense mat formations. These mosquitoes are primary vectors of West Nile Virus among diseases so management targeting this group of mosquitoes is of priority. Reduction of water hyacinth mats should reduce the availability of protective harborage to larval mosquitoes, thus increasing their susceptibility to predation.

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Table 3.1. Cox proportional hazard model. Hazard ratios (HR) with 95% confidence intervals for larval *Culex pipiens* mortality in the presence of *Gambusia affinis* predator in containers with different plant species.

Pair A	Pair B	HR (95% CI)	p-Value
Control	Water Hyacinth	1.24 (1.18, 1.30)	0.0001*
Control	Water Primrose	1.15 (1.09, 1.20)	0.0001*
Control	Brazilian Waterweed	1.14 (1.09, 1.19)	0.0001*
Water Primrose	Water Hyacinth	1.08 (1.03, 1.14)	0.0026*
Brazilian Waterweed	Water Hyacinth	1.08 (1.03, 1.14)	0.0014*
Brazilian Waterweed	Water Primrose	1.00 (0.96-1.05)	0.8657

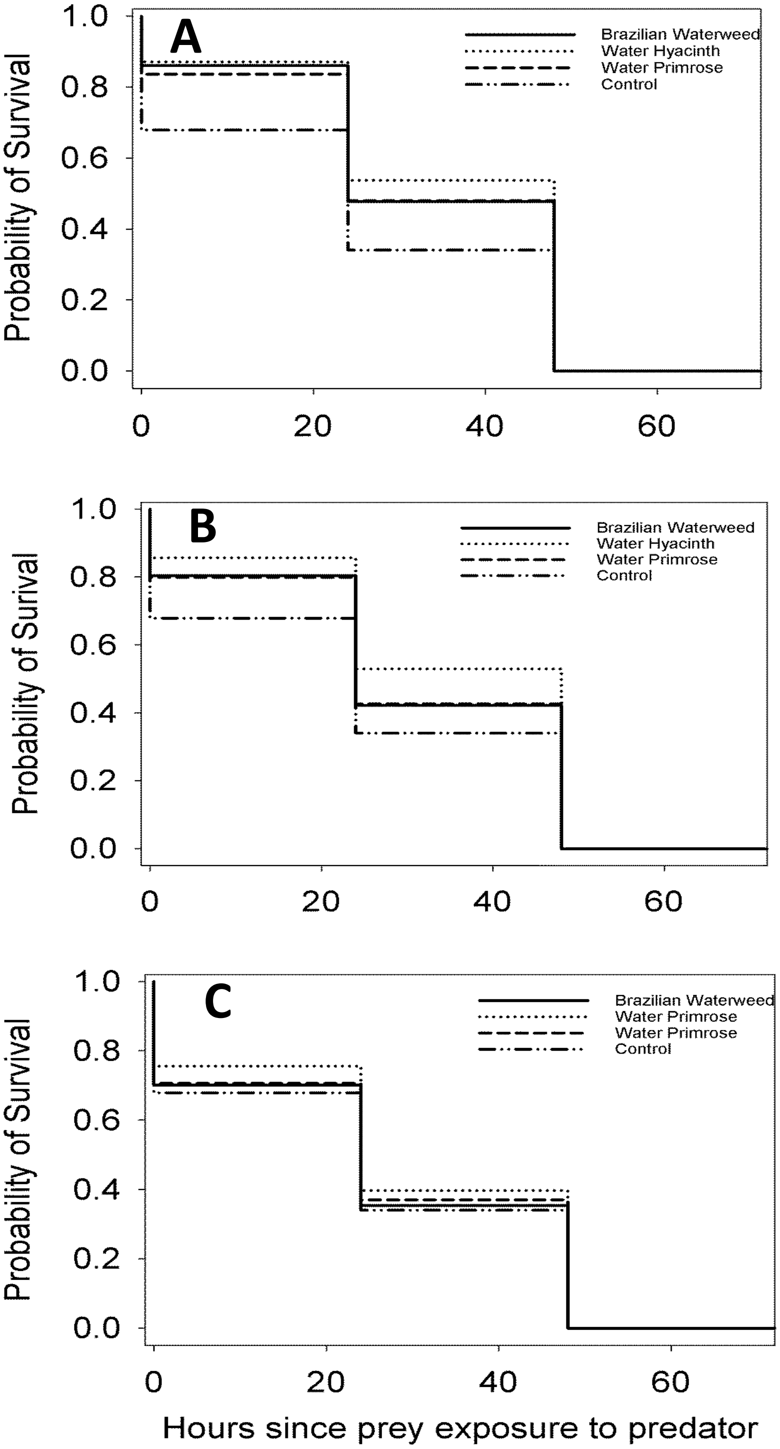
An asterisk (*) denotes a significant difference in risk of mortality between comparisons of the hazard ratio of exposure to the treatment in column A compared to the treatment in column B. Hazard risk ratios containing the value 1.00 within a confidence interval are not significant.

Table 3.2. Cox proportional hazard model. Hazard ratios (HR) with 95% confidence intervals for larval *Culex pipiens* mortality in the presence of *Gambusia affinis* predator in containers with differing densities of invasive aquatic plants.

Group 1	Group 2	HR (95% CI)	p-Value
Low	High	1.14 (1.10-1.19)	0.0001*
Low	Intermediate	1.10 (1.06-1.15)	0.0001*
Intermediate	High	1.04 (0.99, 1.09)	0.0833

An asterisk (*) denotes a significant difference in risk of mortality between comparisons of the hazard ratio of exposure to the treatment in column A compared to the treatment in column B. Hazard risk ratios containing the value 1.00 within a confidence interval are not significant.

Figure 3.1. Survival curves for *C. pipiens* mosquito larvae in the presence of Brazilian waterweed, water hyacinth, water primrose, in containers with **(A)** high (80 to 100% water surface coverage, **(B)** intermediate (50 to 80% coverage) or, **(C)** low (10 to 50% coverage) densities of these plants, or with no plants (Control).



Chapter 4

Development time in a mosquito disease vector, *Culex pipiens* (Diptera: Culicidae): Impacts by invasive aquatic macrophytes

Abstract: Invasive aquatic macrophytes can drastically alter ecosystems by impacting nutrient availability to other organisms. Mosquitoes, which initially undergo aquatic development, are known to derive nutrients directly and indirectly from aquatic plants. Here we test a hypothesis to predict a potential impact of invasive aquatic macrophyte species and density on mosquito larval to adult development time and survival. Three species of weeds, *Eichhornia crassipes* (floating water hyacinth), *Ludwigia hexapetala* (emergent water primrose), and *Egeria densa* (submergent Brazilian waterweed), were compared at three densities for their relative impacts on the larval to adult development time and adult survival of *Culex pipiens*, a primary West Nile Virus mosquito vector. The study revealed that average *C. pipiens* adult emergence time was significantly shorter across all weed species and density levels by one to two days compared to the control tank with no vegetation. The average number of eggs completing development to adulthood was significantly higher (two to three-fold) among tanks containing vegetation than those that not containing vegetation. Most surprising was that different plants species shortened mosquito development time significantly over other plants depending on plant density level; there was no consistent trend across all density levels. Thus it is more likely that impacts by plants are due to physical rather than chemical characteristics of the plants.

Introduction

A variety of factors influence the development and survival of organisms (Couret, Dotson, and Benedict 2014). The availability of food and nutrients is one of several factors that can significantly influence both the length of time it takes for an organism to complete development as well as its survival (Dell, Pawar, and Savage 2011; Flenner, Richard, Suhling 2010; Yang and Rudolf 2010; Yang et al. 2009; Farnesi et al. 2009). The diet of an organism in turn will impact the types of foods and nutrients consumed. Specifically, diet has been shown to significantly alter development time in insects, among other factors including density and temperature (Kingsolver and Huey 2008; Damos 2012; Robinson and Partridge 2001; Mead and Conner 1987). However, past research on insect development focuses on temperature as the main factor responsible for altering the time it takes for species to develop (Arrese and Soulages 2010; Kirby and Lindsay 2009; Kingsolver and Huey 2008; Rueda et al. 1990; Laudien 1973; Wigglesworth 1972). More studies are needed to investigate the effect of other factors such as diet on insect development and survival, particularly for insect groups that threaten public health.

Mosquitoes, which are notorious vectors of human diseases, develop in numerous types of aquatic habitats prior to reaching the adult stage (Skiff and Yee 2014). Aquatic vegetation can be a diet source for mosquito larvae, which consume detrital particles originating from plants directly (Merritt et al. 1992; Dahl et al. 1988), or consume microorganisms that obtain nutrients from detritus provided by aquatic plants (Walker et al. 1991). For example, Yee et al. (2004b) demonstrated that in laboratory settings *Culex pipiens* fed mainly by collecting detrital particles when available compared to other food sources. Additionally, the structure of the environment could impact this process and thus, the overall development time and survival of a mosquito (Timmermann and Briegel 2003; Briegel 2002). Invasive aquatic plants in particular are known for altering the structure of habitats (Gan et al. 2010) due to their often unique physical structures. Here we investigate the impact of three physically distinct invasive aquatic weeds -- water hyacinth (*Eichhornia crassipes*), water primrose (*Ludwigia hexapetala*), and Brazilian waterweed (*Egeria densa*) – on the development time and survival of immature mosquitoes to adult emergence. By understanding the potential environmental parameters impacting mosquito biology we can better inform management of these disease vectoring organisms.

Materials and Methods

Biological materials. Eggs of *C. pipiens* were obtained from laboratory colonies at the San Joaquin Mosquito and Vector Control District in Stockton, CA, and were reared as described in Gerberg, Barnard, and Ward (1994). Floating water hyacinth, *Eichhornia crassipes* (Mart.) Solms. (Pontederiaceae) was obtained from a greenhouse colony at the USDA-Western Regional Research Center, Albany, CA, and were maintained as in Moran et al. (2016). Emergent, rooted water-yellow primrose (*Ludwigia hexapetala* (Hook. & Arn) Zardini et al, *Ludwigia grandiflora* (Michx.) Greuter & Burdet), or *Ludwigia peploides* subsp. *montevidensis* (Spreng.) P.H. Raven/*Ludiwigia peploides* subsp. *peploides* (Hoch and Grewell 2012) (Onagraceae) and Brazilian waterweed (*Egeria densa* Planch. (Hydrocharitaceae) were collected from one field site in the Sacramento-San Joaquin Delta and maintained under water nutrient conditions similar to those used for water hyacinth.

Experimental design. A 3x3 factorial design outdoor cage-enclosure study investigating the effect of plant species (water hyacinth, water primrose, and Brazilian waterweed) and plant density (high, intermediate, and low) on mosquito survival was conducted. The study was conducted during June and July 2015 at the Aquatic Weed Laboratory of the USDA-Agricultural Research Service, Exotic and Invasive Weeds Research Unit, in Davis, CA where average outdoor temperatures averaged 29.9 °C (high), (12.6 °C (low). Within one mesh lumite cage (3 m long x 2.0 m wide x 2.2 m tall) (Bioquip, Rancho Dominquez, CA), a total of 10 clear plastic containers (100 L volume; 85.7 cm long x 49.2 cm wide x 33.9 cm deep) were filled with 2/3rd of a bag (12 kg) of sand (KolorScape, Atlanta, GA) and 2/3rd of a bag (12 kg) of rock pellets (Vigoro, Atlanta, Ga). Dechlorinated water was added to each container to a height of 7 cm from the top. To standardize measurement of plant abundance across species, an estimation of percent area coverage was used as follows: 80-100% tank cover = high density, 50-80% tank cover = intermediate density and 10-50% tank cover = low density. The following treatment combinations were established: 1) water hyacinth – high density; 2) water hyacinth – intermediate density; 3) water hyacinth – low density; 4) water primrose – high density; 5) water primrose – intermediate density; 6) water primrose – low density; 7) Brazilian waterweed – high density; 8) Brazilian waterweed – intermediate density; 9) Brazilian waterweed – low density; or 10) control – no plant added. Plants were allowed one month before the first replicate to root (water primrose and Brazilian waterweed) and acclimate to container conditions, and 1-2 wk before subsequent replicates to acclimate to recover from culling-related disturbance. Plant densities were maintained by replacing (water hyacinth) or trimming (water primrose and Brazilian waterweed).

At the start of each experimental replicate, 50 eggs of *C. pipiens* were introduced into each container. Each tank was observed once every 48 hours over a span of two weeks. Starting at day six the total number of *C. pipiens* adults emerging was determined. The experiment was conducted five times (completely randomized block design with start dates as blocks), and containers were moved haphazardly within the cage between replicates, resulting in a total of five replicates for each treatment combination (N larvae per treatment per replicate = 50 ; N total larvae per treatment = 450).

Data analysis

To examine the effect of aquatic weed species and density on time to adult emergence (= time to complete immature development), data for counts of adult emerged at each sampling point (7, 8, 9, 11, and 14 days after addition of 50 eggs to each tank) were analyzed with SAS software (version 9.4.1) (SAS Institute, Cary, NC). Specifically, PROC LIFETEST was used, with survival estimates generated via the Kaplan-Meier method, and log-rank tests of significance to compare survival curves among aquatic weed species (water hyacinth, water primrose and Brazilian waterweed) and densities (high, low and intermediate). Tukey-corrected pairwise comparisons of survival curves were used to determine differences among survival curves. The initial analysis considered a total of 10 curves (three weeds at each of three densities, and the control). Follow-up analyses compared survival curves among the three weed species and control separately at each weed density (four curves in each test), the effect of weed species across all three densities excluding the control (three curves), and the effect of density across all three weed species excluding the control (three curves). Final counts of total adults emerged were compared across weed species and density using an analysis of variance in SAS PROC GLIMMIX, with a Poisson distribution assumption for count data. Follow-up ANOVAs considered the effect of weed species and the control at each weed density separately, the effect of weed species across all three densities, and the effect of density across all three weed species.

Results

Immature development time. Time for *C. pipiens* to complete immature development and emerge as adults varied significantly across the three weed species and densities (log-rank test, $\chi^2 = 63.0$, df= 9, $P < 0.0001$). The average time to adult emergence, across all three aquatic weeds and three density levels, was (mean ± SE) 10.4 ± 0.2 days (n = 1,453 emerged adults). In the control tank (no vegetation), time to emergence was 12.0 ± 0.3 days (n = 64 emerged adults). Survival curves did not vary across the three aquatic weed species when data for all densities were combined ($\chi^2 = 3.4$, df = 2, $P = 0.185$) (Fig. 4.1). Survival curves varied only slightly according to density when data for all three aquatic weed species were combined ($\chi^2 = 6.2$, df = 2, $P = 0.042$). Development was slightly slower after the 9th day in weeds of any species under intermediate as compared to low density (Fig. 4.1). Average time to emergence at high density, regardless of aquatic weed species, was 10.5 ± 0.1 (n = 471); at intermediate density, 10.6 ± 0.1 (n = 501); and at low density, 10.2 ± 0.1 (n = 481).

Adult emergence was compared between the three aquatic weed species and the control lacking vegetation separately for each weed density. At high density, time to emergence varied significantly ($\chi^2 = 42.5$, df = 3, $P < 0.0001$). Average time to emergence was 10.8 ± 0.2 days on water hyacinth (n = 160), 10.7 ± 0.2 days on water primrose (n = 167), 9.9 ± 0.2 days on Brazilian waterweed (n = 144), and 12.0 days in the absence of vegetation as noted above. In pairwise comparisons of survival curves, development time from egg to adult emergence was significantly shorter (by 1 to 2 days) in tanks containing Brazilian waterweed ($P < 0.0001$) or water primrose ($P = 0.041$) but not water hyacinth ($P = 0.31$), compared to tanks lacking vegetation (Fig. 4.2). Development time was significantly (by almost one day) shorter in tanks containing Brazilian waterweed than in those containing water hyacinth ($P = 0.001$) or water primrose ($P = 0.024$). At intermediate density, time to emergence varied significantly ($\chi^2 = 18.7$, df = 3, $P = 0.0003$). Average time to emergence was 10.5 ± 0.2 days on water hyacinth, (n = 166), 10.4 days on water primrose (n = 175), and 11.0 ± 0.2 days on Brazilian waterweed (n = 160). In pairwise comparisons, the presence of aquatic weed vegetation again significantly decreased time to adult emergence, but the pattern differed from what was observed at high density. At intermediate density, development time was significantly (by 1.5 days) shorter in tanks containing water hyacinth ($P = 0.001$) or water primrose ($P = 0.019$) than in control tanks, while emergence time did not differ in tanks containing Brazilian water weed compared to open water controls (Fig. 4.2). A 0.5-day difference between water hyacinth and Brazilian water weed was significant ($P = 0.028$). At low density, time to emergence varied significantly ($\chi^2 = 38.0$, df = 3, $P < 0.0001$). Average time to emergence was 10.0 ± 0.2 days on water hyacinth, (n = 153), 10.6 ± 0.2 days on water primrose (n = 216), and 9.7 ± 0.2 days on Brazilian waterweed (n = 112). In pairwise comparisons of the low-density tanks, the presence of water hyacinth ($P < 0.0001$) or Brazilian waterweed ($P < 0.0001$) but not water primrose ($P = 0.936$) decreased development time by 2 or more days compared to the control tanks (Fig. 4.2). The 0.5 to almost 1 full day shorter development time on water hyacinth or Brazilian waterweed at low density, compared to water primrose at low density, represented significant differences ($P = 0.005$ in both cases).

Total adult emergence. Total number of emerged adults were counted for each tank 14 days after egg addition, in each of five replicate tests. In two-factor analysis of variance, the effect of aquatic weed species was significant (F = 10.47, df = 2, 40, $P = 0.0002$), as was the weed species-density interaction (F = 3.87, df = 4, 40, $P = 0.009$) but not the density factor (F = 0.80, df = 2, 40, $P = 0.458$). The effect of aquatic weed species was investigated separately for each density. At high density, emergence was significantly higher in the presence of any of the three aquatic weed species than in the control (F = 15.78, df = 3, 16, $P < 0.0001$) and there was no difference between the three weeds (Fig. 4.3). The presence of aquatic vegetation increased adult emergence 2.2-fold to 2.6-fold. The same pattern of differences occurred at intermediate plant density (F = 17.65, df = 3, 16, $P < 0.0001$) (Fig. 4.3.), as aquatic weed vegetation increased adult emergence 2.5-fold to 2.7-fold. At low density, adult emergence varied significantly (F = 28.24, df = 3, 16, $P < 0.0001$), but in this case, 1.4-fold more adults emerged from tanks with water primrose at low density than from tanks with water hyacinth at low density, and 1.9-fold more than from tanks with Brazilian waterweed at low density. The presence of any aquatic weed at low density increased adult emergence at least 1.8-fold over the control tanks. [(P<0.0001), (P<0.0001)]

Discussion

This study investigated the influence of both invasive aquatic plant density and type (species) on mosquito development time and total adult mosquito survival. Our hypothesis that differences in plant density and type would lead to differences in larval *C. pipiens* development time and survival was supported. I found that the presence of aquatic weed vegetation reduced the development time of *C. pipiens* by 1 to 2 days. Additionally, the presence of aquatic weed vegetation increased the number of eggs successfully completing development by 2-fold to almost 3-fold in some cases.

I found that exposure of *C. pipiens* to submerged Brazilian waterweed at a high (80 to 100% surface coverage) density resulted in the shortest time to adult emergence than in the presence of floating water hyacinth or emergent water primrose (or control tank with no vegetation at this density. The reverse pattern was observed at the intermediate (50 to 80% surface coverage) density; exposure of C. *pipiens* to floating water hyacinth and emergent water primrose resulted in the shortest time to adult emergence than in the presence of submerged Brazilian waterweed and the control tank with no vegetation. A third pattern was observed at the low (10 to 50% surface coverage) density; exposure of *C. pipiens* to floating water hyacinth and submergent Brazilian waterweed resulted in the shortest time to development than in the presence of emergent water primrose and the control tank with no vegetation. Overall, while mosquito development time was reduced in the presence of vegetation compared to the control, there was no consistent pattern of a plant species effect across all three plant densities.

These findings are consistent with other studies that found that specific types (Grutters et al. 2015; Valinoti, Ho and Armitage 2011; Martin and Valentine 2011; Rantala et al. 2004) or densities (Savino and Stein 1989) of aquatic vegetation resulted in higher survival for macroinvertebrate prey in aquatic systems. In contrast to this study in which prey survival was highest at intermediate plant densities of water hyacinth, other studies report that predator foraging success rather than prey survival is highest at intermediate plant densities of macrophyte species (Valley and Bremigan 2002; Wiley et al. 1984; Savino and Stein 1982; Crowder and Cooper 1982). Some studies found that prey survival increases only at high vegetation densities, i.e lacking open water (Coen et al. 1981; Stoner 1982; Orth et al. 1984; Nelson and Bonsdorff 1990 ; Orr and Resh 1991), whereas others found that higher plant densities does not necessarily result in the same effect (Canion and Heck 2009). Thus, more studies are needed to make generalizations about the effect of plant density on prey survival across multiple systems.

The maximization of survival among intermediate densities, particularly among water hyacinth, may be explained by similar effects of aquatic macrophytes on mosquito larvae and mosquitofish in the experiment. The intermediate plant density was likely high enough to disguise *C. pipiens* but low enough to make *G. affinis* remain conspicuous. It is possible that at high densities submerged plant shoots (Brazilian water weed), shoots and roots in combination (water primrose) or roots (water hyacinth) had a disguising effect on *G. affinis* that cancelled out the same disguising effect on *C. pipiens* reducing the time windows for prey to avoid the predator. It was expected, as observed, that mosquito survival would be higher across all vegetative treatments at sufficient density compared to the control treatment containing no vegetation, as a lack of refuge resulted in prey being more conspicuous. However, the observation of a protective effect of water hyacinth, even at low levels of refuge, illustrate the dependence of predator-prey interactions on plant species (Grutters et al. 2015) as some prey may be more successful at avoiding predators in low refuge settings if the prey moves relatively fast, and benefits from aquatic plant species-specific habitat structure. Thus, the impact of habitat complexity on prey survival may depend on predator and prey microhabitat use (Klecka and Boukal 2014; Power 1992). Detailed behavioral studies would be required to determine the specific benefit of water hyacinth to either reduce *C. pipiens* apparency or increase *G. affinis* apparency, and to determine resulting effects on predator search time. The results demonstrate the importance of testing various plant species within prey survival experiments in order to generalize or refute conclusions about the benefits of invasive aquatic macrophytes or other aquatic plants for mosquito larval survival. (Grutters et al. 2015; van Kleunen et al. 2014).

Paragraph needed on implications of results for water hyacinth and mosquito management. Include reference that water hyacinth is a major problem. Include reference that *Culex* mosquitos are a major problem. Then make conclusion about how the results could inform management of water hyacinth, and of C. *pipiens*.

Acknowledgments

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Figure 4.1. Emergence curves (probability of <u>not emerging</u>) for *C. pipiens* mosquito egg to adult stage (A) in the presence of Brazilian waterweed, water hyacinth, water primrose (independent of density) and (B) at different plant density levels (independent of plant species).

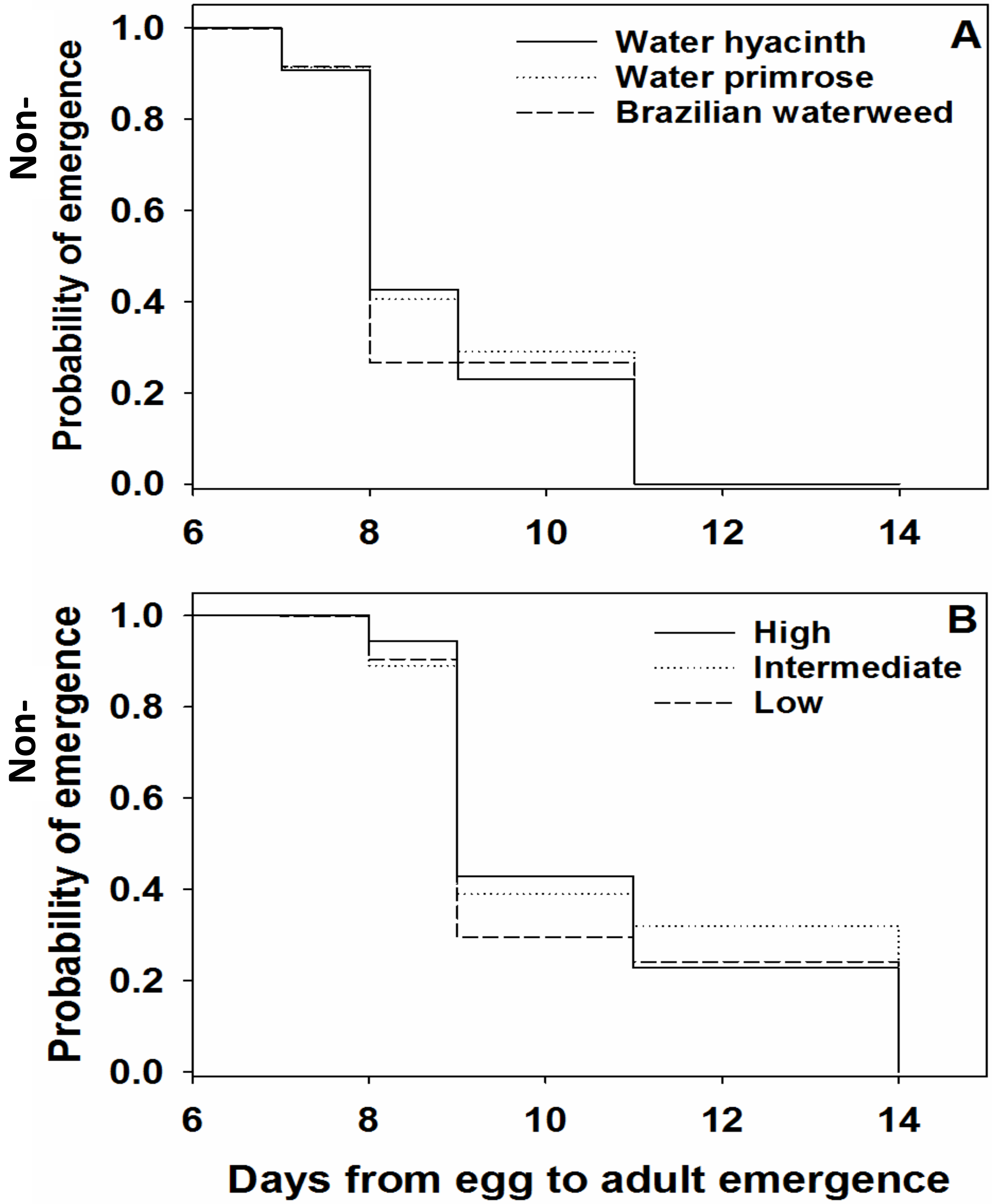


Figure 4.2. Emergence curves (probability of not emerging) for *C. pipiens* mosquito egg to adult stage in the presence of Brazilian waterweed, water hyacinth, water primrose, in containers with **(A)** high (80 to 100% water surface coverage), **(B)** intermediate (50 to 80% coverage) or, **(C)** low (10 to 50% coverage) densities of these plants.

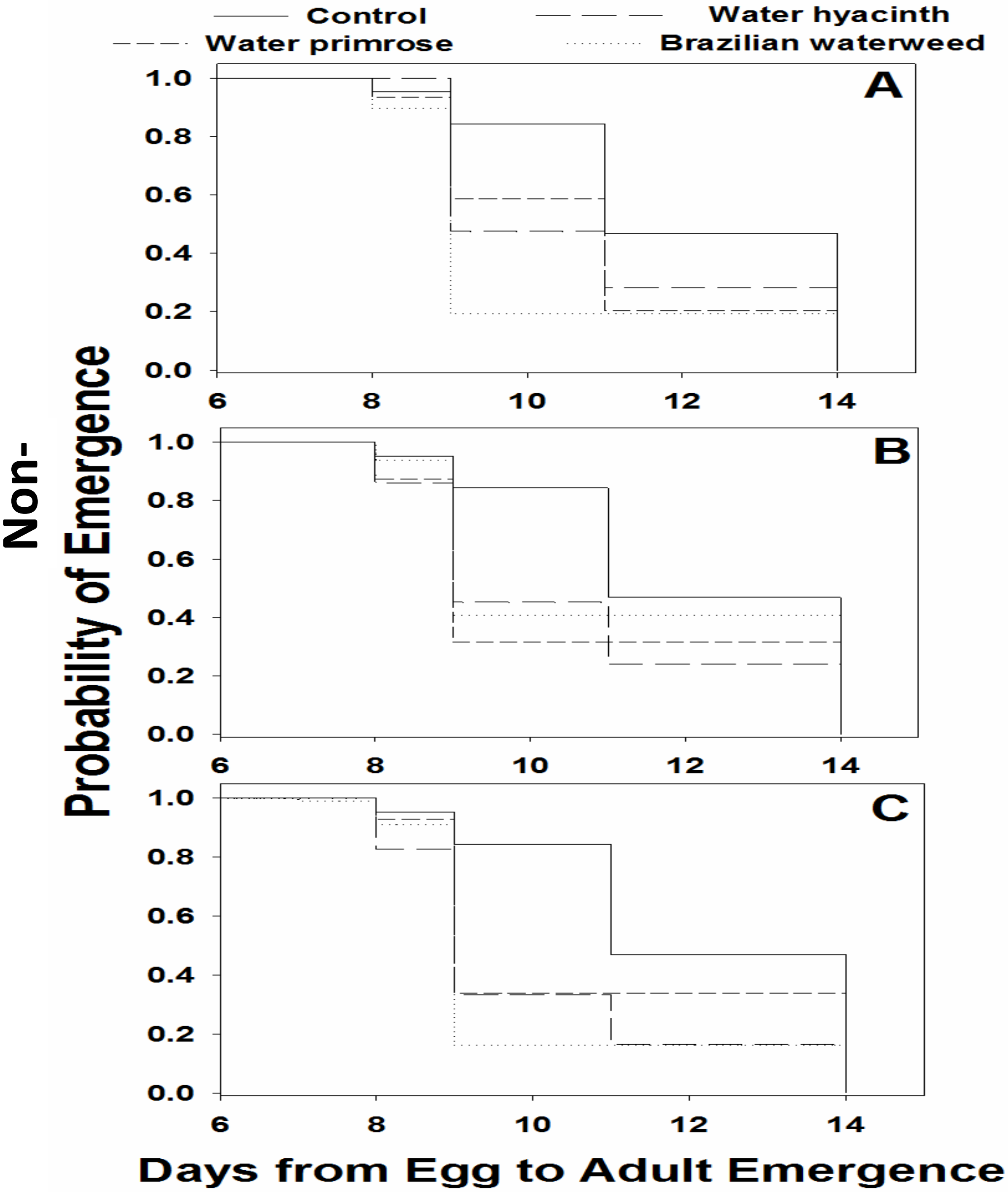
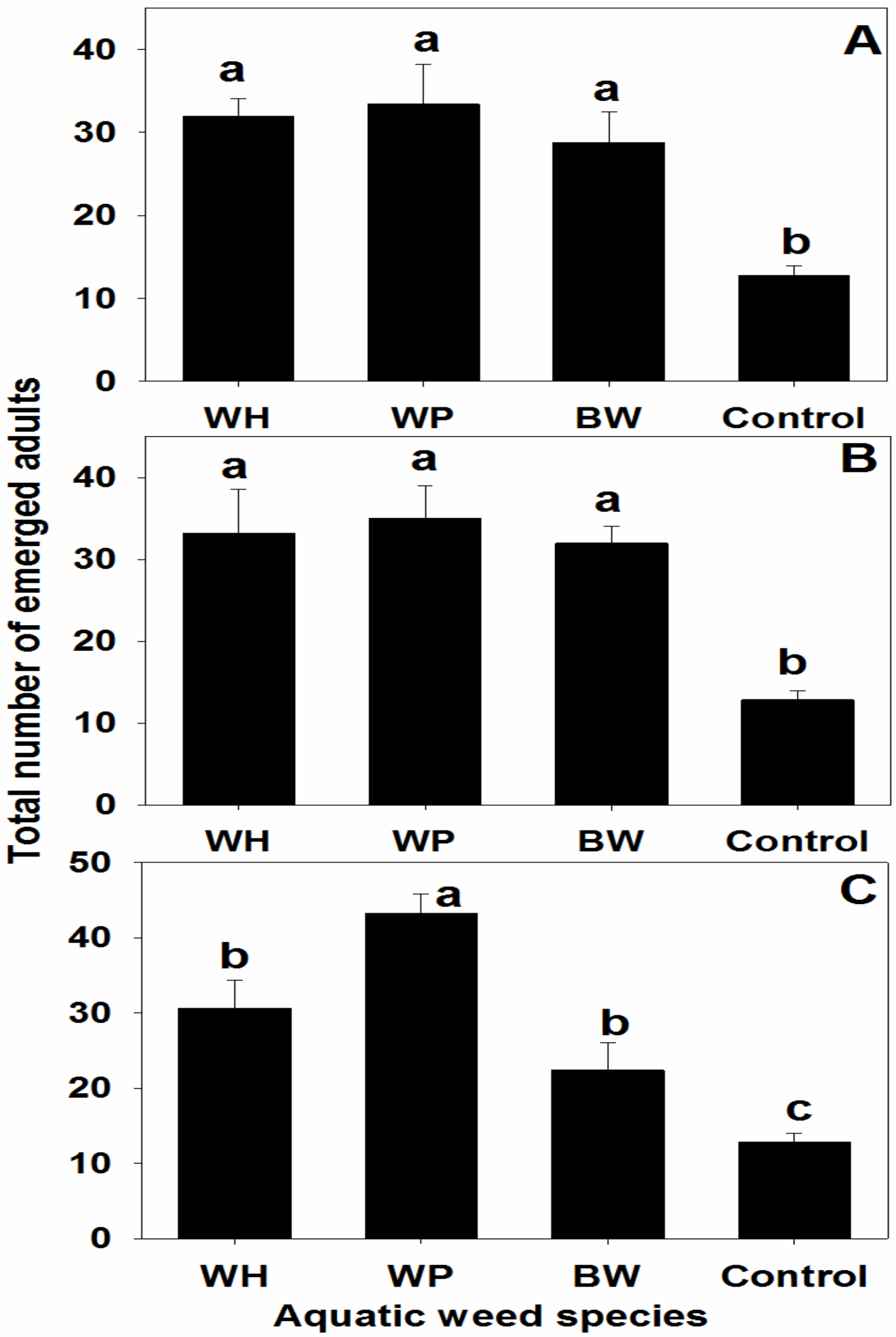


Figure 4.3 Adult emergence outcomes for *C. pipiens* mosquito when reared on Brazilian waterweed, water hyacinth, water primrose, and control water in containers with **(A)** high (80 to 100% water surface coverage), **(B)** intermediate (50 to 80% coverage) or, **(C)** low (10 to 50% coverage) densities of these plants.



Chapter 5

Flight and oviposition responses in mosquito disease vectors, *Culex quinquefasciatus, Aedes aegypti,* and *Anopheles quadrimaculatus* (Diptera: Culicidae): Impacts by invasive aquatic macrophytes and their infusions

Abstract

Mosquitoes use a variety of cues to assess whether a habitat is conducive for the development of their offspring. In addition to factors such as temperature, time of day, and turbidity, the types of plants present in an aquatic environment can influence mosquito behavior. In the present study the attractiveness of aquatic plants and their infusions to adult mosquitoes, *Culex quinquefasciatus, Aedes aegypti,* and *Anopheles quadrimaculatus*, were evaluated as measured by either oviposition behavior and/or flight visits to ports containing one of five species of plants (including an infusion). The species of plants used in the study included *Eicchornia crassipes* (floating water hyacinth), *Pista stratioles* (floating water lettuce), *Myriophyllum aquaticum* (emergent parrotfeather), *Hydrocotyle umbellata* (floating pennywort), and *Oryza sativa* (hay infusion). In a laboratory cage choice bioassay, the mean proportion of egg rafts laid was significantly greater in bowls containing infusions of water hyacinth, water lettuce, and a hay infusion compared to bowls with water only by over 10-fold. In bowls containing pennywort and parrotfeather there was no significant difference in the mean proportion of egg rafts laid compared to bowls with water only. In the olfactometer experiment, ports containing water hyacinth, water lettuce, and the hay infusion each received significantly greater visits by the three mosquito species collectively compared to ports water alone by three-fold, half-fold, and 10-fold, respectively. These results suggest that water hyacinth and water lettuce may possess specific chemical or a blend of chemicals that are attractive to adult mosquitoes.

Introduction

Plants are known to provide various resources to insects including habitat, protection from predators, shading, food, and oviposition sites. Through evolution different groups of insects have developed an ability to associate various plant-derived volatiles with specific types of plants (Nyasembe and Torto 2014; Paré and Tumlinson 1999; Berkov et al. 2000; Schiestl et al. 2000; Loreto and Velikova 2001; Schiestl and Ayasse 2001; Sharkey et al. 2001; Van Poecke et al. 2001). Examples of these plant-derived volatile organic compounds include terpenoids, benzenoids, and other nitrogenous compounds (Knudsen et al. 1993). These compounds have been used widely in agricultural pest management to attract insect pests to traps and repel them away from crops. However, while plant-derived odorous compounds have been widely exploited for use in agricultural pest management their use in the management of pests of medical significance, particularly mosquitoes, has not been sufficiently investigated (Nyasembe and Torto 2014).

Since the early to mid-1900's there has been evidence supporting the theory that mosquitoes can use both visual and olfactory cues to locate specific plants as demonstrated by Sandolhm and Price (1962) which showed that bright-colored flowers with distinct aromas attracted mosquitoes. Joseph (1970) demonstrated that *Culex, Aedes, Anopheles, Psorophora*, and *Culiseta* mosquitoes could be lured into traps by exploiting their attraction to damaged fruits in crop settings. Similarly, in the 1980s studies were able to parse out the roles of visual and olfactory cues in mosquito attraction to plants by use of wind tunnel experiments (Jepson and Healy 1988; Healy and Jepson 1988). Several other studies have demonstrated the attractiveness of plant-derived odors *to Culex, Aedes*, and *Anopheles* mosquitoes (Vargo and Foster 1982; Mauer and Rowley 1999; Otienoburo et al. 2012; Nyasembe et al. 2012).

Mosquitoes may associate specific odorous compounds or blends of compounds with substrate on which they can feed and oviposit (Pichersky and Gershenzon 2002; Bruce et al. 2005; Bruce and Pickett 2011). Within a blend the dominant compound is not necessarily the most influential on plant attractiveness to insects (Webster et al. 2008; Riffell, Christensen, and Hildebrand 2009). Either specific odorous compounds or a blend of compounds may determine levels of attraction or repellency (Rajkumar and Jebanesan 2005; Pushpanathan et al. 2006; Foster 2008; Stone and Foster 2013). Additionally, the emission of different concentrations of the same compounds within and across plant species likely influences attractiveness to mosquitoes (Bruce et al. 2005; Najar-Rodriguez et al.

2010; Gols et al. 2012). Several studies document emissions of varying concentrations of the same compound between interspecific and among intraspecific plants (Jhumur et al. 2008).

Ascertaining particular plants and plant-associated materials (e.g., plant infusions) that are attractive to mosquitoes could improve management strategies for these medically important pests (Ferguson et al. 2010; Govella and Ferguson 2012). Plant infusions could be used to develop more effective mosquito traps to monitor oviposition activity in the field (Reiter et al. 1991; Chadee et al. 1993; Polson et al. 2002; Sant' Ana et al. 2006; Ponnusamy et al. 2010). Several studies have demonstrated the attraction of gravid female mosquitoes to hay and other plant infusions, which have been used in ovitraps to monitor mosquito activity (Isoe et al. 1995b; Mboera et al. 2000; Isoe and Millar 1995; Polson et al. 2002). The attractiveness of an infusion in part depends on the plant species from which it was derived (Ponnusamy et al. 2010).

In the first study I examined the impact of infusions from four aquatic and one terrestrial plant species on oviposition of egg rafts in cups by *C. quinquefasciatus*. The five plant species from which infusions were obtained were two invasive species -- water hyacinth and parrotfeather -- and three native species -- water lettuce, pennywort, and hay. I hypothesized that the proportion of egg rafts laid by *C. quinquefasciatus* would be higher in cups holding plant infusions and lower in cups holding non plant-infused water (control). In the second study I compared the collective flight landing responses of *C. quinquefasciatus*, *A. aegypti,* and *A. quinquefasciatus* to chambers housing water hyacinth, parrotfeather, water lettuce, pennywort, and hay infusion in an olfactometer. I hypothesized that the chambers housing plants (and hay infusion) would receive a higher proportion of visits by *C. quinquefasciatus, A, aegypti.,* and *A. quinquefasciatus* (collectively) than the chambers housing only water.

Materials and Methods

Biological materials. Adults of *C. quinquefasciatus, A. aegypti, and A. quinquefasciatus* were obtained from laboratory colonies at the United States Department of Agriculture Center for Medical, Agricultural, and Veterinary Entomology (CMAVE) facility in Gainesville, FL, and were reared as described in (Gerberg, Bernard, and Ward 1994). Adult mosquitoes were reared on 5% sugar solutions provided continuously. Cages were maintained at a temperature range of 27-29°C Floating invasive water hyacinth, *Eichhornia crassipes* (Mart.) Solms. (Pontederiaceae), rooted invasive parrotfeather, *Myriophyllum aquaticum,* (Vell.)

Verdc. (Haloragaceae), floating native pennywort, *Hydrocotyle umbellata* (Linnaeus) (Araliaceae), floating native water lettuce, Pistia stratioles L. (Pontederiaceae) and hay, Oryza sativa L. (Poaceae) were obtained from ponds near the USDA-CMAVE facility and the Department of Entomology and Nematology at the University of Florida, Gainesville, FL. Plant infusions were prepared as described in Polson et al. (2002) using tightly sealed plastic bags.

Plant infusion bioassay experimental design. A cage enclosure choice study was performed to investigate the effect of plant infusions (water hyacinth, parrotfeather, water lettuce, pennywort, and hay) on mosquito oviposition, measured as the proportion of egg rafts laid. The study was conducted in a laboratory environment at the USDA-ARS Center for Medical, Agricultural, and Veterinary Entomology, Insect Behavior and Biocontrol Research Unit in Gainesville, FL. Temperature was maintained at 27-29°C and relative humidity at 70-85%. A photoperiod of 14 hours of light and 10 hours of dark was used in the study. A total of 144 clear glass bowls (10 oz, Pyrex) were used to hold plant infusions and dechlorinated control water; 60 cups were each filled with 9 ounces of a plant infusion (each of a set of 12 cups received infusions of either water hyacinth, water lettuce, pennywort, parrotfeather, or hay) and 72 cups were each filled with 9 ounces of control water. Each pair of cups, one containing a plant infusion and the other control water (or a pair of cups each holding control water), were placed in a standard mosquito rearing cage (12x12x12'', BioQuip), one on either side directly across from the other. A total of 12 replicates were run per treatment-control pair (six total treatment pairs: water hyacinth infusion and control; parrotfeather infusion and control; pennywort infusion and control; water lettuce infusion and control; hay infusion and control; and control and control).

At the start of each experimental replicate each day 50 *C. quinquefasciatus* adult, blood-fed females were placed into each of 24 cages; each day I initiated four replicates of each of the six treatment pairs over a span of three days. Each cage was initially observed for 15 minutes to ensure that adult mosquitoes remained in suitable condition upon release. Thereafter data was collected after 24 hours and a new block repeating the same steps was performed over the following two days to obtain 12 replicates per treatment-control pair; within each cage water was replaced during mosquito replacement each day. At each observation time the number of egg rafts in each of the two cups per cage was determined and converted into proportions, along with the plant species from which the infusion was derived.

Olfactometer experimental design. The responses of adult mosquitoes were evaluated in a triple-cage olfactometer with dual ports (Posey et al. 1998). The olfactometer consisted of a large, rectangular acrylic arena containing three chambers in a tiered configuration, each containing a pair of ports with removable sleeves from which air flowed. External air was charcoal-filtered under a designated temperature and humidity (27-29°C and relative humidity at 60-62%), with flow through the ports occurring at a rate of 28.0 cm/s. Within the test arena 100 adult female *C. quinquefasciatus* mosquitoes were released and allowed to acclimate to the environment for 1 hour. After 1 hour each of two 10 ounce glass bowls (Pyrex) were added to one of the two ports, a treatment port (holding one of four plant species in 9 ounces of water or the hay infusion) and a control port (holding 9 ounces of water). The contents of each port were masked from view of mosquitoes to cancel out visual effects. Adult mosquitoes had the option to remain in the arena or follow an upwind current to either the treatment port or control port in each of the three chambers, allowing for three replicates to run simultaneously.

After introducing 100 adult female C. quinquefasciatus mosquitoes into the arena I observed the mosquitoes for 15 minutes to ensure that the specimens remained in suitable condition following release. Thereafter the chamber was observed for one hour at which time three additional replications were initiated until 10 replications were completed for each treatment-control pair (and control-control pair). Because only three treatment-control pairs could run during any given replication the pairs were randomly ordered.

Data analysis. In the bioassay experimental design a paired-samples t-test was conducted to compare the mean proportion of egg rafts laid by *C. quinquefasciatus* mosquitoes in bowls holding plant infusions and bowls holding control water ($P<0.05$) Before analyses data were arcsine transformed. In the olfactometer experiment a paired-samples t-test was conducted to compare the mean proportion of adult *C. quinquefasciatus*, *A. aegypti*, and *A. quadrimaculatus* landing inside of treatment ports containing plants and ports containing no plants ($P<0.05$). There was no effect of mosquito species on the proportion visiting ports so this factor was removed from the analyses and collective proportions across all three species were used. Subsequent analysis of variance (ANOVA) was performed to compare the differences in the mean proportion of mosquitoes visiting the treatment-control ports across plant species.

Results

Plant Infusion Bioassay Experiment

The mean difference in the proportion of egg rafts laid (M=86.85, N=12) among the water hyacinth infusion (M=93.425, SD=6.34) and a paired control (M=6.575, SD=6.34) was significantly greater than zero; t(11)=23.69, two tail p<8.61e-11, indicating that water hyacinth infusion facilitated oviposition more than the control (Figure 5.1). The mean difference in the proportion of egg rafts laid (M=26.72, N=12) among the parrotfeather infusion (M=53.325, SD=25.22) and a paired control (M=38.34, SD=22.36) was not significantly greater than zero; t(11)=1.37, two tail p<0.199, indicating that there was no significant difference in oviposition among the parrotfeather infusion and paired control (Figure 5.2). The mean difference in the proportion of egg rafts laid (M=91.17, N=12) among the water lettuce infusion (M=95.58, SD=6.03) and a paired control (M=4.42, SD=6.03) was significantly greater than zero; t(11)=26.17, two tail p<2.94e-11, indicating that the water lettuce infusion facilitated oviposition more than the control (Figure 5.3). The mean difference in the proportion of egg rafts laid (M=20.95, N=12) among the pennywort infusion (M=57.14, SD=17.01) and a paired control (M=40.36, SD=19.49) was not significantly greater than zero; t(11)=1.64, two tail p<0.13, indicating there was no significant difference in oviposition among the pennywort infusion and the control (Figure 5.4). The mean difference in the proportion of egg rafts laid (M=96.06, N=12) among the hay infusion (M=98.03, SD=3.62) and a paired control (M=1.97, SD=3.62) was significantly greater than zero; t(11)=45.96, two tail p<6.32e-14, indicating that the hay infusion facilitated oviposition more than the control (Figure 5.5). The mean proportion of egg rafts laid (M=43.05, N=12) among one control (M=47.16, SD=33.21) and the other control (M=44.51, SD=32.85) was not significantly greater than zero; t(11)=0.15, two tail p<0.88, indicating that neither control facilitated oviposition more than the other (Figure 5.6).

Olfactometer Experiment

There was a significant difference in the percent of mosquitoes attracted into the port containing water hyacinth (M=82.1, SD=9.29) and the control port (M=17.9, SD=9.29); t(9)=10.91, p=1.72e-6, indicating that water hyacinth attracted visits by all three mosquito species more than the control (Figure 5.7). There was not a significant difference in the percent of mosquitoes attracted into the port containing parrotfeather (M=50.42, SD=23.86) and the control port (M=49.56, SD=23.83);

t(9)=0.05, p=0.956, indicating that parrotfeather did not influence visits by all three mosquito species any more than the control (Figure 5.8). There was a significant difference in the percent of mosquitoes attracted into the port containing water lettuce (M=63.72, SD=16.78) and the control port (M=41.28, SD=21.36); t(18)=2.61, p=0.017, indicating that water lettuce attracted visits by all three mosquito species more than the control (Figure 5.9). There was not a significant difference in the percent of mosquitoes attracted in the port containing pennywort (M=53.33, SD=23.25) and the control port (M=46.67, SD=23.25); t(9)=0.45, p=0.66, indicating that pennywort did not influence visits by all three mosquito species any more than the control (Figure 5.10). There was a significant difference in the percent of mosquitoes attracted in the port containing hay infusion (M=91.4, SD=6.03) and control chamber (M=8.26, SD=6.03); t(9)=21.9, p=4.06e-9, indicating that the hay infusion attracted visits by all three mosquito species more than the control (Figure 5.11). There was not a significant difference in the percent of mosquitoes attracted in the port containing the first control (M=43.33, SD=43.88) and the port containing the second control (M=36.67, SD=42.16); t(9)=0.66, p<0.53, indicating that neither of the controls attracted visits by all three mosquito species more than the other (Figure 5.12).

A one-way between subjects ANOVA was conducted to compare the effect of plant species on the difference in proportion of adult mosquitoes visiting chambers containing a particular plant species in water and a control chamber containing only water. There was a significant effect of plant species on the difference in proportion of adult mosquitoes visiting chambers containing plants and chambers containing only water at the p<0.05 level for the three conditions [F(4,45)=9.125, p=1.74e-05] (Figure 5.13). Post hoc comparisons using a t-test with Bonferroni correction indicated that the difference in the mean proportion of adult mosquito visits in the hay infusion port and its paired control port (M=83.5, SD=12.05) and the difference in the mean proportion of adult mosquito visits in the water hyacinth port and its paired control port (M=64.2, SD=18.59) were significantly greater than the differences in the mean proportion of adult mosquito visits in the parrotfeather port and its control port (M=34.7, SD=30.65); pennywort port and its control port (M=35.3, SD= 28.73); and water lettuce port and its control port (M=33.3, SD=23.49).

Discussion

This study explored the influence of five species of plants and their infusions on mosquito oviposition behavior and attraction to plants where visual cues were removed. In the first experiment, my hypothesis that the proportion of egg rafts laid by *C. quinquefasciatus* would be higher in cups holding plant infusions and lower in cups holding non plant-infused water (control) was supported. In the second experiment, my hypothesis that the chambers housing plants (and hay infusion) would receive a higher proportion of visits by *C. quinquefasciatus, A, aegypti.,* and *A. quinquefasciatus* than the ports housing only water was also supported. The proportion of egg rafts laid on average in infusions of water hyacinth, water lettuce, and hay were all significantly greater than that laid in their respective control pairs suggesting. However, the proportion of egg rafts laid on average in infusions of parrotfeather and pennywort were not significantly different than that laid in their respective control pairs.

These results suggest that certain plant species and their infusions possess more attractive properties and thus facilitate oviposition more than others. These attractive properties may include specific volatile odorous compounds or a blend of these compounds that adult mosquitoes associate with oviposition sites (Pichersky and Gershenzon 2002; Bruce et al. 2005; Bruce and Picket 2011). Parrotfeather and pennywort may not have the specific compounds or blends of compounds that are present in water hyacinth, water lettuce, and Bermuda hay. Additionally, parrotfeather and pennywort may possess the same compound present in water hyacinth, water lettuce, and Bermuda hay but in ratios that make the plant and its infusion less attractive. The ratios in which odorous compounds are released from interspecific and intraspecific plants can impact their attractiveness to insects (Bruce et al. 2005). Additionally, bacteria present in plants and their infusions may influence their attractiveness to mosquitoes. Ponnusamy et al. (2015, 2010) demonstrated that some bacterial isolates from bamboo plant infusions significantly influenced the attraction of gravid *A. aegypti* and *A. albopictus* females to potential oviposition sites. The influence of bacteria isolated from hay infusion on *A. aegypti* and *C. quinquefasciatus* has also been documented (Hazard, Turner, and Lofgren 1967). Other studies have reported similar results (Rockett 1987; Benzon and Apperson 1987; Hasselschwert 1988; Pavlovich and Rockett 2000).

Anopheles mosquitoes, the primary vectors of malaria, have been associated with water hyacinth for several decades. Minakawa et al. (2012) demonstrated that within and around Lake Victoria, the second largest lake in the world, *A. rivulorum* dominated sites invaded by water hyacinth compared to open water sites and sites with other types of vegetation as measured by larval sampling and malaria incidences among residents. Additionally, this larval A. rivulorum are highly associated with water lettuce in this area (Lesson 1937). *A. funestus* is another malaria vector that primarily occupies bodies of water invaded by aquatic vegetation in wetlands adjacent to Lake Victoria (Gillies and DeMeillon 1968; Gimnig et al. 2001; Evans and Symes 1937). Among this aquatic vegetation, water hyacinth is considered to be the putative factor associated with *A. funestus* in this area (Ofulla et al. 2010; Minakawa, Seda, and Yan 2002).

The results from this research demonstrate the importance of investigating not only the structural impacts but also the non-structural impacts plants have on mosquito behavioral ecology. Not surprisingly, the results from the bioassay experiment align with those from the olfactometer experiment. Water hyacinth and hay infusion in both experiments facilitated mosquito oviposition and visits into ports relative to the control. Interestingly, water lettuce did not significantly impact mosquito visits into its olfactometer port relative to the control even though there was a significant impact of the water lettuce infusion on mosquito oviposition relative to the control. This may indicate that compounds within water lettuce attract mosquitoes from very close range but not from further ranges; the olfactometer chamber was larger in size compared to the rearing cages in which the boassay experimental replicates were run. The strong attractiveness of hay infusion to adult mosquitoes in this study is supported by Polson et al. (2002) who demonstrated that hay infusion-baited traps are a more accurate indicator of A. aegypti activity than traps baited with water alone. However, a study by Eneh et al. (2016) demonstrated that *A. gambiae* preferred tap water alone over water with hay infusion; the attraction of *Aedes* and *Culex* mosquitoes to hay infusions is documented in the literature and in this present study. While adult *A. quadrimaculata*, collectively with *C. quinquefasciatus* and A. aegypti, more readily visited ports containing plants over ports containing water alone, it is possible that a reverse effect would have been seen if oviposition preference had been measured. It is possible that the olfactory cues used to locate a plant are not identical to those used to stimulate egg-laying. Additional investigations are needed to ascertain the impact of specific and blends volatile compounds present across plant species on different mosquito groups.

Effective management of mosquito populations relies on a combination of integrated approaches that include the application of larvicides, adulticides, and source reduction. However, novel methods for suppressing mosquitoes remain a priority as diseases with which these pests are associated continue to exist in different parts of the world (The malERA Consultative Group on Monitoring 2011). In addition to the management approaches mentioned above, plant-derived chemicals, or phytochemicals, may be exploited for their attractive properties to be used in ovitraps or as a mixture with pesticides (Rajkumar and Jebanesan 2005; Pushpanathan et al. 2006; Foster 2008; Stone and Foster 2013). Water hyacinth continues to invade the Sacramento-San Joaquin River Delta region as does parrotfeather and pennywort, though to lesser extents and occurring at different points in the season. The results from this research suggest that water hyacinth is conducive for mosquito biology and behavior and thus provide more reason to believe that this invasive weed should be targeted for management.

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Figure 5.1. *C. quinquefasciatus* mosquito oviposition preferences in the presence of two bowls containing either a water hyacinth infusion or tap water (control). Graph shows the mean proportion of egg rafts laid in each bowl. There was a statistically significant difference in the mean proportion of egg rafts laid among the two bowls (p<8.61e-11).

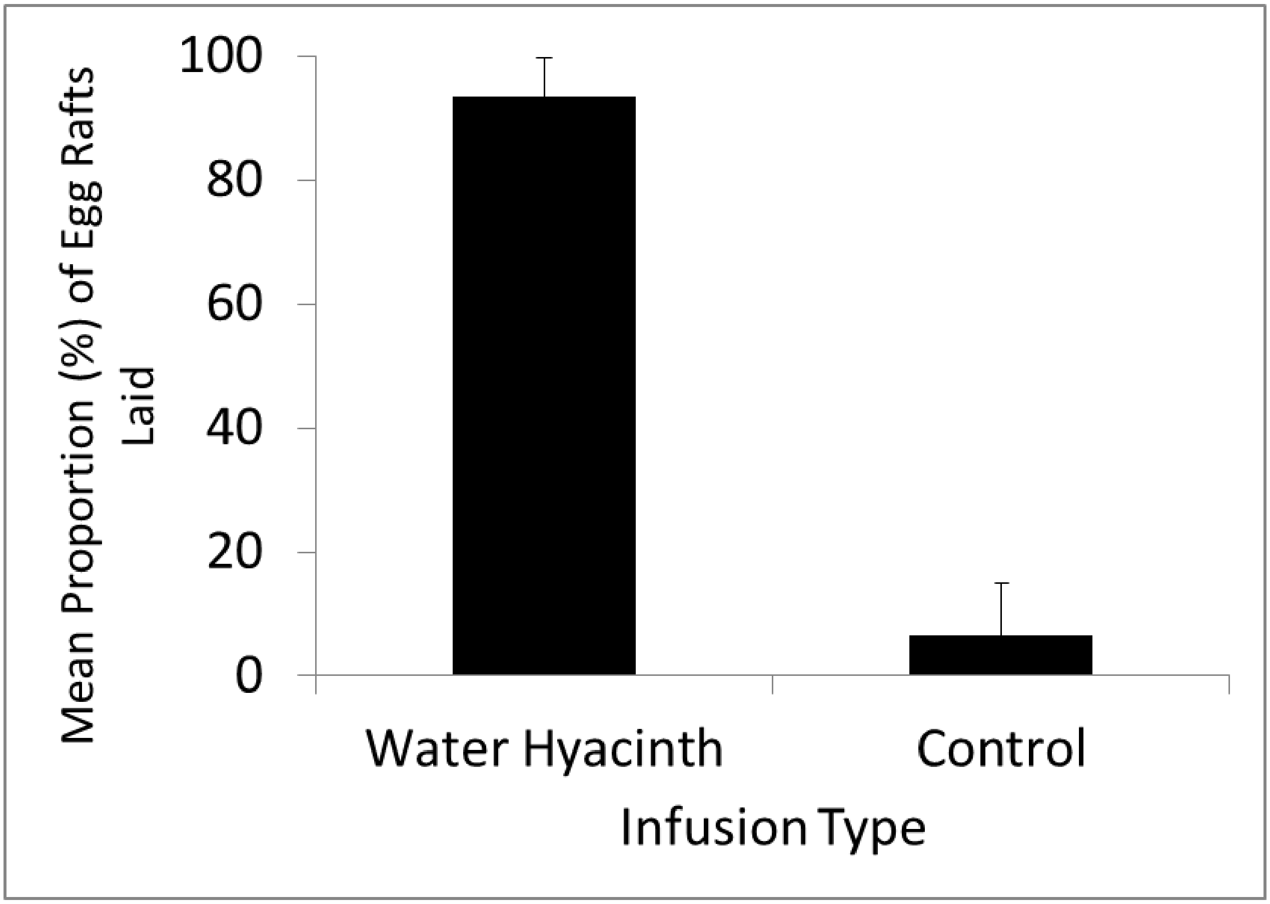


Figure 5.2. *C. quinquefasciatus* mosquito oviposition preferences in the presence of two bowls containing either a parrotfeather infusion or tap water (control). Graph shows the mean proportion of egg rafts laid in each bowl. There was not a statistically significant difference in the mean proportion of egg rafts laid among the two bowls (p=0.199).

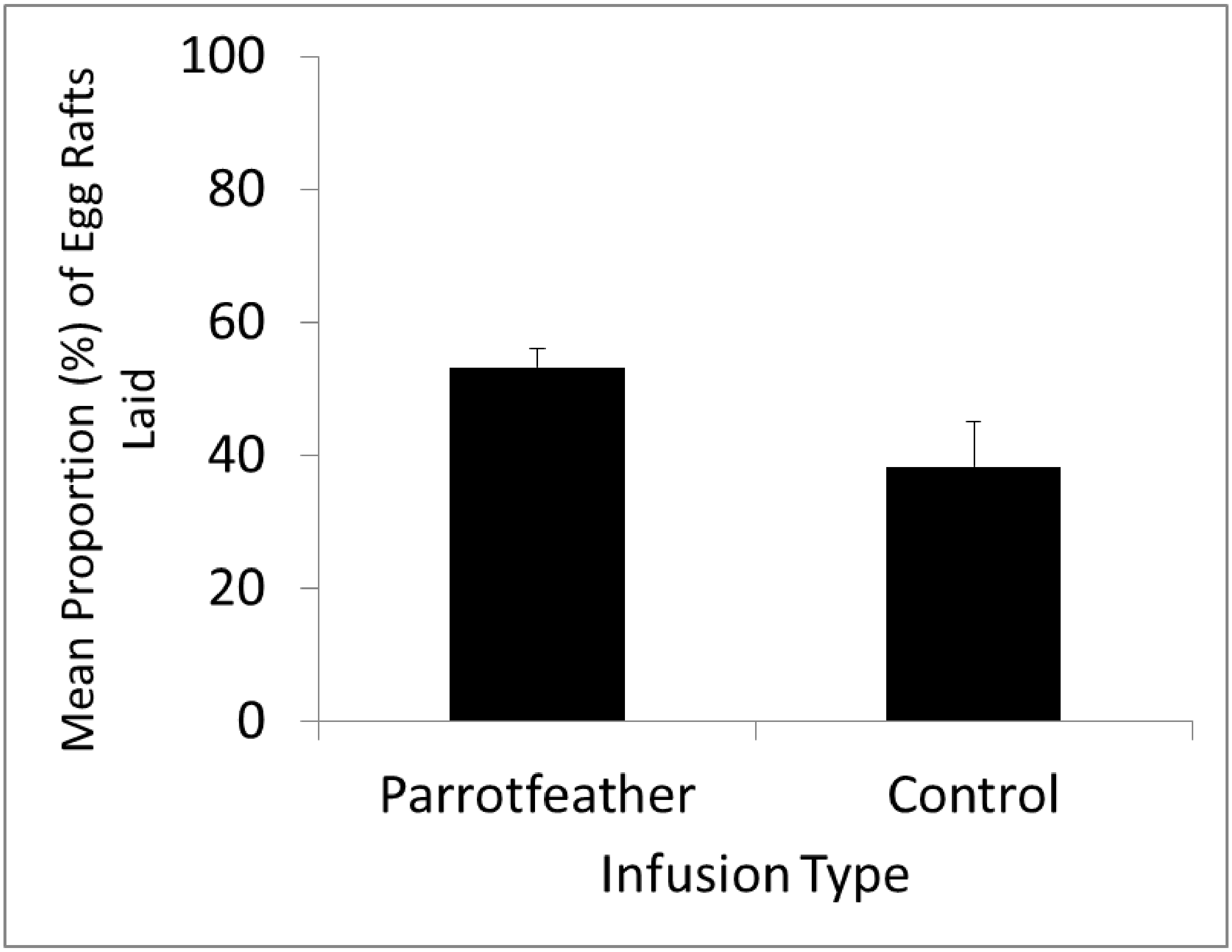


Figure 5.3. *C. quinquefasciatus* mosquito oviposition preferences in the presence of two bowls containing either a water lettuce infusion or tap water (control). Graph shows the mean proportion of egg rafts laid in each bowl. There was a statistically significant difference in the mean proportion of egg rafts laid among the two bowls (p<2.9e-11).

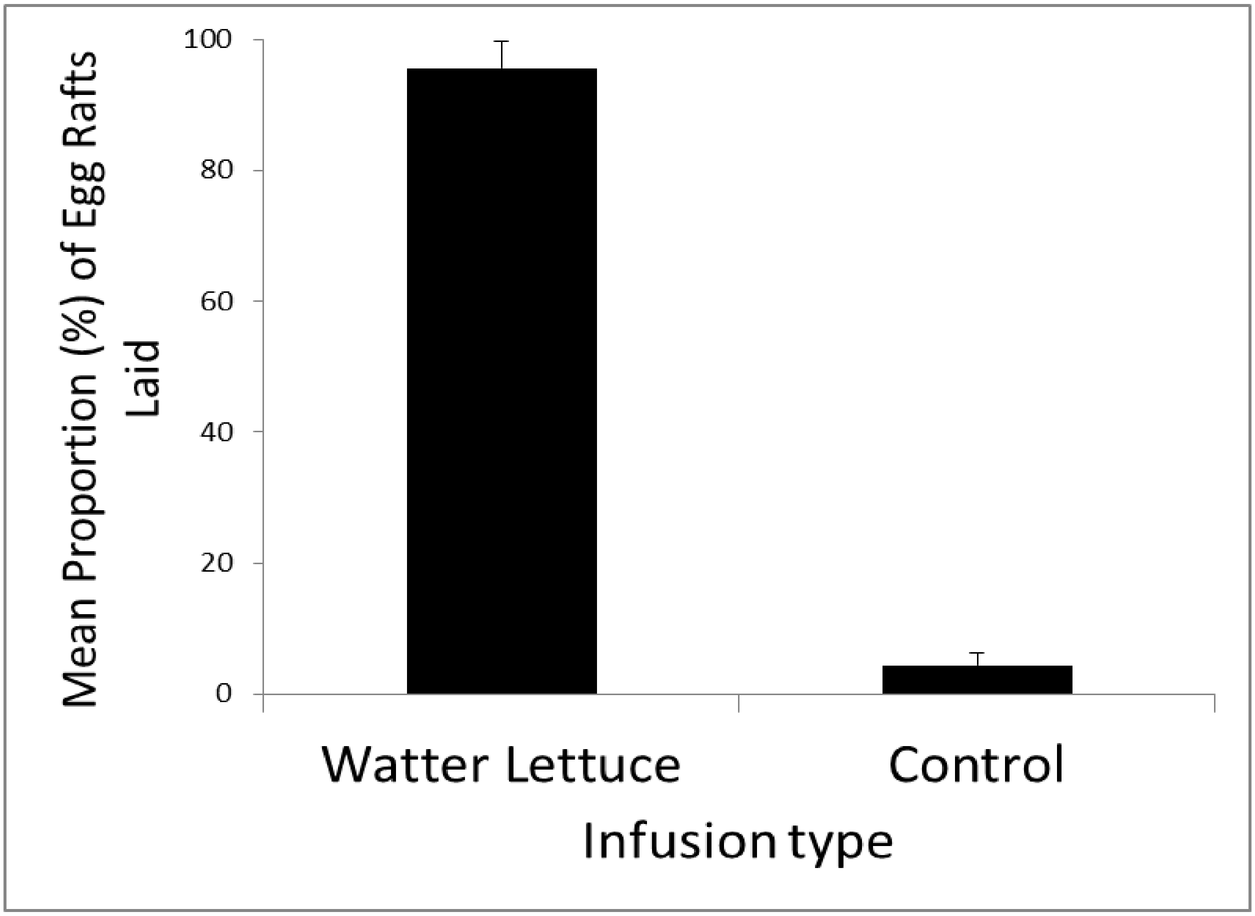


Figure 5.4. *C. quinquefasciatus* mosquito oviposition preferences in the presence of two bowls containing either a pennywort infusion or tap water (control). Graph shows the mean proportion of egg rafts laid in each bowl. There was not a statistically significant difference in the mean proportion of egg rafts laid among the two bowls (p=0.13).

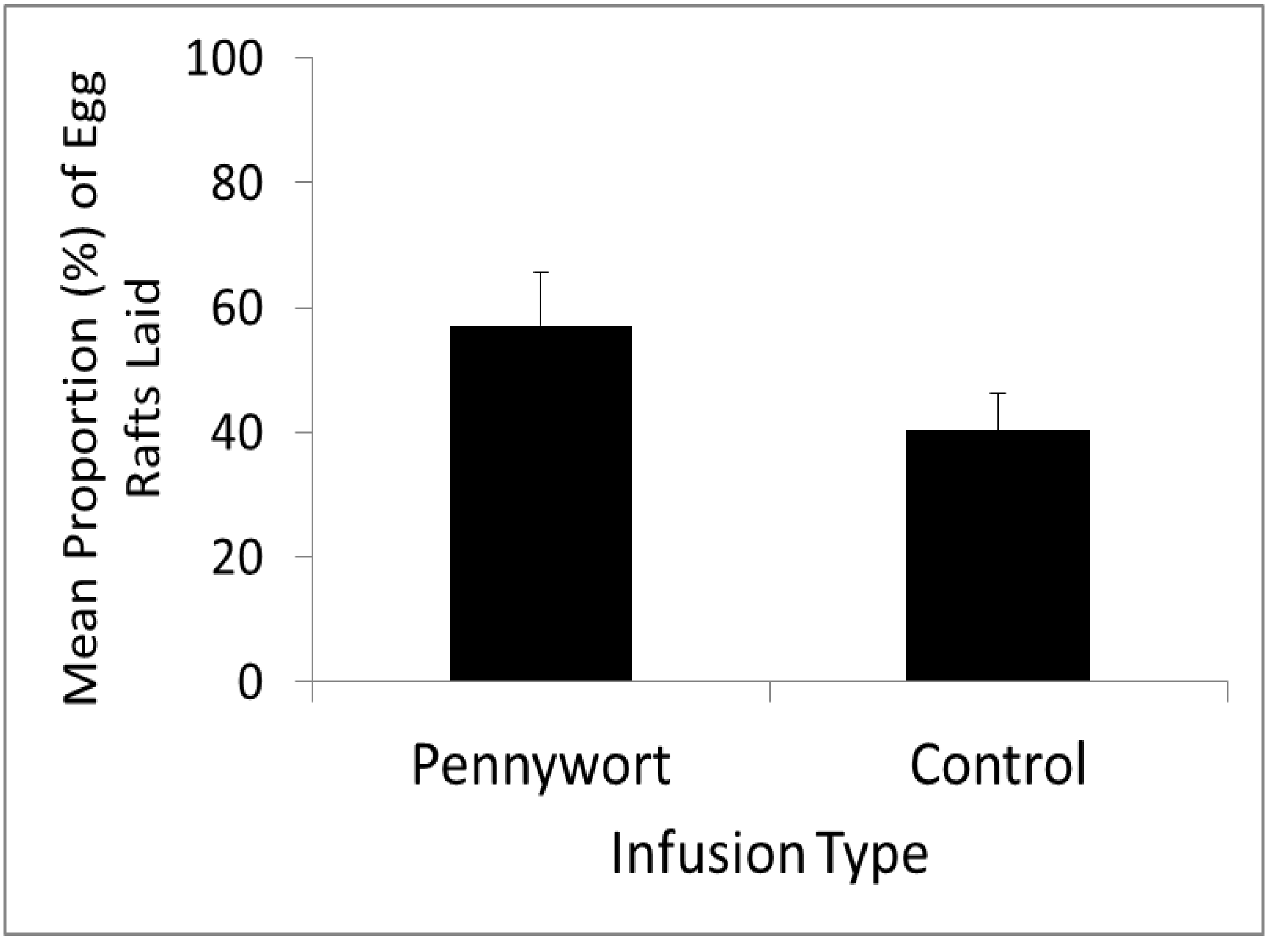


Figure 5.5. *C. quinquefasciatus* mosquito oviposition preferences in the presence of two bowls containing either a hay infusion or tap water (control). Graph shows the mean proportion of egg rafts laid in each bowl. There was a statistically significant difference in the mean proportion of egg rafts laid among the two bowls ($p<6.32e-14$).

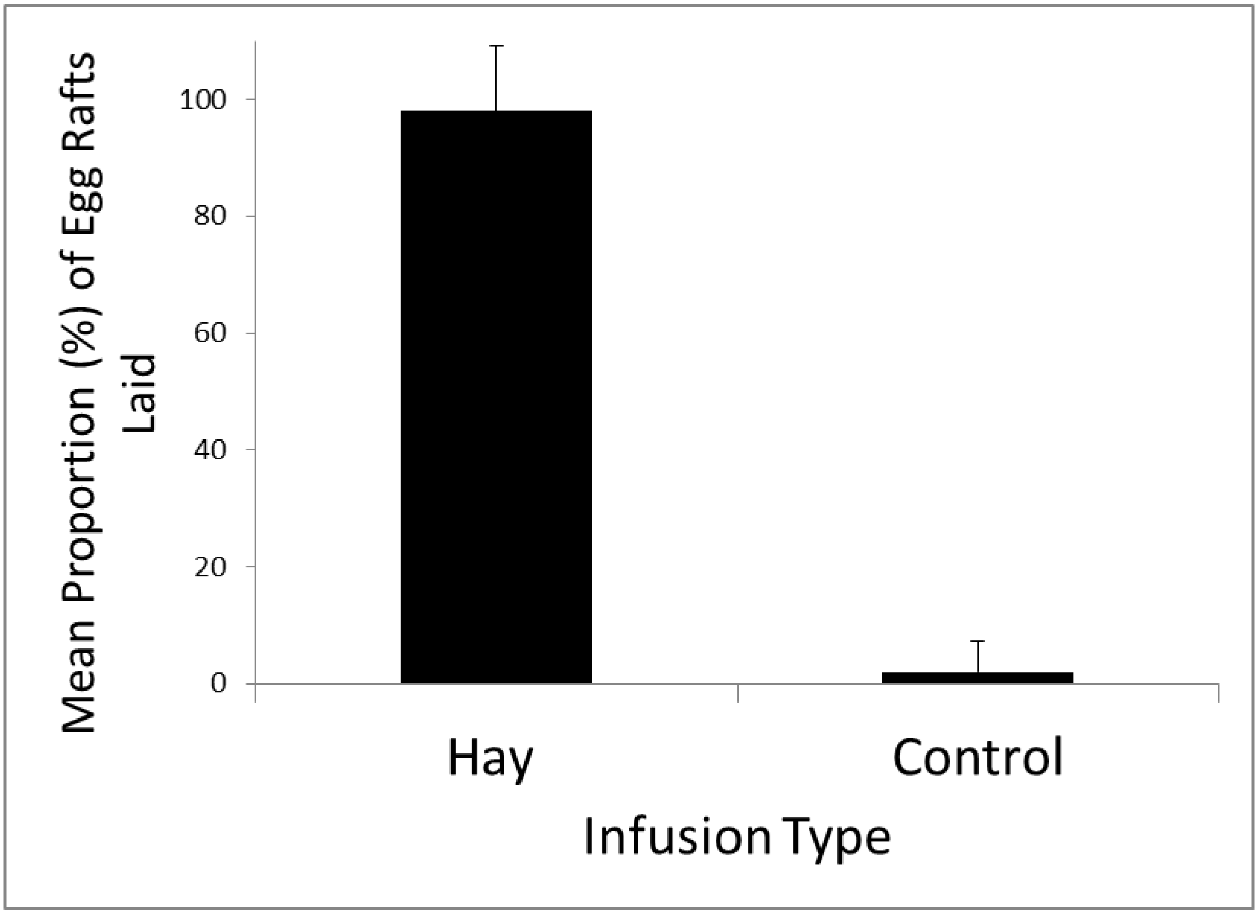


Figure 5.6. *C. quinquefasciatus* mosquito oviposition preferences in the presence of two bowls containing tap water (both controls). Graph shows the mean proportion of egg rafts laid in each bowl. There was a not statistically significant difference in the mean proportion of egg rafts laid among the two bowls (p=0.88).

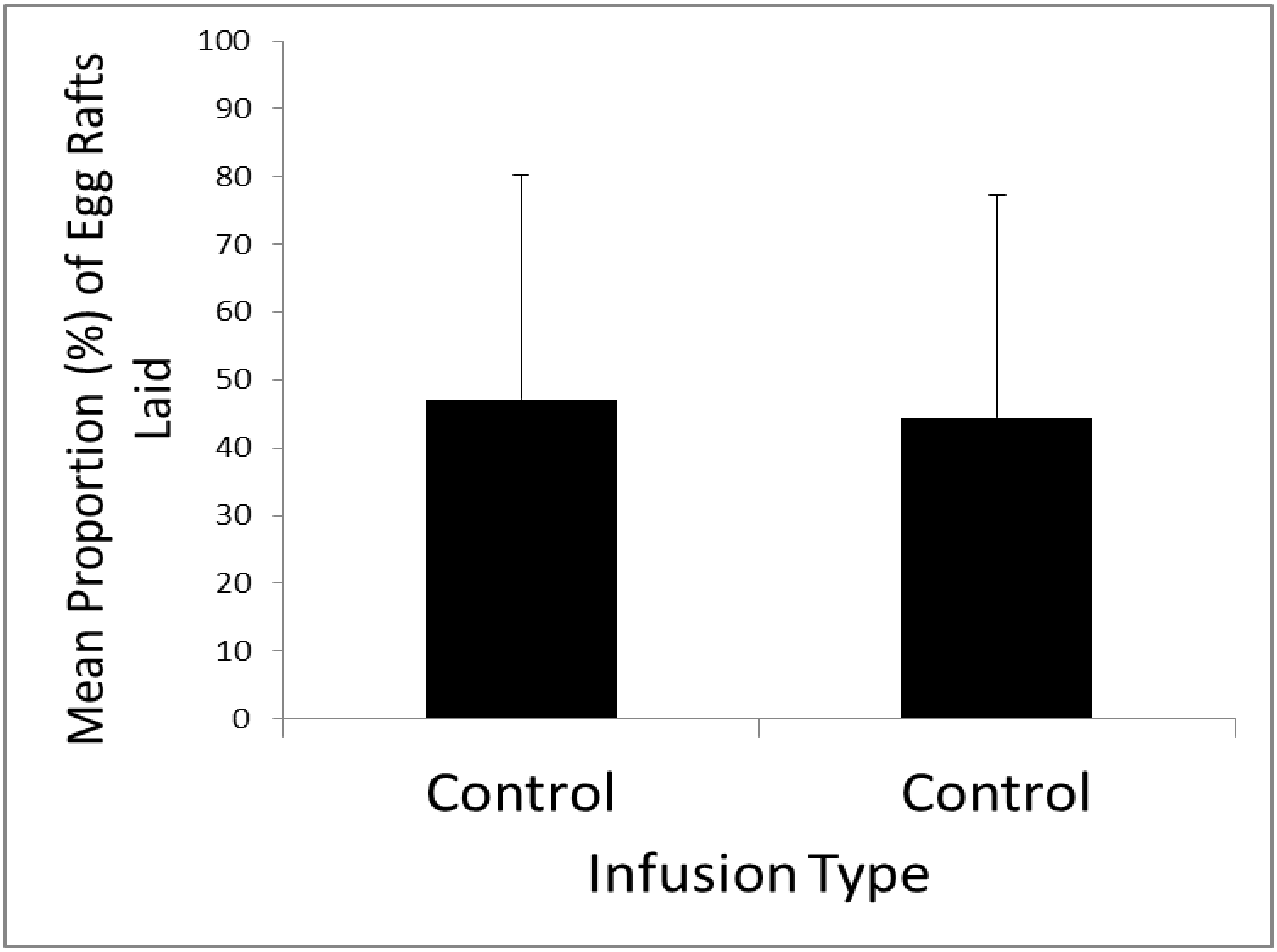


Figure 5.7. *C. quinquefasciatus, A. aegypti*, and *A. quadrimaculatus* mosquito visit preferences into two ports, one containing water hyacinth and the other containing tap water (control). Graph shows the mean proportion of collective visits by the three mosquito species into both ports. There was a statistically significant difference in the mean proportion of collective visits by the three mosquito species into ports (p<1.76e-6).

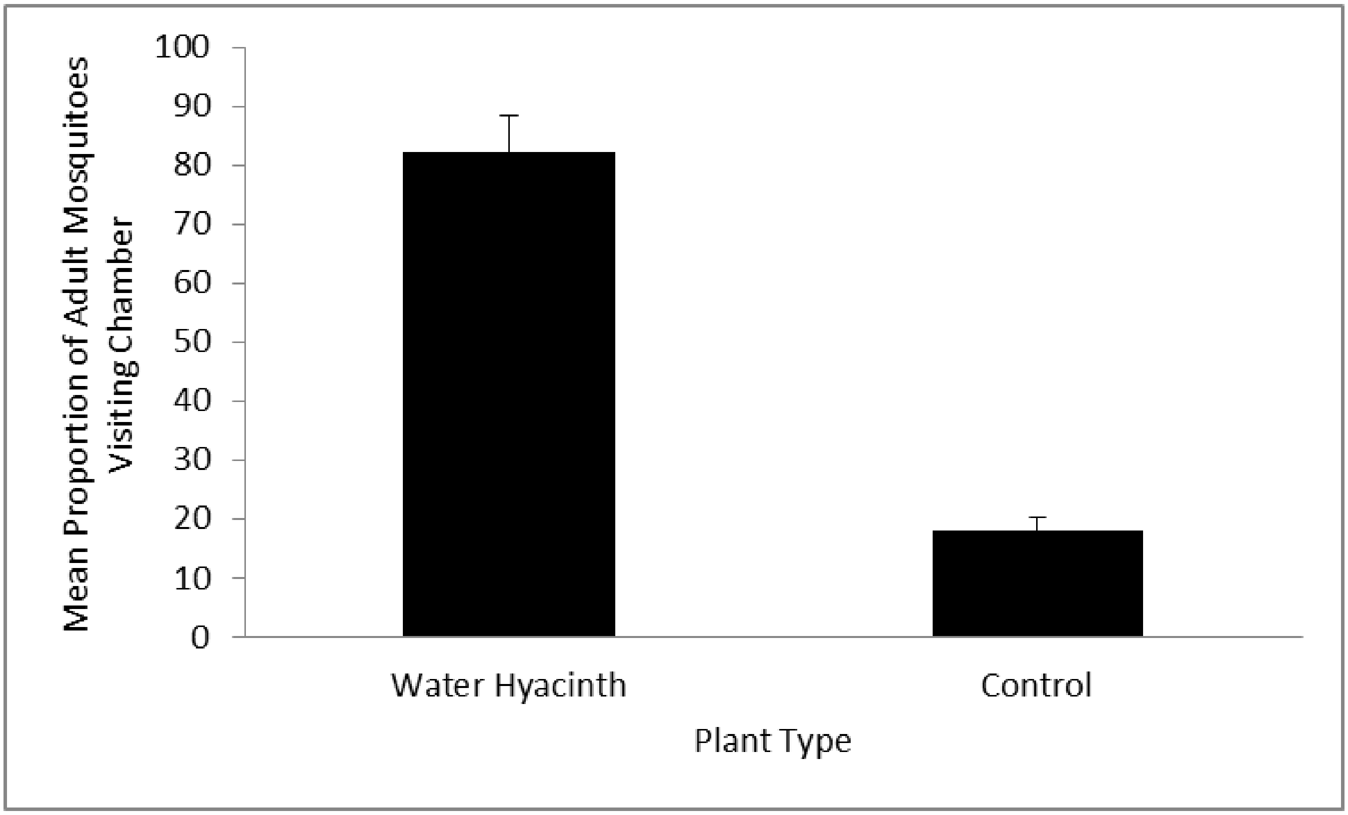


Figure 5.8. *C. quinquefasciatus, A. aegypti,* and *A. quadrimaculatus* mosquito visit preferences into two ports, one containing parrotfeather and the other containing tap water (control). Graph shows the mean proportion of collective visits by the three mosquito species into both ports. There was not a statistically significant difference in the mean proportion of collective visits by the three mosquito species into ports (p=0.96).

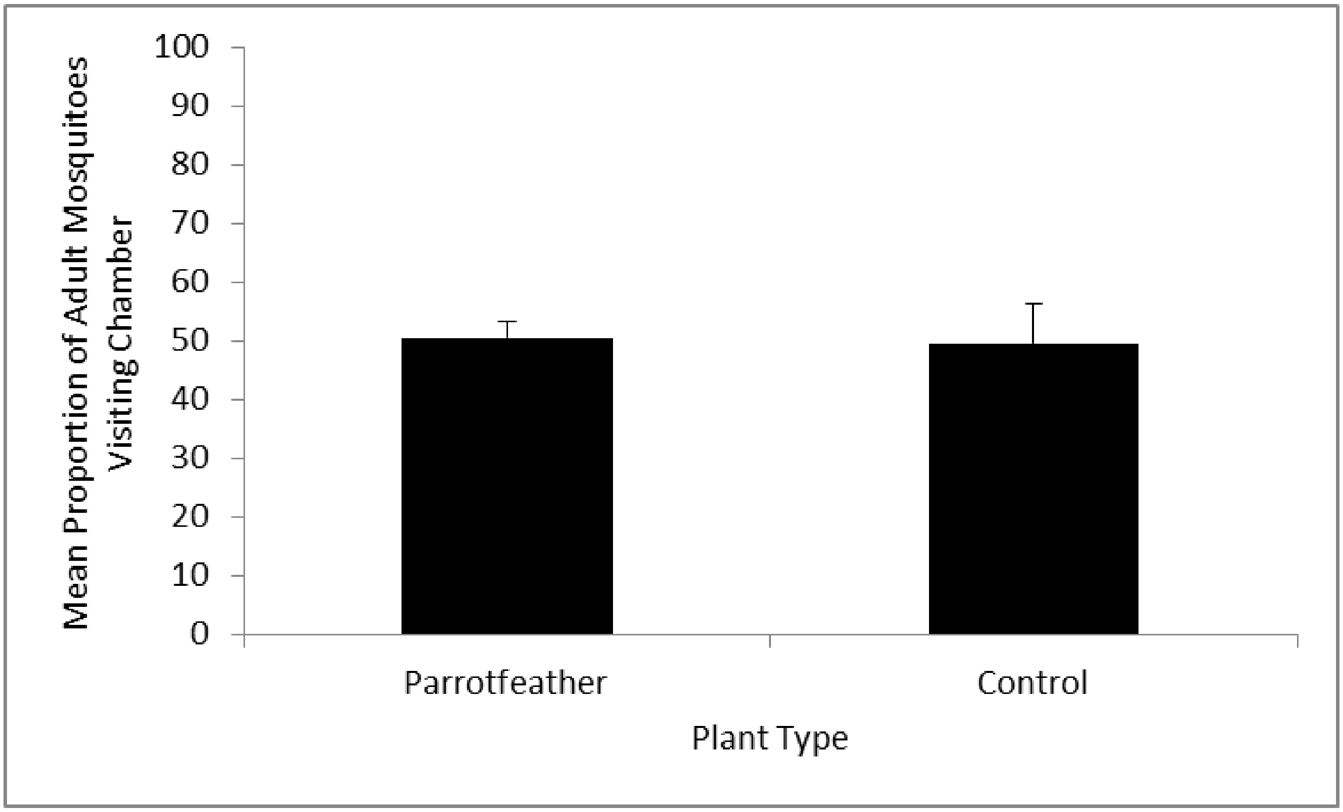


Figure 5.9. *C. quinquefasciatus, A. aegypti,* and *A. quadrimaculatus* mosquito visit preferences into two ports, one containing water lettuce and the other containing tap water (control). Graph shows the mean proportion of collective visits by the three mosquito species into both ports. There was a statistically significant difference in the mean proportion of collective visits by the three mosquito species into ports (p<0.017).

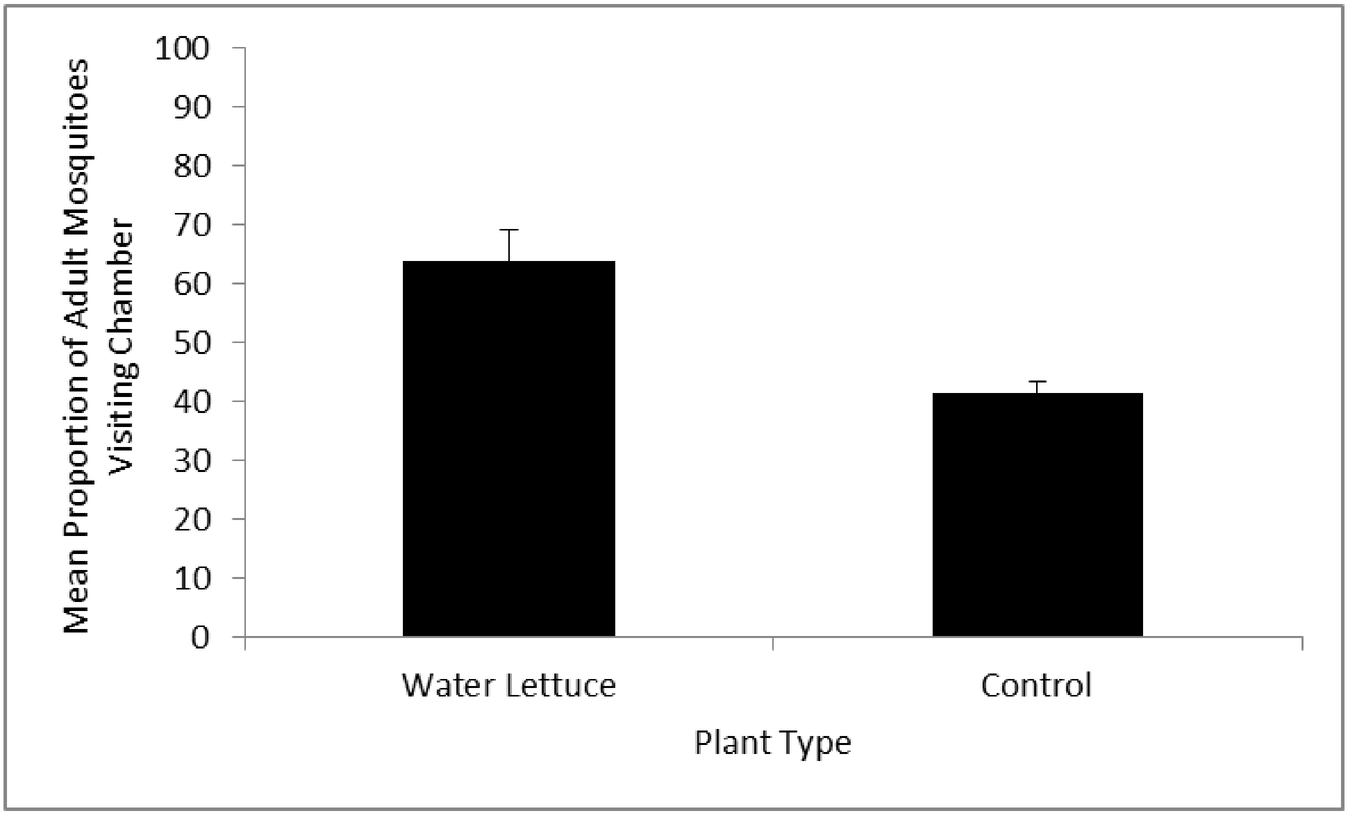


Figure 5.10. *C. quinquefasciatus*, *A. aegypti,* and *A. quadrimaculatus* mosquito visit preferences into two ports, one containing pennywort and the other containing tap water (control). Graph shows the mean proportion of collective visits by the three mosquito species into both ports. There was not a statistically significant difference in the mean proportion of collective visits by the three mosquito species into ports (p=0.66).

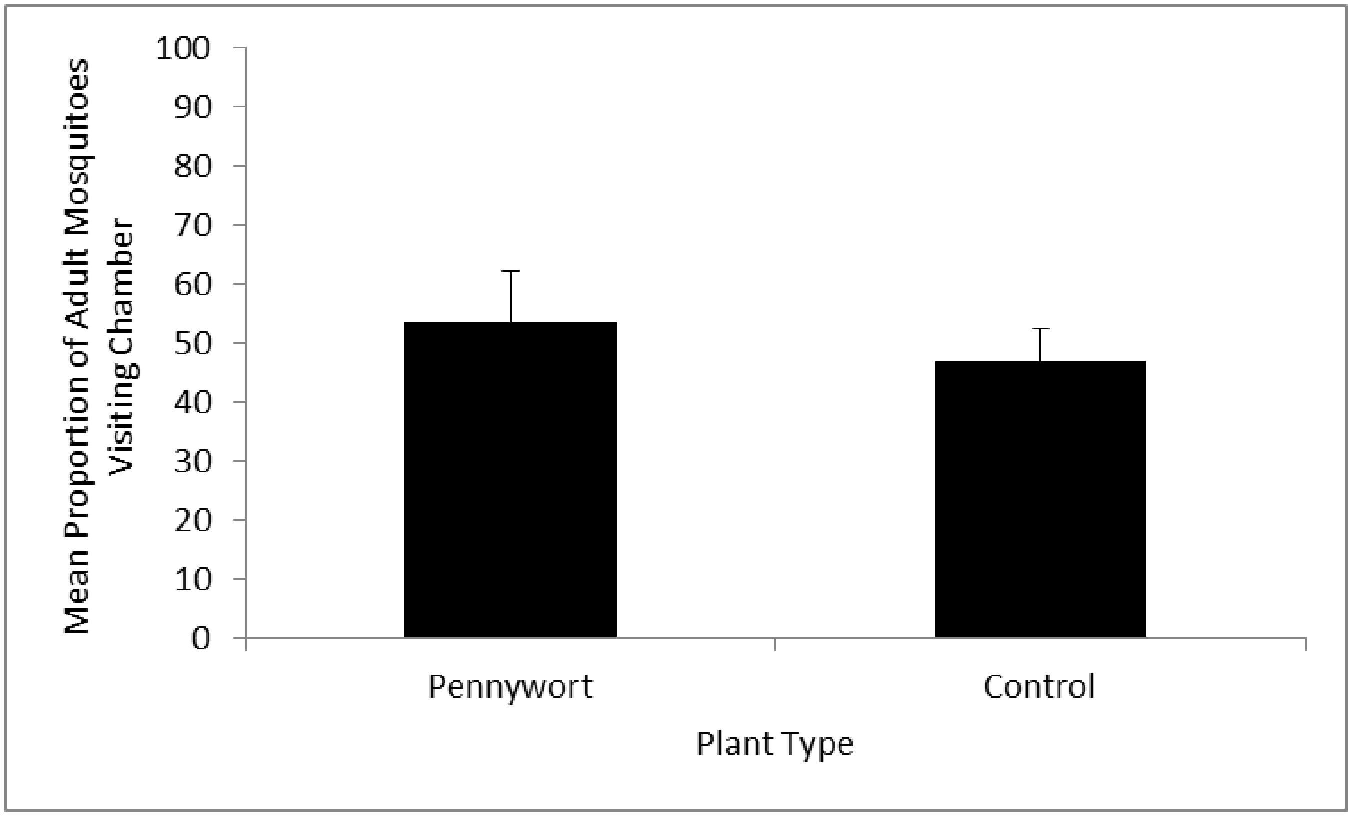


Figure 5.11. *C. quinquefasciatus*, *A. aegypti,* and *A. quadrimaculatus* mosquito visit preferences into two ports, one containing hay infusion and the other containing tap water (control). Graph shows the mean proportion of collective visits by the three mosquito species into both ports. There was a statistically significant difference in the mean proportion of collective visits by the three mosquito species into ports (p<4.07e-9).

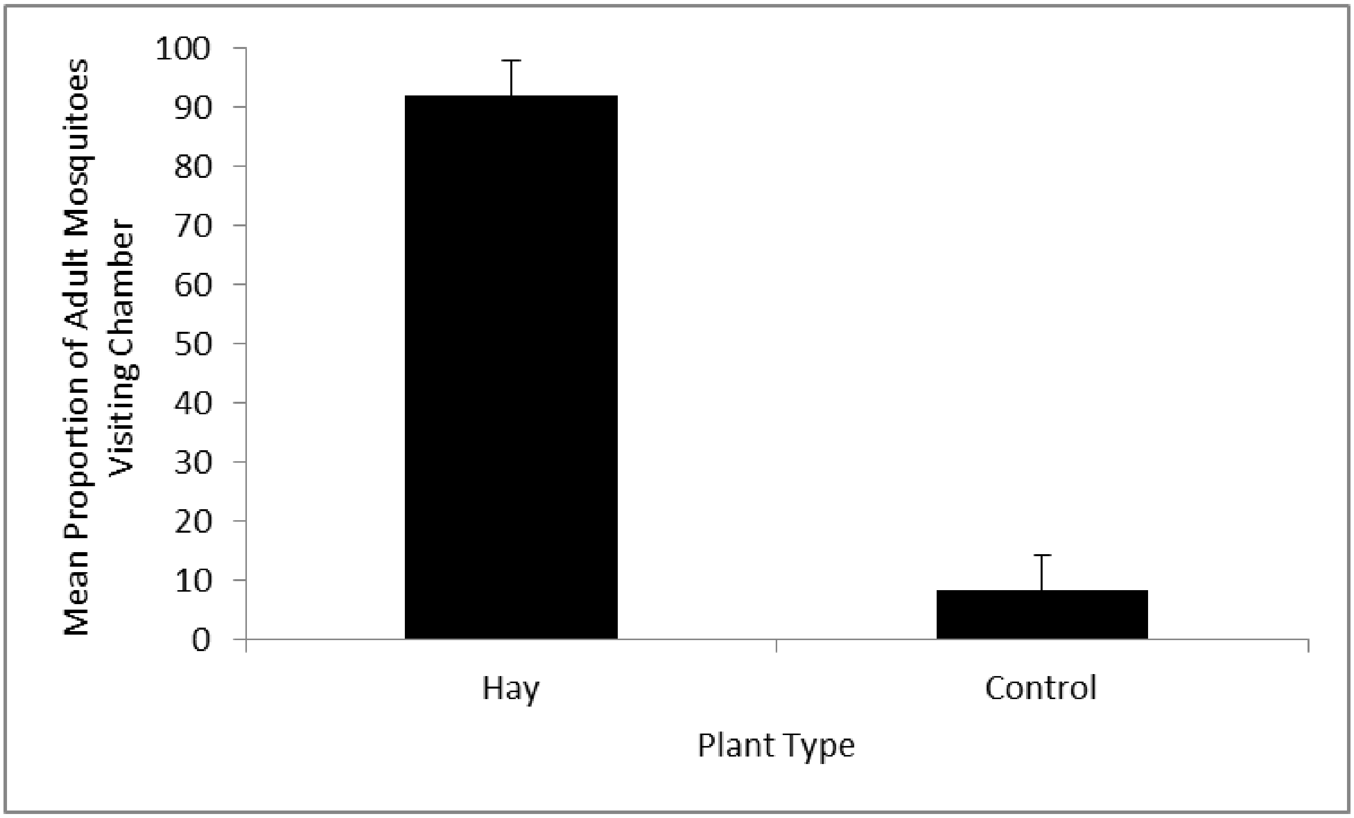


Figure 5.12. *C. quinquefasciatus*, *A. aegypti*, and *A. quadrimaculatus* mosquito visit preferences into two ports, both containing tap water (control). Graph shows the mean proportion of collective visits by the three mosquito species into both ports. There was not a statistically significant difference in the mean proportion of collective visits by the three mosquito species into ports (p<1.76e-6).

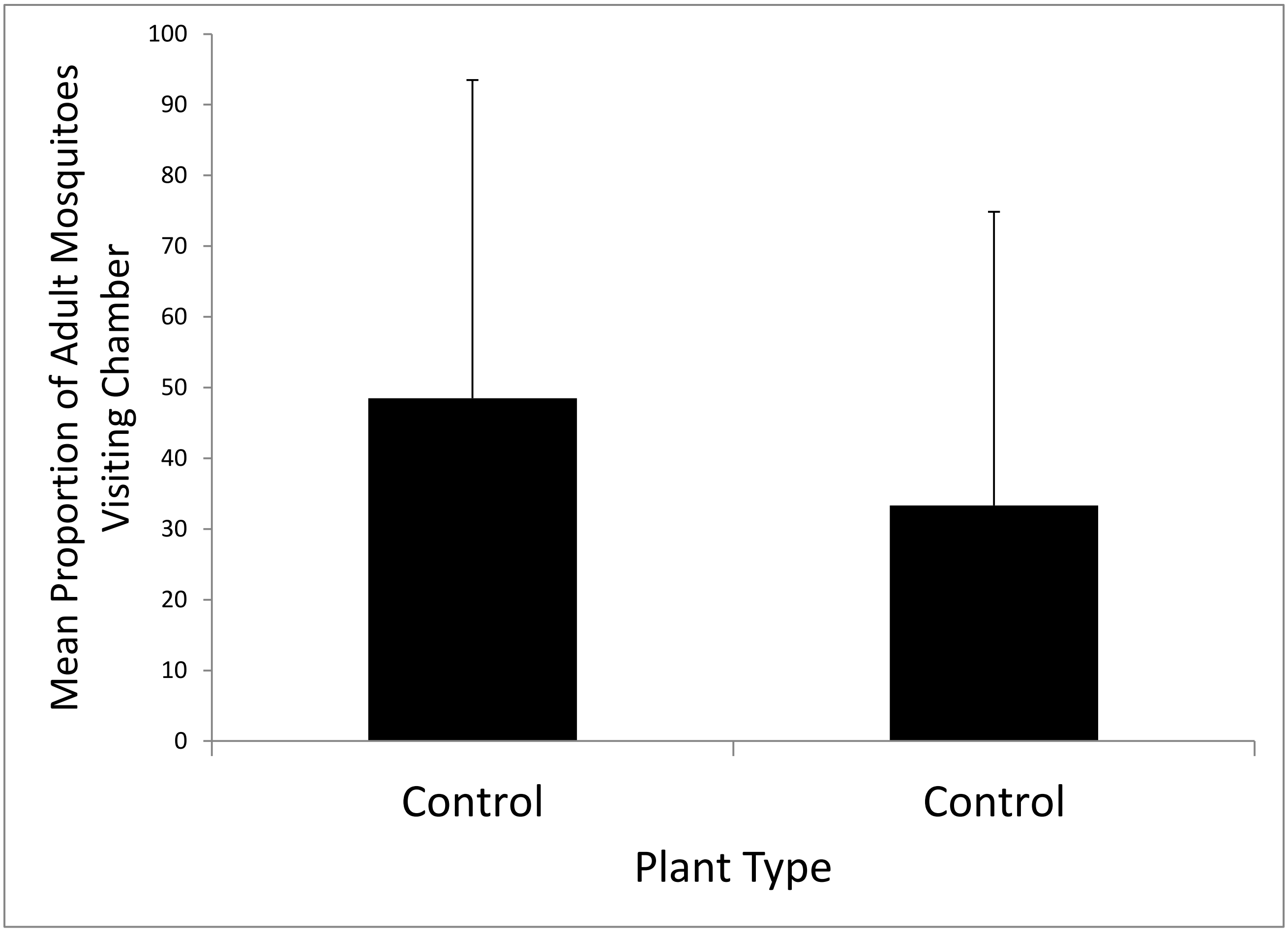
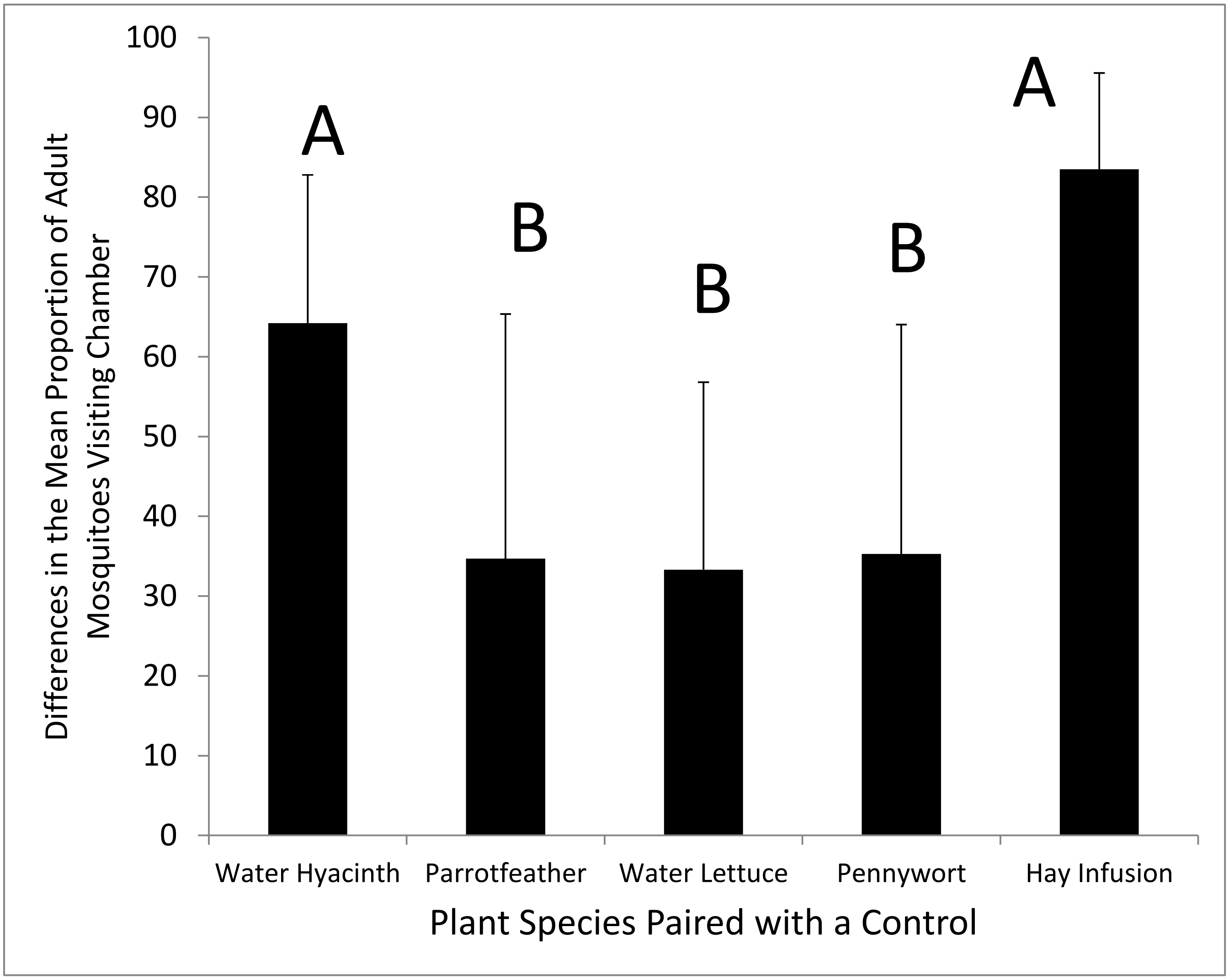
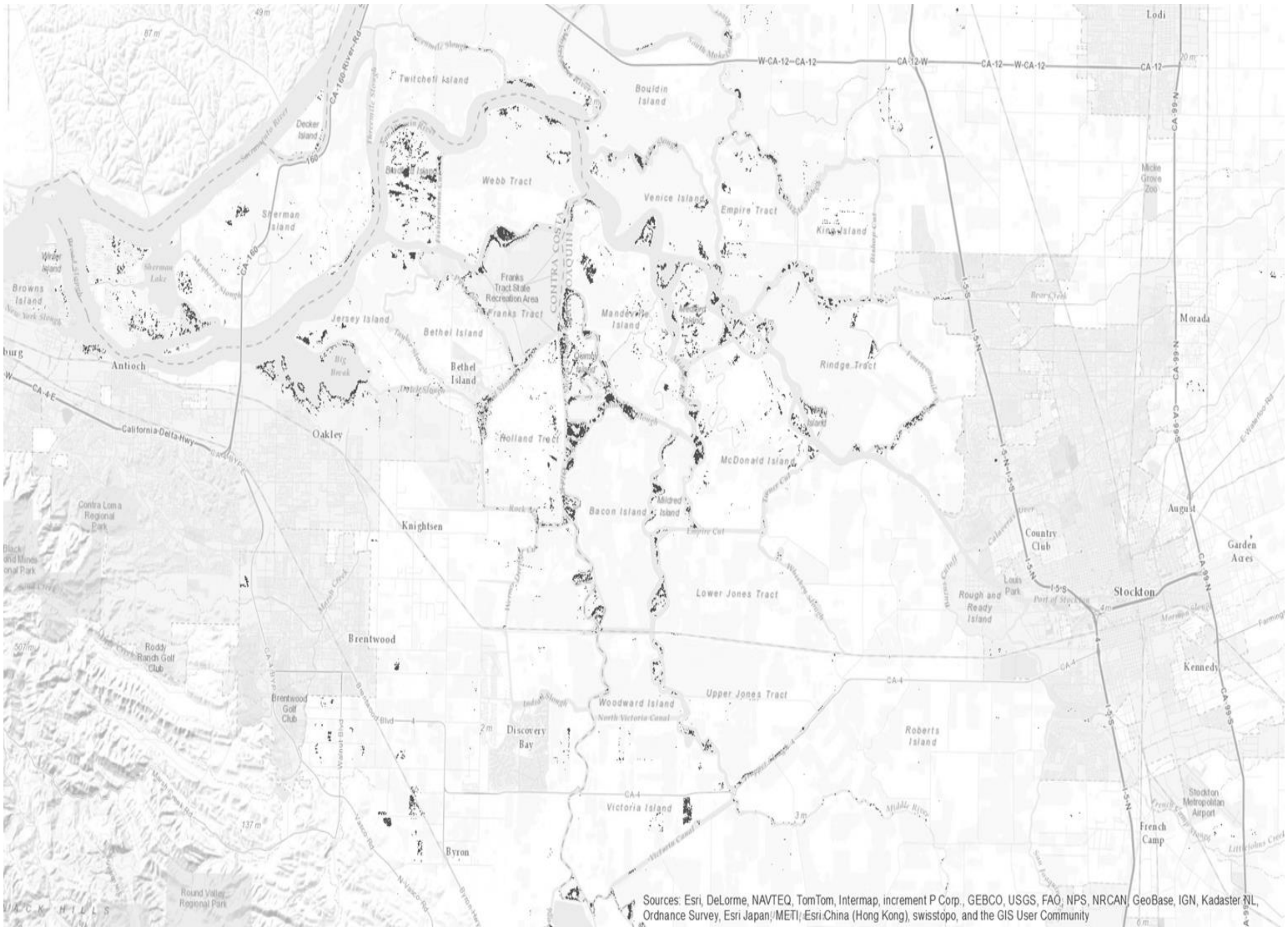


Figure 5.13. Comparison of differences in the mean proportion of collective visits by *C. quinquefasciatus*, *A. aegypti,* and *A. quadrimaculatus* between two ports, one containing a plant species or hay infusion and the other containing control tap water. There was a statistically significant difference of the differences in the mean proportion of collective visits by the three mosquito species into ports (P<1.4e-5)



Appendices

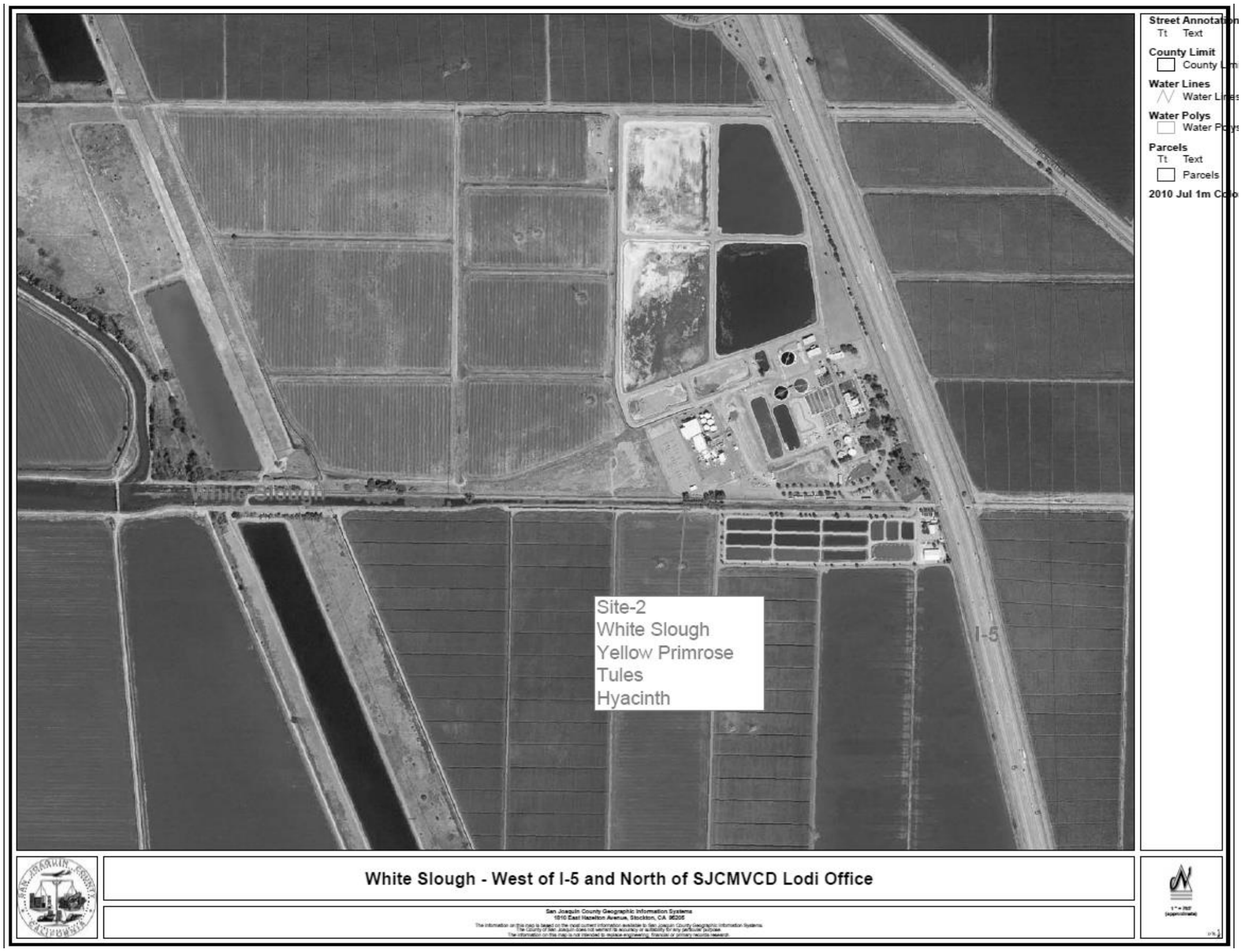


Sources: Esri, DeLorme, NAVTEQ, TomTom, Intermap, increment P Corp., GEBCO, USGS, FAO, NPS, NRCAN, GeoBase, IGN, Kadaster NL, Ordnance Survey, Esri Japan, METI, Esri China (Hong Kong), swisstopo, and the GIS User Community

Appendix Figure 1. Aerial view of sites in the Sacramento-San Joaquin River Delta invaded by water hyacinth and where *C. pipiens* mosquitoes were found (represented in black).

Appendix Figure 2. Aerial view of all sites/passageways investigated in the Sacramento-San Joaquin River Delta (represented in black).

Appendix Figure 3. Mosher Slough, a residential site in the Sacramento-San Joaquin River Delta containing water hyacinth, yellow water primrose, parrotfeather, and tules, and where *C. pipiens* was found.



Appendix Figure 4. White Slough, a rural site in the Sacramento-San Joaquin River Delta invaded by yellow water primrose, water hyacinth, and tules, and where *C. pipiens* was found

Appendix Figure 5. Upland Canal, a rural site in the Sacramento-San Joaquin River Delta invaded by water hyacinth and tules, and where *C. pipiens* was found.