ECOLOGY AND MANAGEMENT OF MOSQUITOES IN CONSTRUCTED WASTEWATER TREATMENT WETLANDS

by

SAMUEL W. WOOLFORD

(Under the Direction of C. Ronald Carroll)

ABSTRACT

Throughout the world, the release of untreated or partially treated wastewater into natural basins is common practice; this has significant effects on ecosystem function, nutrient transport, microbial evolution, pathogen transmission, and human health. Constructed wetlands are an attractive wastewater treatment option for some communities and institutions because of relatively low construction and maintenance costs, additional social benefits, and potential conservation advantages over other treatment methods. Wastewater treatment wetlands are also often excellent habitat for immature aquatic-stage mosquitoes. Because of the economic incentive to build these wetlands close to human populations, municipal treatment wetlands can become a source of nuisance pests and disease vectors in the communities they serve. Immature mosquitoes were present in all but one of 19 municipal treatment wetlands sampled in Georgia, and showed a strong preference for habitats characterized by emergent aquatic macrophytes. Container experiments indicated that mosquito production is increased in partially treated wastewater and in emergent macrophytes, but that the two habitat characteristics may not have a multiplicative effect on mosquito abundance. In vegetated areas, exclusion of Gambusia affinis (Mosquitofish) suggested the fish were far more effective predators of larval mosquitoes in sections with lowest stem density. Mesocosm experiments showed that another fish native to the southeast, Heterandria formosa (Least Killifish), may be an effective predator of mosquito larvae in densely vegetated habitats. These results have strong design and maintenance implications for integrated mosquito management in treatment wetlands.

INDEX WORDS: wastewater treatment, constructed wetland, larval mosquito, disease vector, mosquito control, biological control, larvivorous fish, *Gambusia*, emergent vegetation, duckweed

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TREATMENT WETLANDS

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DEDICATION

To thousands of municipal public servants across America, in recognition of the unglamorous, subterranean, and irreplaceable work of managing our communities' water transport and treatment

systems.

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CHAPTER 1

INTRODUCTION

Constructed wetlands for wastewater and stormwater treatment have been used for decades throughout the world as an efficient and cost-effective way to filter contaminated water before release to natural basins (Perry and Kleinmann 1991, Reed 1991, Hench et al. 2003, Kadlec and Wallace 2008). The first published experimental use of plants to treat wastewater was in the 1950s at the Max Planck Institute in Plön, Germany: Käthe Seidel studied the ecological characteristics and potential socioeconomic importance of *Scirpus lacustris* (bulrush) in sub-surface constructed wetlands (Seidel 1955, Vymazal 2010). Today, we differentiate wetland treatment into three main types (although hybridization between types is possible): Free Water Surface (FWS), Horizontal Sub-surface Flow (HSSF), and Vertical Subsurface Flow (VSSF) wetlands (Figure 1.1). Each of these treatment methods uses a combination of biological, chemical, and physical processes to remove pollutants from wastewater influent, including microbial nitrification and denitrification, phosphorus adsorption in sediments, organic degradation and colloidal settling and filtration (Table 1.1).

As natural wetlands have long been recognized as the 'kidneys of the Earth' because of their capacity to absorb and remove pollutants from through-flowing water, constructed wetlands are engineered to enhance these functions (EPA 2004). Municipal wastewater, agricultural runoff, urban storm water, and many other types of wastewater have been successfully diverted through constructed wetlands for treatment (Kadlec and Wallace 2008). In North America, wetland treatment technology began in the 1970s with the engineering of natural wetlands to receive and polish partially treated wastewater (Odum 1975, Kadlec et al. 1979). In part because of these beginnings, FWS constructed wetlands have long been the most common type of constructed wetland used for wastewater treatment in North America.

Constructed wetlands are used in almost all cases to serve the purposes of secondary wastewater treatment or higher, to comply with the amount of treatment required by United States law for municipal wastewater before release. This usually results in at least a significant reduction of biochemical oxygen demand and suspended solids below certain regulated thresholds, and often includes nutrient and coliform bacteria reduction. Pre-treatment and primary wastewater treatment consists of screening and separating large particles, grit, oils, and grease from raw wastewater, and allowing for initial sedimentation in ponds or lagoons. These primary steps are undertaken before almost all types of further biological wastewater treatment technologies (including suspended growth, attached growth, and land application), and allow constructed wetlands to function properly without fouling or infilling.

CONSTRUCTED WETLAND USAGE CONSIDERATIONS

Larger areal requirements than alternatives can make wetlands for secondary treatment most successful and cost-effective in small communities with relatively low volumes of wastewater, or as tertiary treatment (reduction in nutrient and suspended solids beyond secondary treatment) in larger communities with more conventional secondary treatment (Green and Upton 1994, Kadlec and Wallace 2008). Changing regulatory requirements, expanding populations, aging sewerage infrastructure, and changes in climate can create conditions where the addition of a constructed wetland could prove beneficial to a community or institution that already has wastewater treatment technology in place. Furthermore, their relatively low operating costs make wetlands viable treatment options for smaller communities in many parts of the world that currently conduct no treatment or only primary treatment before releasing partially treated wastewater to receiving surface waters (Haberl 1999, Kivaisi 2001).

Initial construction costs of constructed wetlands include engineering and design, site analysis, land, earth-moving, piping and water regulation tools, plants and substrate, and human use facilities. Including labor and materials, these costs are usually comparable to alternative treatment technologies for achieving the same level of water treatment (Kadlec and Wallace 2008). Operation and maintenance costs for a constructed wetland, however, are usually much lower than for a mechanical system. Energy costs in particular for most FWS and HSSF constructed wetlands are comparatively low, as most function best as gravity fed systems; additionally, even though VSSF systems require periodic pumping of wastewater onto the substrate, energy use is usually much lower than alternatives (Brix 1999). Some basic cost analyses have placed the total cost of constructed wetlands lower than alternative 'concrete-and-steel' technologies by a factor of 2-10 over the course of the site's lifetime, with land area as the primary economic tradeoff for energy (Campbell and Ogden 1999, Kadlec and Wallace 2008, Vymazal and Kröpfelová 2008, Vymazal 2010). The life expectancy of constructed wetlands can also be double or longer than that of a standard treatment alternative; most FWS wetland life expectancies are estimated at 40-50 years with some maintenance required to prevent infilling and mechanical fouling, although some have lasted up to 70 years (Kadlec and Wallace 2008).

In addition to energy savings, constructed treatment wetlands invariably provide a suite of secondary benefits beyond water treatment that can include storm water buffering, water resource conservation, vegetation harvestable for biofuel production, educational and recreational opportunities to human visitors, and resources for wildlife communities (Knight 1997, Mitsch and Gosselink 2000, Knight et al. 2001, Hsu et al. 2011). Most cost-benefit analyses of constructed wetlands have not included the direct and indirect (via ecosystem services) economic offsets of these ancillary wetland characteristics. Besides wastewater treatment, many ecosystem services associated with natural wetlands, such as flood control, groundwater replenishment, biological control, water supply, and cultural and recreational benefits, apply also to constructed wetlands. Costanza et al. (1997) calculated the average economic value of these same services in natural wetlands to be over \$6,500 per hectare per year. While not all of these services may be provided by every constructed wetland, great potential exists to design future wetlands to enhance secondary benefits in addition to wastewater treatment.

Furthermore, some direct economic offsets of constructed wetlands are achievable through certain design and management practices. Liu et al. (2012) created experimental treatment wetland plots to measure the potential production of cellulosic biofuels from vegetation grown in a constructed wetland treating domestic wastewater. Compared to alternative sources of biofuels, the study found energy production in constructed wetlands was second only to micro-algae systems, and constructed wetlands

ranked first in a cost-benefit analysis that incorporated other ecosystem services with biofuel production. Seasonal or periodic biomass harvest from FWS constructed wetlands also has the potential to improve wastewater treatment capabilities, as nutrients taken up by macrophytes are usually returned to the water after senescence and decomposition. Harvesting the shoots and leaves of these plants prior to this creates a positive (rather than neutral) effect on nutrient removal, and extends the life-cycle of the wetland due to reduced infilling.

Finally, constructed wetlands have been shown to be an effective water conservation tool and hedge against drought conditions. This strategy works especially well for larger wetlands used for tertiary treatment, or polishing, after another secondary treatment method has been applied. Effluent from constructed wetlands can be diverted into a reservoir or catchment and reused in communities with water conservation goals and/or restrictions due to drought or resource availability. This type of water reclamation has allowed the Clayton County Water Authority (CCWA) in Georgia to increase the amount of water they are permitted to withdrawal from county reservoirs, and to in turn sell water to nearby municipalities. During severe drought conditions in 2000, the CCWA was even able to provide potable water to Spalding County and the City of Griffin, GA, when water storage in these areas was insufficient (Clayton County Water Authority, personal communication).

WILDLIFE AND HUMAN USE: BENEFITS AND DRAWBACKS

While the primary objective of treatment wetland designers and operators is the removal of excess nutrients, pathogens, toxins, and organic pollutants from wastewater, the use of these wetlands by wildlife and humans merits consideration. Wetland habitat worldwide continues to be lost or degraded due to agricultural and urban expansion, and with fewer resources on the landscape, constructed treatment wetlands may provide refuge for obligate and facultative wetland species (Frayer et al. 1989, Reisner 1993, Lemly et al. 2000, Zedler and Kercher 2005, Casas et al. 2012). Various design and operational conditions can provide differing amounts of functional habitat for a diversity of fauna in constructed wetlands, and in so doing can be beneficial to wildlife and human communities (Worrall et al. 1997, Knight et al. 2001, Fleming-Singer and Horne 2006, Hsu et al. 2011). Indeed, some constructed wetlands

in Georgia offer use of their land to citizen scientists and hobbyists interested in recording bird or insect diversity, and others use their facilities as educational spaces for students and researchers (Augusta, Clayton County, Glennville, Gordon, Lakeland, Pine Mountain).

This situation presents potential conservation benefits, but may also pose problems for wetland managers, increase the chance of wildlife encountering pathogens and toxins in wastewater, or create habitat for nuisance species and disease vectors that breed in aquatic habitats. Little research has been conducted into the risks to wildlife that use the wetlands as habitat. On-site treatment of wastewater clearly presents toxin and pathogen transmission risks to wildlife using the site, especially to acutely sensitive fauna such as many amphibians (Hamer and McDonnell 2008, Snodgrass et al. 2008).

Growing concern regarding the effects of persistent organic chemicals of commerce (POCs) in the environment, such as pharmaceuticals, pesticides, and herbicides, should perhaps be heightened in wastewater treatment systems: they could potentially be a sink for these materials persisting in human waste or discarded into municipal sewer systems. Even very small amounts of POCs have been shown to bio-accumulate to higher trophic levels, and this effect certainly warrants more study in wastewater treatment wetlands (Mackay and Fraser 2000). Preliminary research, however, has shown some tentatively promising signs that these systems may offer relatively high reduction of some microconstituents of wastewater such as antibiotics, caffeine, and anti-inflammatory pharmaceuticals (Pahl, J., unpublished data). More research is necessary to investigate the processes of this removal, but treatment wetlands may at least offer higher quality effluent release to natural basins with regards to POC levels.

A final drawback of wastewater treatment wetlands, and the major concern of this document, is the potential creation of additional breeding habitat for unwanted or medically dangerous animals. In particular, treatment wetlands are often excellent breeding habitat for mosquitoes that lay their eggs on or above standing, vegetated water. Some characteristics common to wastewater treatment wetlands can have a positive effect on mosquito production, including water rich in nitrogen and organic matter, elevated bacterial abundance, and dense vegetation; see Walton (2012) for a thorough review of the

effects. Because of the economic incentive to build these wetlands close to the residential and commercial areas they serve, municipal treatment wetlands without a mosquito management plan can become a source of nuisance mosquitoes in their communities, and create human and wildlife health risks by providing habitat for mosquitoes that vector zoonotic diseases such as Malaria, West Nile Virus, St. Louis Encephalitis, Eastern Equine Encephalitis, and others. In the southeastern United States, mosquitoes of the genus *Culex* are the most likely to thrive in the low-oxygen, high-nutrient conditions of a treatment wetland, and are by far the most likely to transmit disease (Kramer and Mulla 1979, Turell et al. 2005, Yadav et al. 2012).

RESEARCH OVERVIEW

Treatment wetlands can offer manifold environmental, economic, and social benefits to certain communities as a water treatment option. It is paramount, however, that these advantages not come at the cost of human and wildlife health through the creation and maintenance of a habitat for vectors of zoonotic disease. Due to worldwide ecological and community health dynamics, vectored pathogens threaten human populations inconsistently throughout the world; this document is focused on the health risks of mosquito habitat creation in constructed wetlands in the sub-tropical and temperate United States.

In the Unites States, West Nile Virus (WNV) continues to be a significant threat to human populations: in 2012, cases of WNV were documented in all 48 of the contiguous states and Washington, DC for the first time, and major outbreaks were reported in at least four states (CDC 2013). 2012 was the deadliest year on record in the United States (286 deaths), although incidence of the disease was not as high as either 2002 or 2003. WNV is a blood-borne zoonotic arbovirus that can present as fever, encephalitis, meningitis, and poliomyelitis in humans. The virus replicates primarily in many avian species and is vectored between individuals by female mosquitoes seeking multiple blood meals before ovipositing. Because some species of mosquitoes indiscriminately feed on multiple classes of vertebrates, birds, mammals, and less commonly reptiles are all at risk (Burkett-Cadena 2013). Other arboviruses and pathogens such as Eastern Equine Encephalitis Virus, St. Louis Encephalitis Virus, La Crosse Virus, and filarial heartworm are vectored by adult mosquitoes in the United States and continue to threaten the health of significant numbers of people and wildlife nationwide.

Due to the benefits associated with constructed wastewater treatment wetlands detailed above, existing and planned wetlands often adopt some kind of mosquito control strategy to avoid breeding habitat creation and/or to reduce mosquito production. To aid wetland designers and managers in strategic knowledge, and to build upon a broad base of ecological research of immature mosquito behavior and predation, I conducted field observations and experimentation during the period of May through October 2013 in Georgia, United States. I used 19 constructed municipal treatment wetlands throughout the state as well as several locations in urban Clarke and rural Oconee counties as research sites. The following six chapters detail the observations, experimentation, analysis, and conclusions from this research.

The second and third chapters concern mosquito production in treatment wetlands, and the effects of various wetland and habitat characteristics on mosquito oviposition and production. Chapter two describes the results of field sampling at all nineteen constructed treatment wetlands during three sampling intervals throughout the research period. I used immature mosquito abundance and spatial distribution at these sites to model mosquito production based upon habitat characteristics, larvivorous fish presence, and landscape characteristics. Chapter three summarizes a container experiment I conducted at several wetland sites to evaluate the additive effects of partially treated wastewater and senesced wetland vegetation on immature mosquito production.

The fourth, fifth, and sixth chapters review the procedures and results of three experiments concerning the biological control of mosquitoes in wastewater treatment wetlands. Chapter four details a second container experiment to investigate the chemical and structural effects of *Lemna minor* (Duckweed) on mosquito emergence. Duckweed is a common volunteer floating macrophyte in many wastewater treatment systems in Georgia, and often forms thick mats in some areas; the chapter concludes with management recommendations for the plant with regards to mosquito control. Chapter five describes an experiment conducted in August and September to exclude *Gambusia affinis* (Mosquitofish) from some areas of a treatment wetland in which they had been introduced for mosquito control. The goal of

the experiment was to quantify the efficacy of *G. affinis* predation on immature mosquitoes in differing vegetation densities within the wetland, and develop wetland design and management recommendations to improve mosquito control plans that include larvivorous fish.

Chapter six also investigates the potential effectiveness of using a live-bearing fish, *Heterandria formosa* (Least Killifish), to control mosquitoes in habitats common to treatment wetlands. Dire ecological consequences of the introduction of *Gambusia* spp. throughout the world for the intended purpose of mosquito control have been thoroughly detailed (Miura et al. 1984, Ghate and Padhye 1988, Simberloff and Stiling 1996, Peck and Walton 2008, Pyke 2008). Like other non-native species introduced for biological control, Mosquitofish have caused systemic changes through unintended and indirect effects on non-target species. The experiment in chapter six details preliminary research of mosquito predation by another fish native to the coastal plain of the Southeastern United States, and could be a model for experimentation with native larvivorous fish for future wetland designers or operators. Finally, chapter seven concludes this document by synthesizing the results of all field observations and experimentation, compiling a series of management conclusions and recommendations, and outlining promising directions for relevant future research.

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Figure 1.1: Diagrammatic view of three constructed wetland types: A. Free water surface (FWS) constructed wetland; B. Horizontal sub-surface flow (HSSF) constructed wetland; C. Vertical sub-surface flow (VSSF) constructed wetland. Arrows indicate direction of water flow. Adapted from Vymazal (2007).



Wetland type	Water position	Flow direction	Vegetational characteristics	Primary Microbial Substrate	Main treatment processes	Oxic conditions	Relative usage cost (per unit area)
Free Water Surface	Surface flow	Horizontal, to effluent release	Emergent macrophytes, submerged aquatic vegetation, floating vegetation	Plant shoots and leaves; Secondarily roots and rhizomes	Colloidal settling; nitrification of ammonium; nitrification; denitrification	Primarily hypoxic, oxic in shallow regions	Low
Horizont al Sub- Surface Flow	Sub- surface	Horizontal, to effluent release	Emergent macrophytes	Roots and Rhizomes	Colloidal filtration; denitrification; phosphorus adsorption; macrophytic nutrient uptake	Primarily anoxic	Low
Vertical Sub- Surface Flow	Sub- Surface	Vertical, infiltration	Emergent macrophytes	Roots and Rhizomes	Colloidal settling; nitrification; phosphorus adsorption; macrophytic nutrient untake	Oxic in drained conditions, hypoxic in saturated conditions	Higher mainten- ance/ energy costs

Table 1.1: Characteristics of three main constructed wetland types.

CHAPTER 2

DISTRIBUTION AND ABUNDANCE OF IMMATURE MOSQUITOES IN NINETEEN CONSTRUCTED WASTEWATER TREATMENT WETLANDS IN GEORGIA INTRODUCTION

Researchers, resource managers, and others have voiced concerns for decades about the potential for constructed wastewater treatment wetlands to serve as high-quality habitat for breeding and immature mosquitoes, and a large body of literature concerning mosquito production and control in these systems exists (Martin and Eldridge 1989, Tennessen 1993, Walton and Workman 1998, Russell 1999, Thullen et al. 2002, Knight et al. 2003, Workman and Walton 2003, Karpiscak et al. 2004, Mayhew et al. 2004, Sanford et al. 2005, Peck and Walton 2008, Walton 2012, Walton et al. 2012, Walton et al. 2013). Humans and wildlife are susceptible to a multitude of mosquito-borne pathogens, including Malaria, West Nile Virus, Dengue Virus, Ross River Virus, St. Louis Encephalitis, Eastern Equine Encephalitis, and filarial heartworm. Creation of suitable mosquito habitat close to human communities can increase transmission risk of mosquito vectored illness, especially where wildlife using wetlands serve as reservoirs or amplifiers for transmissible pathogens.

Walton (2012) provides a thorough review of wetland design factors believed to influence mosquito production, including water quality parameters commonly targeted for wastewater treatment. Biochemical Oxygen Demand (BOD), a proxy for organic matter content, has been correlated with mosquito production in treatment wetlands, as organic matter can serve as an important food resource for immature mosquitoes (Merritt et al. 1992). Likewise, experimental ammonium-nitrogen enrichment of wetlands has been shown to increase mosquito production (Sanford et al. 2005); this is believed to be due to preferential uptake of biologically available NH₄-N by mosquito food resources such as phytoplankton.

Many researchers have also found emergent macrophyte stands to be concentrated areas of mosquito production (Walton and Workman 1998, Russell 1999, Thullen et al. 2002, Keiper et al. 2003).

Typically, multiple mechanisms are cited for this effect: refuge from predators, increased food resources, poor invertebrate predator diversity in monotypic stands, and mosquito oviposition cues are all believed to contribute to increased production. Walton et al. (2013) found more mosquitoes emerged from the center of wide vegetated bands in a wastewater treatment wetland than from the edges and from narrow bands of vegetation, which the authors attributed to diminished mosquito-predator interaction, as well as decaying vegetation that attracted ovipositing mosquitoes and provided enriched nutrition to their offspring.

Much of the research investigating mosquito production in treatment wetlands has been conducted in demonstration or experimental wetland systems and mesocosms, which have some distinct research advantages over wetlands functioning exclusively for municipal wastewater treatment. Namely, experimental manipulation of wetland characteristics is possible with fewer regulatory standards for water quality effluent. Because operational treatment systems in the United States have a primary obligation to meet Clean Water Act National Pollution Discharge Elimination System (NPDES) water quality requirements, mosquito control is often a secondary concern. Adaptive management of vegetation and water quality parameters for reduced mosquito production is usually not feasible or cost-effective. There has been limited analysis of mosquito spatial distribution and abundance in constructed wetlands functioning as long term wastewater treatment systems. Currently, 21 treatment wetlands in the state of Georgia have NPDES permits to treat municipal effluent. For this study, I sampled mosquito production in 19 of these wetlands (Table 2.1) during three periods over the course of five months, to determine if mosquito production in a cross-section of functioning treatment wetlands can be linked to observed and experimental results in the literature.

The 19 constructed wetlands in this study differ greatly in area, wastewater flow, and treatment type (Table 2.1). Two of the municipalities (Patterson and Rentz) have a regular Pyrethroid-based adult mosquito control program that is likely to affect mosquito production in the wetlands, so data from these two wetlands were removed from most analyses. Larvivorous *Gambusia* spp. are present in six wetlands; three were stocked with Mosquitofish (Huie, Ochlocknee, and Panhandle), and three were colonized

naturally (Augusta, Glennville, and Richmond Hill) by *Gambusia* spp. and other fish species during flooding events or through other dispersal mechanisms. While no empirical evidence existed, anecdotes from researchers and managers characterized some of the 19 wetlands as being prone to a mosquito 'problem,' while others had no such reputation. The goal of this cross-sectional sampling was to describe the immature mosquito spatial distribution and abundance among and within Georgia's constructed wetlands, and evaluate correlations with wetland characteristics to illuminate the causes of some of these differences.

I chose several relatively permanent wetland features from the literature to investigate as potentially influential covariates to mosquito abundance. One of these covariates was the presence of larvivorous fish throughout the wetland, which I predicted would have a strong negative effect on mosquito abundance. Additionally, each wetland in Georgia consisted of varying amounts of emergent vegetation and open water habitat, and I predicted that many more mosquitoes would be present in vegetated habitats. Finally, I investigated whether any effect of landscape development or isolation from natural wetlands was potentially responsible for mosquito presence or abundance. I analyzed the correlations between these covariates and the total immature mosquito abundance, as well as immature *Culex* spp. abundance, as *Culex* is the most medically relevant genus in Georgia and transmits West Nile Virus and St. Louis Encephalitis to humans.

To estimate the effects of these parameters on mosquito populations, I used a Bayesian statistical approach with Markov chain Monte Carlo (MCMC) sampling for several reasons. Sampling from posterior distributions of covariate coefficients provides direct inference of the probability that each parameter affected mosquito occurrence or abundance (or both). This approach also allowed for simpler incorporation of error due to multiple sampling periods, as well as random effects influencing mosquito abundance. Finally, estimation of the posterior distributions of missing data points in the OpenBUGS software (BUGS Project, MRC Biostatistics Unit, Cambridge, UK) allows for k-fold cross-validation to facilitate model selection.

MATERIALS AND METHODS

Sites and Landscape Analysis:

The 19 constructed wetland systems I sampled range in area from less than one hectare to over 130 hectares, are capable of treating between 50 thousand and 46.1 million gallons of wastewater per day, and represent a range of wetland design and management (Table 2.1). I collected samples from these treatment wetlands during three sampling periods over the course of five months from May through September 2013, and conducted multiple site visits at 15 locations. Confirmation of larvivorous fish presence or absence at each site was conducted through discussions with wetland managers, visual observation, active capture by a D-frame sweep net, and/or the placement of several mesh funnel minnow traps.

Landscape analysis was conducted using ArcMap GIS software (ArcMap v. 10, Esri, Redlands, CA, 2010). Aerial photos from NAIP (National Agriculture Inventory Program, USDA-FSA Aerial Photography Field Office, 2010) were used within one kilometer of the wetland boundary to measure two World Bank indicators of development and human density: area of intact forest and total road density. These factors were incorporated in to create a simple index of landscape development:

$$L_i = -\left(F_i + \left(\frac{1}{R_i}\right)\right)$$

Where F_i Is the proportion of the one kilometer buffer around each wetland *i* that is intact forest, and R_i is total road length per hectare of land. *L* is negative so the lowest values reflect low landscape development

I also used wetland spatial data from the National Wetland Inventory (United States Fish and Wildlife Service, 2012) within a one kilometer buffer of each constructed wetland to measure the area of natural wetlands that are at least seasonally flooded, and the edge-to-edge distance of each natural wetland to the constructed wetland site.

These were incorporated into a metric of connectivity to measure the wetland isolation of each site (Hanski and Thomas 1994, Boughton et al. 2010):

$$S_i = -\left(\sum_{i=1}^n \exp(-\alpha d_{ij}) * A_j\right) / B_i$$

Where α is a constant equal to 1 in conservative isolation estimates, d_{ij} is the distance between the constructed wetland *i* and natural wetland *j*, A_j is the area of each natural wetland within one kilometer of the constructed wetland, and B_i is the area of the one kilometer buffer around each constructed wetland (to normalize the statistic for land area).

Sampling Procedure:

I sampled larval mosquito abundance and spatial distribution in each wetland. Over three distinct sampling periods, I took at least ten 500mL water samples per visit from randomly selected locations within the constructed wetland cells. Because the presence, size, and type of aeration pond or settling lagoon was inconsistent from site to site, I never sampled in these features. I sampled all nineteen wetlands in the first period (Early summer: May 13th-June 4th), eleven in the second period (Mid-summer: July 1st-July 24th), and ten in the third period (Late summer: September 10th-October 3rd). Because returning to all nineteen wetlands three times was either not feasible or unlikely to be informative (e.g. due to mosquito adulticide programs onsite), wetlands sampled in the second and third periods were chosen to represent a range of relevant capabilities and characteristics.

Mosquito samples were collected using a standard 350 mL mosquito dipper (methods consistent with Silver (2008)) within 3 meters from the wetland edge, from areas of either planted emergent vegetation (EV) or open water (OW) with or without floating vegetation (such as *Lemna* spp.). For each 500 mL sample, two 250 mL dips were taken within one meter of each other, combined, and subsequently filtered through an approximately 20 micron paper filter (N = 471). Contents remaining on the filter were washed into a 10% ethanol solution, and later preserved in a 70% ethanol solution for identification of mosquito larvae. Second, third, and fourth instar larvae were identified to genus, and fourth instar *Culex* spp. larvae to species.

Statistical analysis:

Immature mosquito abundance is expressed as the number of larvae per 500mL. Larval mosquito count data from 500mL water samples were not normally distributed; nearly half of the samples from eligible sites (N=220 of 441) contained no mosquito larvae. This point mass at zero may reflect separate processes driving mosquito occurrence and mosquito abundance at each micro-site within the wetland. Additionally, the data contained a number of extremely high abundance counts (mean = 13.1, sd = 43.1). I modeled mosquito abundance with a two-component Zero-inflated Poission (ZIP) distribution with a random effects term, to account for overdispersed count data. Alternatives to this approach to overdispersion include using a quasi-Poisson or negative binomial distribution (Ver Hoef and Boveng 2007, Wenger and Freeman 2008).

The ZIP model incorporates a binary distribution to account for excess zeroes (mosquito presence/absence at a specific micro-site), and a Poisson distribution to reflect counts of larval mosquitoes (mosquito abundance) at sites suitable for occupation (Wenger and Freeman 2008, Zeileis et al. 2008). In this instance, mosquitoes were present in every wetland sampled, so no site is thought to be particularly 'unsuitable' mosquito habitat; rather, mosquitoes were absent from randomly selected micro-sites within wetlands, so the occupancy portion of the model can be considered a model of the ubiquity of mosquito presence within a wetland, and the predictive covariates as potential drivers of this process.

I conducted statistical analysis with the R and OpenBUGS software packages (R Foundation for Statistical Computing, Vienna, Austria; (Lunn et al. 2000)). OpenBUGS uses Bayesian Markov chain Monte Carlo (MCMC) sampling methods to estimate posterior distributions for model parameters. I ran three Markov chains per model, and evaluated convergence by visually assessing chain mixing, as well as with the built in Brooks-Gelman-Rubin diagnostic in OpenBUGS. Typically, chains exhibited high autocorrelation and were slow to converge, so standard runs included approximately 300,000 samples from the Markov Chain, with the initial 50,000 samples discarded (Congdon 2006).

I modeled both immature mosquito count (per 500mL) and immature *Culex* spp. count (per 500mL) as observed dependent variables, and investigated correlations with potential drivers of

occurrence and abundance at both the micro-site and wetland scales. I also included two random effects terms in the model: one to account specifically for the effects of temporal variation over the three sampling periods and another to account for random spatial patterns of abundance that affected the overdispersion of the count data. The global model is below, including all covariates I included in various combinations:

$$N_{i(j)t} \sim K_{i(j)t} \times P_{i(j)t}$$
$$K_{i(j)t} \sim \text{Poisson}(\lambda_{i(j)t})$$
$$P_{i(i)t} \sim \text{Bernoulli}(\psi_{i(i)t})$$

 $\log (\lambda_{i(j)t}) = a_0 + a_{hab} (Habitat_{i(j)}) + a_{fish} (Fish_j) + a_{int} (Habitat_{i(j)}) \times (Fish_j) + \delta_t + \varepsilon_{i(j)}$ $\log t(\psi_{i(j)t}) = b_0 + b_{hab} (Habitat_{i(j)}) + b_{fish} (Fish_j) + b_{land} (Landscape_j) + b_{wet} (Wetland_j)$

These covariates apply to wetland *j*, micro-site *i*, and sampling period *t*, and are as follows: *Habitat*: a categorical variable signifying emergent vegetation or open water (observed at the micro-site level); *Fish*: categorical variable signifying presence of absence of fish (observed at the wetland level); *Landscape*: landscape development value (wetland level); *Wetland*: wetland isolation value (wetland level). The terms δ_t and $\varepsilon_{i(j)}$ are random effects drawn from normal distributions; δ_t represents the effect of sampling period *t*, and ε_i is hyper-parameterized with a mean and variance with vague normal prior distributions to account for overdispersion of mosquito count data.

I hypothesized that vegetation and fish presence were likely drivers of immature mosquito abundance, and that multiple combinations of covariates could influence immature mosquito distribution throughout the wetlands. I evaluated six combinations of covariates for predictive capability in the occupancy component of the ZIP model, and retained the same set of predictive covariates in the abundance component across all models (see global model above). Model covariates were considered predictive if the 95% credible intervals of their coefficients did not overlap zero (Tables 2.3 and 2.4). Following selection of the two models of total mosquito abundance and three models of *Culex* spp. abundance with the fewest non-predictive covariates, I used k-fold cross-validation to determine the model with best fit. For each fold (k = 10), I created datasets with missing validation data, and used the

chosen models to analyze the training data in OpenBUGS. Posterior distributions for each validation point were drawn by the program based on model training data. I then compiled the means of the posterior distribution for each missing data point into an equally sized data set to the original. I evaluated the predictive capability of each model by comparing the mean squared error between these compiled 'validation' data-sets and the original data, and chose the model with the least error (Tables 2.7 and 2.8) RESULTS

Models 1 and 2 in Tables 2.3 and 2.4 shared similar predictive power for both overall mosquito abundance (Table 2.3) and *Culex* spp. abundance (Table 2.4). Emergent vegetation and larvivorous fish presence were significant predictors of mosquito abundance. For overall mosquito abundance, interaction between these two covariates was not significant in Model 2. In predicting only *Culex* spp. abundance, a significant interaction effect existed between the two in both Models 1 and 2. The wetland isolation covariate in Model 2 negatively predicted site occupancy (the binomial component of the model), indicating that higher wetland isolation may lead to lower micro-site occupation (or lower spatial ubiquity).

The main difference between models for overall mosquito abundance and models for *Culex* spp. abundance can be seen in Models 3 and 6. While fish presence predicted lower probability of site occupancy in these two models for all types of mosquitoes (Table 2.3), the 95% credible interval of the coefficient overlapped zero, and the effect is interpreted as not significant. In models of *Culex* spp. only (Table 2.4), fish presence has a significant negative effect on *Culex* spp. occurrence at a specific sampling site within a constructed wetland. Emergent vegetation is not a significant predictor of occurrence in Model 6, so Model 3 is the more powerful of the two. The mean effect of the presence of fish on the probability of *Culex* spp. mosquito presence reduces modeled site occupancy from 0.94 (fish absent) to 0.21 (fish present) (Table 2.4).

I analyzed the effect of early, mid-, and late summer sampling by including a random effect term in the mosquito abundance component of the model. In every model for overall mosquito abundance, the posterior distribution mean for the early summer sampling term consistently had a negative value, with a

mean effect of approximately 2 fewer mosquitos per 500mL (Table 2.5). The mid-summer term was never different from zero, and the late summer effect was approximately opposite that of early-summer (2 more individuals/500mL). Sampling season affected *Culex* spp. abundance similarly, although more strongly: the model-mean predicts approximately 4 fewer *Culex* spp. mosquitoes per 500mL in the early season (Table 2.6).

Cross validation of Models 1 and 2 for overall mosquito abundance led to similar mean squared error (MSE) values between predicted validation data and original data (Table 2.7). Model 1 had a lower MSE by 172. Both models tended to under-predict very high values of mosquito abundance, which led to high MSE values. Median squared error values were much lower. I ran cross-validation for Models 1, 2 and 3 for *Culex* spp. abundance, and Model 3 had by far the least MSE, followed by Models 1 and 2, respectively (Table 2.8).

DISCUSSION

Main drivers of overall mosquito abundance:

Both Models 1 and 2 for overall mosquito abundance reveal the significant effects of emergent vegetation (positive) and larvivorous fish presence (negative) on immature mosquito numbers in constructed wetlands. The interpretive difference between the models lies in the significance of an interaction effect between vegetation and fish presence, as well as in the role of wetland isolation in predicting presence or absence of immature mosquitoes. The significant interaction in Model 1 predicts that fish presence should have a larger negative effect on immature mosquito abundance in habitats characterized by emergent vegetation than in open water. This may at first seem counter-intuitive, as fish predation efficacy is often reduced in stands of vegetation due to a reduced visual field and increased mosquito refuge (Manatunge et al. 2000, Willems et al. 2005, Walton 2007). An interaction plot of the mean immature mosquito abundance in these habitats shows that interaction exists, however (Figure 2.1; means in Table 2.2).

The interpretation of this interaction is twofold: 1. Larvivorous fish have a much greater negative effect on mosquito abundance where mosquito density is highest; and 2. Fish likely put more effort into
foraging behavior in habitats where prey is most likely to be found (Pyke et al. 1977). *Gambusia* spp. in open water habitats may feed on invertebrates more concentrated in these areas (e.g. *Amphipoda*, see chapter 5) or engage in other types of behavior, such as courtship and mating (Pyke 2005).

Wetland isolation was the only covariate to be an effective predictor of immature mosquito site occupancy for the entire mosquito community, as seen in Model 2. A negative coefficient for wetland isolation indicates decreased mosquito ubiquity at sites that are more isolated from natural wetlands. As stated above, because immature mosquitoes are present in every wetland, occupancy modeling in this case is not a measure of the suitability of a particular random micro-site, but rather the spatial ubiquity of immature mosquitoes throughout the wetland. Immature mosquitoes may be more likely to be distributed throughout all parts of constructed wetlands that are less isolated.

Previous experimental research in Missouri has shown that mosquito densities can actually be higher in wetlands that are more isolated, a trend attributed to a loss of diversity in mosquito predator meta-communities in landscapes with few wetlands (Chase and Shulman 2009). Spatial ubiquity of immature mosquitoes is related to density, but likely also reflects patterns of mosquito dispersal from nearby wetlands. Especially for larger constructed wetlands, oviposition location by adult mosquitoes dispersing from a nearby natural wetland may depend in part on the wetlands' direction and distance, and in turn impact the probability of mosquito occupancy of micro-sites within a constructed wetland.

Furthermore, the magnitude of the mean coefficient for wetland isolation in Model 2 for both overall (-2.61) and *Culex* spp. (-2.29) indicates that only very isolated constructed wetlands ($S_i > -1$ in Eq. 2) are likely affected by this process. Wetland isolation may play a more important role in driving spatial ubiquity and density within constructed wetlands in more arid climates, where constructed wetlands can be some of the only refuge for immature mosquitoes.

Special considerations for *Culex* species:

Fish presence is a significant predictor of site occupancy for immature *Culex* spp. mosquitoes, but this signal is lost when considering counts of all types of mosquitoes. This difference coincides with life history specializations of *Culex* spp. ovipositing in wetland environments. A broad body of literature

exists demonstrating predator avoidance behavior during oviposition by *Culex* spp., which commonly oviposit in environments with potential invertebrate and vertebrate predators (Angelon and Petranka 2002, Eitam et al. 2002, Kiflawi et al. 2003, Eitam and Blaustein 2004, Blaustein et al. 2005, Ohba et al. 2012). This effect is attributed to the evolutionary advantage of the adult mosquito ability to detect predator-released kairomones. Oviposition in areas without predators, with lower predator densities, or with high-quality predator refugia raises the probability of offspring survival.

The significance of fish presence in determining site occupancy of *Culex* spp. within Georgia's constructed wetlands indicates that fish predator cues likely drive oviposition habitat selection by these mosquitoes, and fish presence deters gravid females from ovipositing. Vegetation has a significant positive effect on mosquito abundance, indicating that where *Culex* spp. do oviposit, emergent macrophytes likely serve as refugia from predators (including predaceous invertebrates) and a potential food source for mosquitoes, through nutrient and organic matter release and support of microbial communities (Merritt et al. 1992, Walton 2012). Finally, the significant interaction between macrophytes and fish presence indicates that when *Culex* spp. do oviposit in wetlands with larvivorous fish, fish have negative effects on immature mosquito abundance even in habitats characterized by emergent vegetation.

Model Validation:

Stochastic variation in mosquito abundances and spatial distribution in constructed wetlands led to large error in predicting validation data for both models of overall mosquito abundance. Both models reinforce the notion that emergent vegetation and biological control of mosquitoes by fish are important in driving immature mosquito abundance across a large cross-section of constructed treatment wetlands, but likely predict actual abundances with little accuracy. Because both vegetation and fish presence are binary covariates, stochastic effects play a large role in prediction accuracy. While using a different method and/or distribution to account for data overdispersion (e.g. a quasi-Poisson or a negative binomial distribution) may have yielded slightly different results, I conducted limited analysis of some models with original data fitted to a negative binomial distribution without substantial predictive changes.

Regardless, Model 1 was the best predictor of overall mosquito abundance, while Model 3 was the best predictor of *Culex* spp. abundance (Table 2.7 and 2.8). As stated above, this implies that larvivorous fish presence plays a role in decreasing *Culex* spp. ubiquity within a constructed wetland (likely due to oviposition deterrence), but the significance of this effect is lost when considering the entire mosquito community. Model 1 also supported the predictive power of an interaction between fish and vegetation in determining overall mosquito abundance, which is clearly observed through graphical analysis (Figure 2.1); Model 2 did not. All models predicting only *Culex* spp. abundance incorporated significant interaction.

While the observed mean occupancy of micro-sites by juvenile mosquitoes across all wetlands was only approximately 0.5, Model 1 predicted a much higher occupancy (mean overall effect = 0.94). This suggests multiple possibilities for interpretation: 1. Imperfect capture within a large wetland due to mosquito avoidance behavior led to false zero counts; 2. Absence of mosquitoes at micro-sites could be the result of random effects of spatial distribution of a small, short lived organism with minimal space requirement; or 3. Many of the mosquito absences contained in the dataset could be 'true zeroes,' meaning that the covariates governing abundance (i.e. fish presence and vegetation) led to an abundance of zero mosquitoes. Each of these processes likely contributed to the data structure, although I find the latter two alternatives most convincing, as research in both constructed and natural wetlands corroborates rare mosquito presence in open water habitats (SWS 2009, Walton 2012).

Management Applications:

These results suggest that integrated mosquito management using both vegetation planting design and biological control with larvivorous fish is likely to be most effective in constructed treatment wetlands, and represent important considerations for wetland designers and managers. Larvivorous fish such as *Gambusia* spp. are effective at reducing mosquito abundance, and most importantly, seem to reduce the ubiquity of mosquitoes most likely to transmit diseases such as West Nile Virus and St. Louis Encephalitis to humans (*Culex* spp.). Many studies suggest that mosquito taxa that commonly co-occur with predators exhibit avoidance of a broad suite of fish and invertebrate mosquito predators (see review

in Vonesh and Blaustein 2010). This indicates that, especially in regions where mosquitoes are vectors for pathogens that threaten humans and wildlife, larvivorous fish in constructed treatment wetlands may reduce health risks by depleting larval abundance, either through oviposition deterrence or direct consumption. Due to the ecological harm often caused by *Gambusia* spp. outside their native range, I strongly advocate that only native larvivorous fish be used for biological control (see Chapters 5 and 6).

Clearly, in wetlands with and without fish, mosquitoes are present and often abundant in areas characterized by emergent vegetation. These areas are crucial for wastewater treatment, as macrophyte stems provide structural support for epiphytic microbes that perform much of the nutrient transformation and extraction that constitutes secondary treatment (Kadlec and Wallace 2008). Macrophytes also increase the complexity of hydraulic pathways, increase water retention time, and can uptake nutrients themselves, all of which benefit water treatment. Proper vegetational design, however, may be able to find tradeoffs between water treatment and mosquito control, and in so doing increase habitat for mosquito predators. The following chapters of this document explore the nature of these tradeoffs further, but some suggestions include reducing the width and/or radius of dense stands of macrophytes, periodic harvest or removal of shoots and leaves, incorporation of deep water refuge for mosquito predators, and thinning vegetation to reduce mosquito refugia.

From a 'big picture' perspective, data from southwestern Georgia in 2012 suggest that average mosquito production in the state's treatment wetlands is less than in undisturbed natural wetlands (Botello et al. 2013). While constructed wetlands may not usually be expected to produce mosquitoes in artificially high numbers, however, the addition of suitable habitat for common disease vectors to any landscape poses health risks to humans and wildlife. Additionally, regions with the most need for inexpensive, easily managed wastewater treatment are often areas of the world with higher incidence of vectored human pathogens. The remaining chapters of this document further investigate processes that drive immature mosquito abundance in and among constructed wastewater treatment wetlands, and how explicit knowledge of these processes may be applied to management strategies to limit risks to human and wildlife health.

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Wetland	Active treatment area (ha)	Max. permitted flow (monthly avg. MGD) ¹	Treatment type	Fish	Mean mosq. larvae/500mL	Ν	Sampling period(s) ²	Landscape Development	Wetland isolation
Alamo	4.49	0.375	Secondary	No	9.3	20	1,3	-0.62	-8.19
Augusta	139.18	46.1	Tertiary	Yes	0.6	20	1,2	-0.45	-20.37
Baconton	1.12	0.1	Secondary	No	1.2	10	1	-0.18	-0.26
Edison	1.51	0.25	Secondary	No	0.75	20	1,2	-0.31	-7.75
Folkston	3.85	0.5	Secondary	No	5.2	10	1	-0.75	-15.38
Glennville	12.71	2	Secondary	Yes	0.6	20	1,3	-0.35	-16.30
Gordon	4.32	0.75	Secondary	No	8.3	30	1,2,3	-0.31	-7.19
Harrison	2.56	0.065	Secondary	No	15.9	50	1,2,3	-0.50	-2.71
Huie	97.85	17.4	Tertiary	Yes	0.4	20	1,3	-0.51	-9.89
Lakeland	4.28	0.5	Secondary	No	1.8	10	1	-0.46	-15.10
Lavonia	5.85	1.32	Secondary	No	61.8	47	1,2,3	-0.43	-4.32
Ochlocknee	0.82	0.05	Secondary	Yes	4.5	20	1,2	-0.51	-3.10
Panhandle	21.77	4.4	Tertiary	Yes	4.2	50	1,2,3	-0.62	-8.97
Patterson*	4.16	0.208	Secondary	No	0	10	1	-0.51	-10.46
PineMt	1.98	0.3	Secondary	No	3.9	30	1,2,3	-0.37	-0.31
Rentz*	1.16	0.12	Secondary	No	1.7	20	1,2	-0.34	-0.70
Richland	1.05	0.3	Secondary	No	29	30	1,2,3	-0.32	-4.36
Richmond Hill	27.93	1.5	Secondary	Yes	3.8	24	1,2	-0.64	-16.79
Tignall	1.78	0.078	Secondary	No	16.2	30	1,2,3	-0.61	-0.92

Table 2.1: Characteristics of 19 wetlands sampled for immature mosquitoes. Rentz and Patterson were not used in statistical analysis because both had mosquito adulticide programs that likely affected onsite conditions. *Not included in statistical analysis because of mosquito adulticide programs.

¹MGD is Million Gallons per Day

²Sampling period 1: May 13th-June 4th; 2: July 1st-July 24th; 3: September 10th-October 3rd

Table 2.2: Mean and median immature mosquito abundance by habitat and fish presence. Emergent Vegetation (EV) and Open Water (OW) in wetlands with and without fish. Error of the mean is standard deviation.

Micro- Habitat	Fish Presence	Mean mosquito abundance	Median mosquito abundance	Median <i>Culex</i> spp. abundance	Median <i>Culex</i> spp. abundance	Ν
OW	Y	0.76 ± 2.55	0	0.34 ± 1.49	0	50
EV	Y	3.22 ± 13.36	0	1.46 ± 8.43	0	124
OW	Ν	0.82 ± 1.56	0	0.39 ± 0.98	0	57
EV	Ν	26.25 ± 60.50	3	12.63 ± 30.14	1	215

Figure 2.1: Interaction plots of immature mosquito abundance by habitat and fish presence. Mean overall and mean *Culex* spp. abundance in Emergent Vegetation (EV) and Open Water (OW) in constructed wetlands with and without larviviorous fish. Error bars represent standard error of the mean.



Table 2.3: Coefficients of covariates in models for immature mosquito occurrence (A) and abundance (B). Values are means of posterior distributions, 95% credible intervals (CI's), and interpreted effect of mean coefficient value. **Bold** coefficients have a CI that does not overlap zero, and asterisked* coefficients are either significant or have higher order significance. Mean effects in parentheses are not assumed to be different from zero, as evaluated by the coefficient CI.

		A. Occ	urrence			B.	Abundance	
Model	Covariate	Mean	95% Credible Interval	Mean effect (occupancy probability)	Covariate	Mean	95% Credible Interval	Mean Effect (mosquitoes/ 500mL; log transformed from coefficient)
1	Intercept*	2.84	1.63 - 4.69	0.94	Intercept*	-1.39	-2.190.61	-4.01
					Vegetation*	2.85	2.04 - 3.64	17.29
					Fish*	-1.10	-2.33 - 0.13	(-3.00)
					FishxVegetation*	-1.74	-3.160.34	-5.64
2	Intercept*	0.30	-1.09 - 2.28	0.57	Intercept*	-1.39	-2.270.14	-4.01
					Vegetation*	2.77	1.35 - 3.67	15.96
	Wetland_Iso*	-2.61	-4.850.81		Fish*	-1.35	-3.100.06	-3.86
					FishxVegetation	-1.60	-3.06 - 0.20	(-4.95)
3	Intercept*	2.71	1.67 - 4.30	0.94	Intercept*	-1.28	-2.090.24	-3.60
					Vegetation*	2.77	1.53 - 3.60	15.96
	Fish	-1.57	-3.45 - 1.90	(0.75)	Fish	-0.59	-2.02 - 1.25	(-1.80)
					FishxVegetation	-1.47	-3.10 - 0.24	(-4.35)
4	Intercept*	1.78	-0.05 - 4.20	0.86	Intercept*	-0.93	-1.87 - 0.47	(-2.53)
					Vegetation*	2.30	0.81 - 3.17	9.97
	Vegetation	1.68	-0.65 - 4.04	(0.97)	Fish	-1.23	-2.57 - 0.20	(-3.42)
					FishxVegetation*	-1.58	-3.140.17	-4.85
5	Intercept*	2.31	0.51 - 4.53	0.91	Intercept*	-1.49	-2.430.42	-4.44
					Vegetation*	2.89	1.93 - 3.67	17.99
	LandDevelop	-1.68	-4.68 - 1.29		Fish*	-1.07	-2.31 - 0.03	(-2.92)
					FishxVegetation*	-1.75	-2.960.40	-5.75
6	Intercept*	1.87	0.38 - 3.77	0.87	Intercept*	-1.09	-2.10 - 0.10	(-2.97)
	Vegetation	1.09	-0.59 - 3.11	(0.95)	Vegetation*	2.56	1.48 - 3.59	12.94
	Fish	-1.81	-3.65 - 1.54	(0.51)	Fish*	0.24	-2.00 - 2.43	(1.27)
					FishxVegetation*	-2.21	-4.200.32	-9.12

Table 2.4: Coefficients of covariates in models for immature *Culex* spp. occurrence (A) and abundance (B). Values are means of posterior distributions, 95% credible intervals (CI's), and interpreted effect of mean coefficient value. **Bold** coefficients have a CI that does not overlap zero, and asterisked* coefficients are either significant or have higher order significance. Mean effects in parentheses are not assumed to be different from zero, as evaluated by the coefficient CI.

		currence		B. Abundance				
Model	Covariate	Mean	95% Credible Interval	Mean effect (occupancy probability)	Covariate	Mean	95% Credible Interval	Mean Effect (<i>Culex</i> mosquitoes/ 500mL; log transformed from coefficient)
1	Intercept*	2.00	0.84 - 3.88	0.88	Intercept*	-2.52	-3.421.30	-12.42
					Vegetation*	3.23	2.25 - 4.11	25.28
					Fish*	-1.12	-2.80 - 0.56	(-3.06)
					FishxVegetation*	-2.53	-4.380.55	-12.55
2	Intercept*	0.35	-1.28 - 2.75	0.59	Intercept*	-2.83	-3.452.05	-16.95
					Vegetation*	3.16	2.35 - 3.81	23.57
	Wetland_Iso*	-2.29	-4.570.32		Fish*	-1.41	-3.10 - 0.04	(-4.10)
					FishxVegetation	-2.37	-3.950.72	-10.70
3	Intercept*	1.60	0.85 - 2.71	0.94	Intercept*	-3.00	-3.841.59	-20.09
					Vegetation*	3.53	2.64 - 4.65	34.12
	Fish*	-2.92	-4.001.95	0.21	Fish*	2.40	-0.43 - 5.04	(11.02)
					FishxVegetation*	-3.00	-5.570.12	-20.09
4	Intercept*	0.94	-0.84 - 3.62	(0.72)	Intercept*	-2.00	-3.550.30	-7.39
					Vegetation*	2.43	0.79 - 3.82	11.36
	Vegetation	1.82	-0.66 - 4.19	(0.94)	Fish	-1.36	-3.37 - 0.57	-3.90
					FishxVegetation*	-2.21	-4.23 - 0.00	-9.12
5	Intercept*	1.49	-0.26 - 3.87	(0.81)	Intercept*	-3.26	-4.272.08	-26.05
					Vegetation*	3.88	2.72 - 4.84	48.42
	LandDevelop	-1.81	-4.67 - 1.04		Fish*	-0.58	-2.5 - 1.16	(-1.79)
					FishxVegetation*	-3.19	-5.230.94	-24.29
6	Intercept*	0.91	-0.32 - 2.43	(0.71)	Intercept*	-2.21	-3.780.92	-9.12
	Vegetation	0.80	-0.39 - 1.99	(0.84)	Vegetation*	3.09	1.89 - 4.46	21.98
	Fish*	-2.78	-4.041.44	0.13	Fish*	2.78	-0.88 - 5.74	(16.11)
					FishxVegetation*	-3.68	-6.610.40	-39.65

Table 2.5: Effect of sampling period on immature mosquito abundance. Means of posterior distributions of seasonal random effects in ZIP model for overall mosquito abundance; NA represents intercept only. Early season: May 13th-June 4th; Mid-season: July 1st-July 24th; Late season: September 10th-October 3rd.

Occurrence	Abundance	Early	Mean Effect (mosquitoes/ 500mL)	Middle	Mean Effect (mosquitoes/ 500mL)	Late	Mean Effect (mosquitoes/ 500mL; log transformed from coefficient)
NA	Veg, Fish, Interaction	-0.61	-1.85	-0.02	-1.02	0.64	1.89
Wetland_Iso	Veg, Fish, Interaction	-0.55	-1.74	-0.05	-1.05	0.60	1.83
Veg	Veg, Fish, Interaction	-0.62	-1.86	-0.04	-1.04	0.66	1.93
Fish	Veg, Fish, Interaction	-0.71	-2.03	0.05	1.05	0.66	1.93
Land	Veg, Fish, Interaction	-0.62	-1.87	-0.02	-1.02	0.65	1.91
Veg, Fish	Veg, Fish, Interaction	-0.75	-2.12	0.10	1.11	0.64	1.91
Mean		-0.64	-1.90	0.00	-0.43	0.64	1.90
Standard Deviation		0.07		0.06		0.02	

Table 2.6: Effect of sampling period on immature *Culex* spp. abundance. Means of posterior distributions of seasonal random effects in ZIP model for *Culex* spp. mosquito abundance; NA represents intercept only. Early season: May 13th-June 4th; Mid-season: July 1st-July 24th; Late season: September 10th-October 3rd.

Occurrence	Abundance	Early	Mean Effect (mosquitoes/ 500mL)	Middle	Mean Effect (mosquitoes/ 500mL)	Late	Mean Effect (mosquitoes/ 500mL; log transformed from coefficient)
NA	Veg, Fish, Interaction	-1.17	-3.22	0.30	1.35	0.86	2.36
Wetland_Iso	Veg, Fish, Interaction	-1.21	-3.35	0.32	1.38	0.89	2.44
Veg	Veg, Fish, Interaction	-1.24	-3.47	0.34	1.40	0.91	2.48
Fish	Veg, Fish, Interaction	-1.52	-4.57	0.66	1.94	0.86	2.35
Land	Veg, Fish, Interaction	-1.19	-3.28	0.30	1.35	0.89	2.43
Veg, Fish	Veg, Fish, Interaction	-1.49	-4.42	0.63	1.87	0.86	2.36
		1 20	2.((2.40	1 51	0.00	2.42
Mean		-1.29	-3.66	0.40	1.51	0.88	2.42
Standard Deviation		0.15		0.17		0.03	

Table 2.7: Mean squared error of validation data for immature mosquito abundance. Squared difference of mean of predicted posterior distribution for each data point with respect to original data, with **overall mean immature mosquito abundance** as the response variable. Obtained from k-fold cross validation of two chosen models (k = 10).

Occurrence	Abundance	Mean Squared Error	90% Confidence Interval	Median Squared Error
NA	Veg, Fish, Interaction	2579	±918	79.6
Wetland_Iso	Veg, Fish, Interaction	2751	± 891	83.7

Table 2.8: Mean squared error of validation data for immature *Culex* spp. abundance. Squared difference of mean of predicted posterior distribution for each data point with respect to original data, with **mean** *Culex* spp. abundance as the response variable. Obtained from k-fold cross validation of three chosen models (k = 10).

Occurrence	Abundance	Mean Squared Error	90% Confidence Interval	Median Squared Error
Fish	Veg, Fish, Interaction	647	± 209	29.2
NA	Veg, Fish, Interaction	1430	± 261	13.9
Wetland_Iso	Veg, Fish, Interaction	1884	± 319	21.4

CHAPTER 3

EFFECTS OF HABITAT CHARACTERISTICS ASSOCIATED WITH WASTEWATER TREATMENT WETLANDS ON MOSQUITO PRODUCTION IN EXPERIMENTAL CONTAINERS INTRODUCTION

Raw sewage from 1.5 billion people worldwide is released untreated to natural waterways and basins; 60% of the world's population lives without any reduced risk of contact with human excreta (Baum et al. 2013). As global population density increases, the effects of inadequate wastewater treatment are expected to have progressively more dire consequences on both the environment and human health (Mara 2004). The need for more extensive wastewater treatment will likely give rise to construction of larger numbers of treatment wetlands, due to economic and environmental advantages over other common methods of wastewater treatment in some communities, particularly in the developing world (Haberl 1999, Kivaisi 2001, Belmont et al. 2004). Because of the incentive to construct these wetlands close to the communities they serve, however, a mosquito management plan (MMP) is important to minimize the creation of nuisance and health risks to human populations. MMP's in constructed wetlands can incorporate design elements prior to wetland creation to discourage mosquito production, but it is unknown whether certain habitat characteristics in existing constructed wetlands, or the additive effect of multiple factors, have more impact on mosquito production than others.

Habitat characteristics can extensively influence mosquito oviposition and larval production. Ovipositing female mosquitoes respond to cues such as food sources, presence of refugia, intra- and interspecific competition, and predator presence to select sites that increase the chances of survival and reproduction for their offspring (Kramer and Mulla 1979, Eitam et al. 2002, Reiskind and Wilson 2004, Bond et al. 2005, Chaves et al. 2009, Nguyen et al. 2012). Larval development and adult emergence is related to these same characteristics, as immature mosquitoes cannot easily migrate away from poor-

quality habitats. Larval production has important biological and medical implications for terrestrial organisms, as adult mosquitoes are vectors of numerous pathogens that affect wildlife and humans.

Free water surface (FWS) wastewater treatment wetlands are typically high-quality immature mosquito habitat, and both mosquito production and oviposition tend to be elevated in wastewater and sewer infrastructure, particularly among mosquitoes of the medically important genus *Culex* (Martin and Eldridge 1989, Tennessen 1993, Russell 1999, Knight et al. 2003, Walton 2012). A constant flux of partially treated wastewater contains nutrients and organic matter that provide larval nutrition, through direct particulate consumption by mosquitoes and development of microbial prey communities (Merritt et al. 1992, Walton 2012). In addition, dense stands of emergent macrophytes can provide refuge from immature mosquito predators, and senesced vegetation can be a secondary source of nutrients and organic matter. Many *Culex* species are competent vectors of zoonotic arboviruses common in the United States such as West Nile Virus and St. Louis Encephalitis (Turell et al. 2005). The most common vector of WNV to humans in the southeastern United States, *Culex quinquefasciatus*, has previously been associated with heavily vegetated, poor-water-quality environments such as treatment wetlands (Workman and Walton 2003, Peck and Walton 2005, Chaves et al. 2009, Nguyen et al. 2012).

I conducted this study to assess the impact on mosquito production of two features common to treatment wetlands: partially treated wastewater, and senesced emergent macrophytes. Characteristics of wastewater, such as high organic carbon content and nutrient enrichment, are thought to provide and enrich important food resources to developing mosquitoes; emergent and decaying macrohytes provide similar food resources through nutrient release and support of microbial communities, and additionally provide refuge from mosquito predators (Merritt et al. 1992, Walton 2012). Both of these characteristics have been associated with increased oviposition and production by certain mosquitoes and in some habitats, but it is unknown whether they interact in habitats such as treatment wetlands to drive an overabundance of immature mosquito production.

I used a randomized block factorial design in containers placed at constructed wetlands with two fully crossed treatments (wastewater and vegetation) and two levels of each treatment (present/absent). I

expected containers with partially treated wastewater and vegetation would exhibit increased mosquito production, and a significant interaction would drive mosquito abundance higher in containers with both present. I also conducted the same experimental procedure in an urban environment to investigate the response of a different mosquito community with potentially differing life histories than those ovipositing in wetland habitats.

MATERIALS AND METHODS

Sites and experimental design:

Eighteen-quart black plastic dishpans (44cm x 36cm x 18cm) were placed in three replicate groups of four containers at four separate sites (12 total containers per site): In randomly-selected locations adjacent to three constructed treatment wetlands (in Jonesboro, Lavonia, and Pine Mountain, GA), and in an urban neighborhood in Athens, GA (Figure 3.1). The three constructed wetland sites represented various designs and management conditions (Table 3.1). Two common sources of nutrients and organic matter in wastewater treatment wetlands were used as treatments to evaluate the effect on mosquito production in the containers: 1. Partially treated wastewater from the treatment wetland; and 2. Senesced wetland vegetation.

Factorial design:



Each treatment consisted of two levels (present and absent) and treatments were fully crossed, so each container held either 5.5L distilled water or partially treated wastewater, and either 50 grams (dry weight) senesced wetland vegetation (*Typha* spp.) or no vegetation (see factorial diagram above). In the case of the containers in Athens, GA, water from treatment wetlands in Lavonia, GA was used.

Mosquitoes were allowed to oviposit in containers for three consecutive weeks during the months of June-August 2013, and containers were sampled weekly by dipping twice with a standard 350mL mosquito dipper to remove a total of 500mL water. Containers were left uncovered for the duration of the experiment. At the PineMt site, evaporative dry-down in the containers prevented sampling in the third week.

250 mL dips were taken at least five minutes apart to minimize the effects of active larval avoidance on count data, combined into one 500mL sample, and subsequently filtered through an approximately 20 micron paper filter. After filtration, water was returned to the container, and the container refilled with either distilled or wetland water to the original 5.5L. Contents remaining on the filter were washed into a 10% ethanol solution, and later preserved in a 70% ethanol solution for identification of mosquito larvae. Second, third, and fourth instar larvae were identified to genus, and fourth instar *Culex* and *Aedes* larvae to species.

Statistical analysis:

Larval mosquito abundance is expressed as number of individuals per 500mL dip sample. Abundance was averaged across multiple samples taken from each container. I conducted a two-way analysis of variance (ANOVA) with the mean immature mosquito abundance sampled from each replicated experimental unit (container), with wetland site as a blocking variable (N = 36) (R Foundation for Statistical Computing, Vienna, Austria). The crossed vegetation and wastewater treatments served as independent variables. Additionally, I performed a two-way ANOVA on the mean immature mosquito abundance from containers in the urban site (separated due to differing mosquito communities), without a blocking variable (N = 12).

RESULTS

At each location, total mosquito production was highest in containers with organic matter (wastewater, vegetation, or both). The three sets of containers that were adjacent to constructed wetland cells exhibited similar patterns of mosquito production (Figure 3.3). Specifically, mean larval mosquito abundance per 500mL sample was highest in containers treated with senesced *Typha* spp., and mosquito production was elevated in containers with only partially treated wastewater (relative to distilled water) at two sites (Huie and PineMt). ANOVA results (Table 3.2) indicated that both wastewater (P = 0.04) and vegetation (P << 0.001) had significant effects on mosquito abundance, but there was no interaction between the two (P = 0.45). The significance of the blocking variable is due to very low mosquito abundances sampled from containers at one wetland site (Lavonia); interaction plots show the differential impact of treatments at this particular site (Figure 3.2). Conversely, mosquito production in the Lavonia constructed wetland itself was highest of the three wetland sites (Table 3.1; see chapter 2).

At each of the wetland sites, the majority of identifiable mosquito larvae sampled from containers were of the genus *Culex*, and at least 30% of fourth instar larvae I sampled in each location were *Culex quinquefasciatus* (Table 3.4 and 3.5). *Culex* production in each of the three wetland locations followed a similar pattern to overall mosquito production (Figure 3.4).

Very few *Culex* mosquitoes were sampled from the containers in an urban neighborhood in Athens (2.3% of identifiable larvae), and most fourth instar mosquitoes sampled were the container breeding species *Aedes albopictus* and *A. japonicus*. The response by mosquitoes in an urban environment indicated a significant preference for containers containing wastewater (P = 0.004), and unlike in containers adjacent to wetlands, a significant interaction existed between the wastewater and vegetation treatments (P = 0.01) (Table 3.3). The vegetation treatment alone was not significant at P<0.05 in these containers (P = 0.08).

DISCUSSION

The pattern of mosquito production was similar in containers placed at each of the three wetland sites, and overall mosquito production was highest in containers treated with both senesced macrophytes

and partially treated wastewater. Both treatments exhibited a statistically significant effect on mosquito abundance in two-way ANOVA models, but there was no significant interaction between the treatments, indicating that the multiplicative effects of both sources of organic matter likely do not contribute to abnormally high mosquito production.

Although it is difficult to draw conclusions from the containers at the Lavonia wetland site due to low mosquito abundance (total immature mosquitoes sampled = 18), ANOVA results and patterns at the Huie and PineMt sites suggest that mosquito response to senesced vegetation in treatment wetlands could be stronger than to nutrient-enriched partially treated wastewater. For mosquitoes ovipositing in wetlands, macrophytes in wetland cells that could provide both refugia and food sources may be a stronger signifier of potential offspring survival than merely organic matter content of the water. In the absence of vegetation, however, wastewater remains a significant factor aiding mosquito production.

This result is supported by literature describing oviposition and predator avoidance cues used by some *Culex* and *Anopheles* mosquitoes, which made up the majority of the genera found in containers at wetland sites (Workman and Walton 2000, Bond et al. 2005, Ohba et al. 2012). Many species in these genera occur in habitats that can be populated by predators of immature mosquitoes, and preferentially oviposit or move to areas with fewer predators or more potential refuge (Sih 1986, Eitam et al. 2002, Eitam and Blaustein 2004, Blaustein et al. 2005). Conversely, mosquitoes that breed in ephemerally flooded containers typically without predators, such as *Ae. albopictus* and *Ae. japonicus*, have been shown to exhibit weaker larval response to either predator or refugia cues (Sih 1986, Kesavaraju et al. 2007, Ohba et al. 2012).

Mosquito production in containers I placed in an urban neighborhood reflected this characteristic: the vast majority of immature mosquitoes sampled were container breeding *Aedes* species, and only the presence of wastewater and the interaction of vegetation and wastewater were statistically significant predictors of mosquito abundance at P<0.05 (Table 3.3). Thus, mosquito response in these containers was to organic matter content with a weaker response to potential refugia. This major difference in mosquito

response in containers placed at wetland sites and in Athens was likely due to life history characteristics of the mosquito community ovipositing in the various locations.

Life histories of mosquitoes most associated with Georgia's treatment wetlands, such as *Anopheles* spp., *Culex* spp., *Culiseta* spp., *Mansonia* spp., and *Uranotaenia* spp., are such that predator avoidance strategies in oviposition and larval stages are evolutionarily advantageous. Refuge availability is thus a likely driver of mosquito production in treatment wetlands, where larvivorous fish and predacious macroinvertebrates often co-exist, and where food resources provided by partially treated wastewater are abundant. Results of this study support previous research by Walton and others that shows elevated mosquito production in high nutrient aquatic environments such as wastewater (Sanford et al. 2005, Chaves et al. 2009, Walton 2012). Indeed, previous research in treatment wetlands has shown elevated mosquito production where macrophytes were felled in place and left to serve as a carbon source for denitrifying bacteria, similar to the senesced vegetation in this study (Walton and Jiannino 2005).

These results of this study do offer some specific management implications for constructed wastewater treatment wetlands to reduce mosquito production, and potentially reduce local risk of pathogen transmission to humans. Specifically, high mosquito production in containers with senesced vegetation suggests that vegetated habitats within wetlands should be confined to as small an area as possible to still meet water treatment goals. Walton et al. (2012) suggested planting macrophyte beds of no more than 5 meters in width, as most predation pressure occurs along the open water-vegetation boundary. Thullen et al. (2002) advocated for increasing open water area by creating a series of vegetated hummocks surrounded by deeper open water zones, which had the additional benefit of improving water treatment in a high ammonia system.

Because vegetation is an integral structural attribute for biological treatment in wetlands, at times a more practical management technique may be to seasonally remove shoots and leaves of some aquatic macrophytes from wetland areas before senescence and decomposition. This prevents senesced vegetation from returning organic carbon and nutrients to the system, as well as removes some structural refugia offered by decomposing macrophytes. In current practice, this management strategy is often

approximated by winter-time burning of wetland vegetation, but this can return much of the organic material and nutrients taken up by plants to the water in the form of ash. Harvesting vegetation in other ways, however, is often impractical, expensive, and can risk damage to the continued health of the macrophyte community (Kadlec and Wallace 2008).

A better tactic could be to design wetlands so that shallower, vegetated areas of cells can be drained and the vegetation harvested. This would be a more effective mosquito management strategy through limitation of mosquito refuge and food resources. Mayhew et al. (2004) found that periodic draining of constructed wetland mesocosms reduced mosquito emergence, and retaining habitat for mosquito predators during these dry downs in deeper sections could prevent local resurgence of mosquito populations after re-flooding (Walton 2012). Additionally, this management strategy would likely extend the life of the treatment wetland by preventing addition of excess nutrient sources and in-filling material, and could provide a potential economic or energy resource through fermentation of harvested vegetation into cellulosic biofuel (Liu et al. 2012).

Several uncertainties remain regarding the effect of the habitat characteristics I studied on mosquito oviposition and production. It is unknown why so few mosquitoes colonized the containers at the Lavonia wetland site (18 total sampled versus a mean of 170 at the other two wetland sites), especially when the wetland itself exhibited by far the highest immature mosquito abundance (see Chapter 2; Table 3.1). Containers were placed at the Lavonia site one month after those at Huie and PineMt (mid-June vs. mid-July), but seasonal effects of mosquito production within the treatment wetlands also run counter to this trend (abundances were higher in wetlands in July than June).

Results from the previous chapter of this document detail reduced production in treatment wetlands with larvivorous fish, which is likely the result of oviposition habitat selection by adult mosquitoes, predation of immature mosquitoes by fish, and larval mosquito avoidance behavior (Sih 1986, Angelon and Petranka 2002). I expected that chemical cues from fish predators in wastewater could affect mosquito production in tubs at the Huie site (Table 3.1). I found no discernable pattern in this study, however, to suggest differences in production between wetlands with and without fish, or those

conducting secondary or tertiary treatment (which contributes to nutrient and organic matter content of the wastewater). Including more sites with these characteristics in future analysis could clarify these effects.

Future research is also needed to tease apart the differential responses of mosquito oviposition and mosquito larval development due to the effects of wastewater and senesced vegetation. Some cues may influence where a gravid female mosquito oviposits, such as the presence of refugia; other factors may be responsible for survival, development, and emergence of her offspring, such as abundance of food resources. Both of these processes affect the actual adult mosquito production from a specific site. I only measured total abundance of immature mosquitoes per dip sample, so the effects of these two biological processes were combined in this particular study. Finally, aerial colonization of containers by aquatic predators such as beetles (dytiscids and hydrophilids) and backswimmers (notonectids) was not accounted for in this study. While I observed these animals in some containers, I made no effort to remove them so as to not alter immature mosquito populations in the process. Other researchers have shown rapid invertebrate aerial colonization to be quite extensive, so future research on the effect of aerial colonizers on mosquito production in similar containers may be warranted (Tronstad et al. 2007).

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Figure 3.1: Location of container sites within the state of Georgia. Athens: Athens, Ga. residential neighborhood; Huie: Clayton County Huie constructed wetlands complex, Jonesboro, Ga.; Lavonia: Town of Lavonia constructed wetlands, Lavonia, Ga.; PineMt: Town of Pine Mountain constructed wetlands, Pine Mountain, Ga.



Table 3.1: Wetland site characteristics. Mean mosq. larvae from random dip sampling in each wetland (Huie N=20, Lavonia N=47, PineMt N=30)

Wetland	Active treatment area (ha)	Max. permitted flow (monthly avg. MGD)	Treatment type	Fish	Mean mosq. larvae/500mL
Huie	97.85	17.4	Tertiary	Yes	0.4
Lavonia	5.85	1.32	Secondary	No	61.8
PineMt	1.98	0.3	Secondary	No	3.9

Table 3.2: ANOVA table for the effects of vegetation and wastewater on container mosquito abundance at wetland sites. *Statistically significant (P<0.05)

Factor	df	Sum Sq	Mean Sq	F value	Pr(>F)	
Vegetation	1	299.5	299.5	32.2	3.51E-06	*
Wastewater	1	41.2	41.2	4.4	0.04	*
Vegetation x Wastewater	1	5.6	5.6	0.6	0.45	
Block	2	201.2	100.6	10.8	0.0003	*
Error	30	279.2	9.3			

Table 3.3: ANOVA table for the effects of vegetation and wastewater on container mosquito abundance at an urban site. *Statistically significant (P<0.05). *Statistically significant (P<0.05); *Statistically significant (P<0.1)

Factor	df	Sum Sq	Mean	F value	Pr (> <i>F</i>)	
			Sq			
Vegetation	1	57.8	57.8	3.9	0.083	х
Wastewater	1	228.2	228.2	15.5	0.004	*
Vegetation x Wastewater	1	149.3	149.3	10.1	0.013	*
Error	8	117.9	14.7			

Figure 3.2: Interaction plots of immature mosquito abundance by vegetation and water type.



Interaction plot of Wetland site and Vegetation treatment

Interaction plot of wetland site and water treatment



Clean (Distilled) Water (C) or Wastewater (W)

Figure 3.3: Mean immature mosquito abundance per sample in experimental containers. Treatments are: A. 5.5L distilled water; B. 5.5L Distilled water and 50g senesced *Typha* spp.; C. 5.5L partially treated wastewater; D. 5.5L partially treated wastewater and 50g senesced *Typha* spp. Error bars are 95% confidence intervals.



Site	Total Mosquitoes collected	Total identifiable larvae	Total <i>Culex</i> Larvae	Total <i>Aedes</i> larvae	Fourth Instar Larvae	Fourth instar Culex quinquefasciatus
Huie	221	139	95	8	61	19
PineMt**	119	85	57	2	37	16
Lavonia	18	16	12	2	6	2
Athens	571	427	10	401	151	1

Table 3.4: Total counts of larval mosquitoes collected from experimental containers.

Table 3.5: Counts of 4th instar larval mosquito species collected from experimental containers.

Species	Huie	PineMt	Lavonia	Athens
Aedes albopictus	4	1	2	139
Aedes japonicus	0	0	0	9
Anopheles spp.	18	13	2	2
Culex nigripalpus	0	3	0	0
Culex quinqefasciatus	19	16	2	1
Culex restuans	15	3	0	0
Culex territans	5	1	0	0

Figure 3.4: Mean immature *Culex* spp. abundance per sample in experimental containers. Treatments are: A. 5.5L distilled water; B. 5.5L Distilled water and 50g senesced *Typha* spp.; C. 5.5L partially treated wastewater; D. 5.5L partially treated wastewater and 50g senesced *Typha* spp. (Error bars are 95% confidence intervals.



CHAPTER 4

PHYSICAL AND CHEMICAL EFFECT OF *LEMNA MINOR* (COMMON DUCKWEED) COVER ON CULICINE MOSQUITO EMERGENCE

INTRODUCTION

Lemna minor (common duckweed) is a floating small-leaved freshwater aquatic plant that is native or naturalized throughout the world. One of the smallest flowering plants, *L. minor* undergoes extremely rapid vegetative propagation, and successfully disperses among aquatic habitats by affixing to mobile organisms such as birds, wetland mammals, and often humans. Given available nutrient and light sources, it typically outcompetes other freshwater plants in stagnant or slow-moving freshwater bodies, and has been suggested as a suitable candidate for phytoremediation of wastewater polluted with heavy metals such as cadmium, copper, and zinc (Hou et al. 2007).

L. minor was present in every constructed wastewater treatment wetland I sampled in Georgia from May-October 2013 (N = 19). It often forms thick monospecific mats (or carpets) that can be over 1 inch in thickness, due to the high-nutrient content of partially treated wastewater (see Figure 4.1). These mats can obstruct water intakes and drainage pipes, and can become a nuisance for wetland managers who must maintain adequate water flow between wetland cells and to effluent discharge points. The density and thickness of these mats, however, may prevent larval mosquitoes from accessing atmospheric oxygen with their siphons, and mosquito pupae from emerging from the water. Because *L. minor* is such a common volunteer plant species in constructed treatment wetlands, I conducted this study to investigate its potential contribution to mosquito management strategies in these systems.

The literature evaluating the effects of the Lemnoideae (duckweeds) on mosquito populations is conflicting, and somewhat sparse. Anecdotal reports of early mosquito control efforts using floating vegetation suggested that complete surface coverage of Lemnoideae acts as an oviposition deterrent to adult mosquitoes, as well as a barrier to atmospheric oxygen exchange for immature mosquitoes in the
water; when even a small portion of the surface was uncovered, however, *Culex* and *Anopheles* mosquitoes frequently bred (Matheson 1930). Some researchers have suggested that *L. minor* decreases oviposition by adult mosquitoes through alteration of the color of the water (potentially used as an oviposition cue), by decreasing the amount of algal particulate matter available as larval food resources, and by lowering the temperature of the water (Sjogren 1968, Angerilli and Beirne 1980). Additionally, some research suggests *L. minor* may produce a volatile allomone that deters oviposition by *Culex pipiens*, and can cause malformation and mortality in larval-stage *C. pipiens* (Eid et al. 1992b, a); Judd and Borden (1980), however, found that *L. minor* extracts had a volatile compound that deterred oviposition of *Aedes aegypti*, but had no effect on *C. pipiens*.

These results are complicated by research that suggests positive or neutral relationships between mosquito populations and presence of floating duckweed mats. Burton (1960) reported that first instar *Mansonia uniformis* regularly perforate the roots of *Lemna polyrrhiza* in India to obtain oxygen, and are supported by high *Lemna* densities. Other research has suggested that some *Anopheles* spp. are attracted to sites with dense and diverse floating vegetation, but that this effect diminishes to neutral in times or areas of *Lemna* spp. dominance (Hopkins 1936, Rejmankova et al. 1991). Finally, some species of mosquitoes are known to lay their eggs on the edges of floating *Lemna* spp. leaves, particularly *Culex erraticus*, and so are thought to exhibit a positive relationship with duckweed presence (Burkett-Cadena 2013).

Because *L. minor* forms thick mats in constructed treatment wetlands in Georgia, I was interested in evaluating the role it plays in mosquito control in these systems. To do this, I conducted a container experiment to test the emergence of mosquitoes from water with various surface coverage of *L. minor*, as well as from water that previously contained *L.minor* to investigate the potential role of plant allomones. As immature mosquitoes are present in all of Georgia's constructed treatment wetlands, meaning oviposition has already occurred, this study evaluates only the effect of *L. minor* on mosquito emergence.

Most immature mosquitoes in these wetlands occur in habitats with emergent macrophytes such as *Typha latifolia* (see chapter 2 of this document), while *L. minor* proliferates mainly in open water areas

where sunlight is abundant. Thus, any effect of duckweed on mosquitoes would necessarily be an additional factor to consider in a comprehensive Mosquito Management Plan (MMP) that addressed the entire wetland. I hope this study can be informative for strategic wetland management that accounts for the tradeoff between the potential maintenance nuisance caused by *L. minor* and its contribution to mosquito control.

MATERIALS AND METHODS

To evaluate the effect of *Lemna minor* on larval development and mosquito emergence, I placed a random mixture of fifteen 3rd- and 4th-instar *Aedes* spp. and *Culex* spp. mosquito larvae in each of nine containers (three-liter buckets, 22.8cm diameter opening) with 2L of fresh water and differing amounts of duckweed on the surface. Juvenile mosquito stage was evaluated based on size observation. Duckweed was harvested from a constructed wetland, washed thoroughly, and stored wet for no more than 24 hours before being placed in containers with larval mosquitoes.

Initially, three levels of *L. minor* cover were used, each with three container replicates: 0% *L. minor* surface cover, 50% *L. minor* surface cover, and 100% *L. minor* surface cover. Emergence traps were placed on all containers, which were kept outside in a covered area to maintain a normal photoperiod and outdoor temperature (Figure 4.2). I sampled these traps and removed all adult mosquitoes over a period of 13-days, until some duckweed began to sink from the water surface. This experiment was repeated one week following its completion with one additional treatment level, in order to assess purely chemical effects of *L. minor* on mosquito production: Three additional containers contained 2L of fresh water that had contained *Lemna minor* for 24-hours prior to the experiment, and was subsequently removed before mosquito introduction.

Results from common treatment levels over the two experimental periods were analyzed graphically for variance among common treatments, and combined when no significant differences were observed (Figure 4.3). I used the mean total emerged adult mosquitoes in a one-way analysis of variance (ANOVA) model with *L. minor* treatment level as the explanatory factor to evaluate the relationship between duckweed and mosquito emergence (R Foundation for Statistical Computing, Vienna, Austria).

When significant difference existed, I used Tukey's Range Test with a 95% family-wise confidence interval to evaluate differences between levels of *L.minor* treatments. Since all larval mosquitoes could not be assumed to be of the same initial development stage on Day 1, I did not conduct statistical analysis on the speed of development. To visualize the rate of mosquito emergence from the experimental containers, I plotted the interaction between time elapsed and immature mosquitoes remaining in the container (Figure 4.4).

RESULTS

Mosquito emergence was significantly less in containers with 100% *Lemna minor* cover, from which 2 adult *Aedes albopictus* mosquitoes emerged out of 90 total larvae (P << 0.001; Figure 4.5). No differences were found in total emergence between other levels of *L. minor* cover (Tables 4.1 and 4.2). Overall, an average of $64\% \pm 17.2\%$ (SD; N = 15 containers) of larval mosquitoes eclosed from containers not treated with 100% surface *L. minor*, which is within the range of other emergence trap studies of *Culex* and *Aedes* mosquitoes (Tun-Lin et al. 2000, Walton 2009, Hamer et al. 2011). When analyzed on their own, both genera of mosquitoes exhibited no significant difference from the overall pattern.

DISCUSSSION

The results of this study suggest that *L. minor* acts as a physical barrier to mosquito emergence when it forms mats that completely cover the surface of water bodies. This mechanical barrier does not appear to influence emergence when there is 'patchy' surface coverage, however, as adult mosquitoes can still emerge from uncovered areas. These results do not support the notion that *L. minor* produces a chemical compound that acts as an antagonist to the types of mosquito larvae in this study, which included individuals from the *Culex pipiens-quinquefasciatus* complex previously reported to have been affected. Similar numbers of mosquitoes emerged from containers with 50% duckweed cover, no duckweed cover, and water that had contained duckweed for 24 hours before removal. Eid et al. (1992b) suggested that *C. pipiens* first instar larvae were most susceptible to the insecticidal effects of the duckweed compound, but found mortality effects with each larval stage; results of this study suggest that

more research is needed to understand the chemical effects of the plant on mosquito development and emergence in real world conditions.

As a mosquito control strategy in constructed treatment wetlands, these results suggest that *L. minor* may only be effective when forming mats on the water surface and creating a physical barrier to larval gas exchange and emergence. More research is needed, however, to evaluate the role of duckweed as a facet of a comprehensive MMP. Tariq et al. (2009) investigated the synergistic effects of larvivorous fish (*Poecellia reticulata*) with experimentally introduced *L. minor* and another duckweed (*Spirodella* spp.), and found near complete mosquito control at all duckweed and fish densities. Their analysis concluded that partial duckweed surface coverage caused mosquito larvae to concentrate in areas where atmospheric gas exchange was possible, and predation by *P. reticulata* then became more efficient. Unfortunately their study did not evaluate mosquito response in a control site or with fish or duckweed alone. The synergistic effects of duckweed and biological control with fish should be explored further, especially where duckweed does not completely cover the water surface.

Duckweed management strategy in treatment wetlands needs to balance any positive mosquito control effects with potential maintenance costs of infrastructure fouling. Often the latter is of no concern, but treatment wetlands with narrow discharge pipes or conveyances sometimes have incentives to reduce duckweed growth. Given the results of this study, it is unlikely *L. minor* offers enough of a mosquito control benefit in these systems to advocate for infrastructure adaptation to support its growth. Maintenance of complete *L. minor* surface cover is likely to be difficult, due to limited light penetration in dense emergent vegetation, waterfowl consumption, and weather effects (particularly strong wind). More research is needed, however, to investigate the effects of *L. minor* chemical release on mosquito development and condition, as Eid et al. (1992a) reported malformation in mosquitoes reared in water containing duckweed extracts. If *L. minor* at densities lower than complete surface coverage can be a cause of poor body condition in mosquito larvae or adults, benefits to mosquito management may increase. Fully-controlled experimental study of these sub-lethal effects of duckweed, as well as

synergistic effects with other control strategies in an MMP, will be useful to fully analyze tradeoffs for

duckweed management in constructed treatment wetlands.

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Figure 4.1: Examples of constructed treatment wetlands with *L. minor* coverage. Clockwise from top left: Rentz, Ga.; Folkston, Ga.; Clayton County, Ga ; Alamo, Ga.



Figure 4.2: Containers after attachment of emergence traps.





Figure 4.3: Comparison of mean total emerged mosquitoes over two 13-day sampling periods. Error bars indicate one standard deviation. A: No *L. minor*; B: 50% initial surface *L. minor* coverage; C: 100% initial surface *L. minor* coverage.

Figure 4.4: Mean larval mosquitoes remaining in containers with differing *L. minor* levels over 13-day sampling period. Error bars indicate 95% confidence interval. A: No *L. minor*; B: 50% initial surface *L. minor* coverage; C: 100% initial surface *L. minor* coverage; D: 100% surface *L. minor* coverage for 24-hour period prior to experiment, *L. minor* completely removed immediately before mosquito introduction.



Temporal effects of L. minor on mosquito emergence

Figure 4.5: Mean total mosquitoes emerged from water with differing *L. minor* levels over 13-day sampling period, with error bars indicating 95% confidence interval. A: No *L. minor*; B: 50% initial surface *L. minor* coverage; C: 100% initial surface *L. minor* coverage; D: 100% surface *L. minor* coverage for 24-hour period prior to experiment, *L. minor* completely removed immediately before mosquito introduction.



Effects of L. minor on Mosquito Emergence

Treatment

Table 4.1: One-way ANOVA table of total mosquito emergence from containers with different levels of *L. minor* coverage.

	df	Sum Sq	Mean Sq	F value	Pr(>F)
L. minor Level	3	380.6	126.9	24.42	2.15E-06
Residuals	17	88.3	5.2		

Table 4.2: Results of Tukey's Range Test with 95% family-wise confidence interval. A: No *L. minor*; B: 50% initial surface *L. minor* coverage; C: 100% initial surface *L. minor* coverage; D: 100% surface *L. minor* coverage for 24-hour period prior to experiment, *L. minor* completely removed immediately before mosquito introduction. Only level C (complete cover) is significantly different from others.

Levels	Difference	95% conf. lwr.	95% conf. upr.	Adjusted P value
B-A	-1.67	-5.41	2.07	0.60
C-A	-9.83	-13.57	-6.09	0.00
D-A	0.50	-4.08	5.08	0.99
С-В	-8.17	-11.91	-4.43	0.00
D-B	2.17	-2.42	6.75	0.55
D-C	10.33	5.75	14.92	0.00

CHAPTER 5

EXPERIMENTAL EVALUATION OF DIRECT PREDATION BY GAMBUSIA AFFINIS ON IMMATURE MOSQUITOES IN THREE VEGETATION DENSITIES

INTRODUCTION

Some ecological characteristics of wastewater treatment wetlands have been correlated with increased populations of some mosquitoes, including several that are medically important as zoonotic disease vectors. Specifically, high levels of inorganic nutrients, particulate organic matter, dense stands of monospecific emergent vegetation, and hypoxic conditions have been shown to increase mosquito oviposition and/or immature mosquito production (Walton and Workman 1998, Thullen et al. 2002, Sanford et al. 2005, Chaves et al. 2009, Walton 2012, Yadav et al. 2012). Therefore, mosquito management in wetlands treating municipal wastewater often involves multiple strategies, which can include limitation of larval mosquito food resources (usually through targeted reduction of Biological Oxygen Demand and Total Suspended Solids as surrogates for particulate organic material), vegetation management and planting design, hydrologic alteration, and biological control (Russell 1999, Kadlec and Wallace 2008, Walton 2012).

Most constructed treatment wetlands in the state of Georgia feature dense, low-diversity stands of emergent macrophytes, often *Typha* spp. (cattail) and *Schoenoplectus* spp. (bulrush). Both of these genera have been shown to provide effective refuge for immature mosquitoes, and decomposing macrophytes can be an additional source of organic matter for mosquito nutrition (Walton et al. 1990, Keiper et al. 2003, Jiannino and Walton 2004). Dense vegetation also typically provides less habitat for larval mosquito predators such as carnivorous invertebrates and larvivorous fish (Gerberich and Laird 1985, Walton 2007, Walton et al. 2012).

In Georgia, *Gambusia affinis* and *G. holbrooki* (Western and Eastern Mosquitofish) are by far the most common larvivorous fish species in existing wetlands treating municipal wastewater. The two

species are commonly considered together because of their similar biology, appearance, and taxonomy, and I will use *Gambusia* in this chapter unless referring specifically to only one of the species (Pyke 2005). *Gambusia holbrooki* is native to the eastern United States and *Gambusia affinis* to the Mississippi River Basin, but both species have been introduced throughout much of the globe for mosquito control and together are now the most common freshwater fish in the world. Unfortunately, *Gambusia* have often had disastrous unintended and/or indirect effects on non-target species (Pyke 2008). I study them here because of their relevant native range and ubiquity in Georgia, but do not advocate their introduction outside their native range in practically any scenario.

Gambusia are visually-feeding fish that prefer open water habitats to dense vegetation (Loftus and Kushlan 1987, Pyke 2005). Many studies have described very limited mosquito production in these areas, even in the absence of larvivorous fish (Batzer and Resh 1992, Orr and Resh 1992, Walton and Workman 1998, Walton 2012). This is likely due to gravid female mosquitoes selecting vegetated areas for oviposition due to refugia and food resources offered by aquatic macrophytes, as well as increased predation pressure on larvae in open water environments. Practical considerations limit open water areas in treatment wetlands, however, as vegetation serves important nutrient uptake and structural functions (Kadlec and Wallace 2008). Some studies have suggested management for narrow bands of vegetation, or hummocks of vegetation surrounded by deep water zones to reduce mosquito production, as well as to potentially increase water treatment capabilities (Thullen et al. 2002, Walton 2012). To add to the strategic knowledge base for constructed treatment wetlands undergoing mosquito management, I tested the effect of *Gambusia* predation within varying macrophyte stem densities in an existing constructed wetland.

I conducted a fish exclusion experiment in a constructed treatment wetland with existing areas characterized by dense emergent macrophytes, sparse emergent macrophytes, and open water. By excluding fish from areas of the wetland, I evaluated their direct predation effects on immature mosquitoes in different vegetation densities. I also conducted limited evaluation of *Gambusia affinis* predation of other macroinvertebrates in the same habitats. Willems et al. (2005) found reduced mosquito

predation by *Gambusia holbrooki* in dense simulated vegetation in laboratory mesocosms, albeit with artificially high stem densities. I hypothesized that *Gambusia* predation, as signified by difference in mosquito abundance between closed frames (exclosures) and open frames, would be highest in sparse emergent macrophytes, due to mosquito preference for vegetated habitats, as well as increased visual acuity of *Gambusia* over habitats with higher stem densities.

MATERIALS AND METHODS

Site Characteristics:

Prior to installation of fish exclosures, I conducted a site survey of vegetation density variation within cells of a constructed treatment wetland in Lovejoy, GA (Panhandle Constructed Wetlands Facility, operated by the Clayton County Water Authority). During the course of sampling across all wetland cells at this wetland (N = 21; see Chapter 2), several were found to contain immature mosquitoes. Of these cells, I visually identified one that had clear differentiated zones of Open Water, Sparse Vegetation (intermediate density), and Dense Vegetation (OW, SV, and DV, respectively). To quantify vegetation density, I counted stem densities in a $1m^2$ quadrat in at least five randomly selected points in the areas I identified. The actual stem densities I found were: DV: >53 stems/m² over an area approximately $25m^2$; SV: 11-26 stems/m² over the same area; and OW: 0 stems/m² over the same area. From these larger areas, I selected two locations with each vegetation density to install fish exclosures.

Fish Exclosures:

I assembled eighteen 16 inch x 18 inch x 36 inch frames from polyvinyl chloride (PVC) piping, nine of which I enclosed on four sides with 3 millimeter nylon screen to function as fish exclosures, and nine of which remained open. The bottom and top of each exclosure was left open, and 4-inch vertical PVC studs were attached to the bottom to hold the frames in place (Figure 5.1). All 18 frames were installed by digging 16 inch x 18 inch rectangular trenches in the sediment, and sinking each frame at least 1 inch past the base (5 inches below the sediment, including the height of the vertical studs). Open and closed frames were placed in randomly chosen groups of three in an appropriate vegetation density, so that two groups of three frames were in each type of vegetation density (Figure 5.2). This was to

minimize any stochastic effects of location within the wetland cell, but also retain ease of access to the exclosures. Emergent vegetation consisted of a mix of five species of wetland plant (Table 5.1), and at least two species were present in each exclosure in DV and SV. No vegetation was added to or removed from any exclosure area, and all exclosures and control frames were exposed at least six inches above the water surface.

Following installation, I removed all fish and larval amphibians from each exclosure within 24 hours using a D-frame sweep net. Both were visible beneath the water surface, but I made a good faith effort to remove unseen individuals by sweeping the net 20 additional times after the last visible individual was removed. All control and treatment exclosures were left in place for 29 days in August and September 2013, and fish were only observed inside a treatment (screened) exclosure on one instance: during the first round of sampling for immature mosquitoes, two *G. affinis* individuals were found and removed. I also on three occasions removed partially-mature Pig Frogs (*Rana grylio*) that had grown legs and leapt into an exclosure.

Sampling procedure:

I sampled each exclosure for immature mosquito abundance every 72-96 hours while the exclosures were installed, consistent with *C. quinquefasciatis* oviposition periodicity seen by Chaves and Kitron (2011) in Atlanta (approximately 20 miles from the study site). Immature mosquitoes could enter closed frames either through oviposition and hatching, or active immigration by young instars small enough to pass through the screen (emigration was thus possible as well). Larval mosquitoes were collected by taking two 250mL water samples with a standard 350mL mosquito dipper. Samples were taken at least five minutes apart to minimize the effects of active larval avoidance on count data, combined into one 500mL sample, and subsequently filtered through an approximately 20 micron paper filter. Contents remaining on the filter were washed into a 10% ethanol solution, and later preserved in a 70% ethanol solution for identification of mosquito larvae. Second, third, and fourth instar larvae were identified to genus, and fourth instar *Culex* larvae to species.

Following the eighth sampling event, I sampled the free-swimming and planktonic invertebrate community within in each exclosure by pushing a D-frame sweep net unidirectionally at random three times through the water column. Contents of the net were washed into a 10% ethanol solution, and later preserved in a 70% ethanol solution for identification to at least class, and most often to order or family.

Statistical analysis:

Larval mosquito abundance in exclosures is expressed as number of individuals per 500mL dip sample. Because I sampled each frame repeatedly through time (N = 8), I used mean abundance and mean *Culex* spp. abundance in repeated measures analysis of variance (ANOVA) models for each vegetation density (R Foundation for Statistical Computing, Vienna, Austria). These modeled the difference in mean abundance between frame types (i.e. Closed vs. Open) in each density, as well as within individual frames across time to discern any trends. I also used frame type within each vegetation density as the explanatory variable in ANOVA models for mean macroinvertebrate abundance sampled following the four-week mosquito sampling period, as well as for mean abundance of each type of macroinvertebrate sampled. RESULTS

I found no effect of repeated mosquito sampling across time in within frame analyses in any of the vegetation densities (Table 5.2 and 5.3). I recorded a significant difference in mean larval mosquito abundance between open and closed frames in both SV (P = 0.0015) and OW (P = 0.035), although the magnitude of the effect was very small in OW (Figure 5.3). Approximately $10.04 \pm 3.81 (1146\% \pm 397\%)$ more immature mosquitoes per 500mL were sampled from closed frames (fish exclosures) in SV than in open frames. In OW, both closed and open frames exhibited abundances of <1 individual/500mL, although $0.29 \pm 0.3 (725\% \pm 750\%)$ more mosquitoes were sampled from closed frames. In DV, there was no significant difference in mean larval mosquito abundance between control and treatment exclosures at $\alpha = 0.05$ (Table 5.4). Mean immature mosquito abundance was higher in DV than in OW by 3.75 ± 2.71 individuals per 500mL (N = 48 samples per habitat; P << 0.001).

Most immature mosquitoes sampled and identified were *Culex* spp. (Table 5.5). In general, production of mosquitoes identifiable as *Culex* spp. followed the same pattern as total mosquito

production, and the only significant difference in *Culex* spp. abundance between open and closed frames was found in SV (Figure 5.3). The overall mean of *Culex* spp. mosquitoes sampled from closed frames was higher than from open frames by over 3 individuals per 500mL. There was a slight opposite trend in exclosures in DV, so this overall trend was driven entirely by the differential in exclosures in SV.

The mean macroinvertebrate abundance sampled from the water column of each frame after the four week mosquito sampling period was higher in the closed frames (fish exclosures) than the open frames for each vegetation density, but the effect was only statistically significant in OW (Figure 5.4; OW: P = 0.001; SV: P = 0.13; DV: P = 0.14). By far the most numerous invertebrates sampled from the exclosures were crustaceans: Amphipods (Order *Amphipoda*) and Seed Shrimp (Class *Ostracoda*) (Table 5.6). Interestingly, overall mean *Amphipoda* abundance was significantly affected by frame type (open vs. closed), while mean *Ostracoda* abundance was not (P < 0.02 and P > 0.19, respectively), although both were more abundant in closed frames (Figure 5.5). Mean abundance for every other invertebrate type was less than five individuals per sample, even in closed frames alone, and no significant differences were found between frame types or vegetation densities in other invertebrate types.

DISCUSSION

As predicted, these results suggest *Gambusia affinis* have a much larger predatory impact on immature mosquitoes in sparsely vegetated areas of a wastewater treatment wetland than in densely vegetated areas. Mosquito production in open water areas was <1 individual per 500mL even inside fish exclosures, and overall not significantly different from zero. Dense vegetation likely provides a refuge for immature mosquitoes, especially from Mosquitofish that preferentially occupy open water and have limited visual hunting ability within dense stands of emergent macrophytes. This study substantiates similar laboratory results found by Willems et al. (2005) showing reduced *Gambusia holbrooki* predation on immature mosquitoes in dense simulated vegetation.

Immature mosquito abundance was highest in treatment exclosures in sparse vegetation. This result suggests that the density difference between DV and SV micro-habitats may not greatly influence mosquito oviposition by adults and/or predation avoidance by immature individuals. This is surprising,

given that long-term effective predation by fish in these habitats would likely exert evolutionary pressure to select for mosquito attraction to dense vegetation. More research is required to verify the accuracy of this result, and eliminate possible alternative causes.

Placement of the exclosures in dense vegetation caused the most disturbance of the three vegetation densities, so possible cage effects could have impacted the absolute mosquito abundances in dense vegetation. The difference in overall mosquito abundance between DV and SV could also be due to random effects of a relatively small sample size, however, as certain exclosures consistently produced higher abundances than their counterparts in different geographic locations in the wetland cell. Similarly, a combination of random effects and potential cage effects/vegetation disturbance could have contributed to the higher abundance of immature mosquitoes in DV open frames than closed exclosures. Repetition of the experiment with greater replication and in other wetlands would provide verification of absolute mosquito abundances.

While very few mosquitoes were sampled from exclosures in OW areas, I identified most that were found in OW as *Anopheles* spp. This has important implications for research into biological control of the *Anopheles* Malaria vectors, especially because some research suggests *Gambusia* may prefer *Culicini* mosqitoes over *Anopheles* (Hess and Tarzwell 1942). Globally, Malaria is by far the most medically important mosquito-vectored pathogen, responsible for hundreds of thousands of human deaths annually (WHO 2014).

G. affinis likely consume many invertebrates apart from immature mosquitoes, based on differences between samples from open and closed exclosure frames (Figure 5.4; Table 5.6). Much of the difference in this study was seen in the greater numbers of Amphipods in closed frames. This same effect was seen with seed shrimp in SV and OW exclosures, although not in DV, where *G. affinis* were presumably visually limited by macrophytic refugia. The absolute difference between mean Amphipod abundance in DV open and closed frames was also the smallest of the three micro-habitats, by a factor of half. These results seem to support the reported notion that *Gambusia* spp. are generalist predators on small macroinvertebrates (as well as some fish and larval amphibians), that nevertheless can be effective

mosquito control agents in their native range due to the magnitude of their consumption (Bence 1988, Peck and Walton 2008).

The results of this study support recommendations made by Thullen et al. (2002) and Walton et al. (2012) concerning vegetation structure and planting in wastewater treatment wetlands. As part of an integrated mosquito management strategy it is beneficial to increase the amount of interface between open water and vegetation, either by limiting stem density of emergent macrophytes (as shown in this study), planting shallow vegetated hummocks surrounded by deeper water zones, or planting narrow bands of emergent vegetation bounded by open water. Each of these strategies reduces mosquito refugia and food resources, and creates additional habitat for mosquito predators. While this study investigated *Gambusia* predation, recent studies have found the vegetation-open water interface to be the location of the highest predation pressure by invertebrate predators such as notonectids (Peck and Walton 2008, Walton et al. 2012). Increasing open water area can also have water treatment benefits, especially for systems high in ammonia, as increased wind mixing allows for oxic nitrification.

As an additional mosquito control tactic, larvivorous fish introduction will be most effective in this or a similar type of planting scheme, focusing on minimizing dense stands of emergent macrophytes. This creates additional habitat for mosquito-consuming fish, and maximizes efficiency of fish visual predation by removing mosquito refugia. This study only concerns the habit and effectiveness of *Gambusia* spp., which have become notorious for unintended ecosystem effects when introduced outside their native range. It may be that other larvivorous fish such as *Heterandria formosa* (see chapter six of this document) are more successful mosquito predators in dense vegetation. Due to common indirect and unforeseeable ecological effects of transplanting species, I advocate for use of native fish, thorough study of predation effects, careful planning, and thoughtful introduction.

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Figure 5.1: Open (left) and closed exclosure frames. Center foreground is size of upper frame opening.

Table 5.1: Plant list of emergent macrophyte species in wetland cell with fish exclosures. At least two species were present in each exclosure in SV and DV.

Species Name	Common Name
Pontederia cordata	Pickerelweed
Schoenoplectus americanus	Three-square Bulrush
Schoenoplectus californicus	Giant Bulrush
Sagittaria latifolia	Arrowhead
Typha latifolia	Common Cattail



Figure 5.2: Locations of closed (exclosure) and open frames within one wetland cell at the Panhandle treatment wetlands in Lovejoy, GA.

	df	SS	MS	F-value	Р
Open Water					
Between frame types					
Treatment	1	1.02	1.02	9.8	0.035*
Residuals	4	0.42	0.10		
Within frame type					
Time	7	2.15	0.31	1.24	0.31
Treatment x Time	7	2.81	0.40	1.63	0.17
Residuals	28	6.92	0.25		
Snorra Vagatation					
Sparse vegetation Potucon frame tunes					
Treatment	1	1210	1210	50.60	0.0015*
Residuals	1 4	81.1	20.3	57.07	0.0015
Within frame type	-	01.1	20.5		
Time	7	216.5	30.93	0.57	0.78
Treatment x Time	7	146.5	20.93	0.38	0.91
Residuals	28	1530.9	54.68		
Damas Wassets from					
Dense vegetation					
Between jrame types	1	11.00	11.02	0.27	0.62
I reatment	1	11.02	11.02	0.27	0.63
Residuals	4	165.67	41.42		
within frame type	7	40	7	0.00	0.00
Time	/ 7	49	12 (4	0.22	0.98
Treatment x Time		95.5	13.64	0.43	0.88
Residuals	- 28	893.7	31.92		

Table 5.2: Repeated Measures ANOVA table for mean immature mosquito abundance in closed and open frames in three vegetation densities. *represents significance at $\alpha = 0.05$.

	df	SS	MS	F-value	Р
Open Water					
Between frame types					
Treatment	1	0.021	0.021	1	0.374
Residuals	4	0.08333	0.021		
Within frame type					
Time	7	0.146	0.021	1.000	0.452
Treatment:Time	7	0.146	0.021	1.000	0.452
Residuals	28	0.583	0.021		
~					
Sparse Vegetation					
Between frame types					
Treatment	1	553.5	553.5	18.71	0.012*
Residuals	4	118.3	29.6		
Within frame type					
Time	7	95.3	13.62	0.423	0.88
Treatment:Time	7	103.3	14.76	0.459	0.856
Residuals	28	901	32.18		
Dense Vegetation					
Between frame types					
Treatment	1	16.33	16.33	1.352	0.31
Residuals	4	48.33	12.08		
Within frame type					
Time	7	17.3	2.476	0.147	0.99
Treatment:Time	7	13.7	1.952	0.116	0.997
Residuals	28	473	16.893		

Table 5.3: Repeated Measures ANOVA table for mean immature *Culex* spp. abundance in closed and open frames in three vegetation densities. *represents significance at $\alpha = 0.05$.



Figure 5.3: Mean immature mosquitoes per 500mL and mean immature *Culex* spp. per 500mL in open (blank columns) and closed (shaded columns) frames in three vegetation densities. Error bars represent 95% confidence interval.

Table 5.4: Difference between mean immature mosquitoes per 500mL in closed and open frames (C - O) in three vegetation densities. P-values from analysis of variance with H_0 : No difference between treatments.

Micro-Habitat	Exclosure	Mean immature mosquitoes/500mL	C - O	P-value
OW	Open	0.04 ± 0.2		
OW	Closed	0.33 ± 0.3	0.29	0.035
SV	Open	0.96 ± 2.7		
SV	Closed	11 ± 3.81	10.04	0.0015
DV	Open	4.42 ± 2.1		
DV	Closed	3.46 ± 3.0	-0.96	0.63

Table 5.5: Total number of immature mosquitoes sampled from open and closed frames. Totals summed in each of three vegetation densities collected over entire four week experiment.

Micro- habitat	Exclosure	Total larvae	Identifiable to genus	<i>Culex</i> spp. larvae	Fourth instar larvae	Culex quinquefasciatus larvae
DV	Open	106	83	70	38	13
DV	Closed	83	61	42	15	5
OW	Open	1	1	0	0	0
OW	Closed	8	6	1	1	0
SV	Open	23	14	5	5	2
SV	Closed	264	201	168	70	14

Figure 5.4: Mean individual macroinvertebrates collected from water column of open (blank columns) and closed (shaded columns) frames in three different vegetation densities. Error bars represent 95% confidence interval.



Habitat	DV	V	OW		SV	
Exclosure Frame	Open	Closed	Open	Closed	Open	Closed
Total	34.67 ± 30.76	62.67 ± 34.51	28.33 ± 19.13	109.33 ± 27.05	85.00 ± 48.60	131.67 ± 68.67
Amphipoda	6.00 ± 22.22	21.67 ± 31.42	3.00 ± 14.57	61.67 ± 20.61	50.67 ± 25.81	83.67 ± 36.51
Gastropoda	0.00	0.67 ± 0.92	0.00	2.00 ± 3.21	0.00	0.00
Coleoptera	0.00	0.67 ± 0.92	0.00	0.00	0.67 ± 1.85	1.67 ± 2.62
Odonata	0.67 ± 2.35	2.00 ± 3.34	1.00 ± 4.67	4.00 ± 6.61	6.00 ± 0.00	3.00 ± 0.00
Trichoptera	0.00	2.67 ± 2.45	0.00	0.00	0.67 ± 0.92	0.67 ± 1.31
Plecoptera	0.67 ± 0.65	0.00	0.67 ± 0.65	0.00	0.00	0.00
Ephemeroptera	0.00	0.00	2.00 ± 3.64	2.67 ± 5.15	5.67 ± 4.89	4.67 ± 6.93
Hemiptera	0.00	0.67 ± 0.92	1.00 ± 1.13	0.00	0.00	1.00 ± 1.60
Chironimidae	1.67 ± 5.92	7.00 ± 8.38	4.00 ± 1.13	1.00 ± 1.60	4.00 ± 2.36	6.67 ± 3.33
Tipulidae	0.00	0.00	0.67 ± 1.85	1.67 ± 2.62	0.00	0.00
Annelida	0.00	1.00 ± 0.00	0.00	4.67 ± 0.92	1.67 ± 1.85	0.67 ± 2.62
Ostracoda	25.67 ± 32.22	27.00 ± 45.58	16.00 ± 31.68	31.67 ± 44.80	15.67 ± 23.41	29.67 ± 33.11
Decapoda	0.33 ± 0.66	0.00	0.00	0.00	0.00	0.00

Table 5.6: Mean invertebrate abundance sampled from water column of exclosures. Animals classified at least to Class, most to Order (Voshell and Wright 2002).





CHAPTER 6

PREDATION BY HETERANDRIA FORMOSA (LEAST KILLIFISH) ON IMMATURE MOSQUITOES IN MESOCOSM HABITATS WITH VARYING MACROPHYTE COVERAGE INTRODUCTION

Biological control of mosquitoes—the use of predators, parasites, pathogens, competition, or microbial toxins to reduce mosquito population abundance—has been recognized as a potential abatement mechanism since at least the late 19th century (Lamborn 1890, Becker 2010). Before recent development of target-specific toxins produced by certain spore-forming bacteria, the introduction of larvivorous fish for consumption of immature mosquitoes was long seen as the most efficient method. For this reason, the Poeciliids *Gambusia affinis* (Western Mosquitofish, native to the Mississippi River basin) and *Gambusia holbrooki* (Eastern Mosquitofish, native to the eastern and southeastern United States), were introduced throughout the world beginning in the early 20th century (Walton 2007, Chandra et al. 2008). Today, *Gambusia* spp. are collectively the most common freshwater fish worldwide, having been introduced to over 60 countries on every continent except Antarctica for mosquito control (Chandra et al. 2008, Pyke 2008). The two species are commonly considered together because of their similar biology, appearance, and taxonomy, and I will use *Gambusia* in this chapter unless referring specifically to only one of the species (Pyke 2005).

While evidence of mosquito population reduction by *Gambusia* is not hard to find (Krumholz 1944, Hoy and Reed 1970, Bence 1988, Peck and Walton 2008), a large body of literature documents the adverse effects of these introduced fish on native animal species and ecosystem processes. Hoy et al. (1972) found that ponds experimentally stocked with low densities of *Gambusia affinis* actually exhibited higher mosquito production than control ponds, which was attributed to the fish preferentially feeding on invertebrate mosquito predators such as backswimmers (notonectidae) and predaceous beetles (dytiscidae). In general, *Gambusia* are thought to be somewhat indiscriminate invertebrate consumers,

and introduction has led to change in invertebrate communities in many systems (Hurlbert and Mulla 1981, Miura et al. 1984, Schaefer et al. 1994). Some researchers have reported direct effects on fish and amphibian populations through *Gambusia* consumption of eggs or juveniles (Ghate and Padhye 1988, Belk and Lydeard 1994, Schaefer et al. 1994). For an extensive review of many more unintended consequences of *Gambusia* introduction throughout the world, see Pyke (2008).

This study arose from research aimed at improving biological mosquito control in constructed wetlands used for the treatment of municipal wastewater, habitats that are often extremely favorable to immature mosquitoes (Walton 2012). Out of nineteen active constructed treatment wetlands in Georgia, *Gambusia* are present in six. Chapter 5 of this document details reduced predation efficiency by *Gambusia* on immature mosquitoes in dense stands of macrophytes, a result supported by laboratory experiments by Willems et al. (2005). Dense stands of macrophytes are common in most Free Water Surface (FWS) treatment wetlands, as emergent vegetation serves an important structural function: epiphytic microbes that grow on roots, rhizomes, shoots, and leaves perform much of the nutrient transformation necessary for wastewater treatment. In some systems, nutrient uptake by vegetation can also play an important role in water treatment (Kadlec and Wallace 2008).

Mosquito Management Plans (MMPs) can include vegetation design and management strategies to reduce the area or density of these stands of macrophytes, due to their attractiveness to mosquitoes as refuge from predators such as *Gambusia* (Walton 2012). Complex vegetation management schemes, however, are often labor intensive and/or economically expensive. The use of a mosquito predator that is a more efficient control agent in dense vegetation could provide an inexpensive improvement in mosquito control.

To this end, I sought to test immature mosquito predation in dense vegetation by *Heterandria formosa* (Least Killifish), a Poeciliid fish related to *Gambusia* spp. *H. formosa* is native to the southeastern coastal plain from southern North Carolina south to Florida and west to Louisiana. The fish are livebearers that grow to a maximum size of about 3 centimeters. Anecdotal evidence from older literature and communication with breeders suggests the fish prey on mosquitoes, and may be less
aggressive and occupy vegetated habitats more often than *Gambusia* spp. (Seal 1910, Reimer 1970, Nico et al. 2013). I chose this fish because its native range overlaps with many of Georgia's constructed wetlands; this study could serve as a basis or model for future investigations of immature mosquito consumption by native fish species worldwide prior to introduction for mosquito control.

MATERIALS AND METHODS

Mesocosms and experimental design:

I created wetland mesocosms in eighteen 14.5-gallon plastic tubs (59.7cm x 42.9cm x 31.1cm). Each tub was prepared with two inches of packed sand and ten gallons of fresh water. This scale is similar to that used in various previous studies of non-container breeding mosquitoes and other *Diptera*, as well as of *H. formosa* (Schaefer et al. 1994, Eitam et al. 2002, Petranka and Doyle 2010, Chaves and Kitron 2011, Nguyen et al. 2012). Each tub was assigned one of three vegetation treatment levels: A. No vegetation (sand substrate only); B. 50% emergent macrophyte coverage; and C. 100% emergent macrophyte coverage. Giant Cutgrass (*Zizaniopsis miliacea*) was harvested from a constructed wetland, washed thoroughly, and rhizome fragments buried in the sand substrate for appropriate vegetation coverage and density in each tub. I subsequently trimmed the cutgrass to approximately 4-6 inches above the water surface, so that an aluminum mesh cover could be placed over each tub to prevent predation and/or oviposition from mosquitoes and non-target organisms (Figure 6.1). Mesocosms were kept outdoors in rural Oconee County, GA.

Following mesocosm assembly on September 24, 2013, 96 *H. formosa* purchased from an aquarium vendor were introduced to twelve of the tubs (four tubs of each treatment level, eight fish per mesocosm) and allowed to acclimate for 24 hours without food. In all eighteen mesocosms I then introduced 120 live immature mosquitoes across all developmental stages harvested from a constructed wetland in Lavonia, GA. Previous sampling (N = 46 samples from June-September 2013) had shown this wetland to have a relatively high mean immature mosquito abundance (61.8 \pm 99.4 larvae/500mL), and a high proportion (84.9%) of those identifiable to genus (2nd-, 3rd-, and 4th-instar larvae) were *Culex* mosquitoes. This mesocosm experiment took place during the wetland sampling period, so immature

mosquitoes introduced into mesocosms were not identified but assumed be representative of the source community.

Every ninety minutes during daylight hours I recorded the location of *H. formosa* in mesocosms with 50% emergent macrophyte coverage, for a total of eight observations per tub (Table 6.3). These observations were taken after sitting at least ten feet from the tubs for ten minutes, and subsequently counting those fish visible in the open water portion of the mesocosm. After 24-hours I removed all macrophytes from the substrate and dipped each several times in the mesocosm to prevent accidental removal of larval mosquitoes. I then immediately removed all fish from the tubs. I filtered all water from each of the tubs through a 20-micron cloth filter, and washed the contents of the filter onto a white plastic surface. All remaining live mosquitoes were removed and counted, and the proportion of 4th instar larvae and pupae recorded.

Statistical analysis:

The analysis of mosquito survival consisted of two stages, conducted using the R statistical software (R Foundation for Statistical Computing, Vienna, Austria). To evaluate whether fish indeed affected larval survival, I used a two sample Student's t-test to test for significant difference between the mesocosms without fish (N = 6) and those with fish (N = 12). Due to the limited number of mesocosms without fish per vegetation treatment level, I could not statistically resolve differences in mosquito survival between levels when no fish were present, although there were no discernable trends upon graphical inspection (Figure 6.2).

After determining a significant effect of fish predation, I used a one-way analysis of variance (ANOVA) model to evaluate the effect of the vegetation treatment level on *H. formosa* predation of immature mosquitoes in wetland mesocosms. When a difference existed, I used Tukey's Range Test with a 95% family-wise confidence interval to assess differences in *H. formosa* predation of immature mosquitoes in differing levels emergent macrophytes.

RESULTS

In six control mesocosms without fish, an average of $84.7\% \pm 7.7\%$ (101.67 ± 9.27) of live immature mosquitoes introduced to the mesocosms were collected live after 24 hours. There were no visual differences between macrophyte treatment levels (Figure 6.2). This survival rate was significantly higher than in mesocosms with fish, in which an average of $5.1\% \pm 5.6\%$ (6.1 ± 6.7) survived (t = 22.51; P << 0.01).

Average mosquito mortality due to *H. formosa* (either by direct predation or indirect effects of fish presence, such as stress) across all twelve mesocosms with fish was 94.9%. Mosquito survival in mesocosms with 100% emergent vegetation was significantly higher than the other treatment levels (P = 0.01; Table 6.1). On average 10.75 ± 8.8 more immature mosquitoes survived, a $9.0\% \pm 7.3\%$ decrease in mosquito consumption by *H.formosa* in densely vegetated mesocosms. This was the only significant difference found between treatment levels (Table 6.2).

Fish in mesocosms with both emergent vegetation and open water habitats showed a clear preference for the portion with emergent macrophytes. An average of 7.1 ± 1.2 *H.formosa* were observed in the macrophyte habitat (N = 32), an average of 88.3% \pm 15.0% of fish in the mesocosms.

DISCUSSION

For fish that are visual predators, lower predation efficiency is expected in habitats with lower light intensity, greater complexity, and that offer physical refugia for prey (Confer et al. 1978, Manatunge et al. 2000). Dense stands of emergent vegetation, common in wastewater treatment wetlands, meet all of these conditions. In particular, dense macrophytes can reduce the swimming speed and visual field volume of fish, and thus the frequency of prey encounters (Manatunge et al. 2000). While this has been shown to result in a sharp decline in predation by some fish (such as *Micropterus salmoides* and *Gambusia* (Savino and Stein 1982)), predation of immature mosquitoes by *H. formosa* in mesocosms with dense emergent macrophytes was reduced compared to open water habitats, but by only approximately 9%. The limited decline I observed in *H. formosa* is promising for their potential for biological control of mosquitoes in constructed wetland habitats.

Furthermore, I observed the vast majority of fish in the vegetated half of mesocosms containing both open water and emergent macrophytes. This result suggests that *H. formosa* may prefer habitats characterized by dense vegetation rather than open water areas. As these habitats are preferred oviposition and refuge sites for immature mosquitoes, *H. formosa* likely would have a higher mosquito encounter rate than some fish currently used for biological control in treatment wetlands (such as *Gambusia*, which prefer open water).

By replicating the vegetated habitats found in many existing treatment wetlands, I attempted to evaluate the possibility of immature mosquito predation by *H. formosa* as part of a Mosquito Management Plan. *Gambusia* have typically been used in this type of biological control, but chapter 5 of this document details their sharp predation declines in dense vegetation in an existing constructed treatment wetland, and their preference for open water habitats. Results of this study were consistent with the notion that emergent vegetation provides refugia for immature mosquitoes, but *H. formosa*'s small size and preference for vegetated habitats may have contributed to relatively high consumption rates even in mesocosms with dense macrophytes. No direct comparison between *Gambusia* and *H. formosa* is possible from these results, however, because of the difference in environments between studies (mesocosm vs. actual wetland).

Further steps need to be taken to fully evaluate the potential effectiveness of *H. formosa* for biological control. In particular, studies similar to this one with a full community of aquatic invertebrates co-existing with fish are essential. This study only investigated the direct predator-prey relationship in a two-animal system, but many more direct and indirect effects are likely when a more fully developed aquatic community is present. Results from a mesocosm study with an invertebrate community composition corresponding to a specific treatment wetland could inform managers of that wetland about the value of future field trials or experimental fish introduction.

This study can be seen as an initial step in a process for evaluating the usefulness of a particular fish for biological control, and serve as a model for other regions, systems, and pests. I have used a fish native to the southeastern United States, as well as habitats and plants commonly observed in Georgia's

treatment wetlands. Native organisms should be used whenever possible for biological control, as the history of environmental and economic consequences of exotic species introduction of is long and tragic (Pimentel et al. 2005). *Gambusia* introduction worldwide for mosquito control has resulted in a variety of negative effects on non-target organisms, and the fish are considered invasive in many parts of the world. Many fish throughout the world are thought to be antagonists of immature mosquitoes; hopefully this experiment can be a template for evaluating the potential effect of a native fish species on mosquito populations in a constructed wetland. As more treatment wetlands are planned and constructed, biological control is likely to be one facet of an inexpensive MMP to reduce the threats of mosquito vectors to human health.

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Figure 6.1: Experimental mesocosms with three macrophyte treatments and two individual *H.formosa*. Penny for scale.



Figure 6.2: Surviving mosquitoes in all six mesocosms without introduced *H. formosa*. Three treatment levels: A: No emergent macrophytes; B: Half emergent macrophytes, half open water; C: Entirely emergent macrophyte habitat.



Treatment Level

Table 6.1: ANOVA table for mosquito mortality in mesocosms with *H. formosa*. Treatment levels are differing proportion of emergent macrophytes.

	df	Sum Sq	Mean Sq	F value	Pr(>F)
Treatment	2.00	310.17	155.08	7.72	0.01
Level					
Residuals	9.00	180.75	20.08		

	Difference	95% conf. lwr.	95% conf. upr.	Adjusted p- val
B-A	-1.00	-9.85	7.85	0.95
C-A	10.25	1.40	19.10	0.03
C-B	11.25	2.40	20.10	0.02

Table 6.2: Results of Tukey's Range Test with 95% family-wise confidence interval. A: No emergent macrophytes; B: Half emergent macrophytes, half open water; C: 100% emergent macrophyte habitat.

Figure 6.3: Mean survival of immature mosquitoes in mesocosms with *H. formosa* and different proportions of emergent macrophytes. A: No emergent macrophytes; B: Half emergent macrophytes, half open water; C: 100% emergent macrophyte habitat.



Table 6.3: *H. formosa* location during daylight hours of experimental period in mesocosms with both open water and emergent macrophyte habitats.

Habitat	Open Water	Emergent Macrophytes
Average H. formosa count	0.94	7.06
Standard deviation		1.20
Proportion of H. formosa	11.72	88.28
Standard deviation		14.97

CHAPTER 7

SYNTHESIS AND CONCLUSIONS

Worldwide, sewage generated by 1.5 billion people, almost one quarter of the global population, is released untreated directly into natural rivers, lakes, and coastal oceans (Baum et al. 2013). This is only a portion of the two million tons of untreated municipal, agricultural, and industrial waste per day that is directed into natural basins that people depend on for food and drinking water, hygiene, and irrigation. Wastewater pollution leads to significant effects on ecosystem function, aquatic biodiversity, nutrient transport, microbial evolution, and pathogen transmission (Baker-Austin et al. 2006, Corcoran 2010). Additionally, approximately 760,000 children die each year from diarrheal disease as a result of contanct with contaminated water (WHO 2013). Constructed wetlands are an attractive wastewater treatment option for some communities and institutions because of relatively low operation advantages over other treatment methods. Effective control of disease-carrying mosquitoes that use these systems as habitat is paramount, so as to not created unintended threats to human and wildlife health.

The results of this research suggest that mosquito management considerations should be incorporated into the design and planning of constructed wastewater treatment wetlands in order to facilitate the most cost-effective, comprehensive mosquito control. Ecological processes driving mosquito spatial distribution and abundance, as well as wastewater characteristics and water treatment goals, need to be considered to determine the best mosquito control strategies. Cross-sectional sampling of constructed wetlands in Georgia supported previous research indicating that highest mosquito production occurs in areas characterized by emergent macrophytes, rather than open water. In the six constructed wetlands in which larvivorous fish were present (primarily *Gambusia* spp.), statistical analysis of mosquito abundance suggested that fish are likely effective at reducing immature mosquito numbers, and at reducing *Culex* spp. ubiquity in these habitats.

Three biological mechanisms are likely governing these results: 1. Oviposition by adult female mosquitoes in areas that provide greater chances for offspring survival; 2. Predator avoidance and refuge-seeking behavior by immature mosquitoes in the wetlands; and 3. Mortality caused by direct and indirect effects of predators of immature mosquitoes. It is difficult to separate the relative importance of these processes for the purpose of better mosquito management, but some insight can be gleaned from this research.

Based on results of a container experiment (chapter 3), as well as previous supporting research (Bond et al. 2005), it is likely that oviposition by mosquitoes within treatment wetlands is heavily dependent on vegetation cues. I found that containers with only senesced wetland vegetation exhibited a significant increase in mosquito colonization and production relative to containers without vegetation. This only applied to containers located at treatment wetlands, supporting the notion that many mosquitoes ovipositing in treatment wetlands (such as *Culex* and *Anopheles* spp.) possess life history traits that include predator avoidance and refuge seeking (Blaustein and Kotler 1993, Eitam and Blaustein 2004, Blaustein et al. 2005). Vegetation was also the most substantial predictor of mosquito abundance within treatment wetlands. While this is likely a combination of the biological mechanisms listed above, as high stem density can provide predator refuge and plant decomposition may increase larval mosquito food resources, it is clear that vegetation management is key for mosquito control.

Both oviposition and larval survival are also likely to be influenced by larvivorous fish presence. Chapter 5 details the differential direct predation effects of *Gambusia* in differing vegetation densities within a wetland, and results of cross-sectional sampling indicated that fish have a significant negative effect on mosquito abundance (Chapter 2). These results have important implications for biological mosquito control, as vegetation management and biological control may work synergistically to reduce mosquito production from constructed wetlands.

MANAGEMENT IMPLICATIONS

There are specific tradeoffs that must be considered when managing vegetation in a Free Water Surface constructed wetland. Emergent macrophytes provide structure for epiphytic microbes that

perform much of the nutrient transformation required for adequate wastewater treatment. Oxic nitrification, and anoxic nitrification and denitrification are essential processes facilitated by the presence of emergent vegetation; in some cases nutrient uptake by the plants themselves can be significant (Kadlec and Wallace 2008). Alternatively, this research supports evidence that larger and denser macrophyte stands foster increased mosquito production; these stands may also limit the amount of oxic nitrification possible in the treatment process (transformation of NH_4^+ -N to NO_3^- -N) (Thullen et al. 2002, Thullen et al. 2005).

Several options for balancing treatment needs with creation of immature mosquito refuge have been suggested in the literature: shallow vegetated hummocks surrounded by deeper water zones (Thullen et al. 2002), or narrow bands of emergent vegetation bounded by open water (Walton et al. 2012) both increase the area of interface between vegetation and open water. These strategies support high levels of predator-prey interaction between immature mosquitoes and both vertebrate and invertebrate predators, while also reducing mosquito refugia and food resources and providing better mosquito predator habitat.

Some techniques to reduce mosquito production take place in wetland management, rather than design. Volunteer Duckweed (Lemnoideae) can form thick carpets on the water surface that prevent mosquito emergence, so may be beneficial for mosquito control if other issues are not of management concern, such as infrastructure fouling and reduced dissolved oxygen (see chapter 4). Seasonal harvest of emergent vegetation likely limits the positive effect of macrophytes on mosquito populations, and prevents nutrients taken up during the growing season from re-entering the system through the decomposition of senescent plants. In practice, periodic vegetation harvest in constructed wetlands is often not cost-effective, and is sometimes approximated by annual or seasonal burning of macrophyte stems. Additional consideration during wetland design could make vegetation harvest more economically feasible, as complete cell dry-down and specific machinery or low-cost harvest techniques may be necessary. A bio-fuel end product for harvested vegetation, such as cellulosic ethanol or biomass pellets, could offset some of the harvest costs.

Vegetation design and management tactics for mosquito control can work in concert with biological control strategies in an integrated Mosquito Management Plan. This research has shown that *Gambusia* exhibit reduced predation effectiveness on immature mosquitoes in dense vegetation, so planting schemes that increase edge:area ratio of emergent vegetation, or management to limit vegetation density, would create better environments for direct predation by those particular species. Additionally, some mosquito species important to human and wildlife health may respond to predator presence more strongly: Chapter 2 suggested *Culex* spp. oviposition may be reduced where fish are present. Mosquito management goals need to be considered alongside mosquito ecology in comprehensive mosquito management plans in order to most efficiently reduce threats to human and wildlife health.

In considering biological control by larvivorous fish in wetlands outside the United States, care should be taken to use species only in their native range. Some species may be more effective predators in dense vegetation than *Gambusia*, and research such as the predation experiment detailed in chapter 6 is recommended to investigate the mosquito control potential of native fish. Finally, there may be synergistic effects of biological control and Duckweed surface coverage, but little empirical research supports or debunks this possibility.

FUTURE RESEARCH DIRECTIONS

The potential for constructed wetlands as a low-cost wastewater treatment alternative in areas of the world without adequate wastewater treatment has been acknowledged for decades. Some major barriers to implementation have been local awareness and technical knowledge of potential treatment system operators, as well as restricted foreign aid projects that tend to favor 'concrete-and-steel' technology (Kivaisi 2001). Application of mosquito control research to geographical regions in greater need of basic wastewater treatment will serve to aid appropriate design and management strategies for future constructed wetlands, especially in tropical regions, where some of the greatest potential exists for the application of treatment wetland technology.

Humans and wildlife in various regions of the globe also are at risk of contracting differing mosquito-vectored pathogens. Pathogens of most concern in some areas currently pose little threat in the

United States (Malaria, for instance), and are vectored by mosquitoes with specific physiologies and life histories. The effectiveness of various mosquito control strategies studied here may be different with different mosquito communities, or less effective at reducing a specific focal mosquito type. Local application of mosquito control research in constructed wetlands to mosquitoes of medical and ecological importance is key. Toxins produced by the spore-forming bacteria *Bacillus thuringiensis israelensis* (*B.t.i.*) and *B. sphaericus* (*B.s.*) are some of the most promising larval control mechanisms due to their target-specificity and relatively neutral ecosystem impacts, and more research should be done to evaluate their role in constructed wetland mosquito management. Additionally, predators of immature mosquitoes worldwide need to continue to be identified. Research to investigate the potential of native fish and invertebrates for biological mosquito control in constructed wetlands around the world will be important for limiting future risk of mosquito-borne illness due to the creation of mosquito habitat.

Finally, further research is necessary to elucidate the biological mechanisms driving mosquito spatial ecology and abundance in constructed wetlands. If predator signals can be an effective cue to limit oviposition by adults, perhaps chemical cues can be added to treatment systems to mimic these effects without costs to managers for information, stocking, and possible unintended consequences of biological control. Conversely, if the effect of direct predation is stronger than existing oviposition cues, vegetation management and planting design will be the most promising strategy for limiting mosquito populations. A synthesis of basic ecological knowledge of immature mosquitoes in natural and constructed wetland environments throughout the world would provide an excellent foundation for the future control of mosquitoes in treatment wetlands, and illuminate knowledge gaps that deserve research attention.

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