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Seasonal Influence of Nutrient Dynamics and Conspecific Interference on Culex quinquefasciatus Oviposition and Environmental Parameters of Combined Sewage Overflow (CSO) Habitats

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An abstract of
a thesis submitted to the Faculty of Emory College of Arts and Sciences
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Abstract

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A study of factors that influence mosquito oviposition and aquatic ecology in Tanyard Creek, an urban stream in Atlanta, GA that receives combined storm and waste water discharge, was conducted in 2008 and 2009. The effects of combined sewage overflows (CSOs) on Culex quinquefasciatus activity were determined by directly manipulating nutrient density in CSO habitats. Nutrient enrichment of the aquatic habitats was positively correlated with oviposition. Secondly, availability of oviposition habitats to conspecific (members of the same species) use was controlled to study the effects of conspecific presence on Culex oviposition. Conspecific presence was an important deterrent to oviposition in June. There may not have been enough oviposition activity in the fall and spring to accurately quantify conspecific effects. Next, nutrient dynamics in isolated pools (container habitats) and free-flowing body (CSO stream) were compared. Ammonia and nitrate, known to be oviposition attractants, accumulated in higher quantities in isolated pools relative to the stream. Flushing of the stream following precipitation events expectedly washed out nutrient densities. Lastly, weather was assessed throughout the study to quantify the effects of temperature and precipitation on oviposition. Temperature and time of year were clear and consistent predictors of egg raft abundance for all treatments in this experiment. It is unclear whether rainfall was important by itself, but in combination with other factors, the interaction with precipitation was significant. Culex quinquefasciatus is the main vector for West Nile Virus in the southeastern United States, and thus understanding mosquito dynamics can lead to better understanding of urban WNV transmission.

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Introduction

West Nile Virus (WNV) is the most widely distributed arbovirus in the world, present on all continents except Antarctica (Kramer, Styer, and Ebel, 2008). First reported in the West Nile region of Africa, WNV has since spread all over the United States, causing epidemics in several urban centers (Turell et al., 2001). In the southeastern US, the most common vector for West Nile Virus is *Culex quinquefasciatus* (Godsey et al., 2005). Members of this species breed primarily in drainage and sewage systems of municipal centers where, coincidentally, outbreaks often occur (Vazquez-Prokopec et al., 2010; Calhoun et al., 2007; Hayes, 1973). Atlanta, Georgia employs a combined sewage and storm water system to treat and dispose wastewater. A combined sewer is a single pipe sewer system in which stormwater runoff and domestic sewage are mixed and treated together. Following heavy rainfall events, large volumes of untreated wastewater, rich in organics and eutrophics, exceed the maximum capacity of the combined sewer system (CSS) and discharge into urban streams. The CSS is a relic regime that continues to operate in many older cities. In Atlanta, CSO facilities are located in residential areas with high human population densities.

A study conducted by Calhoun et al. (2007) demonstrated that combined sewage overflows (CSOs) from Atlanta facilities increase *Culex quinquefasciatus* abundance by providing high-nutrient mosquito breeding grounds. *Culex quinquefasciatus* also oviposited more egg rafts in water with elevated nutrient levels (Bentley and Day, 1989). Oviposition was enhanced in habitats containing nutrient-rich water specifically from combined sewage overflows (Chaves et al., 2009).

Glossary

Arbovirus: virus transmitted by arthropod vectors, including mosquitoes
Conspecific: of or belonging to the same species
Gravid: the mated or pregnant condition
Oviposition: the process of laying eggs by a gravid female mosquito
Site Selection: the preference of certain ovipositional habitats over others
Vector: an agent (person, animal or microorganismm) that carries and transmits an infectious agent

Vazquez-Prokopec et al. (2010) showed that WNV infections in both *Culex quinquefasciatus* and humans were significantly higher near CSO-affected streams. Residential streams receiving combined storm and waste water effluent present a plethora of opportunities to study the activities of disease-carrying mosquito species operating in such close proximity to humans.

Three of the four stages (egg, larvae, and pupae) in the life cycle of a mosquito are aquatic (Fig. 1). Thus, the aquatic stages are important in terms of mosquito dynamics. We investigated factors influencing oviposition and site selection in aquatic habitats. Oviposition is of particular interest to WNV-related studies because it is known to demonstrate a strong correlation with abundance, distribution, biting rate, and pathogen transmission ability (Spencer et al., 2002; Jones, 1999; Reiskind and Wilson, 2004).

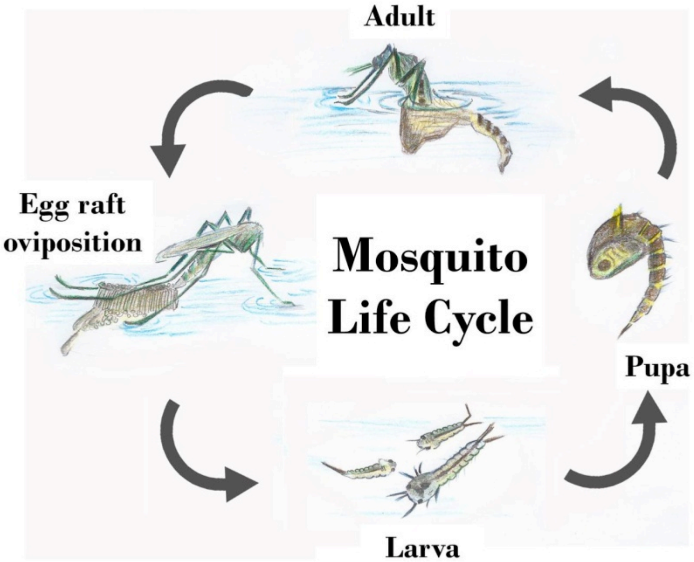

FIGURE 1. Life cycle of female mosquito. Image created by author.

The act of oviposition can be divided into 3 phases: ovipositional flight, site selection, and egg deposition. First, the initiation of flight is dependent upon a variety of environmental factors including precipitation, relative humidity, and air temperature (Bentley and Day, 1989). Next, the location and selection of a habitat involve several visual, olfactory, and tactile responses. Insects with aquatic larvae, such as *Culex quinquefasciatus*, influence the outcome of their offspring with this important decision because juveniles may be unable to move or relocate to more suitable habitats if the choice was an unfavorable one. Studies have shown that gravid female mosquitoes are quite discriminating in selecting sites for egg deposition and may demonstrate bias depending on a habitat's suitability to support the species (Bentley and Day, 1989; Mangel, 1987; Beehler et al., 1994; Spencer et al., 2002; Reiskind and Wilson, 2004). The discriminatory process may be related to a variety of factors including, but not limited to, nutrient availability, chemical cues, conspecific presence, and climatic variability (discussed below).

We examined the impact of nutrient dynamics, conspecific interference, climatic factors, and their corresponding interactions on oviposition behavior of *Culex quinquefasciatus* in urban aquatic habitats. Our primary aims were: (i) to investigate whether gravid mosquitoes select oviposition sites based on nutrient richness; (ii) to evaluate chemical measures of water quality in isolated pools versus free-flowing stream; (iii) to investigate if oviposition is hindered, enhanced, or unaffected by the presence of conspecifics; and lastly (iv) to evaluate the effects of time of year, temperature, and precipitation on oviposition. An additional point of interest was to determine how these biotic and abiotic factors interacted as predictors of *Culex* oviposition.

Nutrient and chemical dynamics

Environmental mechanisms involved in oviposition are still poorly understood. We investigated the effects of organic enrichment on abundance of egg rafts. Higher levels of nutrients were expected to enhance mosquito oviposition by way of habitat selection. Walker et al. (1991) suggested that nutrient-rich oviposition habitats stimulate microbial metabolism and consequently mosquito productivity. In terms of this study, the most pertinent nutrients that affect *Culex* growth and survivorship are phosphate, ammonia, and nitrate (Carpenter, 1982; Sunish and Reuben, 2001; Walker et al., 1991).

Ammonia is the most common form of environmental nitrogen in cultivated land and can often be found downstream from water treatment plants where sewage is discharged

(Leisnham et al., 2005). Ammonia is frequently detected in areas such as farms, parks, and lawns following heavy application of nitrogenous fertilizers. Ikemoto and Sakaki (1979) found a positive correlation between ammonia concentration in stagnant water and mosquito abundance. Furthermore, Sunish et al. (1998) showed ammonia to be an ovipositional attractant. The adaptive capability of gravid females to discriminate reservoirs high in ammonia may be attributed to developmental advantages associated with nitrate nitrogens.

Bacteria, in time, decompose ammonia into nitrate in a process known as nitrification. In the case of synthetic fertilizer application, runoff seeping into nearby water bodies increases firstly the concentration of ammonia and subsequently that of nitrate. Ammonia attracts adult mosquitoes, while nitrate nitrogens help stimulate the growth of larvae (Carpenter, 1982; Sunish and Reuben, 2001; Kaufman and Walker, 2006). Sunish and Reuben (2001) found nitrate concentrations to one of the best predictors of immature abundance consistently within and between seasons. Nitrates amplify the density of microorganisms, subsidizing the main diet of mosquito larvae; thus, nitrates exert a positive influence on culicine populations (Sunish and Reuben, 2001). Furthermore, nitrates have also been found to increase the total adult mass of mosquitoes (Victor and Reuben, 2000; Kaufman and Walker, 2006).

Phosphates similarly have a positive influence on juvenile mosquito development, mostly in the latter aquatic stages, specifically pupation (Sunish and Reuben, 2001; Carpenter, 1982). Like nitrate and ammonia, phosphates and other phosphorous containing compounds common in fertilizers can percolate into waterways following precipitation events. CSOs can also transfer high levels of phosphate from fecal matter and industrial wastes into polluted urban streams.

In Georgia, sporadic release of nutrient-rich wastes into metropolitan waterways is characteristic of Atlanta’s antiquated water treatment systems. Each overflow inundates the normally stagnant CSO stream with large pulses of contaminated water over a short period of time. Such pulses flush out the old aquatic body and new nutrients are brought in. These new nutrient levels play a role in the regrowth of biotic and abiotic factors in the stream, which may serve as fresh habitats for larvae. In this way, discharge continually renews the system. Thus, nutrient composition over time in isolated (or stagnant) pools was expected to differ from that of a free-flowing water body.

Conspecific presence

The next factor considered to influence site selection was conspecific presence. A study by Kiflawi et al. (2003) suggested that gravid mosquitoes avoid negative interactions with other species or conspecifics when choosing among reservoirs for oviposition. When given the choice between high or low densities of competitors and predators, the majority of females oviposited in pools with a lower density of both (Blaustein and Kotler, 1993; Kiflawi et al., 2003). In this study, overused habitats were predicted to deter further conspecific oviposition. The hypothesis that mosquitoes oviposit selectively to avoid potential competitors received support from research that indicated changes in oviposition behavior are related to chemical cues and pheromones emitted by members of the same species (Bentley and Day, 1989; Takken, 1999; Millar et al., 1994).

Climate

Finally, abiotic factors directing oviposition activity were also considered. The abundance and proliferation of *Culex* populations is seasonal (Speilman, 2001). In particular, time of year, temperature, and precipitation are known to affect the fecundity, size, and ovipositional behavior of mosquitoes (Bock and Milby, 1981; Rajagopalan et al., 1976; Nayar and Sauerman, 1970). Alto and Juliano (2001) conducted several experiments examining the effects of temperature and precipitation on mosquito production. They found that high temperatures and humidity generally result in greater production of mosquitoes, but within a certain optimum range. For example, egg mortality was higher with extreme hot and cold temperatures. Alto and Juliano concluded that mosquito populations occurring in regions with relatively high summer temperatures are likely to have high rates of population growth with populations peaking early in the season whereas populations occurring in regions with low summer temperatures are likely to experience slow, steady product of adults throughout the season. Likewise, mosquito abundance is often positively related to precipitation (Ho et al., 1971; Lounibos, 1981; Sulaiman and Jeffery, 1986). It is important not to consider temperature and precipitation separately as they often work together to influence mosquito dynamics. For example, optimum temperatures are actually detrimental to adult production when there is too little moisture (Alto and Juliano, 2001).

The southeastern part of the United States is highly seasonal, experiencing a mixture of snowfall, torrential downpours, humid weather, and drought. In Atlanta, Georgia, mosquito breeding occurs in fluctuating cycles. The temperature gap between the warm and cold seasons in this region is considerable and has been linked to differences in *Culex* development and disease transmission, but a clear understanding of these processes has yet to be established. Because both temperature and precipitation vary from one region to the next, their interactions make generalizing seasonal effects on mosquito activity difficult. Studies on annual fluctuations are needed to determine how abiotic factors influence the species active in the region of interest.

Methods

Study Site

Map and satellite images of the location for this experiment are shown in Figure 2. The aquatic body represented is Tanyard Creek, a CSO-polluted stream flowing through various Northwest Atlanta neighborhoods (Calhoun et al., 2007). The study site, situated on an embankment parallel to the stream, is bordered by the Bobby Jones Golf Course to the north, Ardmore Park to the south, and several residential homes to the west. A treatment compound 1260 meters downstream of the site is the smallest CSO wastewater facility in Atlanta, yet handles the largest amount of water. An overflow from this facility was triggered by >0.1 in of precipitation.

Oviposition Habitats

We chose to conduct a semi-natural experiment where conditions could be taken from the natural environment but where manipulation of such conditions was also possible, similar to the laboratory setting. The semi-natural experiment consisted of 24 dark blue 18.93 L (5 gallon) Rubbermaid Roughneck containers positioned in a four-row by six-column arrangement (Fig. 3). Each container was filled with 8 L of water collected from Tanyard Creek within 48 h following an overflow event. Although overflow duration and amount of nutrient release is always variable, CSO water is still highly eutrophic under these conditions (Bernhardt et al. 2008). Twelve of these containers received an additional infusion of 12 g of crushed Purina One Brand dog biscuits (21% protein content). This served as an ovipositional attractant (Chaves et al., 2009). The purpose was to quantify site selection in gravid female mosquitoes choosing between enhanced and unenhanced habitats to oviposit egg rafts. Eighteen of the containers were covered during the first week of the experiment with clear plastic tarp to prevent insects, debris, detritus, leaf litter, and rainfall from adulterating the contents (Fig. 4). The six containers that remained exposed consisted of three with additional nutrient content and three without (Fig. 3).

Procedures

Egg rafts oviposited in each exposed container were counted and disposed within one day following the initial experimental setup to minimize external influences on oviposition (e.g. chemical cues and presence of conspecifics). One day was chosen based on the incubation period for *Culex* egg hatching (de Meillon et al. 1967).

Each subsequent week for four weeks, the tarp was removed from six successive containers, 3 including and 3 excluding protein enrichment (Fig. 5). Egg raft removal was performed twice weekly on all open containers. The purpose of gradually uncovering the containers was to quantify the effects of conspecific presence, which weighed more heavily in containers with longer exposure time than those uncovered later on in the experiment. Thus, exposure time, a key variable inherent in the third aim of this study, is defined as the maximum length of time in weeks for which a group of containers was exposed. For example, Group A containers were uncovered from the start of the experiment and represent the treatment with the greatest exposure time. Conversely, Group D containers were uncovered the fourth and last week of the experiment, thus representing the treatment with the lowest exposure time. Group B and C represent corresponding intermediate exposure times (Fig. 3). The distinction between exposure time and chronological time (Week 1-4, or Visit 1-8, of the experimental period) must be emphasized. To reiterate, at the start of the fourth week, all tarps were removed and all containers were sampled that week for egg rafts.

To measure nutrient levels, a 15 mL surface water sample was collected from each uncovered container and three 15 mL surface water samples were collected from various points along the stream, placed on ice, and filtered to remove solid materials during each semi-weekly visit. Continuous flow calorimetric assays measuring ammonium, nitrate, and phosphate concentrations were completed for all water samples by a separate lab at the University of Georgia (see Appendix for complete description).

These procedures were conducted starting on October 6, 2008 (Fall) and repeated on April 7, 2009 (Spring) and June 1, 2009 (Summer) using the same experimental setup. A winter trial was not included because there is too drastic a decline in *Culex quinquefasciatus* activity for adequate oviposition comparison with respect to nutrient addition and conspecific presence.

Rainfall and air temperature data for all visits were collected from the Weather Underground Weather Station KGAATLAN32 and used to assess the influence of time of year on ovipositional activity.

Statistical Analysis

To quantify the effects of time of year (Season: Spring, Summer and Fall), medium age (Week: 1, 2, 3, 4), and nutrient addition (Treatment: Y and N) on the concentration of chemical species, an analysis of variance (ANOVA) was performed for the concentration of nitrate [NO_3^-], ammonium [NH_4^+], and phosphate [PO_3^-] as a function of time of year, week, and treatment, which can be expressed as [C] = f(S, W, T, S:W, S:T, W:T, S:W:T, E).

To account for the lack of independence, or pseudoreplication, associated with taking multiple measurements and samples from the same containers, linear mixed effects (LME) models were employed (Chaves, 2010). These models analyzed number of egg rafts oviposited as a function of nutrient addition, nutrient concentrations, temperature, season, precipitation, and exposure time as fixed factors. Random factors included the nested variability due to the time of year, the week and visit. Linear mixed effects models (LMEMs) were fitted with restricted maximum likelihood (REML), which served as a way of estimating the parameters of the statistical model.

The Akaike Information Criterion (AIC) was used to select the best model for analysis. The model with the lowest AIC value comprised of a set of observed variables which most parsimoniously and accurately predict the response variable (Anderson, Burnham, and Thompson, 2000), number of oviposited egg rafts in this case. Subsequently, a bootstrap analysis was performed to calculate the significance of the variables in the model determined to be of highest importance by the AIC. Bootstrapping is a way to assign measures of accuracy to sample estimates by creating a large number of datasets to find confidence intervals for the parameters of the best model.

All of the aforementioned analyses were performed using the R statistical package.

Results

Nutrient Richness

Treatments with protein supplement (Y) received higher oviposition than treatments without supplement (N). Average number of egg rafts per container (±SE) for Season:Treatment was 0.58 ± 0.94 for Fall:N; 12.1 ± 15.78 for Fall:Y, 2.72 ± 1.09 for Spring:N; 14.38 ± 6.78 for Spring:Y; 8.7 ± 3.04 for Summer:N; and 41.73 ±16.85 for Summer:Y (Fig. 9). Oviposition was highest in the June and lowest in October for both treatments. There was also considerable spread in the distribution of egg rafts for the fall treatments (Fig. 9). Overall, significantly more egg rafts were oviposited in nutrient-infused habitats than in habitats containing only CSO water from the stream (Tbl. 2, Fig. 6), indicating mosquitoes preferred enriched oviposition sites. The model selection and bootstrap analysis consistently demonstrated nutrient enhancement to one of the most important predictors of oviposition (Tbl. 1 and 2, $P<0.00001$). According to the bootstrap, enhanced containers were estimated to receive 18.739 more egg rafts relative to unenhanced containers (Tbl. 2, Treatment Y vs. Intercept).

Water Quality

Containers with added nutrients consistently had higher mean values for ammonia and nitrate concentrations across all months than either the stream or the containers with no added nutrients (Fig. 7). Ammonia nitrogens were the dominant form of inorganic nitrogen. Conversely, phosphate concentrations exhibited the opposite trend and were lowest in nutrient-infused containers. Phosphate levels overall were lower than either ammonia or nitrate levels (Fig. 7).

All variables analyzed in the ANOVA (S, W, T, S:W, S:T, W:T, S:W:T) were significant for ammonia nitrate concentrations (Tbl. 3). Only time of year, treatment, and their interaction were significant for the concentrations of nitrate nitrogen and phosphate.

Presence of Conspecifics

Figure 8 show oviposition in groups of containers categorized by exposure time, where Groups A-D represent containers with decreasing exposure time (Fig. 3). These graphs illustrate the extent to which gravid mosquitoes utilized the container habitats for oviposition. No trend was observed for April or October. In June, when mosquitoes deposited more egg rafts overall (Fig. 9), a stepwise trend showed an increase in mean number of egg rafts (A: 21.54±10.03; B: 25.25±9.54; C: 29.08±8.10; D: 32.08±12.66) from most to least exposed groups.

Exposure time, when interacting with time of year, was part of an important model used to predict oviposition (Tbl. 1). The model selection showed synergistic effects in the interaction of time of year and exposure time (Tbl. 1). This was demonstrated in early summer, when oviposition was high. During this time, conspecific deterrence may have played a factor in lowering oviposition in Group A containers compared to Group D (Tbl. 2).

Time of Year

Oviposition in either treatment was highest in early summer (Fig. 9). Higher temperature was also associated with greater numbers of *Culex* egg rafts, and lower temperature was associated with decreases in number of egg rafts (Fig. 10). Oviposition in either treatment was lowest in the fall (Fig. 9). Along with nutrient addition, time of year was the most important predictor of oviposition in our study (Tbl. 1 and 2).

Precipitation negatively influenced oviposition (Tbl. 2), specifically in the fall (Fig. 10). When there was no precipitation, habitats received more egg rafts. When there was >0.01 in rainfall, mosquitoes oviposited less often in containers. Rain was a predictor of oviposition in association with time of year and temperature (Tbl. 1). Abundance of egg rafts was sensitive to rain when other variables became important and did not serve as a predictive variable alone. For example, when the temperature was low ($Temp_{max} < 32.5$), rain became a factor; e.g, rain acted with decreasing temperatures in the fall to drive oviposition downwards (Fig. 10).

Discussion

The positive influence of nutrient on ovipositional density is congruent with findings from previous studies, substantiating the idea that female mosquitoes oviposit selectively. In order to optimize the fitness of their offspring, gravid females discriminated between multiple habitats placed adjacent to one another. Because all containers contained water from a neighboring CSO stream, nutrient infusion was already inherent to all habitats present. Previous research demonstrated that mosquitoes also preferentially oviposited in CSO habitats as compared to tap water as a control (Chaves et al., 2009). This experiment confirmed the same phenomenon with varying levels of moderate and high nutrient concentration.

Studies involving semi-natural conditions have real world application. Modern day sewage treatment facilities, catch basins, and storm drains are aquatic hotspots for vector activity worldwide (Lauret, 1953; Hayes, 1973; Mian and Mulla, 1986; Mogi and Okazawa, 1990). High nutrient levels characteristic to these areas have been found to accelerate juvenile mosquito survival and development (Reiskind et al., 2004). In this study, nutrient-infused CSO significantly enhanced oviposition across all months. These findings provide further evidence that urban areas of aquatic pollution replete with human and industrial waste are attractive to a viable species of WNV. Here in Georgia, 66 mosquito pools were previously identified as WNV-positive, 95% of which contained *Culex quinquefasciatus* (Rosemarie Kelly, Georgia State Health Department). Vazquez-Prokopec et al. (2010) found WNV infection in mosquitoes, corvids, and humans to be spatially clustered around CSO creeks and facilities. This study suggested that CSO-affected areas are significant sources of *Culex quinquefasciatus* that may facilitate WNV transmission to humans in urban centers continuing to use CSO systems in waste management. Corrective action to mitigate nutrient-richness in urban waterways may be a remedy for mosquito problems not only in Atlanta but the other 772 US cities currently employing the CSO system (Billah, 2008).

Nutrient addition also yielded higher levels of ammonia and nitrate, related through the process of nitrification, in isolated pools than in the stream. The CSO stream, though periodically flooded with nutrient-rich waters, was subject to turbulence and did not remain stagnant throughout the study period. Thus, not only were concentrations of these nutrients elevated in containers with protein infusion, but the isolation of such pools allowed the compounds to accumulate over time.

The reverse trend for phosphate can also be explained by main stream flushing. When the CSO creek is inundated with storm and sewage water, new levels of nutrients replenish the biota. Phosphates, commonly found in industrial waste and organic fertilizers, were expectedly high as a result. Though important for latter stages of juvenile development, phosphates were not found to be strong ovipositional attractants. On the other hand, nutrient-infused habitats simultaneously yielded higher levels of oviposition along with ammonia and nitrate concentrations, while that of non-infused habitats were significantly less. These findings paralleled that of Sunish and Reuben (2001), who similarly determined ammonia and nitrate nitrogens to be clear and consistent predictors of mosquito abundance.

Disturbance by physical processes such as stream flow and flushing could have a major influence on the interactions of nutrient dynamics, bacterial abundance, and mosquito productivity in CSO systems. Walker et al. (1991) found that stemflow, or the flow of water down the trunk or stem of a plant, reduced bacterial abundance but increased mosquito productivity. Stemflow was also responsible for the dilution of nutrients (nitrate nitrogens, ammonium, and phosphate) and regulation of nutrient cycling processes including nitrification.

Similarly, it would be advantageous to explore the dynamics of larger aquatic ecosystems to further examine the effects of these biotic processes on mosquito populations.

Minimizing nutrient concentrations in residential habitats may be beneficial for community health for several reasons. Firstly, ammonia is a toxic waste product and one of the most damaging water pollutant in manure. Secondly, the twin roles of ammonia acting as an oviposition attractant and larval growth enhancer following conversion to nitrate are doubly advantageous for breeding. Residential neighborhoods with kempt lawns like those found in the study site are common fixtures of the metropolitan setting. Maintenance requires regular application of fertilizer and manure. These inputs are followed by large spikes in aquatic ammonia concentration when such materials accumulate in stagnant and eutrophic portions of urban waterways, which then become breeding grounds for mosquitoes. Thus, the rich organic contents of the water provide an ample food supply for mosquitoes while toxic nutrient levels and diminished oxygen concentration limit the ability of other organisms to thrive. Fish, amphibians, and invertebrate predators of mosquitoes were rarely seen in the stream near the study site. The rest of the terrestrial surroundings, however, were kept in manicured conditions. These semi-natural city corridors serve as frequent meeting grounds for people, domesticated animals, and mosquitoes, helping to illustrate the importance of vectors as a bridge between human and pathogen.

Nutrient enrichment by itself is not altogether detrimental to pest control. The interplay of several environmental factors added to the robustness of vector activity. Among the parameters studied, both time of year and nutrient richness influenced oviposition in a clear and consistent manner. Other important factors, however, were nested within these parameters. For example, temperature and rain are related to time of year and would be expected to vary depending on the study period. Thus, the combination of such effects is also important to consider.

In this study, oviposition increased in the early summer as temperatures increased and decreased during the fall as temperatures fell. The intermediacy of temperature and egg raft count for the spring also mirrored this trend. In hindsight, it would have also been helpful to record water temperatures during each visit. While air temperatures influence flight, breeding, and oviposition activity, water temperatures can be important for mosquito development and oviposition strategy, i.e., hovering versus depositing eggs directly onto the water surface. *Culex* mosquitoes, in particular, use contact stimuli during attachment of eggs at or below the water line to evaluate water chemistry, temperature, and texture (Bentley and Day, 1989). In addition, due to the time constraints of this project, long term data collection was not possible. It would be most beneficial, however, to have weather and oviposition data for several years in order to compile a conclusive longitudinal seasonality study.

Precipitation, though necessary to some degree for optimal vector activity, was found to thwart mosquito oviposition. In total, there were 7, 12, and 11 rainfall events in the months of October, April, and June, respectively. Rain was not the most significant factor in determining oviposition activity as June, despite being a relatively wet season, was most productive in terms of oviposition (Fig. 9).

The negative influence of rainfall on oviposition appeared to conflict with previous findings that mosquito abundance is positively related to precipitation (Ho et al. 1971, Lounibos 1981, Sulaiman and Jeffery 1986). Heavy precipitation, however, influences insect activity in a host of complex ways. For example, Calhoun et al. (2007) determined in a longitudinal study of mosquito ecology in Tanyard Creek that water effluent from the CSO system helped to regulate mosquito density following large precipitation events. Floods were associated with large immediate reduction in mosquito numbers. However, since mosquitoes (including eggs, larvae, pupae, and adult forms) were not completely eliminated by the discharge, total numbers quickly rebounded only days after flooding, often to significantly higher levels. Therefore, it is possible for precipitation to be both positively correlated with mosquito abundance and negatively correlated with oviposition activity as rain deters mosquito flight. In perspective, sporadic and unpredictable flooding patterns associated with irregular rainfall could increase the risk of urban WNV transmission by allowing rapid buildup of vectors that breed in CSO streams.

Interestingly, the Group variable, which represented exposure time and conspecific presence, was not a significant factor by itself, but was shown to have compounding effects in combination with time of year. The synergy of these variables was illustrated in June, when oviposition was the highest. A decrease in exposure time increased the proportion of oviposition in stepwise increments. To clarify, Group D habitats were uncovered for the least amount of time and thus subjected to the least conspecific presence. These exhibited a 10.32% increase in oviposition relative to Group C habitats. Similarly, Group C habitats showed a 15.18% increase in oviposition relative to Group B habitats, which had 17.21% more egg rafts than Group A. The stepwise increase in oviposition could be attributed to conspecific avoidance of overused habitats. There were no such trends in the spring and autumn. These months also had considerably less oviposition activity, however, meaning containers belonging to Group A, or the "overused" habitats, were not as heavily used in the spring as the Group A habitats in June. Oviposition was so low in October, in fact, that there was not a good distribution across either the treatments or groups to draw conclusions on the effect of conspecific presence.

Thus, it cannot be concluded whether conspecific interference had any effect on oviposition in fall or spring. The interaction of two independent variables, Season and Group, suggested that they enhance the effects of one another when there egg raft abundance is high (as in the summer). The combination of these factors, rather than conspecific deterrence alone, is an important predictor of oviposition. Intraspecific competition during the summer season could have negatively interfered with the gravid female's perception of a habitat's suitability for maximized fitness (Mogi and Okazawa, 1990), thereby increasing the proportion of oviposition in newly exposed habitats. Overused habitats pose certain risks for larvae, some of which include density-dependent and predatory survival costs (Kiflawi et al., 2003). Streams are habitats for other species with aquatic larvae, including other insects, frogs, and toads, which are viable sources of competition and/or predation for mosquitoes. The presence of anuran tadpoles has been shown to cause significant reductions in oviposition rate (Mokany and Shine, 2003). Therefore, the putative trade-off between food resource and conspecific interference must also be considered in respect to gravid response.

Though it is known that mosquitoes use chemical and biology cues to detect ponds where conspecifics are present, behavioral response is not well understood. Contradicting results from other studies state that *Culex quinquefasciatus* oviposited more frequently both in the presence of conspecifics and into water bodies that previously contained conspecifics (Mokany and Shine, 2003; Beehler and Mulla, 1995). This was explained by use of pheromones left over by conspecifics to provide a reliable cue that the habitat offers suitable conditions and/or low predation risk. Thus, the "right decision" on the part of gravid female mosquitoes involves a complex interplay between conspecific competition and conspecific endorsement that is hard to simplify. Interspecific variation must also be taken into account; studies on a broader range of mosquito taxa can be useful to further analyze these mechanisms.

Sound waste management practices on the part of both municipalities and homeowners have the potential to lower the risk of mosquito borne diseases in urban areas. Recognizing nearby breeding habitats is a start. For example, the study site of this experiment was located in the backyard of several homes in a metropolitan neighborhood. People with pets were often seen walking their dogs along the CSO stream where samples were collected. A newly constructed dog walk was also recently erected downstream, and manicured lawns in adjacent parks help make the location attractive to residents. This was worrisome because mosquitoes are known to spread acquired pathogens to a wide range of vertebrate hosts including dogs and other household animals (Hayes et al., 2005). It is unknown if the residents were aware of the amount of mosquito activity in the area. The data from this study and many others provide evidence for the preference of polluted systems by WNV vectors. Open sources of stagnant water found in and around people's homes, especially when rich in nutrients, may be more likely than clean or covered water to attract such mosquitoes. Identifying and subsequently removing these sources can help to decrease the presence of mosquitoes that are potential vectors.

Lastly, it should be noted that only adult mosquitoes, and not egg rafts, have the ability to transmit pathogens to humans. This study focused on oviposition alone and thus did not account for the impact of differential selection on total number of emerged adults. The capacity of a habitat to support a mosquito reaches far beyond oviposition into emergence, fitness, fecundity, vector effectiveness, and so on. Topics branching from this study in combination with the paucity of knowledge concerning vector ecology create a metaphorical sandbox for research and should be explored in further detail.

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Appendix

Procedure for continuous flow colorimetric assays

Text used: Standard Methods for the Examination of Water & Wastewater, 21st Edition. ISBN: 0-87553-047-8.

For assay of NO3-N, 4500-NO3- F. Automated Cadmium Reduction Method is used. All of the nitrate measured has been reduced to nitrite by the open tubular cadmium reactor, and along with the nitrite already present in the sample, is measured as an azo dye following its diazotization with Sulfanilamide and then coupling with N-1-Naphthylenediamine. The reactions are taken out in an acidic (pH 1-2) solution, and the product is measured at 540nm. (RFA Methodology. Alpkem Corporation. NL 7-86. Nitrate + Nitrite Nitrogen A303-S170).

To test for NH4 and Ammonia Nitrogen, many different reagents are employed. Sodium Salicylate/Sodium Nitroferricyanide and Sodium Hypochlorite react with the ammonia present in the sample. This reaction occurs in a buffered alkaline solution (pH 12.8-13) made from combining Potassium Sodium Tartrate, Sodium Phosphate-Dibasic, Sodium Hydroxide, and deionized water. The product is the salicylic acid analog of indophenol blue, which is then measured at 660nm. (RFA Methodology. Alpkem Corporation. Ammonia Nitrogen A303-S021).

For assay of PO4-P, 4500-P F. Automated Ascorbic Acid Reduction Method is used. Here, Ortho-phosphate reacts with Ammonium Molydate and Antimony Potassium Tartrate to form a Phosphoantimonylmolybdenum complex. Next, the complex is reduced by Ascorbic acid to a heteropolyblue. This reaction occurs in an acidic medium, and the product is measured at 880nm. (RFA Methodology. Alpkem Corporation 1987. SA 2-87. Ortho-Phosphate A303-S203).

TABLE 1. Selection of linear mixed effects models (LMEMs) for Akaike Information Criterion.

Model/Covariates	**Nested Random Factors**	**AIC**
Null: no covariates	Week+Visit, and Week	3094
Null: no covariates	Season+Week+Visit, and Season+Week	2970
Treatment+Season*Group+Rain	Season+Week+Visit, and Season+Week	<u>2721</u>
Treatment+Season+Rain1+Group	Season+Week+Visit, and Season+Week	2737
Treatment+Temp(avg)+Rain1+Group	Season+Week+Visit, and Season+Week	2746
Treatment+Temp(max)+Rain1+Group	Season+Week+Visit, and Season+Week	2747
Treatment+Temp(min)+Rain1+Group	Season+Week+Visit, and Season+Week	2749
Treatment+Temp(avg)*Group+Rain	Season+Week+Visit, and Season+Week	2755
Treatment+Temp(max)*Group+Rain	Season+Week+Visit, and Season+Week	2756
Treatment+Temp(min)*Group+Rain	Season+Week+Visit, and Season+Week	2758

* interacting variables (synergistic effects)
\+ non-interacting variables

TABLE 2. Parameter estimates for the best model explaining overall mosquito oviposition. Intercept represents Season:Spring, Season:Fall, Treatment N, and Group A.

Parameter	Estimate	Std. Error	95% CI	P*
Intercept	1.4255	4.3831	-7.414 to 8.136	–
Treatment Y	18.739	4.383	16.552 to 20.911*	P < 0.00001
Summer	11.168	5.980	1.783 to 21.970*	P < 0.00001
Group B	0.781	2.449	-5.811 to 3.945	
Group C	1.611	2.869	-7.061 to 4.379	
Group D	0.618	3.776	-8.032 to 7.080	
Rain	-6.746	3.481	-9.571 to 1.397	

*Obtained by bootstrap

TABLE 3. Analysis of variance (ANOVA) for the effects of time of year (Season, with three levels: Spring, Summer and Fall), week (W, with four levels corresponding to weeks 1 through 4), and treatment (nutrient addition with two levels Y and N) on the concentration of: ammonium [NH_4^+], nitrate [NO_3^-], phosphate [PO_4^{3-}].

[NH_4^+]

Factor	df	Sum Sq.	Mean Sq.	F Value	Pr(>F)
Season	2	832.71	416.36	43.1636	5.272e-15***
Week	1	460.13	460.13	47.702	2.181e-10***
Treatment	3	2621.31	1310.66	135.876	2.2e-16***
Season:Week	6	124.68	62.34	6.4625	0.002129**
Season:Treatment	2	1114.8	278.7	28.8929	2.2e-16***
Week:Treatment	3	445.64	222.82	23.0996	2.840e-09***
Season:Week:Treatment	6	136.47	34.12	3.5369	0.009021**
Residuals	126	1215.39	9.65		

[NO_3^-]

Factor	df	Sum Square	Mean Square	F Value	Pr(>F)
Season	2	751.7	375.85	30.0783	2.094e-11***
Week	1	48.44	48.44	3.8763	0.05117
Treatment	3	1925.11	962.56	77.0315	2.2e-16***
Season:Week	6	4.63	2.31	0.1852	0.83118
Season:Treatment	2	954.45	238.61	19.0957	2.682e-12***
Week:Treatment	3	10.45	5.22	0.4181	0.6592
Season:Week:Treatment	6	1.85	0.46	0.0371	0.99735
Residuals	126	1574.45	12.5		

[PO_4^{3-}]

Factor	df	Sum Square	Mean Square	F Value	Pr(>F)
Season	2	4.8045	2.4022	23.1608	2.716e-09***
Week	1	0.2694	0.2694	2.5971	0.1096
Treatment	3	9.0921	4.5461	43.83	3.555e-15***
Season:Week	6	0.4543	0.2272	2.19	0.1162
Season:Treatment	2	5.2996	1.3249	12.7737	9.315e-09***
Week:Treatment	3	0.1605	0.0802	0.7736	0.4635
Season:Week:Treatment	6	0.6269	0.1567	1.551	0.2029
Residuals	126	13.0688	0.1037		

***statistically significant (P<0.001)
**statistically significant (P<0.01)

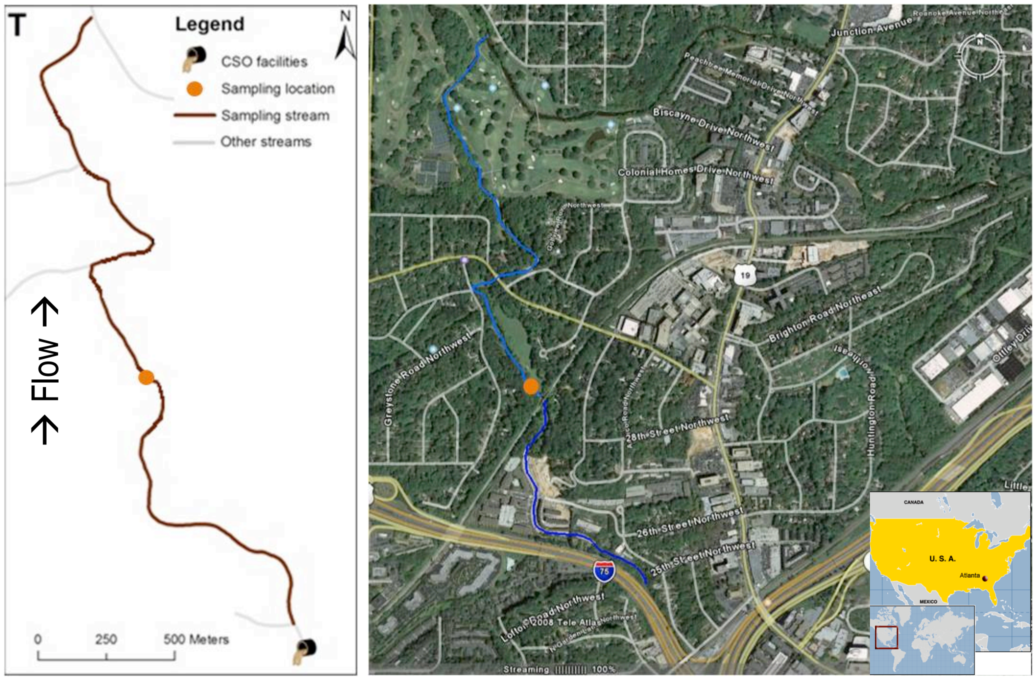

FIGURE 2. Map of the study location (left) showing where semi-natural habitats were constructed in relation to Tanyard Creek CSO facilities. Satellite image (right) used to show urban density in South Buckhead neighborhood of the city of Atlanta, Georgia.

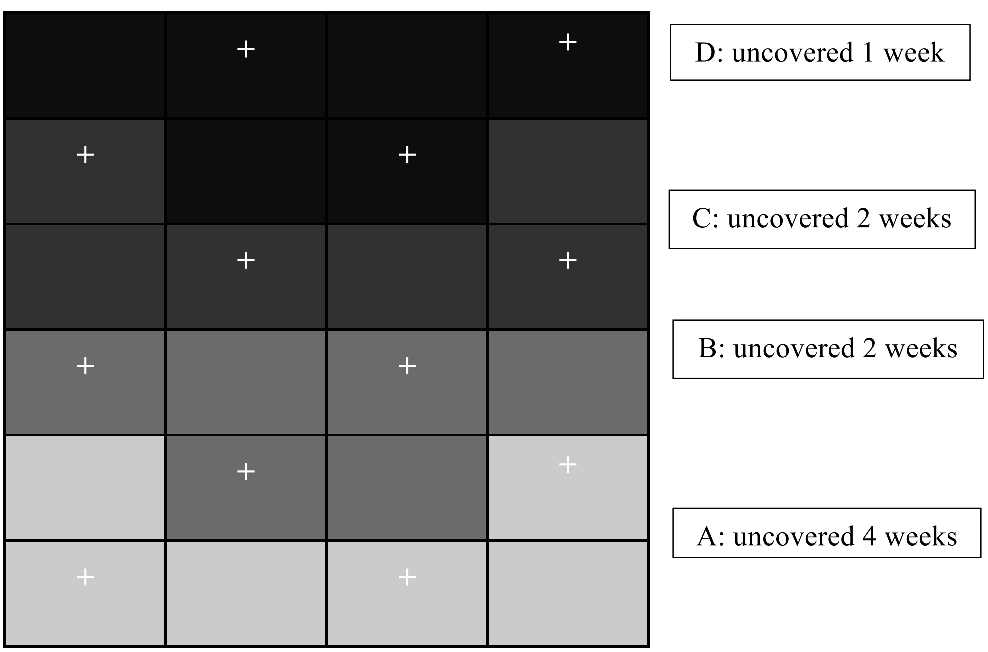

FIGURE 3. 4 x 6 experimental setup of oviposition containers. (+) represents protein addition. Light to darkest grey represents Groups A – D, respectively, where lightest grey (Group A) containers were uncovered for all 4 weeks and darkest grey (Group D) containers were uncovered only the last week of the experiment.

FIGURE 4. Semi-natural treatments with (+) protein addition on the left and (-) protein addition on the right.

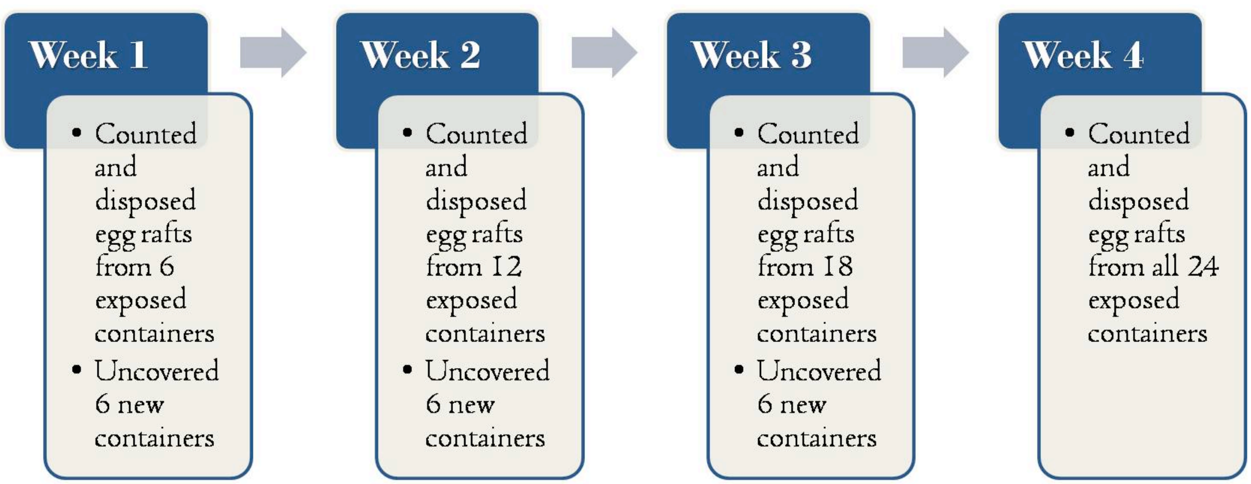

FIGURE 5. Experimental procedure repeated in October, April, and June.

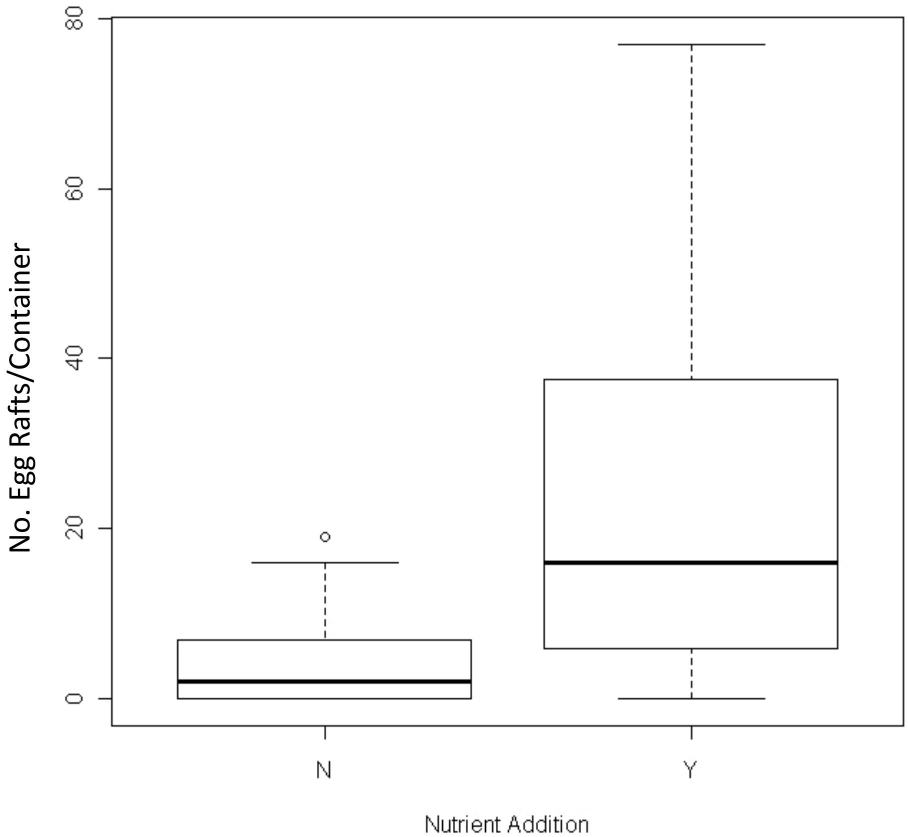

FIGURE 6. Boxplot of *Culex quinquefasciatus* oviposition as a function of nutrient addition to experimental habitats (N – no protein infusion, Y – protein infusion). Bold line represents median. Bottom quadrant of box represents 1st quartile, and top quadrant represents 3rd quartile. 50% of the data lies inside the box. The other 50% are represented by dotted lines and circles, or outlying values and extreme outliers.

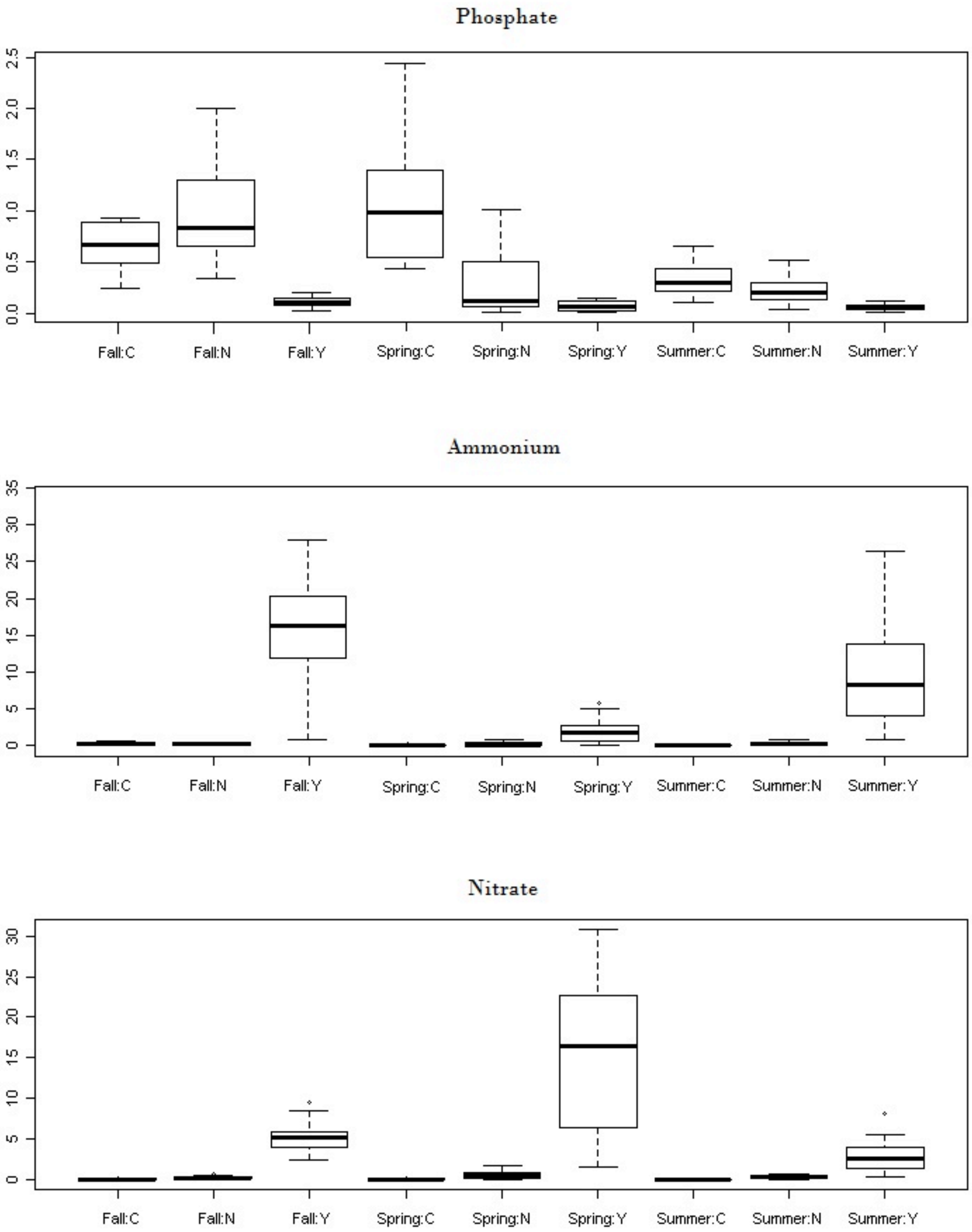

FIGURE 7. Nutrient concentrations (mg/L) in protein-infused (Y) and non-infused (N) containers and stream (C) over the spring, fall, and summer sampling seasons. (A) Boxplot of phosphate (PO_4^{3-}) concentrations (mg/L). (B) Boxplot of ammonium (NH_4^+) and ammonia nitrogens concentrations (mg/L). (C) Boxplot of nitrate (NO_3^-) and nitrite nitrogens concentrations (mg/L). Note difference in scales among panels. Bold line represents median. Bottom quadrant of box represents 1st quartile, and top quadrant represents 3rd quartile. 50% of the data lies inside the box. The other 50% are represented by dotted lines and circles, or outlying values and extreme outliers.

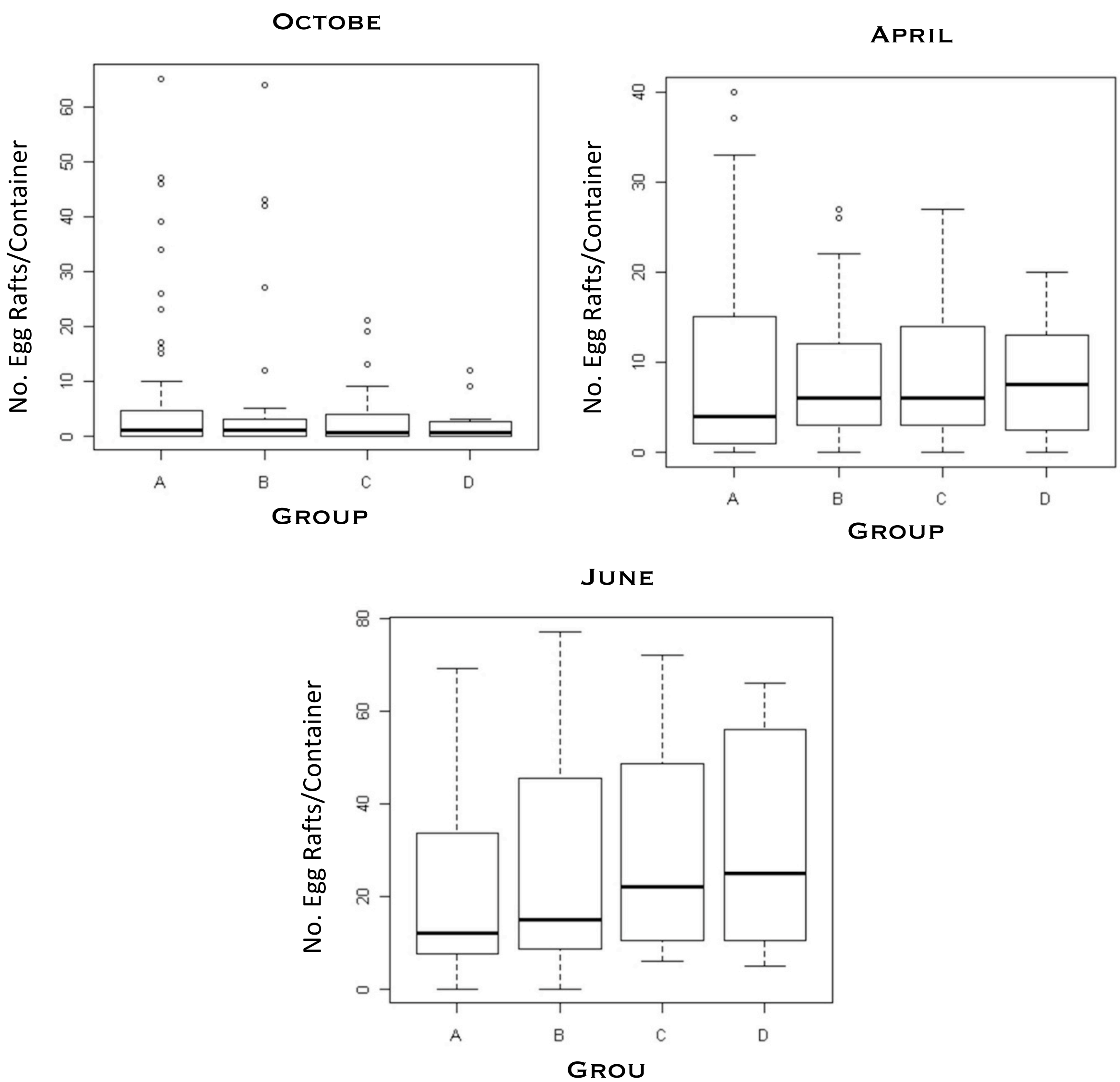

FIGURE 8. Boxplots of *Culex quinquefasciatus* oviposition as a function of exposure time (Groups A-D, decreasing exposure time) for the spring, fall, and summer sampling seasons. Bold line represents median. Bottom quadrant of box represents 1st quartile, and top quadrant represents 3rd quartile. 50% of the data lies inside the box. The other 50% are represented by dotted lines and circles, or outlying values and extreme outliers.

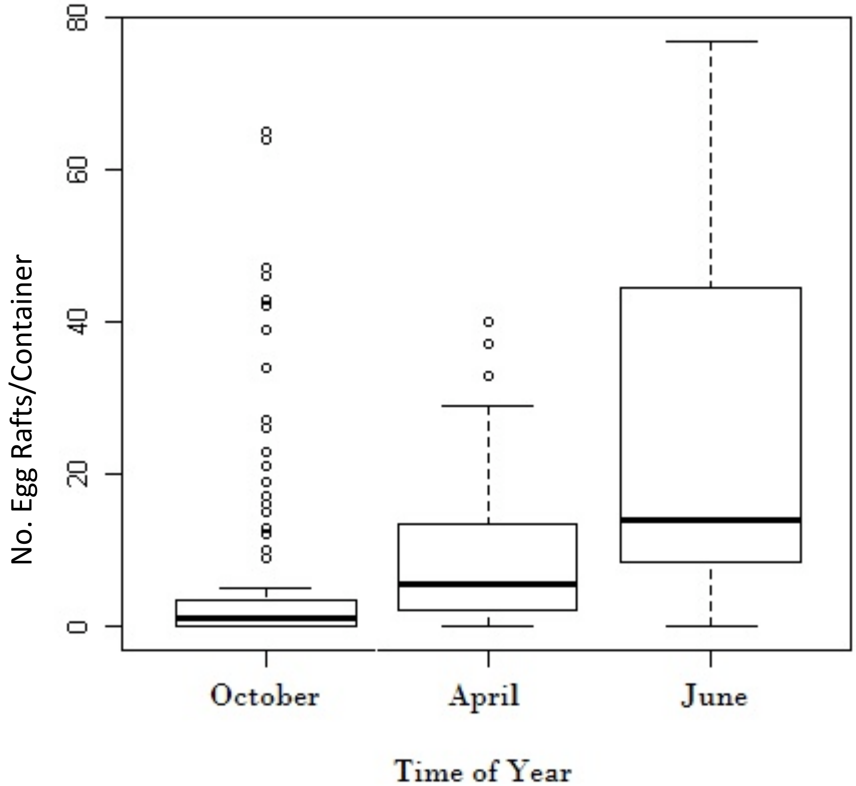

FIGURE 9. Boxplot of *Culex quinquefasciatus* oviposition as a function of time of year. Bold line represents median. Bottom quadrant of box represents 1st quartile, and top quadrant represents 3rd quartile. 50% of the data lies inside the box. The other 50% are represented by dotted lines and circles, or outlying values and extreme outliers.

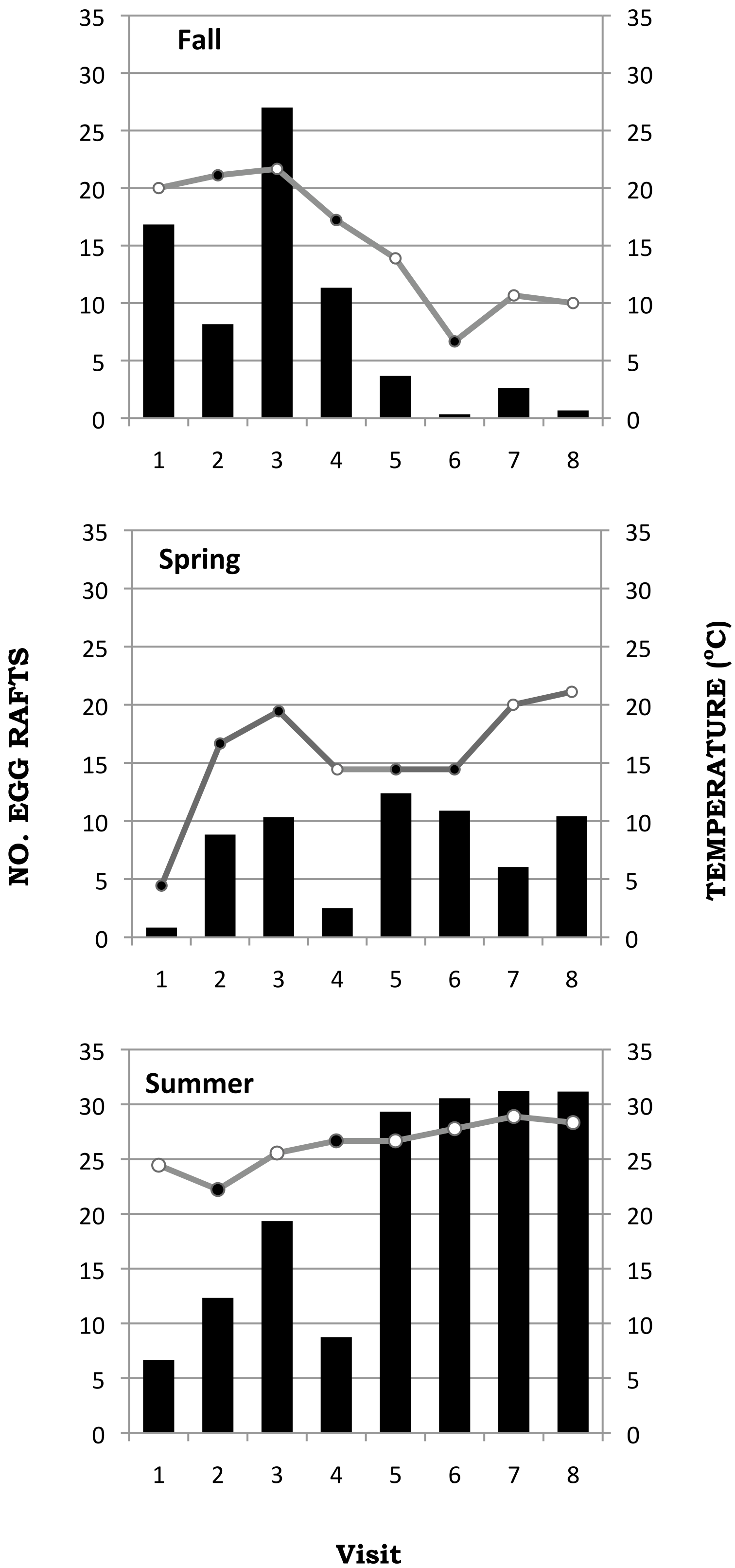

FIGURE 10. Graphs of *Culex quinquefasciatus* oviposition (black bar) and temperature (grey line) across three sampling periods (each month consisted of 8 visits). Black circles represent rainfall of >0.01 in.