Combined Sewage Overflow Enhances Oviposition of *Culex quinquefasciatus* (Diptera: Culicidae) in Urban Areas

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ABSTRACT Ecosystem changes caused by anthropogenic activities have modified the environment in ways that at times promote the emergence of vector-borne diseases. Here, we study the effects of combined sewage overflows (CSOs) from urban streams in Atlanta, GA, on oviposition site selection by *Culex quinquefasciatus* under seminatural field conditions. Counting egg rafts was a reliable indicator of oviposition preferences, and CSO water quality, especially when enriched, was a more attractive oviposition substrate than nonenriched water. Therefore, environmentally sound management of municipal waste water systems has the potential to diminish the risk of *Culex*-borne diseases in urban areas.

KEY WORDS water quality, habitat choice, multilevel analysis, environmental grain, West Nile virus

Aquatic ecosystems are undergoing major changes because of the interactions of humans with the environment. Human-mediated changes range from different dynamics in the balance of elements in the biosphere to the emergence of pathogens in most kinds of living organisms (Levins et al. 1994, Grimm et al. 2008, Johnson and Carpenter 2008). A large body of studies has indeed shown that mosquitoes are successful at breeding in drainage and sewage systems (Lauret 1953, Lumsdem 1958, de Meillon et al. 1967a, Scorza 1972, Hayes 1973, Munstermann and Craig 1977, Mulligan and Schaefer 1982, O'Meara and Evans 1983, Mian and Mulla 1986, Strickman and Lang 1986, Mogi and Okazawa 1990). However, general attention to the effects of sewage management on urban mosquitoes is scarce and merits further attention. As pointed out by Calhoun et al. (2007), many urban areas in the United States, and worldwide, use combined waste and storm waste systems for treating and disposing of water (a.k.a., combined sewage overflows [CSOs]). CSO systems combine water from urban runoff and sewage for processing in wastewater treatment facilities. However, after heavy rainfall events, the systems collapse, resulting in the release into urban streams of untreated water rich in materials responsible for eutrophication and pollution.

Calhoun et al. (2007) showed that streams under the influence of nutrient pulses from CSOs have an increased presence of *Culex quinquefasciatus*, the main vector of West Nile virus in the southeastern United States (Turell et al. 2001). A key factor in determining the local abundance of insect populations is the choice of habitat for preadult development. It has been proposed that habitat choice is determined by the ability of gravid females to distinguish habitats where the fitness of their offspring is optimized (Mangel 1987). In general, given the heterogeneity of natural environments, fitness is a complex function of exogenous (e.g., food sources) and endogenous factors (e.g., intraspecific competition). Therefore, habitat colonization will follow, under ideal conditions, a pattern where average fitness differences are similar across a variety of habitats where females can make oviposition choices, a.k.a. ideal free distribution (Fretwell and Lucas 1970, Ellis 2008). Several factors have been identified in regard to the choice of oviposition habitat in mosquitoes, from purely chemical cues (Ikeshoji 1966, Dadd and Kleinjan 1974, Bentley and Day 1989, Beehler et al. 1994b, Millar et al. 1994, Braks et al. 2007) to the presence of microorganisms (Madder et al. 1980, Rockett 1987, Bentley and Day 1989, Beehler et al. 1994a, Poonam et al. 2002) and conspecific individuals (Reisen and Siddiqui 1978, Bruno and Laurence 1979, Suleman and Shirin 1981, Madder et al. 1983, Wilmot et al. 1987, Dhileepan 1997, Zahiri et al. 1997a) to oviposition medium quantity and quality (Reisen and Meyer 1990, Reiskind and Wilson 2004, Harrington et al. 2008). However, studies describing these factors have either been limited to the laboratory or have not specifically explored the possible effects of combined sewage overflows on oviposition under natural conditions. For *Cx. quinquefasciatus*, there is a gap in knowledge about differences in the number of eggs per raft across oviposition substrates. This information might be important, because it may provide hints about size differences in mosquitoes

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colonizing heterogeneous water bodies and ongoing evolutionary processes (Day et al. 1990, Ranta et al. 2006). Therefore, the goals of our study were to test whether egg rafts are robust indicators of oviposition habitat choice, i.e., to test whether the number of rafts reflects the actual number of eggs being oviposited in a given medium and to investigate the effects of combined sewage overflows on oviposition by *Cx. quinquefasciatus* under seminatural conditions, accounting for water quality and quantity, nutrient availability, and presence of conspecifics in urban landscapes.

Materials and Methods

Experiment 1: Effects of Water Quality and Nutrient Availability on Oviposition Site Selection

This experiment was done along Tanyard Creek, a CSO polluted stream in Atlanta, GA (Calhoun et al. 2007). A two-factor by two-level experiment was designed to test the effects of water quality and nutrient availability on oviposition. The first factor consisted of two levels, including water from Tanyard Creek collected within 48 h after an overflow event (CSO) and tap water as control (6 liters in total for replicate). An overflow event currently happens in the city of Atlanta, GA, when >0.1 in of precipitation occurs. For the specific event we studied, 9,380 kgal of wastewater were released over 135 min. Although overflow duration and amount of nutrient release is highly variable, water eutrophication and decreased dissolved oxygen always occurs (Bernhardt et al. 2008). Levels for the second factor were related to the addition of nutrients: one to which 24 g of crumbled dog food were added (20% protein content) and the other without dog food. Each of the four resulting treatments was replicated three times using 37.85-liter dark blue surface Rubbermaid (Columbus, OH) containers as artificial oviposition habitats. Egg rafts in each container were counted and removed 3 and 6 d after the experiment was set up. Although incubation time for egg hatching of this species under similar conditions is ≈1 d (de Meillon et al. 1967b), we consider our approach valid, because eggs from rafts older than 1 d likely hatched and sank as reported elsewhere (Scorza 1972). Statistical analysis was done using a negative binomial generalized linear model (NB-GLM) selected by backward elimination, using the Akaike information criterion (AIC) (Faraway 2006) of a full model that considered the number of egg rafts per container a function of the interaction between medium age (as categorical variable, one category for each survey), water quality, and presence of nutrients. Results from the NB-GLM were analyzed by using an analysis of deviance, the equivalent of analysis of variance (ANOVA) for generalized linear models.

Effects of Water Quality on Egg Raft Size. The eggs of 15 rafts from each level of water quality were counted under a dissection scope at ×40. The mean number of eggs per raft from each kind of water (with added nutrients) were compared using a *t*-test (Zar 1998).

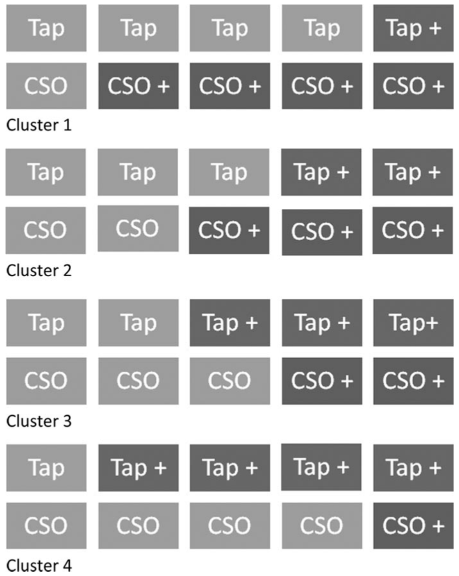

Fig. 1. Experimental design for the evaluation of oviposition factors as scale dependent. Tap and CSO stand for control tap water and combined sewage overflow water, respectively. +, addition of nutrients. For each cluster, the quantity of containers with each kind of water quality and nutrients is constant.

Experiment 2: Evaluation of Oviposition Factors as Scale Dependent

Experiment 2 was done in Baker woodland reserve at Emory University in Atlanta, GA.

Experiment 2A: Effects of Local Quantitative Changes on the Composition of Water Quality. A similar design to experiment 1 was used to evaluate the effects of water quality and nutrient availability as scale dependent, i.e., with varying number of replicates for each treatment across clusters of replicates. A total of 40 19.92-liter dark blue Rubbermaid containers were distributed across four clusters (10 containers/cluster; see Fig. 1). Clusters were separated by at least 75 m along a transect following a stream. The selection of this separation was based on knowledge about *Cx. quinquefascuatus* dispersal, which is mostly limited to distances <2 km (Service 1997, Silver 2008). To control for cluster compositional differences, the total amount of each kind of water (CSO and Tap) and added nutrient (yes and no) were kept constant per cluster. However, the local quantity of containers for a given treatment was varied in a split-plot fashion, because full replication was not feasible. Thus, each cluster contained one, two, three, or four containers of the same treatment, in combinations where the total numbers of containers with each level of nutrients, as well as the total number of containers with each level of water quality was equal to five (Fig. 1). Oviposition medium contents were adjusted to the size of the containers, adding 3 liters of water and 12 g of crumbled dog food (20% of protein) to each container.

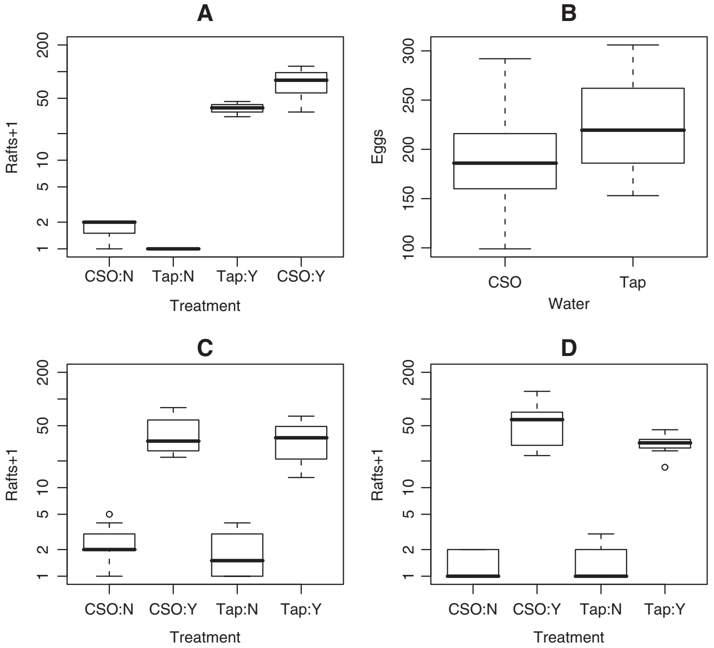

Fig. 2. Effects of water quality and nutrient availabity on *Cx. quinquefasciatus* oviposition preferences. (A) Boxplot (median and quartiles) for the total number of egg rafts in the four treatments, close to a CSO stream. (B) Boxplot for the number of eggs per egg raft from treatments with nutrients and different water quality. (C and D) Boxplots for the total number of egg rafts in the four treatments varying factors as scale dependent (C) without removing the rafts and (D) removing the rafts. The two levels for water quality are Tap and CSO; the levels for nutrients are presence (Y) and absence (N). The *y*-axes for boxplots A, C, and D are in logarithmic scale.

Experiment 2B: Effects of Keeping and Removing Egg Rafts. For two 8-d periods, the total number of new rafts in each of the 40 containers was recorded daily. In the first period (11–18 July 2008), old egg rafts were kept in the containers and were separated under a cylindrical (3.5 cm radius) floating styrofoam device covered with a mesh to avoid double counting. Larvae were allowed to develop, and no pupae were observed during this period. For the second experiment, (19–26 July 2008), after cleaning the containers, the same setup was used, but egg rafts were removed daily from the containers. For the statistical analysis, data corresponding to the last 5 d of each period were analyzed, because no rafts were recorded during days 1–3. The total number of egg rafts were summed, and a split plot linear mixed effects model that considered cluster and error as random factors and the possible interaction between the number of replicates per cluster, water quality, and nutrients as fixed factors was simplified using AIC-based backward elimination. The method used for fitting the models was a restricted maximum likelihood, and inferences about the fixed factors were based on the highest posterior density interval from 100,000 realizations of a Markov Chain Monte Carlo from the model selected as best (Faraway 2006).

Mosquito Identification

In all experiments, fourth-instar larvae coming from egg rafts were identified as *Cx. quinquefasciatus.* For experiments with raft removal, individuals from five randomly chosen rafts were examined per period of sampling; for the experiments without raft removal, 25 individuals from the last day of the experiment were examined.

Results

Experiment 1: Effects of Water Quality and Nutrient Availability on Oviposition Site Selection. Average number of total rafts per container (±SE) were 0.00 ± 0.00 for the tap water; 0.66 ± 0.33 for CSO water; 36.67 ± 4.33 for tap water with nutrients; and 75.67 ± 23.15 for CSO water with nutrients, showing that all treatments with CSO water had more rafts than control treatments with tap water (Fig. 2A). Differences became pronounced when nutrients were added to oviposition medium. The best model considered the additive effects of water quality, nutrients, and medium age as a categorical variable (Table 1). The number of rafts for the first oviposition period, compared with Tap water and absence of nutrients, was increased 2.85 (95% CI: 1.53–5.84) times in the CSO containers, 189 (95% CI: 51.8–1238) times in the presence of nutrients, and 21.2 (95% CI: 10.0–47.1) times during the second observation period (i.e., day 6). For the number of eggs per raft, 14 and 15 rafts from tap and CSO water, respectively, were counted. The mean number of eggs (mean ± SE) of 225 ± 46 for tap water and 192 ± 51 for CSO water (Fig. 2B) were not

Table 1. Negative binomial generalized linear model analysis of deviance of the effects of water quality, nutrient availability, and medium age on *Cx. quinquefasciatus* oviposition (no. of egg rafts) close to a CSO stream

Factor	df	Deviance	LRT	$Pr > \chi$
Water quality	1	27.542	8.925	0.0028
Nutrients	1	136.722	118.106	<0.0001
Medium age (category)	1	84.031	65.415	<0.0001
Residual		18.616		

Negative binomial overdispersion parameter, $\hat{\varphi} \pm \text{SE} = 4.14 \pm 2.66$.

statistically different ($t = 1.80$, df = 27, $P > 0.083$), indicating no differences between mosquitoes choosing each oviposition site.

Experiment 2: Evaluation of Oviposition Factors as Scale Dependent

The best models to explain the number of egg rafts per treatment differed according to whether or not egg rafts were removed during the study period. Figure 2C shows that the total number of rafts was higher for both treatments when nutrients were added. When egg rafts were not removed, model factors included the number of containers of the same treatment in the cluster (α), the addition of nutrients (β), and their interaction ($\alpha \times \beta$), which was the only significant factor (Table 2). The equation describing this model is:

$$Rafts_{ijkl} = \mu + \alpha_i(number) + \beta_j(nutrients) + \alpha \times \beta_{ij}(number \times nutrients) + \sigma_k + \varepsilon_{ijkl} \quad [1]$$

where μ is the mean value, σ represents the variability at the cluster scale, and ε represents the variability at the container scale (or error). When egg rafts were removed, the addition of nutrients increased the number of egg rafts, but the numbers collected were still higher for the CSO water quality (Fig. 2D). Selected variables were the addition of nutrients (β), water quality (γ), and their interaction ($\beta \times \gamma$); both β and $\beta \times \gamma$ were statistically significant (Table 2). The model equation is:

$$Rafts_{ijkl} = \mu + \beta_j(nutrients) + \gamma_i(water) + \gamma \times \beta_{ij}(water \times nutrients) + \sigma_k + \varepsilon_{ijkl} \quad [2]$$

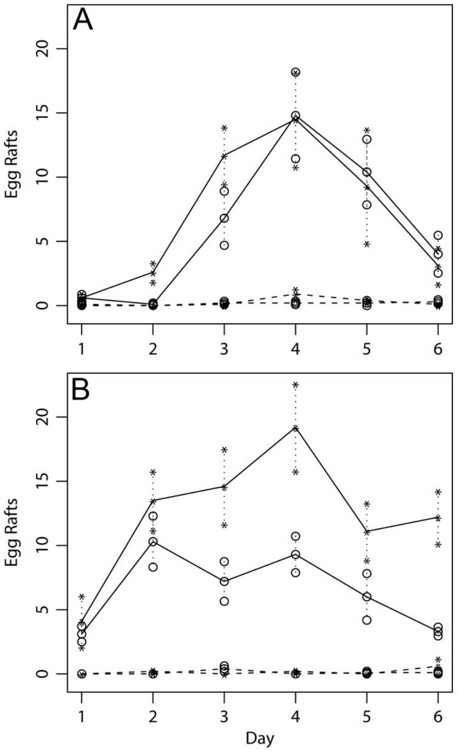

Fig. 3. Temporal dynamics of *Cx. quinquefasciatus* oviposition (A) without removal and (B) with removal of rafts. Lines are for average number of rafts; SE is indicated with dotted vertical lines. Water quality is indicated with signs: *, CSO; ○, Tap; solid lines, added nutrients; dashed lines, absence of additional nutrients.

where μ, σ, and ε have the same interpretation as in equation 1. Both models show that the largest variability is at the individual container scale, and the relative amount of variability (cluster variance/cluster + container variance) that can be attributed to differences across clusters is 7% when egg rafts were not removed and 11% when rafts were removed daily (Table 2). Figure 3 shows the average temporal dynamics of oviposition (daily rafts oviposited) for all treatments across clusters during the last 6 d of each experiment. In the case of nonremoval of rafts (Fig. 3A), oviposition monotonically reached a maximum

Table 2. Parameters for the mixed effects models selected as best for the experiments evaluating factors affecting *Cx. quinquefasciatus* oviposition as scale dependent

Parameter	No raft removal		Daily raft removal	
	Estimate	95% CI	Estimate	95% CI
Tap ($\hat{\mu}$)	3.973	−14.594 to 22.446	1.019	−11.668 to 14.417
No. containers ($\hat{\alpha}$)	−0.925	−6.367 to 4.705	—	—
CSO ($\hat{\gamma}$)	—	—	−1.238	−15.458 to 12.909
Nutrients ($\hat{\beta}$)	12.550	−11.699 to 36.522	29.462	15.643–44.006[a]
Containers × nutrients ($\hat{\alpha} \times \hat{\beta}$)	8.300	0.692–15.977[a]	—	—
Water × nutrients ($\hat{\gamma} \times \hat{\beta}$)	—	—	27.176	6.337–47.586[a]
Cluster variability VAR($\hat{\sigma}$)	10.982	—	28.208	—
Container variability VAR($\hat{\varepsilon}$)	140.124	—	225.569	—

[a] Statistically significant ($P < 0.05$).

and diminished. In contrast, in the removal experiment (Fig. 3B), oviposition maximum was reached nonmonotonically (i.e., with the slope changing sign throughout time) and was higher for CSO+ nutrients water throughout the study period. Originally, we removed and kept rafts expecting to have similar outcomes; the differences were beyond our predictions, and it was unfeasible for us to carry out an experiment making comparisons for this manipulation to rule out a merely temporal effect.

Discussion

Elevated nutrients increased the number of egg rafts oviposited by *Cx. quinquefasciatus*, an observation confirming previous results (Bentley and Day 1989, Silver 2008). Oviposition is additionally enhanced in habitats containing water from combined sewage overflow, when the presence of conspecific egg rafts is not a factor. As shown by the experiment when egg rafts were not removed, the presence of conspecifics and quantity of water (as measured by groups of containers of a particular treatment) may also interact to increase attractiveness to ovipositing females. These data improve the current understanding of oviposition site selection by this West Nile virus (WNV) vector species. It indicates that polluted water pulses coming from CSOs may alter the dynamics of adult populations by enhancing and concentrating oviposition and mosquito production in CSO streams. It is also illustrative of how CSOs may determine oviposition choices by gravid females.

More generally, our results give insights on the mechanisms governing the choice of oviposition habitats by female mosquitoes. The counting of egg rafts is a reliable measure of habitat oviposition preference by *Cx. quinquefasciatus* mosquitoes, because no significant difference in the number of eggs per raft was observed between oviposition substrates. However, it should be noted that number of eggs per raft can change seasonally (Bock and Milby 1981, Madder et al. 1983), thus making comparisons likely to be valid only for synchronous oviposition events. In addition, mosquito perception for oviposition habitat selection seems to be fine grained, given that most variability was observed at the scale of individual containers (Levins 1968, Ranta et al. 2006). Although for the experiment where egg rafts were kept in the containers there was a positive effect of habitat size, the degree of variability was higher at the scale of the individual containers than at the scale of clusters in both experiments, supporting local differences in the oviposition medium as the most important factor governing oviposition habitat choice. This is a common pattern that has been reported for other mosquito species (Kitron et al. 1989, Edgerly et al. 1998). This result also opens the question of how these habitats affect the overall fitness of mosquitoes and whether the fitness traits that are selected increase the vectorial capacity of *Cx quinquefasciatus.* To address this question, further studies on the survival and size at emergence of mosquitoes developing in CSO are needed. This kind of information also would be useful to test predictions of the oviposition preference–offspring performance theory (Ellis 2008), which assumes that individuals evaluate the environment at temporally long scales to distinguish which habitats are most likely to increase the offspring fitness. In general, studies support that the choice of water quality occurs over short time scales, because, for example, *Cx restuans* females were not able to distinguish the presence of added nutrients in containers with hay infusion, even though some fitness components were increased for individuals in containers with higher amounts of nutrients (Reiskind et al. 2004). Therefore, the assumption of choice as determined by an evaluation of increased offspring fitness needs to be revised and used as the best possible null hypothesis to test. Also, *Cx. quinquefasciatus* larvae are known to be washed away from containers after rainfall (Koenraadt and Harrington 2008), which may imply an environmentally mediated dispersal and a violation of the assumption of no dispersal for immature stages behind the ideal free distribution (Fretwell and Lucas 1970). A better understanding of the role of the environmental forcing is necessary, because the degree of temporal resolution used for oviposition decisions is likely to be conditioned by the state of seasonal environmental variables (Day et al. 1990, Edgerly et al. 1998).

Our study also opens questions about the degree to which eutrophication and pollution of CSO water are attractive for oviposition. Further microbiological characterization of CSO water is necessary to determine whetherthe increased oviposition is related to cues originating from microorganisms that thrive in this kind of environment (Reisen and Siddiqui 1978, Suleman and Shirin 1981, Rockett 1987, Poonam et al. 2002), eutrophication alone (Carpenter 1982, Fish and Carpenter 1982, Beehler and Mulla 1993, 1995), the presence of pheromones from conspecifics (Bruno and Laurence 1979, Braks et al. 2007), or the exclusion of natural predators, parasites, and competitors (Chesson 1984, Lowenberger and Rau 1994, Zahiri et al. 1997b, Kiflawi et al. 2003, Bond et al. 2005, Yasuoka and Levins 2007). In general, a better understanding of the factors that determine oviposition across the different components of a heterogeneous environment deserve further attention, especially the possible dynamic interactions among the above mentioned factors. For example, the degree of eutrophication and forms of available nutrients are likely to be influenced by differences in microorganism diversity (Walker et al. 1991). Finally, knowledge of vector oviposition is potentially useful for developing predictive spatio-temporal models of mosquito abundance, which may guide mosquito control activities for WNV and other vector-borne diseases (Reiter 1983, Mulla et al. 1992). It also shows how management of basic natural resources can result in unforeseen influences on other public health issues that at first sight seem to be independent (Mogi and Okazawa 1990). Therefore, our study encourages the use of environmentally sound practices for the management of water as an ecosystem service, because benefits are likely to be beyond what is normally foreseen.

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