Combined sewage overflow accelerates immature development and increases body size in the urban mosquito *Culex quinquefasciatus*

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Abstract

In urban landscapes the crowding of humans and their waste products may alleviate intra-specific interactions of common mosquitoes. Here, we present the results of a semi-natural experiment addressing the effects of water from a sewage overflow stream on density dependent fitness components and phenotypic traits of a common tropical and subtropical urban mosquito, *Culex quinquefasciatus* Say (Diptera: Culicidae). This semi-natural experiment was designed to quantify the relative importance of density dependence, weather forces and water quality on larval mortality, sex ratio and size at adult emergence. Results showed that mortality hazards were independent of larval density, decreased in sewage overflow water and increased with minimum temperatures. Under all rearing conditions adult mosquito size decreased with density. Mosquitoes from sewage overflow water emerged faster, were bigger and had an increased ratio of females to males. All these traits may contribute to the regulation of mosquito populations.

Introduction

Human population growth and concentration in urban centres have led to many changes in ecosystems. One of the best studied changes is the natural cycling of nitrogen (Grimm et al. 2008). More specifically, the concentration of wastewater and runoff in urban centres has modified aquatic environments, as evidenced by excess nitrogen and phosphorous in urban streams (Grimm et al. 2008). The reliance of several relatively old urban centres on relict wastewater management infrastructure intensifies the runoff problem. For example, in countries with strict water management regulations (e.g., USA) some relatively old cities have combined sewer overflow systems (CSOs). These systems collect stormwater, industrial wastewater, and domestic sewage in the same pipe, which is directed to wastewater treatment plants (Bernhardt et al. 2008). During heavy precipitation events, excess stormwater causes the pipes and water storage areas to exceed their volume, dumping a mixture of untreated stormwater runoff, sewage and other waste into urban streams and rivers (Calhoun et al. 2007). Nutrient pulses and extreme flooding shift habitat quality for most native fauna, rendering the environment unsuitable for many resident species and probably conferring fitness advantages to the species able to tolerate the changes. One of the groups more likely to benefit from changes in the balance of nitrogen, especially the accumulation of its organic forms in aquatic habitats are some species of mosquitoes, because of both the increase of basal resources and changes in their inter-specific interactions (Mogi and Okazawa 1990).

Mosquitoes are of special interest because of their role as both nuisances and as vectors of several pathogens to humans and other vertebrates (Hamer et al. 2009; Chaves et al. 2010). Factors affecting the population dynamics and vectorial capacity of mosquitoes are complex, and their delineation is particularly important for understanding and predicting the transmission dynamics of vector-borne diseases (Chaves and Koenraadt 2010).

For example, body size can differentially affect mosquito susceptibility to pathogenic infections. Large female mosquitoes are more susceptible than small ones to infection with the Ross River virus (Nasci and Mitchell 1994). However, sometimes small females are more susceptible to infection than large ones, as documented for Japanese Encephalitis virus (Takahashi 1976), West Nile virus (Baqar et al. 1980), LaCrosse Encephalitis (Grimstad and Walker 1991). Nevertheless, larger females generally imbibe more blood and display higher biting persistence (Nasci 1991) and have an extended life-span (Hawley 1985), all key components of vectorial capacity (Garrett-Jones 1964). In contrast, smaller females are generally less fecund (Nasci 1987), and have a lower rate of parity in the field (Scott et al. 2000), indicating lower blood feeding success and vectorial capacity. Also, seasonal variation in adult body size has been recorded in many mosquito species, across several genera (Fish 1985). There is no consensus on whether changes are due to the canalization of exogenous environmental cues (Day et al. 1990), i.e., the imprinting of the environment on phenotypes (Schmalhausen 1949); or the abundance and stability of larval habitats and concurrent density-dependent regulation (Mori and Wada 1978; Minakawa et al. 2002).

Of great importance in the transmission of emerging zoonotic diseases, mosquito species from the *Culex pipiens* complex are found in human settlements worldwide (Calhoun et al. 2007) and have been associated with the presence of polluted body waters since early studies in medical entomology (Britton 1914). *Culex quinquefasciatus* Say (Diptera: Culicidae) is a common tropical and sub-tropical species within the *Cx. pipiens* complex, as well as a vector of several pathogens, ranging from viruses, like West Nile Virus (Styer et al. 2007), to nematodes that cause filariasis (Sasa 1976). Calhoun et al. (2007) reported high pre-imaginal abundances of *Cx. quinquefasciatus* in streams impacted by CSOs, and previous results have shown this species to preferentially oviposit in these habitats (Chaves et al. 2009). However, whether this preference translates to an overall fitness benefit for this mosquito vector species is still an open question. In this study, we attempted to determine whether water from a CSO stream could alter phenotypic traits, the survival of larval mosquitoes and their size at emergence in a changing environment. We designed a semi-field experiment to quantify the relative effects of density, temperature variation, and water quality on larval mortality, pupation rate, sex ratio, and adult size at emergence. These traits are important in shaping the population dynamics of organisms with complex life cycles, and in mosquitoes they are correlated with the potential success of disease transmission.

Material and Methods

Semi-natural experiment

To understand effects CSOs on *Cx. quinquefasciatus* we conducted a semi-natural experiment. The experiment was designed to study larval survival, time to emergence, sex ratios and adult size at emergence, since all these variables are known to determine the fitness and vectorial capacity of mosquitoes. The experiment had two factors, density with three levels (50, 100, and 150 larvae/150 ml of medium) and water quality which had two levels (combined sewage overflow and tap water); each resulting treatment was replicated three times. Densities were chosen based on abundance values from our own field samples that averaged 0.3 larva/ml, and using estimates from field samples in previous studies at other sites, which although highly variable rarely exceeded 1 larva/ml (Britton 1914; Rajagopalan et al. 1976). For all treatments the media were composed of 150 ml of either tap or CSO water (from Tanyard Creek, Atlanta, GA) with 2 g of ground dog-food (20% of protein content). Media were placed in 500 ml 'food-grade' containers, each with a single balsa wood strip for adults to rest after emergence. We added food because previous trials showed that larvae do not survive past the third instar in CSO water and the second instar in tap water without supplementation. A previous trial also showed no difference in larval survival and adult size at emergence between larvae reared in tap water and water from a non-CSO creek (Peavine Creek, Atlanta, GA). The larvae used in the experiment were hatched from egg-rafts collected in field oviposition traps as described in Chaves et al. (2009), and were representative of a single generation of larvae, instead of the overlapping life stages of larvae found in the field, to allow for measurement

of development and emergence time from a standardized hatching age. Egg rafts were kept in tap water, and resulting first instar larvae were ≤6 h of age when counted and added to containers that were covered with 0.5 mm mesh cloth to prevent escape of adult mosquitoes. Containers were placed close to natural vegetation in a sheltered area in the backyard of the Math and Science Center building of Emory University in Atlanta, GA. The number of surviving pre-adult individuals was recorded daily, and individuals were classified as young larvae (first to third instar), old larvae (fourth instar), pupae or adults. Adults were collected daily, counted, sexed and frozen at −20°C, and then dried for 72 h at 45°C. Only individuals with complete appendages were weighted. Temperature for each density treatment was measured and stored with a Hobo® datalogger model UA-001-08 (Onset Computer Corporation, Bourne, MA, USA) was placed to record water temperature every 2 h. Combined sewer overflow system water was collected following an overflow event on 24th August 2008. Water quality parameters were measured when collecting the CSO water used in the experiment at eight sites of a CSO stream. Water quality was also measured in three sites of a control stream (which did not have statistically significant different effects in mosquito survival when compared to tap water in preliminary trials), both in Atlanta, GA. Water quality parameters were also measured in both streams the week before the overflow event (17th August 2008).

Statistical analysis

Mosquito survival under semi-natural conditions

Daily pre-adult survival (l_X) was computed using the equation $l_X = N_X/N_0$, where N_X indicates the number of survivors at day X, and N_0 the initial number of larvae (Carey 2001). Daily survival was analyzed using Cox proportional hazard models where covariates were included as time dependent variables (Venables and Ripley 2002). Survival was modelled using a baseline hazard function h_0, so that the hazard function $h(t) = h_0 \exp(f(covariates(t)))$ measured the proportional increase in mortality. Studied hazards included water quality, density, and average, minimum, maximum and standard deviation of the daily temperature (24 h before the daily census). Covariates were selected by backward elimination of non-significant factors based on the Wald test for single parameter (Venables and Ripley 2002). The robustness of the results for the time independent significant hazards was further tested by examining

the odds of survival to fourth instar larvae, pupae and adult stage using binomial generalized linear models that were corrected for overdispersion using the standard deviation of the Pearson residuals (Faraway 2006).

Emergence time and adult size

Hierarchical linear mixed effects (LME) models were used to analyze time to emergence and size at emergence, allowing us to consider the variability due to the arrangement used to keep the samples in the field and record temperature, thus modelling explicitly the variability that creates the problem of pseudoreplication (i.e., the failure of a statistical analysis to properly incorporate the true structure of randomness present in the data) an issue of concern within the framework of simpler linear models (Chaves 2010).

Emergence time (E) of individual mosquitoes was studied as function of the rearing water quality, density and sex and analyzed with a LME model:

$$E_{ij} = \mu + \alpha + \beta + \pi_j + \varepsilon_{ij} \qquad (1)$$

where subindex i represents a given individual, subindex j represents a given 0.5-l container, ε represents the individual variability and π the container variability, the parameter μ is the average time of emergence for males in tap water, the parameter α is the additive effect of CSO water and the parameter β is the additive effect on time emergence of being a female. To ease interpretation of results we substracted 6 days to the variable E_{ij} (time to the emergence of the first mosquitoes). Both π and ε are assumed to be independent and normally distributed random variables with variances σ_π^2 and σ_ε^2 respectively. More complex models were discarded since their increased complexity did not increase the likelihood enough to diminish their Akaike Information Criterion (Venables and Ripley 2002). 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 95% confidence intervals from 100000 Markov Chain Monte Carlo (MCMC) realizations of the model selected as best (Faraway 2006). This modelling strategy has the advantage of explicitly modelling the lack of independence of larvae samples that come from the same container, which can be incorporated in the MCMC used to compute the confidence limits for the estimated parameters (Faraway 2006).

Adult size (A) of individual mosquitoes was analyzed as a function of water quality, sex, density and day of emergence using a LME model:

$$A_{ij} = \mu + \alpha + \beta + \gamma + \delta + \pi_j + \varepsilon_{ij} \qquad (2)$$

where subindices i and j, and variables π and ε have the same interpretation as in equation (1). The parameter μ is the average weight for males reared in tap water, α is the additive effect of increasing the density by one individual, β represents the additive effect of rearing in CSO water, γ the additive effect of being a female and δ the additive effect of increasing by 1 day the developmental time.

Sex ratios

Sex ratios were analyzed using a binomial generalized linear model (Faraway 2006) that included water quality, density and their interaction. The lack of significance for all these factors implies a 1 : 1 sex ratio, while significance in any of them implies a distorted sex ratio.

Results

Figure 1 and table 1 show water quality results for both streams, before and after an overflow. The pH

was the only physico-chemical characteristic that showed similar variability within and between streams, with no significant differences in mean value between the streams and following the overflow. Differences in dissolved oxygen were not significant either between streams or following the overflow, although the average value was slightly larger in the control stream. Both NH_4^+ and PO_4^- had significantly larger concentrations in the CSO stream when compared with the control stream. No difference was detectable in the concentration of NO_3^- between the streams, although the variability at the stream level was half that of individual samples.

An average of 62% of the larvae across all treatments emerged as adults. Figure 2 shows the survivorship schedule for the larvae reared at different densities and under different simulated larval habitats. With the exception of 0.66 larva/ml in tap water, no major effect of density on mortality is apparent, with similar average values for the two water quality treatments. However, as shown by the error bars, survivorship variability was higher in the
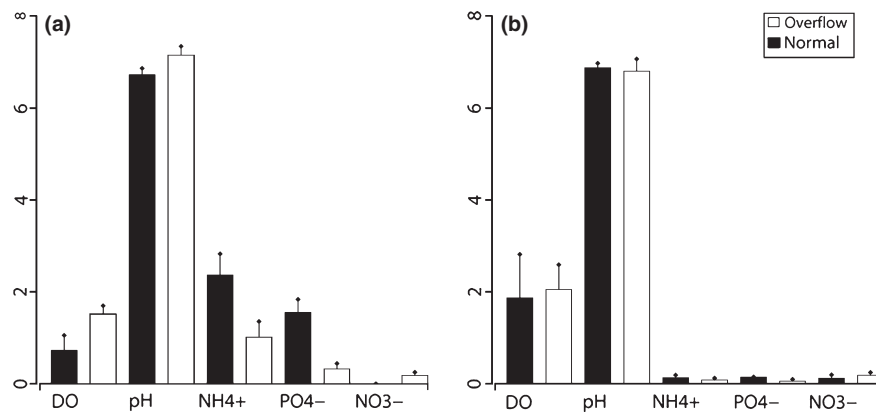

Fig. 1 Physico-chemical variables of water from a combined sewage overflow (a) and from a control stream (b), during normal (white bars) and following an overflow event (black bars) conditions. With the exception of pH all variables were measured in mg/l, lines indicate SE.

Table 1 Parameter estimates for the linear mixed effects models evaluating physico-chemical characteristics and nutrient contents in a combined sewage overflow and a control stream

Parameter (95% CI)	DO (mg/l)	pH	NH$_4^+$ (mg/l)	PO$_4^-$ (mg/l)	NO$_3^-$ (mg/l)
Control ($\hat{\mu}$)	1.64 (0.66, 2.63)*	6.67 (6.26, 7.05)*	0.68 (−0.18, 1.53)	0.62 (0.11, 1.13)*	0.056 (−0.059, 0.170)
CSO ($\hat{\alpha}$)	−0.77 (−1.75, 0.25)	0.13 (−0.28, 0.54)	1.47 (0.61, 2.36)*	0.73 (0.20, 1.24)*	−0.043 (−0.160, 0.073)
Overflow ($\hat{\beta}$)	0.52 (−0.43, 1.62)	0.28 (−0.07, 0.67)	−0.90 (−1.72, −0.10)	−0.82 (−1.30, 0.34)	0.15 (0.05, 0.27)*
Stream $\hat{\sigma}_\pi$	2.96×10^{-6}	0.24	1×10^{-4}	2.10×10^{-6}	0.05
Error $\hat{\sigma}_\varepsilon$	1.04	0.35	0.91	0.54	0.11

Values for parameters $\hat{\alpha}$ and $\hat{\beta}$ are additive in relation to the control.

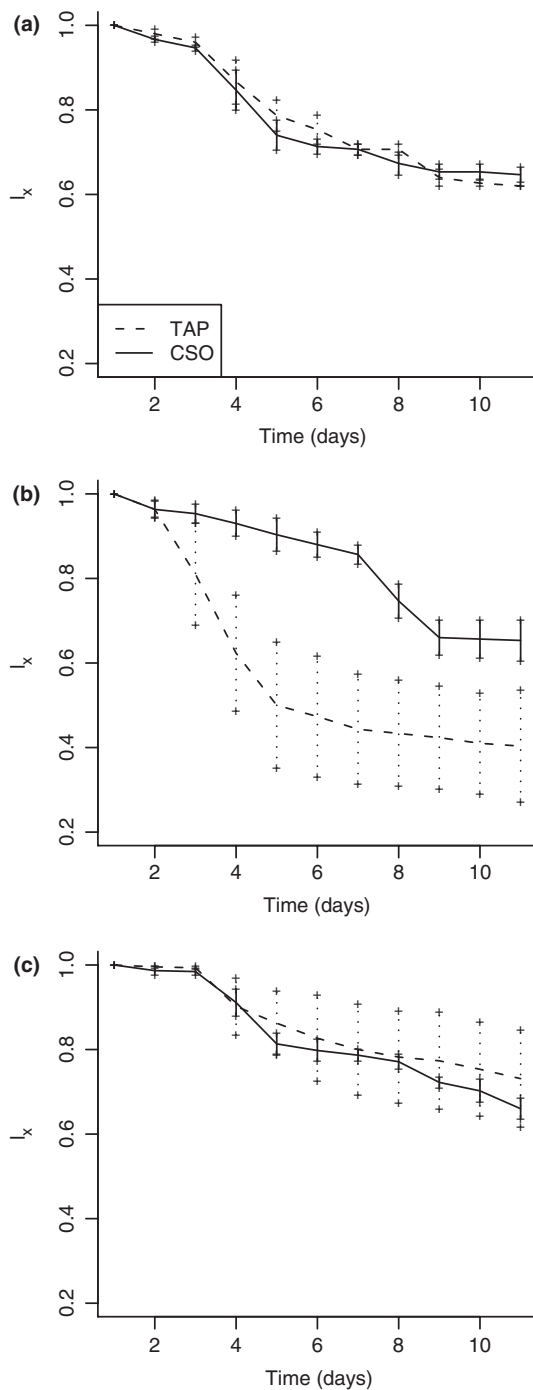CSO, combined sewer overflow system.

*Statistically significant (P < 0.05).

Fig. 2 Survivorship schedule (l_x) for mosquito larvae reared at three densities: (a) 0.33 larva/ml; (b) 0.66 larva/ml and (c) 1.00 larva/ml. Dashed lines indicate larvae reared in tap water, and solid lines larvae reared in combined sewer overflow system water, + indicate SE.

tap treatment across the three densities. Figure 3 shows different measurements of temperature, showing that for the 0.66 larva/ml density treatment, both maximum and minimum temperatures

were higher than for other treatments in days 4–6. Temperature for the 0.66 larva/ml density treatments also had a low variability when compared to other density treatments. However, overall temperatures were very similar for the rest of the study across containers. A more quantitative analysis showed that the model selected as best for the survival analysis included time, minimum temperature and water medium as statistically significant mortality hazards (likelihood ratio test = 89.3, d.f. = 3, P < 0.0001). Each day, mortality hazards [mean (95% CI)] increased 1.20 (1.13, 1.28) times. Each increase of one degree in minimum temperature above its mean increased the hazard 3.09 (2.19, 4.37) times and tap water increased the hazard of mortality 3.08 (2.15, 4.39) times when compared to CSO water. The binomial generalized linear model showed that odds of surviving to the adult stage were increased 1.48 (1.03, 2.11) times in CSO water, there was no significant difference in the odds of surviving to pupae according to water quality (P > 0.07), and odds of surviving to fourth instar larvae were 2.53 (1.56, 4.10) times larger in CSO water when compared to tap water. To compute the odds of survival to a given stage, models were adjusted for over-dispersion, thus making the testing for overall model goodness of fit not feasible (Faraway 2006). In all cases density had no effect either on the mortality hazards, or on the odds of survival to any given stage, and survival was always higher in CSO water, showing the robustness of both patterns.

A total of 1169 individuals emerged from all treatments. A subtotal of 622 emerged from the CSO water (325 females and 297 males), and 547 emerged from the tap water (252 females and 295 males). After drying, 67 individuals were discarded before weighing due to appendage loss, 26 (eight males, 18 females) and 41 (21 males, 20 females) from CSO and tap water, respectively. Figure 4 and table 2 show the effects of density and water quality on weight and time to adult emergence. Independent of water quality, females were bigger than males at emergence and took a longer time to develop (almost 1 day). The average weight at emergence was diminished by 1 μg per additional individual in the larval container. For each additional day of development, in comparison with the average for a given treatment and independent of sex and water quality, weight at emergence was increased by 13 μg. An increase in weight of close to 60 μg per imago was recorded in CSO water, independently of sex. In CSO water emergence was faster for both sexes, by approximately 0.5 days. With the single
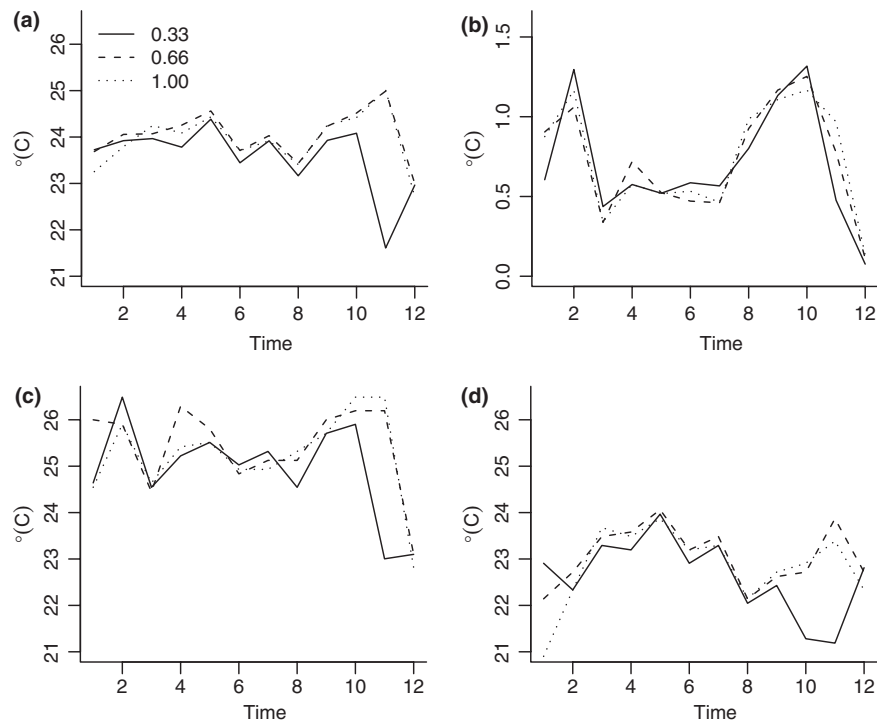
Fig. 3 Temperature (°C) measurements in the containers holding replicates for the three densities: (a) mean; (b) standard deviation (SD); (c) minimum and (d) maximum. Solid lines are for containers with a density of 0.33 larvae/ml, dashed lines for 0.66/ml and dotted lines for 1.00/ml, in a total volume 150 ml of rearing water.

exception of males in CSO water, where emergence was mostly synchronous at day 8, an increase in density delayed the time to adult emergence by 0.04 days for each additional individual. For both time and weight at emergence, about a quarter of the total variability was explained by the container where individuals were reared [i.e., container variability/(container variability + error)] with the other three quarters due to the individuals themselves [i.e., error/(container variability +error)]. We also computed the variability at the scale of the large containers, i.e. those holding the temperature dataloggers and smaller containers used to replicate the treatments, but the variability was nil at about 1 order of magnitude less than that of the small containers (not reported).

Finally, fig. 5 shows the sex ratios for the different levels of density and water quality. For CSO water the sex ratio tends to be more biased towards females with increasing larval density, while in tap water the ratio is more biased towards females with decreasing larval density. In fact, the binomial generalized linear model showed that for emerging adults the odds of being a female increased by 1.006 (1.003, 1.012) as a function of the interaction between density and CSO water. In tap water the odds did not change (P > 0.19). This model had a good fit to the data (deviance = 9.42, d.f. = 14, P > 0.05).

Discussion

Nutrient loading is a major ongoing ecological problem in urban streams worldwide (Grimm et al. 2008). The effects of urban stream pollution are a major determinant of the abundance of mosquito vectors in urban centres and the pathogens they transmit worldwide (Britton 1914; Sasa 1976; Calhoun et al. 2007). However, ecological understanding of the mechanisms explaining mosquito abundance in urban streams is insufficient. Although we have previously shown that water from these streams is more attractive than unpolluted water for oviposition of *Cx. quinquefasciatus* (Chaves et al. 2009), and extensive knowledge supports that chemical cues and pheromones can further enhance oviposition in these sites (Millar et al. 1994; Beehler and Mulla 1995) no association has been shown between the effects of highly attractive oviposition mediums and fitness of mosquito larvae.
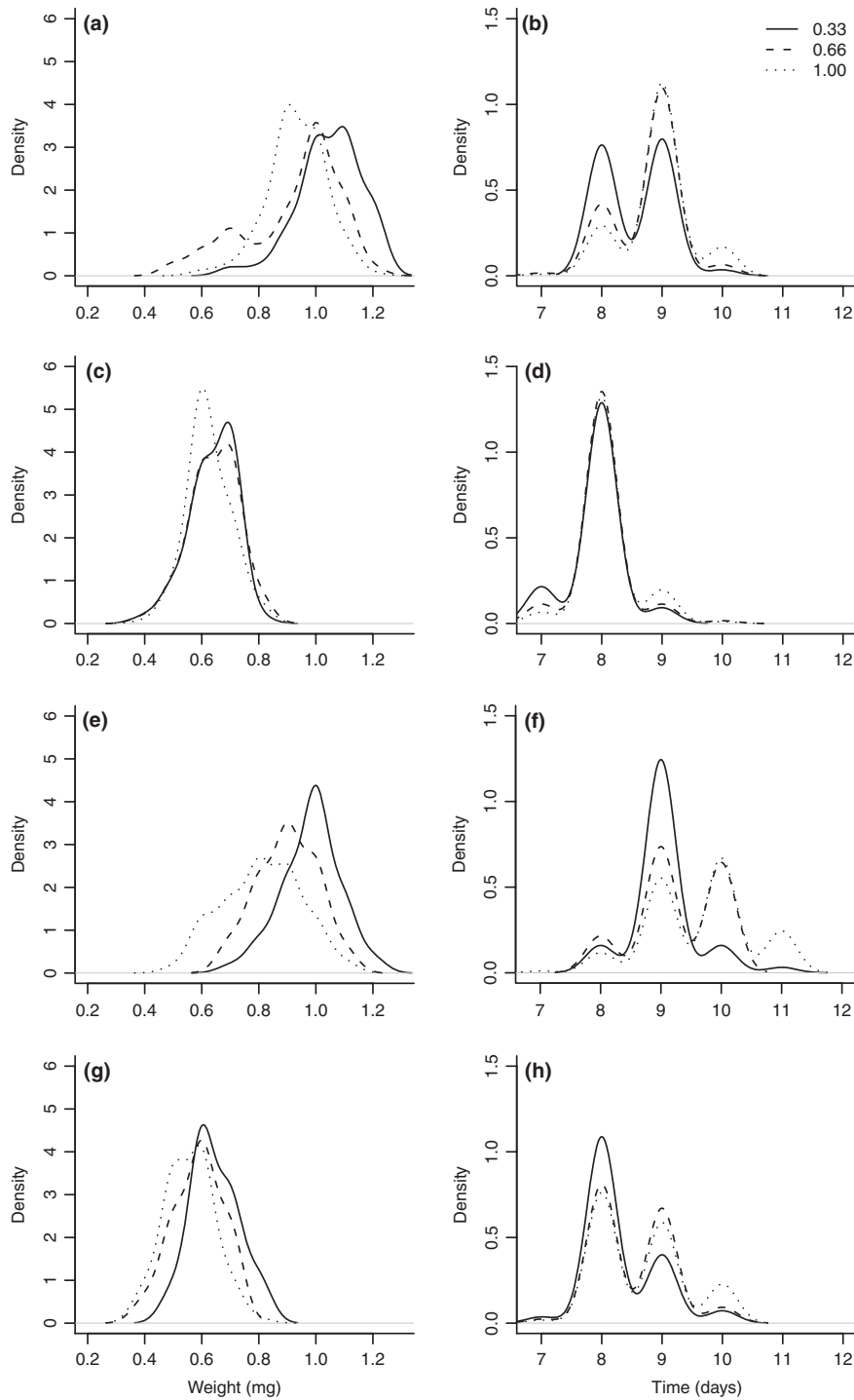
Fig. 4 Effects of density on weight and time to emergence of: females in combined sewer overflow system (CSO) water (a, b); males in CSO water (c, d); females in tap water (e, f) and males in tap water (g, h). The *y* axis show smoothed kernel densities for variables in the *x* axis.

The input of additional nutrients is known to increase the fitness of mosquito larvae in other Culicinae species, e.g., *Aedes triseriatus* (Walker et al. 1991). The CSO stream we studied has considerable large amounts of reduced biological forms of nitrogen (NH_4^+) when compared to other control streams not influenced by sewage overflows, where *Cx. quinquefasciatus* is absent (fig. 1). This nutrient is a major

Parameter	Covariate	Emergence (days) Estimate (95% CI)	Weights (mg) Estimate (95% CI)
$\hat{\mu}$	Males tap water	2.163† (1.871, 2.446)*	0.659 (0.603, 0.715)*
$\hat{\alpha}$	CSO water	−0.561 (−0.765, −0.367)*	0.0598 (0.0231, 0.0952)*
$\hat{\beta}$	Density	0.004 (0.001, 0.006)*	−0.0010 (−0.0013, −0.0005)*
$\hat{\gamma}$	Females	0.834 (0.763, 0.903)*	0.289 (0.274, 0.304)*
$\hat{\delta}$	Time to emergence	–	0.0139 (0.0037, 0.0240)*
$\hat{\sigma_\pi}$	Container variability	0.19	0.034
$\hat{\sigma_\varepsilon}$	Individual variability	0.60	0.102

Values for parameters $\hat{\alpha}$, $\hat{\beta}$, $\hat{\gamma}$ and $\hat{\delta}$ are additive in relation to males in tap water ($\hat{\mu}$).
CSO, combined sewer overflow system.
*Statistically significant (P < 0.05).
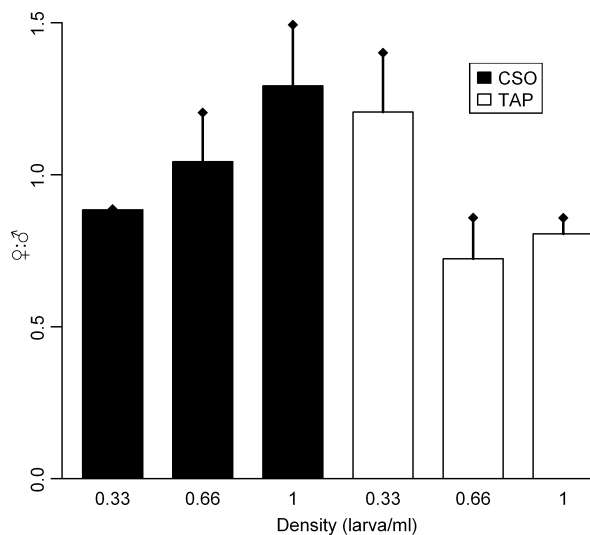†This value is relative to the first mosquito emergence (6 days).

Fig. 5 Sex ratios of emerging mosquitoes (females : males). Bars represent average ratios and lines indicate SE.

limiting resource for bacteria at the base of the food-web supporting culicine mosquitoes (Carpenter 1982). Thus, provided the increased basal availability of resources necessary for mosquito development, in our study enhanced by the protein addition, resource limitation is an unlikely mechanism for the population regulation of this species, and also may explain the increased abundance of positive dips for this species as function of overflow size (Calhoun et al. 2007).

In *Cx. quinquefasciatus* mosquitoes, development in nutrient-poor laboratory conditions results in smaller adults and longer development time, with some individuals delaying pupation in favour of increasing adult body size (Agnew et al. 2000). In *Cx. quinquefasciatus*, the mechanism of ecological interference

has been found in laboratory settings (Ishii 1963; Kurihara 1963; Agnew et al. 2000), and retardant growth factors have been reported for artificially high densities (Ikeshoji and Mulla 1970). Interference emerges as a product of larval density and not resource abundance, in *Cx. quinquefasciatus* proportional increases in resources still led to smaller adults (Agnew et al. 2000). The decrease in adult body size and distorted sex ratio with density may also lead to delayed density dependent effects. For example, in *Aedes albopictus* the reduced body size of adult mosquitoes in laboratory conditions leads to delayed density-dependent effects on population size (Tsuda et al. 1991). This effect could be expected to be stronger if we consider that a female biased sex ratio can reduce the per capita mating success of *Cx. quinquefasciatus*. However, further studies are required to better understand density-dependence regulation of *Cx. quinquefasciatus*, and more generally in mosquitoes before suggesting its absence when effects are not instantaneous.

Our results also highlight the importance of phenotypic plasticity as a product of the differential canalization of environmental factors by different stages of ontogenetic development (Schmalhausen 1949). As recognized in the early 1930s, the effects of the environment are likely to be more stringent at earlier developmental stages, and decrease as organisms become more autonomous of environmental conditions during their ontogeny (Janisch 1932). We observed how small differences in temperature can significantly increase the mortality hazard of *Cx. quinquefasciatus* larvae, especially when they occurred early during the experiment. Our analysis indicates that mosquito larvae are three times more likely to die for each additional degree of increase in the minimum daily water temperature when

compared to the average minimum temperature recorded in our study. A similar phenomenon has been observed for *Aedes aegypti* under experimental circumstances (Bar-Zeev 1958). Population models for mosquitoes under climate change have usually ignored this variable sensitivity of organisms to the external environment (Pascual et al. 2006). In fact, it appears that mosquito larvae in CSO water were more autonomous from external changes in temperature, as shown by the higher mortality and survival variability in treatments with tap water. This pattern further reveals stability as a potential advantage for mosquito survival in stable CSO microhabitats. Our study also reports an age increase in the mortality hazards of larval mosquitoes, which should be considered when formulating realistic dynamical models for *Cx. quinquefasciatus*.

Finally, our results are based on a manipulation, nutrient addition, that probably enhanced effects of the CSO water. We consider that additional studies could overcome this limitation by adding substrate from the stream and see if mosquitoes still show the same patterns and comparing emerging mosquitoes from creeks with different degrees of pollution. Also, comparison of mosquito development in water pre- and post-sewage overflow could further improve the understanding of mosquito development acceleration. However, insights from this study can help to better understand abundance patterns of *Cx. quinquefasciatus* in urban streams.

Acknowledgement

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