AQUEOUS NITRATES AND LARVAL COMPETITION ALTER DISEASE RISK FROM TWO URBAN MOSQUITO SPECIES

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Abstract. Mosquito-borne disease is (re)emerging worldwide and is becoming an increasing public health threat, particular in urban areas. However, many factors influencing the larval dynamics that regulate biting adult populations in urban landscapes are still unknown. This study evaluated how a common urban water pollutant and the sequence of larval hatching in container habitats influence the larval competitive interactions between two urban disease vectors, *C. pipiens* and *A. albopictus*. The study demonstrated strong evidence of asymmetrical competition, where *A. albopictus* developed faster and had higher pupation rates in all treatments. Higher nitrate levels in the larval environment resulted in larger *A. albopictus* females and higher emergence and faster time to emergence for *C. pipiens*. There were also indications of possible interactions between nitrates and priority effects. These results give insight into the abiotic factors important to each species and each species' life strategy, while also providing vital information that can be used to predict future disease risk from these vectors on a local scale.

INTRODUCTION

Mosquito-borne disease is becoming an increasingly important global health issue as their incidence has strikingly increased in the past 30 years (Gubler 2002). In particular, arboviruses (such as Dengue and West Nile virus), have become a larger threat as they have expanded their range globally and caused more frequent and/or larger epidemics (Gubler 2002).

Additionally, urbanization has been linked to increases in mosquito-borne diseases (Leisnham and Slaney 2009). Urban environments tend to have increased presence of water-holding container habitats for larvae stages (Leisnham and Slaney 2009), decreased or absent mosquito larvae predators (Leisnham and Slaney 2009), increased temperatures that promote faster larval development (Paz and Albersheim 2008), and an increased density of humans, and thus blood meals (Wilcox and Gubler 2005). All of these factors lead to higher population levels of mosquitoes and thus increased disease risk. The mosquito species typically found in urban areas are also the species that most frequently transmit disease to humans (Juliano & Lounibos, 2005, Wilcox & Gubler, 2005). As the larval stages are dependent on water habitat, it is likely that they are sensitive to water chemistry. Johnson (unpublished) found that nitrate and phosphorus varied in storm water ponds along an urban-to-rural gradient and that *Culex* species in particular were associated with higher nitrate levels. Similarly, Dowling et al. (2013) reported species-specific associations with water chemistry measures in urban container habitats.

Disease risk to humans from mosquito-borne arboviruses is dependent on local adult mosquito population size, species composition, and vector competency. Interspecies competition in the aquatic larval mosquito stage has also been shown to have significant effects on all three of these factors (Armistead et al. 2008;

Lounibos et al. 2003; Alto et al. 2005, respectively). Competition between mosquito species has been well documented in the lab and in the field (Juliano 2009). Competition is usually asymmetric, with one species being a better competitor than the other, and is often context-dependent (Juliano 2009). The outcome and extent of competition has been shown to vary with abiotic factors such as temperature (Carrieri et al. 2003), nutrient source (Costanzo et al. 2011), and habitat drying (Costanzo et al. 2005a).

One aspect of competition that has received little attention is priority effects, or the inter-stage competition that arises when one species inhabits a container before another. Mosquito larvae already present in a container could have a negative or positive effect on mosquito larvae that hatch later. Few studies have been done on the priority effects of mosquito larvae on mosquito larvae. Livdahl (1982) studied the intraspecies priority effects of a second cohort of the tree hole mosquito *Aedes triseriatus* on an initial cohort of *A. triseriatus* in the field and found that the second cohort had no significant effect on the first, but that the first cohort had severely negative effects on the survival of the second cohort. Sunahara and Mogi (2002) studied intraspecies and interspecies priority effects on *A. albopictus* and *Tripteroides bambusa*. The early presence of *A. albopictus* or *T. bambusa* had a negative effect on survival of *A. albopictus* larvae introduced later while the survival of later introduced *T. bambusa* larvae was less affected by the presence of the early larvae cohort.

Aedes albopictus and *Culex pipiens* are both container-breeding mosquito species known to co-occur widely in urban areas in the United States and parts of Europe (Carrieri et al. 2003, Costanzo et al. 2005b, LaDeau et al. 2013). *A. albopictus* is an invasive species in the United States that was introduced in the 1980s. It takes blood meals mainly from mammals but sometimes from birds (Estrada-Franco and Craig 1995). *A. albopictus* is also a voracious biter of humans and will bite both during the day and at night (Estrada-Franco and Craig 1995) making it a nuisance in the areas it has invaded. *A. albopictus* is a known potential vector for West Nile virus, Yellow fever virus, St. Louis encephalitis, dengue fever, and Chikungunya fever (Estrada-Franco and Craig 1995). *Culex pipiens* is a widespread mosquito in the United States and globally and a known vector of West Nile virus and St. Louis encephalitis in the eastern U.S. (Farajollahi et al. 2011). *C. pipiens* mainly takes its blood meal from birds, but in urban settings will also bite humans (Farajollahi et al. 2011).

Both *A. albopictus* and *C. pipiens* have been shown to inhabit the same containers in urban environments (Costanzo et al. 2005b, LaDeau et al. 2013), so the potential for competition and priority effects is present. Interspecies competition in a laboratory setting between synchronously hatched larvae of *A. albopictus* and *C. pipiens* has been demonstrated (Carrieri et al. 2003, Costanzo et al. 2005b). Carreri et al. (2003) found *A. albopictus* to be a superior competitor for food, though the magnitude of the competition asymmetry was dependent on temperature. Costanzo et al. (2005b) also showed *A. albopictus* to be the stronger competitor and to decrease *C. pipiens* survivorship and increase its development time. Costanzo et al. (2005b) pointed out the need for an inquiry into competition between asynchronous cohorts of these two species, which this study plans to address.

In an urban environment, *A. albopictus* and *C. pipiens* have been associated with specific water quality traits. *A. albopictus* has been positively associated with container water having high levels of nitrite and nitrate and negatively associated with high levels of phosphorus (Dowling et al. 2013). Meanwhile, *C. pipiens* has been negatively associated with high levels of nitrite, nitrate, and phosphorus in container habitats (Dowling et al. 2013), but also with high levels of nitrate in storm water ponds (Johnson and LaDeau, unpublished). It is unclear whether the relationship is due to preferential ovipositioning, occurs as a result of larval presence, or is due to altered competition that favors one species over another under those water conditions. This study will evaluate how different levels of nitrate and the sequence of larval introduction influence the competitive interactions between *C. pipiens* and *A. albopictus*.

The main questions of this study are: 1) Do priority effects alter the survival, time to emergence, or adult size of *A. albopictus* and *C. pipiens*? 2) Do different levels of nitrate alter the survival, time to emergence, or adult size of *A. albopictus* and *C. pipiens*? and 3) Do different levels of nitrate alter the extent or presence of priority effects in *A. albopictus* and *C. pipiens*?

METHODS

Larval *A. albopictus* and *C. pipiens* were raised in mesocosms inside growth chambers to test for priority effects and the effect of varying nitrate levels on survival to emergence, time to emergence, and female wing length.

Mosquitoes were hatched and later raised in a leaf nutrient solution made from combining dried deciduous leaf litter and carbon-filtered well water. The container was sealed and placed outside in ambient temperature for four days during June and July 2013 in Millbrook, NY. The solution from each cooler was strained, mixed together, and then stored inside in a covered plastic bucket at room temperature until used.

Culex pipiens eggs were obtained from Paul Leisnham at the University of Maryland while A*edes albopictus* eggs were obtained from mixed-origin colonies at Rutgers University. Eggs were placed into shallow (less than 5cm deep) white plastic hatching containers with a clear plastic lid that contained leaf nutrient solution. Within 48 hours of hatching, the larval were transferred to their experimental mesocosm. The mesocosms were black plastic cups (opening diameter: 10.0 cm, bottom diameter: 7.4 cm, height: 17.0 cm) filled with 400 ml of leaf nutrient solution. Treatment nitrate concentrations were chosen to represent a nitrate concentrations observed in urban container habitats (Dowling et al. 2013). To achieve high and low nitrate treatments, 2.7 ml of a 300 mg/L nitrate solution was added to the high nitrate treatment cups (for a treatment nitrate concentration of 2.0 mg/L) and 2.7 ml of DI water was added to the low nitrate (no added nitrate) treatment cups.

On Day 1 of treatment, 15 newly hatched larvae of *Culex pipiens* or *Aedes albopictus* were added to both high and low nitrate treatment cups. On Day 3, 15 newly hatched larvae of *Culex pipiens* or *Aedes albopictus* were added. Thus there were four species combination treatments (first cohort/second cohort): *C. pipiens*/*C. pipiens*, *C. pipiens*/*A. albopictus*, *A. albopictus*/*A. albopictus*, and *A. albopictus*/*C. pipiens* and two levels of nitrates, high and low, for eight total treatments. There were six mesocosms for each treatment which were split equally among three climate controlled growth chambers, each treated as a block, for a total of 48 mesocosms. The growth chambers were set for a cycle of 14 hours of light with a temperature of 30.0° C and 10 hours of dark at a temperature of 21.0° C. 30 ml of leaf nutrient solution was added to each mesocosm every third day (Day 3, Day 6, Day 9, etc.). At least two mesocosms of each treatment were checked each day until pupae were seen, and then all cups of a treatment were checked each day for pupae. *A. albopictus*/*A. albopictus* and *A. albopictus*/*C. pipiens* mesocosms were started on June 10th, 2013 while *C. pipiens*/*C. pipiens* and *C. pipiens*/*A. albopictus* were started on June 12th, 2013. Each mesocosm was run until all mosquitoes were emerged or dead or were stopped on August 5th, 2013.

Once a pupae developed in the mesocosm, it would be removed along with 2 ml of solution from its mesocosm and placed into a small (about 50 ml) clear vial or translucent plastic sample bottle with lid. The pupae would be replaced to their respective growth chambers, until they emerged as adults or were determined to be dead (no movement or complete emergence after 3 or more days). Emerged adults were placed into a freezer in their containers for at least 24 hours. Emerged adults from each treatment were pooled and air dried before being examined under the microscope at 60X magnification. For each mosquito date of emergence, sex, species, and wing length was recorded.

All statistical summaries and analyses were computed using the R Statistical Software (Version 2.15.0). Differences between wing length and emergence metrics across treatments were evaluated using alpha = 0.05 using linear and generalized linear models as appropriate (lm and lmer functions).

RESULTS

The results indicate very strong evidence of asymmetrical competition which greatly favored *A. albopictus*. *C. pipiens* females had a larger wing length (average of 1.78) than *A. albopictus* females (average of 1.54) regardless of treatment. *A. albopictus* females emerged in much larger numbers (n=200) than *C. pipiens* females (n=20) and *A. albopictus* females emerged faster than *C. pipiens* females. More *A. albopictus* females emerged from mixed species *A. albopictus* and *C. pipiens* treatments than single species *A. albopictus* treatments, while more *C. pipiens* emerged from single species *C. pipiens* treatments than mixed species treatments.

The addition of nitrate affected number of emergences and time to first emergence of *C. pipiens*. The number of adults emerged was significantly greater for *C. pipiens* females raised in higher nitrate conditions (CC: $p=0.006$; CA: $p=0.011$), although there was no nitrate benefit when *C. pipiens* were the second cohort behind *A. albopictus* (AC: $p=0.831$) (Figure 1). The rate of emergence was also affected by nitrate treatment with *C. pipiens* in CC emerging significantly faster when nitrates were added to their aqueous environment ($p<0.001$), though still slower than *A. albopictus* with or without nitrates (as can be seen in the AA treatment results) ($p<0.001$) (Figure 2).

Figure 3 shows that *A. albopictus* females were larger in the presence of *C. pipiens* than when raised with another cohort of *A. albopictus*, and largest when *A. albopictus* was the second cohort (CA; $p=0.39$). When *C. pipiens* was raised as the second cohort behind *A. albopictus* no *Culex* females emerged (Figure 3). Further, *C. pipiens* females raised with a younger, second cohort of *A. albopictus* were significantly smaller than those raised with other *C. pipiens* ($p=0.001$) (Figure 3).

There was also evidence of an interaction between order and identity cohort (priority effects) and added nitrates. *A. albopictus* females were significantly larger (based on wing length) when added as a second cohort to *C. pipiens* and when nitrates were added to the aqueous larval environment indicating a potential interaction between these biotic and abiotic factors (Figure 4).

DISCUSSION

In this study we were not able to conclude that there are significant priority effects between cohorts of *C. pipiens* and *A. albopictus* under these conditions due to a low number of *C. pipiens* emergences. However, we did find that *C. pipiens* and *A. albopictus* show clear asymmetrical competition that strongly favors *A. albopictus* which supports the results of other studies (Carrieri et al. 2003, Costanzo et al. 2005b). We also found that the addition of nitrates to the ambient levels in our mesocosms did affect both *C. pipiens* and *A. albopictus* in unique ways. *C. pipiens* emerged faster and in larger numbers when nitrates were added, though the females still emerged at the same size. Conversely, when additional nitrates were added to *A. albopictus*, they took the same amount of time to emerge and emerged in similar numbers, but the females that emerged were larger. These contradictory effects of additional nitrates may indicate different life strategies for *C. pipiens* and *A. albopictus*, in which *A. albopictus* tries to get the largest number of offspring to adults as quickly as possible, disregarding size while *C. pipiens* is willing to spend more time in the larval stage and emerge in lower numbers in order to produce larger, more fit adults. In previous mosquito abundance surveys, *A. albopictus* population size has been greatest later in the season and *C. pipiens* population size has remained somewhat constant over the course of the season (Dowling et al. 2013, Costanzo et al. 2005b), which is compatible with the suggested life style differences of these two species. Our study did find a potential interaction between cohort order and identity and the addition of nitrates, with emerged female *A. albopictus* that are part of a second cohort after *C. pipiens* being significantly larger when nitrates are added.

The mechanism for how nitrates effect the outcomes this study measured was not tested, but it is plausible that the nitrates promote bacterial growth and thus provide more food resources to the larvae than when additional nitrates are not added. The differing reactions of the two species thus results from different reactions to increased resources rather than a direct effect of the nitrates in the larvae themselves.

Field studies show that *A. albopictus* and *C. pipiens* continue to co-occur so frequently in urban container habitats (Carrieri et al. 2003, Costanzo et al. 2005b, LaDeau et al. 2013), yet the Lotka-Volterra model of species coexistence would indicate that the two species cannot coexist since interspecific competition of *A. albopictus* is greater than the intraspecific competition of *C. pipiens* (Gordon 2000). Perhaps the different life strategies based on food resource allocation seen in the results of this study could provide a more behavioral explanation for the continued presence of *C. pipiens* in habitats also occupied by *A. albopictus*. Alternatively, the large amount of asymmetrical competition between these two species may lead to *A. albopictus* replacing *C. pipiens* in currently shared environments, which would likely lead to a drastic shift in mosquito-carried disease risk in that area as the two species are competent vectors for different diseases (Estrada-Franco and Craig 1995, Farajollahi et al. 2011).

The effects of additional nitrates on these two species and their interactions could also lead to changes in disease risk patterns. Higher nitrate level could lead to higher numbers of biting adult female *C. pipiens* in urban areas thus increasing the potential for disease transmission, particularly of West Nile virus. The effect of nitrates on increasing the size of *A. albopictus* under select conditions could also potentially alter disease risk patterns. In a different mosquito genus (*Anopheles*) the frequency of mosquito infection with *Plasmodium falciparum* (malaria parasite) was highest among intermediately sized mosquitoes (Lyimo and Koella 1992), indicating a potential correlation between mosquito size and disease transmission risk, which may also be applicable to *A. albopictus* and the diseases it transmits.

Further, knowledge of the species interactions between *C. pipiens* and *A. albopictus* and their reactions to increased levels of nitrates commonly found in the urban areas they inhabit will provide a greater ability to create disease risk models that incorporate data at a more local spatial scale.

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APPENDIX

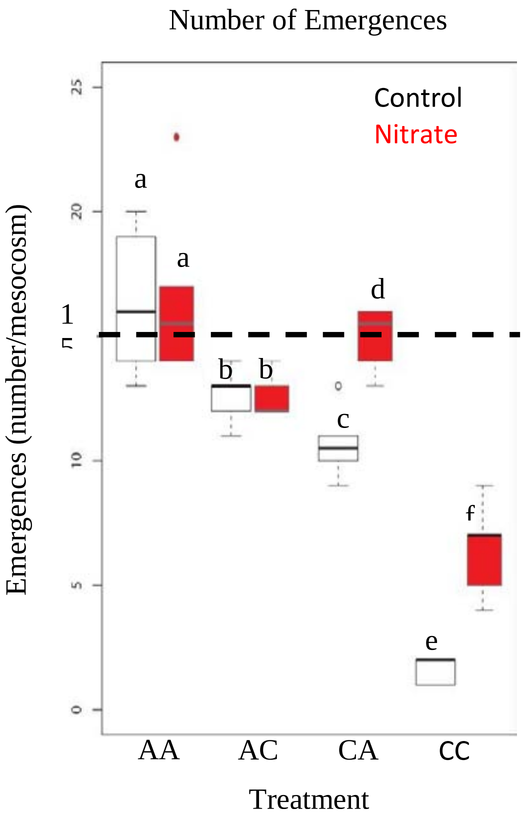

FIGURE 1.

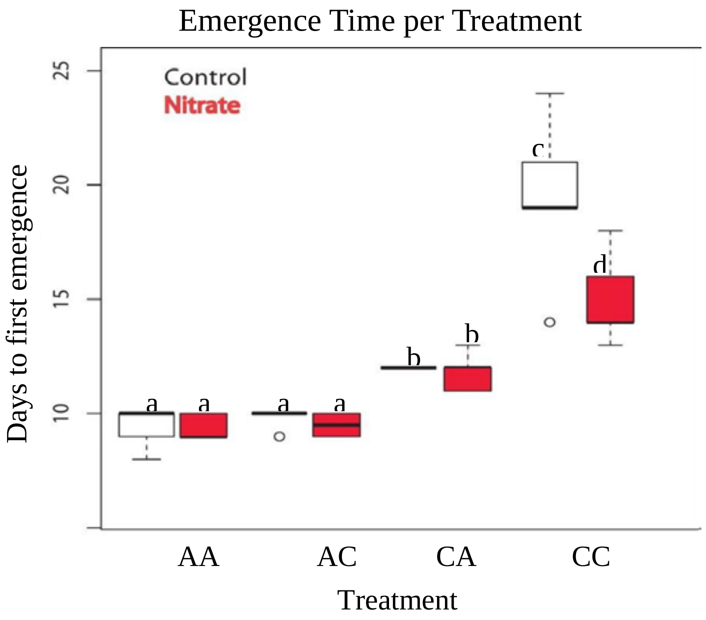

FIGURE 2.

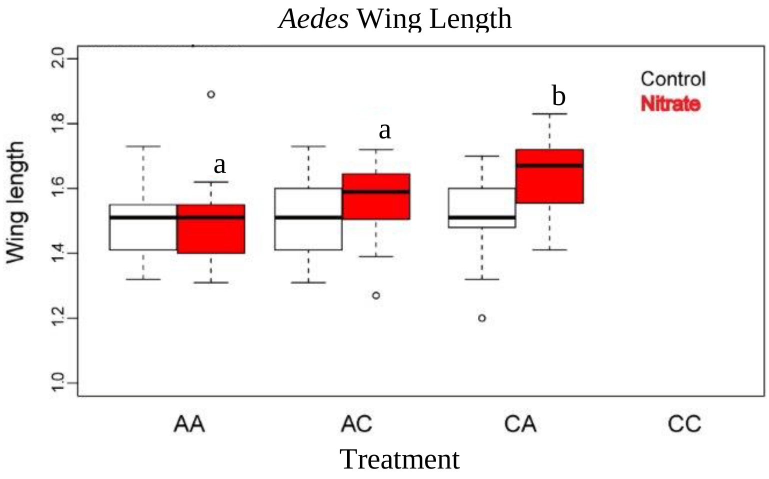

FIGURE 3.

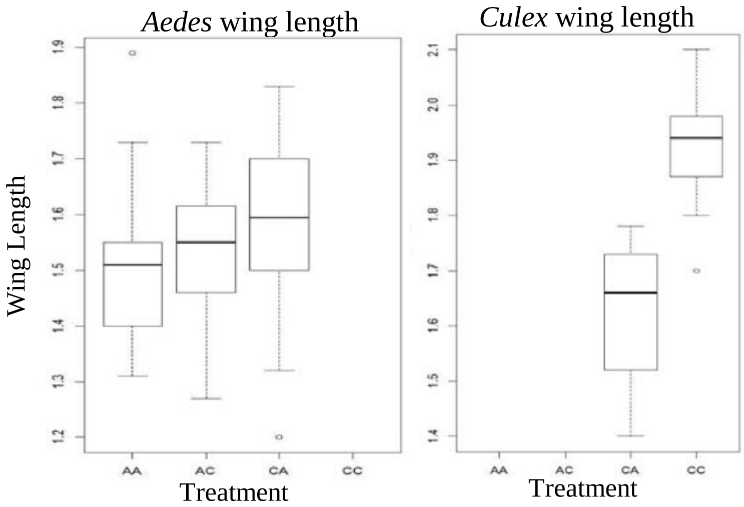

FIGURE 4. Box plot of emerged female wing lengths (on a relative scale) for *A. albopictus* (left) and *C. pipiens* (right). Note different y-axis scale. Treatments: AA = 1st cohort *Aedes*, 2nd cohort *Aedes*, AC = 1st cohort *Aedes*, 2nd cohort *Culex*, CA = 1st cohort *Culex*, 2nd cohort *Aedes*, CC = 1st cohort *Culex*, 2nd cohort *Culex*.