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Environmental Impacts on life history in container breeding mosquitoes

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ENVIRONMENTAL IMPACTS ON LIFE HISTORY

IN CONTAINER BREEDING

MOSQUITOES

Kathleen M. Westby

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In this dissertation I explore different ways that the environment impacts life history in mosquitoes in ways that may alter vectorial capacity. In chapter I, I tested if short-term sugar deprivation experienced after exposure to La Crosse virus altered infection rates in *Aedes albopictus* and if sugar treatment and virus infection status altered blood feeding behavior and fecundity. I found no evidence that sugar deprivation impacted infection rates or fecundity. Sugar deprivation did increase blood feeding. There was no effect of infection status on blood feeding or fecundity. In chapter II, I tested for effects of seasonal cues (temperature and photoperiod) experienced during larval development on *Aedes triseriatus*. I found reduced larval survivorship, increased development rate, and smaller sizes when larvae were reared under warmer conditions with a shorter photoperiod. I found no effect of seasonal rearing temperatures on adult longevity and a significant size and treatment interaction for fecundity. In chapter III, I tested the effects of larval habitat age and prior habitat exploitation by a previous non-overlapping cohort of larvae on female larval survivorship, development rate, size and adult longevity. I found that larvae reared in older habitats had increased survivorship

faster development, and resulting adults had increased longevity. Prior exploitation

significantly prolonged development rate only. In chapter IV, I investigated the impacts

of larval habitat size and habitat drying on mosquito communities. I found that larval

densities differed over time only in the smallest containers and that two weeks was

sufficient for these communities to rebound to densities observed in stable volume

habitats after they were completely dried and refilled. Predatory species density was not

affected by habitat size or drying. Predator density did not affect prey density but did

significantly decrease adult longevity which indicates negative trait-mediated effects of

predation on prey species.

ENVIRONMENTAL IMPACTS ON LIFE HISTORY

IN CONTAINER BREEDING

MOSQUITOES

KATHLEEN M. WESTBY

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ENVIRONMENTAL IMPACTS ON LIFE HISTORY

IN CONTAINER BREEDING

MOSQUITOES

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CHAPTER I

SHORT TERM SUGAR DEPRIVATION: EFFECTS ON LA CROSSE VIRUS

INFECTION WILLINGNESS TO BLOOD FEED, AND FECUNDITY

IN *AEDES ALBOPICTUS* (DIPTERA: CULICIDAE)

Abstract

Resource availability has the potential to impact greatly host life history characteristics and response to parasitism. This effect of resources can arise when the affected host is a vector of the parasite that exploits it. Body condition can alter vector competence (condition-dependent competence), and other phenotypic traits such as blood feeding behavior and fecundity that impact vectorial capacity in multiple vector- parasite systems. Infection with arboviruses can have measurable fitness costs, though costs are not identified in all mosquito-virus interactions. Condition-dependent virulence is observed when usually benign infections become increasingly virulent for the host (or vector) under resource stress. We tested the hypotheses of condition-dependent competence and condition-dependent virulence by manipulating short-term sugar deprivation in *Aedes albopictus* (Skuse) in La Cross Virus (LACV) exposed and unexposed control females. We predicted that infection status interacts with sugar deprivation to alter willingness to blood feed and fecundity in the second gonotrophic cycle (condition-dependent virulence). Sugar deprivation had no effect on body infection or disseminated infection

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rates. Infection status, sugar treatment, and the interaction had no effect on fecundity.

There was a significant effect of sugar treatment on willingness to blood feed with sugar

deprived females being more willing to take a second blood meal. Infection status and the

interaction with sugar treatment had no effect on blood feeding behavior. In this

experiment, we found no evidence of short-term sugar deprivation leading to condition-

dependent competence for LACV or condition-dependent virulence of LACV in *A.

albopictus*.

Introduction

The environmental conditions that mosquitoes experience, in both the larval and adult

stages, can have profound impacts on population growth. Limited resource availability in

the larval habitat, whether due to low resource quantity, poor resource quality, or

crowding, has been shown to reduce larval survivorship, adult longevity, and fecundity

(Costanzo et al. 2005, Reiskind and Lounibos 2009, Gilles et al. 2011, Alto et al. 2012,

Muturi et al. 2012). Sugar and blood meal availability for the adult mosquito can also

impact longevity and fecundity (Nayar and Sauerman 1975, Naksathit and Scott 1998,

Mostowy and Foster 2004, Vaidyanathan et al. 2008, Xue et al. 2010). Resource

availability affects mosquito immune function (Suwanchaichinda and Paskewitz 1998,

Koella and Sorenson 2002, Telang et al. 2012, Ponton et al. 2013) presumably because

immune function is costly (Ahmed et al. 2002, Schwartz and Koella 2004). Resource

availability also can impact vector competence for viruses, *Plasmodium*, and filarial

parasites (Okech et al. 2004, 2007, Vaidyanathan et al. 2008, Alto and Lounibos 2013,

Breaux et al. 2014). This "condition-dependent competence" and the combined effects of

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resource limitation on vector life history traits can influence vectorial capacity (Dye 1986, Moller-Jacobs et al. 2014).

Arbovirus infections of their mosquito vectors were traditionally thought to be necessarily benign (Ewald 1994). For the pathogen, it would be disadvantageous to disable the vector to the point where she is not robust enough so survive the extrinsic incubation period (EIP), which is the time from ingestion of the pathogen until the vector is able to transmit the pathogen, or to successfully locate and to feed on a new host, which is the ultimate criterion for the mosquito to be a functionally competent vector (Hardy et al. 1983, Breaux et al. 2014). A pathogen must, however exploit the vector as a resource to develop or to replicate sufficiently to be infective and to reach the salivary glands to be transmitted (Hardy et al. 1983). Vectors are not passive carriers of their pathogens and insect vectors have multiple immune responses that they can employ to defend against infection (Murdock et al. 2013, Blair and Olson 2014). Experimental evidence is mounting that arbovirus infections can increase mortality, reduce fecundity, and alter feeding behavior in many arbovirus-mosquito systems (Lambrechts and Scott 2009). Costs of arboviral infection are not detected in every vector-virus system and this result may arise because experiments have been conducted under ideal conditions that mask any deleterious effect of infection or any potential fitness cost of mounting an immune response (Tripet et al. 2008). Stressful conditions may turn what is normally a benign infection into one that produces measurable fitness costs of infection (Brown et al. 2000).

This condition-dependent virulence has been described for multiple insect orders and pathogen taxa under both nutritional and temperature stresses (Donegan and Lighthart 1989, Brown et al. 2000, Mitchell et al. 2005). The assumption is that resources are limited and the cost of fighting an infection diverts resources away from other functions such as body maintenance and reproduction, so the more limited resources are, the more pronounced the cost of infection may be (Tripet et al. 2008). Increased virulence and pathogen transmission have been observed for *Aedes aegypti* exposed to a microsporidian parasite across a larval food gradient (Bedhomme et al. 2004). *Anopheles stephensi* infected with *Plasmodium* showed reduced survivorship depending on sugar water availability and parasite genotype (Ferguson and Read 2002). Female *Culex quinquefasciatus* infected with West Nile Virus showed differential mortality across a temperature gradient compared to uninfected controls (Alto et al. 2014). Little is known about the potential relationship between condition-dependent competence and condition-dependent virulence in mosquito vectors.

La Crosse virus (LACV) (Family: *Bunyaviridae*) is an important cause of pediatric encephalitis in the United States and is maintained in the mosquito vector through horizontal and vertical transmission with often high transovarial transmission rates (Hughes et al. 2006). *Aedes triseriatus* is the primary natural vector but invasive *Aedes albopictus* and *Aedes japonicus* may also be vectors (Gerhardt et al. 2001, Erwin et al. 2002, Lambert et al. 2010, Harris et al. 2015, Westby et al. *unpublished data*). Three separate studies have demonstrated condition-dependent competence of LACV for *A. triseriatus*, and all three studies manipulated resource availability for larvae (Grimstad

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and Haramis 1984, Grimstad and Walker 1991, Bevins 2008). LACV infection also decreases egg overwintering success for *A. triseriatus* which is a time of stress due to the cold winter temperatures experienced in the northern parts of *A. triseriatus's* range and could be considered condition-dependent virulence (Mcgaw et al. 1998). LACV infection alters blood feeding behavior in *A. triseriatus* and *A. albopictus*, perhaps due to pathological effects of infection or due to a cost of fighting the infection (Grimstad et al. 1980, Jackson et al. 2012). Under benign laboratory conditions, LACV infection did not affect female survivorship or fecundity in the first gonotrophic cycle for either *A. triseriatus* or *A. albopictus* (Costanzo et al. 2013). Because LACV has altered infection rates under conditions of resource stress for larvae, has increased virulence for the vector under stressful temperature conditions, can alter feeding behavior, and is transovarially transmitted only after the first gonotrophic cycle (Miller et al. 1979) it is then reasonable to test hypotheses about condition-dependent competence and virulence in this vector-parasite system.

We focus on the nutritional stress caused by sugar deprivation in part because that stressor is known to induce both condition-dependent competence and condition-dependent virulence in at least one mosquito-arbovirus system and is an important part of mosquito biology (Vaidyanathan et al. 2008). We tested whether the nutritional stress of short-term sugar deprivation during the extrinsic incubation period in adult females has an effect on LACV vector competence in *Aedes albopictus* and if sugar deprivation interacts with infection status to affect mosquito fitness, measured as willingness to take a blood meal and egg production (condition-dependent virulence).

Materials and Methods

Aedes albopictus used in this experiment were from several generations of a laboratory colony originating from Effingham, IL and maintained in the laboratory for one year. Larvae were reared in 10 cohorts of 250 individuals in one liter of water with 0.15 g bovine liver powder. Water was replaced and 0.085 g bovine liver powder was added every three to four days. Pupae were removed daily and allowed to emerge in 20 mL vials. After emergence, adults from each larval pan were assigned to one of the four treatments: infectious blood/constant access to sugar, non-infectious blood/constant access to sugar, infectious blood/intermittent access to sugar, non-infectious blood/intermittent access to sugar, so that equal numbers of females from each pan were assigned to each treatment daily. Females were housed in groups in one liter paperboard cages by date of emergence with males that were less than or equal to five days old in a roughly 1:1 male:female ratio and given *ad libitum* access to a 20% sugar solution Four day old females were sugar starved and given water only for 48 hours prior to being offered a blood meal. Larvae were reared and adults were held at 25°C with a 14:10 L:D photoperiod.

At six days old, females were offered an infectious or non- infectious blood meal for 40 minutes using an artificial membrane feeder (Hemotek$^{®}$, Lancashire, United Kingdom). Adults were then cold anesthetized and blood fed females were removed and housed in group cages by date of feeding and treatment. Females were given a chance to feed on three consecutive days to maximize sample size. Sugar treatments began the day a female took the initial blood meal. Those in the constant sugar treatment were given continuous

access to 20% sugar solution, delivered via cotton pads. Those in the intermittent sugar group were given 20% sugar for 24 hours and then given only water for the next 72 hours. This regimen was repeated until the end of a 15 day extrinsic incubation period (EIP). All sugar and water pads were replaced daily to minimize any effects of drying on the concentration of sugar available to the mosquitoes. The group cages contained a small, water-filled plastic cup lined with seed germination paper for oviposition.

At 15 days post blood meal, all surviving females were offered a non-infectious blood meal for 40 minutes on each two consecutive days. All females, whether they took the second blood meal or not, were then transferred to individual 300 mL paperboard cages with an oviposition substrate. Five days after the second blood meal females were killed by freezing and stored in individual tubes at -80^OC. All eggs laid in the individual cages were counted. Females were dissected on fresh slides with flame sterilized forceps, and abdomens dissected, retained eggs counted, and bodies and legs stored separately at -80^oC. Eggs laid and eggs retained were summed and analyzed together as total eggs produced in the second gonotrophic cycle. Eggs laid in the group cages during the 15 day intrinsic incubation period were not analyzed due to the unknown infection status of the females. Wings were discarded as they were too damaged to get an accurate size measurement.

Infectious blood meals were prepared by inoculating 400 µL of LACV stock virus for an initial inoculum of 3.94 x 10^6 plaque forming units (LACV/human/1960, GenBank accession numbers EF485030-EF485032) on confluent monolayers of Vero cells in 75 cm^2 vented tissue culture flasks. Flasks were incubated at 37° C with 5% CO_2 for one

hour at which time 15 mL of Leibovitz's L-15 media (plus 1% penicillin-streptomycin and 10% fetal bovine serum) was added and held for 72 hours to allow for virus amplification. To determine blood meal titer 500 µL of the media was stored at -80°C. The medium containing virus was mixed in a 1:1 ratio with defibrinated bovine blood which created blood meals of (mean $\pm$ SE) 3.2 x 10^{6} $\pm$ 1 pfu per mL determined via plaque assay in Vero cells. The non-infectious blood meal was prepared with a 1:1 ratio of uninfected medium prepared in the same manner as the infectious media minus virus and blood.

Bodies and legs were separately homogenized in 200 µL of L-15 medium with one stainless steel BB using a Retch shaker for 4 minutes at 380 rpm. Another 800 µL of medium was added to each sample and then centrifuged for 10 minutes at 1000 rpm. Samples were stored at -80°C. Infection status was determined by inoculating 200 µL of supernatant onto confluent Vero cell monolayers in 24 well plates with a positive and negative control on each plate. After 72 hours, a sample was considered positive if there were visible cytopathic effects. Infection status of those females offered an infectious blood meal was classified as either 1) exposed but not infected 2) exposed and infected but not disseminated (body infected but legs not) or 3) exposed and infected and disseminated infection (both body and legs infected).

Total eggs in the second gonotrophic cycle was analyzed as a two-way ANOVA (SAS PROC GLM) with sugar treatment, infection status, and the interaction as the independent variables. The residuals were approximately normal and met the assumption of homogeneity of variance. The effects of sugar treatment (constant, intermittent),

infection status (not exposed; exposed-not infected; exposed-nondisseminated; exposed-disseminated), and the interaction on the taking of the second blood meal (yes, no) were analyzed with a χ^2 test (SAS PROC CATMOD). The effect of sugar treatment on infection status for those females offered an infectious blood meal was analyzed with a 2 x 4 table χ^2 tests with "died before the end of the EIP", "not infected", "body infection only", and "disseminated infection" as the four possible outcomes (SAS PROC CATMOD). All analyses were performed in SAS 9.3 (SAS User's Guide, SAS Institute Inc., 2011).

Results

 A total of 418 females took the initial blood meal and were entered in the experiment. The four treatment groups had roughly equal number of females (Table 1.1.). A total of 51 females died before the end of the extrinsic incubation period (EIP) (Table 1.1.). The combined body infection rate (i.e., nondisseminated infection) for the two sugar treatments was 41.2% of those taking an infectious meal (Table 1.1.). The combined disseminated infection rate for the two sugar treatments was 20.9% of those taking an infectious meal (Table 1.1.) or about 1/3 of the total number infected (Table 1.1.). Sugar treatment had no effect on the four possible outcomes of infection status (died before EIP, not infected, body infection only, disseminated infection ($\chi^2 = 1.42$; df = 3; p = 0.7002). There was no significant effect of infection status on willingness to take the second blood meal, but there was a significant effect of sugar treatment, with those individuals given intermittent sugar more likely to take a blood meal than those given constant access to sugar. The interaction between infection status and sugar was also not

significant (Table 1.2.; Fig. 1.1.). There was no significant effect of LAC infection status, sugar treatment, or the interaction on the number of eggs produced in the second gonotrophic cycle (Table 1.3.; Fig. 1.2.).

Discussion

The environmental conditions that mosquitoes experience can have profound effects on their responses to parasitism, both for pathogens they transmit and for strict pathogens of mosquitoes. Numerous studies have demonstrated that factors such as nutritional status, temperature, and sublethal effects of insecticides alter infection and transmission rates for vectors of disease (Vaidyanathan et al. 2008, Muturi et al. 2011a, Muturi et al. 2011b, Alto et al. 2014). Additionally, these same factors can alter other life history characteristics that will influence population rate of increase and vectorial capacity (e.g., development rate, longevity, fecundity; Delatte et al. 2009, Muturi et al. 2010). The life stage when the stress occurs, and how long the stress persists, may also be important factors in determining whether there are meaningful effects on vector competence and other life history traits. In this experiment we imposed short-term sugar deprivation, specifically during the extrinsic incubation period, and found no condition-dependent effects on vector competence of or fitness correlates of *Aedes albopictus* exposed to La Crosse Virus (LACV).

Other studies demonstrated higher LACV infection rates in *Aedes triseriatus* when larvae were reared under suboptimal food conditions (Grimstad and Haramis 1984, Grimstad and Walker 1991, Bevins 2007). It was reasonable then to predict that suboptimal adult nutrition would also lead to higher infection rates in our experiment. One reason we may

have not observed an effect of sugar deprivation is that our treatment may have induced

little actual stress or may not have produced females with suboptimal body condition.

Mosquitoes could have been able to compensate for the three days without access to

sugar by imbibing larger sugar meals during the 24 hours they had access to sugar. We

had no means of controlling and did not measure the sizes of sugar meals or frequency of

sugar meals in this study. Experiments using a malaria model found significantly

different infection rates when sugar was withheld for only 24 hours, which indicates that

72 hours should have been long enough to observe an effect if it was biologically relevant

in this vector-parasite system (Ferguson and Read 2002). It could also be that 20% sugar

solution was sufficiently concentrated for the females to store ample reserves to

compensate for the 72 hour "stress" period without sugar. Takahashi (1976) gave *Culex*

tritaeniorhynchus females either 1% or 8% sugar after exposure to Japanese Encephalitis

Virus and found no difference in virus secretion in mosquito saliva between the two

groups. Vaidyanathan et al. (2008) held adult *Culex pipiens* with sugar concentrations

ranging from 2 to 40 % after exposing them to West Nile Virus infectious blood meals.

The only significant difference in competence they found was that females given 2%

sugar had higher transmission rates than the females given greater sugar concentrations

(Vaidyanathan et al. 2008). Their study also only manipulated sugar availability during

the extrinsic incubation period; thus there is some evidence in the literature that adult

sugar deprivation alters vector competence for at least one arbovirus transmitted by

Culex. Short-term nutritional deprivation alters immune function in insects, however after

the stress is alleviated immune function can return to normal levels (Siva-Jothy and

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Thompson 2002). Thus, the length and strength of the stress period may have important implications for vector competence in natural populations.

Several studies have demonstrated that nutrition alters aspects of insect immunity that act against protozoan and filarial parasites so there is evidence that under stress, immune function is altered in mosquitoes (Suwanchaichinda and Paskewitz 1998, Koella and Sorenson 2002, Telang et al. 2012). We do not know of published studies that have tested for altered RNAi activity, which is believed to be the main mechanism regulating viral infections, in mosquitoes given different nutritional regimes (Blair and Olson 2014).

Virus infection has been shown to alter blood feeding behavior in *Aedes aegypti* infected with dengue and in *Aedes triseriatus* and *Aedes albopictus* infected with LACV (Grimstad et al. 1980, Platt et al. 1997, Jackson et al. 2012). LACV infection increased probing and decreased engorgement in *A. triseriatus* (Grimstad et al. 1980). LACV infection also reduced the size of blood meal ingested in both *A. triseriatus* and *A. albopictus* and decreased re-feeding rates in *A. triseriatus* but not *A. albopictus* (Jackson et al. 2012). We found no evidence of LACV infection status influencing the willingness to take a blood meal fifteen days after a preliminary blood meal, corresponding to the extrinsic incubation period, and this is consistent with the findings of Jackson et al. (2012).

We did find a significant effect of sugar treatment on willingness to blood feed. Females that were given only intermittent access to sugar were more likely to take a second blood meal than those that were given constant access to sugar. This finding is not surprising as

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it has been repeatedly demonstrated in laboratory studies that sugar deprivation increases blood feeding rates in multiple mosquito species (Straif and Beier 1996, Canyon et al. 1999, Gary and Foster 2001, Braks et al. 2006). We were specifically interested in the interaction between infection status and sugar treatment which, if significant, would have supported the hypotheses that there are condition-dependent effects of infection on propensity to blood feed. We predicted that infected mosquitoes would show altered feeding rates depending on the sugar treatment they received. Our data did not support our prediction. The lack of interaction between infection and sugar treatment could again be due to the fact that temporary sugar deprivation did not actually induce nutritional stress though it was sufficient to alter feeding behavior. There is currently a lack of published investigations of the interactive effects of arbovirus infection and body condition on blood feeding behavior; thus it is difficult to determine if the results of our study are generalizable.

There was no effect of infection status on egg production in the second gonotrophic cycle. This result is consistent with Costanzo et al. (2013), who found that LACV infection had no effect on egg production in either *A. triseriatus* or *A. albopictus* in the first gonotrophic cycle. This finding is interesting when compared to results from Jackson et al. (2012) who found that both species take smaller blood meals when infected with LACV. There is a positive relationship between blood meal size and number of eggs produced (Briegel 1990).

The effects of sugar feeding on egg production in mosquitoes are not consistent and are based mainly on laboratory experiments. Different studies have found reduced fecundity,

no effect on fecundity, or an effect on daily fecundity but not lifetime fecundity
(reviewed by Stone and Foster 2013). While most of the lipids that go into egg
production come from the blood meal, sugar meals do contribute to egg production and
mosquitoes with low reserves often produce no eggs after an initial blood meal (Nayar
and Sauerman 1975, Klowden 1986). Mosquitoes with large reserves also tend to take
smaller blood meals which is postulated to be the mechanism that leads to lower
fecundity in high reserve or sugar fed females (Mostowy and Foster 2004). We found that
short-term sugar deprivation had no effect on egg production in the second gonotrophic
cycle for *A. albopictus*. The lack of observable difference is likely not due to the sugar
fed females having a crop full of sugar, as all females were starved for 48 hours prior to
being offered the second blood meal (Mostowy and Foster 2004). By the time the females
in this experiment reached the second gonotrophic cycle they were at least 21 days old.
The sugar manipulation was applied when females were 6-21 days old. It could be that by
the time the females reached the second cycle they had had sufficient time to recover
from the period they were denied sugar and had equal body condition compared to the
females given sugar *ad libitum* by taking large and frequent sugar meals when it was
available.   We did not measure size of blood meals or lipid and glycogen stores in this
experiment so we cannot say with any certainty why there was no observable difference
among groups.

As with willingness to blood feed, we were particularly interested in the interaction
between infection and sugar treatment on egg production and found no evidence for it.
There was also no interaction between food treatment and viral infection on fecundity in

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Western-tent caterpillars (Lepidoptera: Lasiocampidae) (Myers et al. 2011). Significant interactive effects between glucose concentration and infection status were observed to affect survival in *Anopheles stephensi* infected with *Plasmodium* (Lambrechts et al. 2006). Thus it may be that these interactive, or condition-dependent effects, are only present in some vector-pathogen systems, perhaps those where infection has a high cost for the vector. Several studies published recently have demonstrated multiple interactive effects of mosquito and virus genotype and the environment on vector competence demonstrating that there is a suite of factors that work together to determine competence (Mitchell et al. 2005, Richards et al. 2009, 2010). Future research should investigate the interactive effects of infection and environment on other traits (e.g., survivorship and fecundity) that are likely to impact mosquito population growth and vectorial capacity.

The main goal of this study was to test for condition-dependent competence of LACV and condition-dependent virulence effects on important life-history traits such as blood feeding behavior and fecundity. We found no evidence that short-term sugar deprivation during the extrinsic incubation period interacted with infection status to alter blood feeding behavior or fecundity in *Aedes albopictus*. It is possible that effects may have been expressed in other traits that we did not measure such as probing, blood meal size, or egg quality.

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Table 1.1. Number of females that: entered the experiment in each treatment group; died during the extrinsic incubation period (EIP); were not successfully infected; had a nondisseminated infection; and had a disseminated infection; offered a second blood meal after the EIP; and took the second blood meal when offered.

LACV	Constant Sugar	Number	Died in EIP (% of fed)	Not infected (% of fed)	Body only positive (% of fed)	Legs positive (% of fed)	Offered 2nd blood meal	Took 2nd Blood meal (% those offered)
Yes	Yes	105	14 (13.3%)	27 (25.7%)	43 (41.0%)	21 (20.0%)	91	19 (20.1%)
Yes	No	106	14 (13.2%)	25 (23.6%)	44 (41.5%)	23 (21.7)	92	38 (41.3%)
LACV Total		**211**	**28 (13.3%)**	**52 (24.6%)**	**87 (41.2%)**	**44 (20.9%)**	**183**	**57 (31.1%)**
No	Yes	106	16 (15.1%)				90	22 (24.4%)
No	No	101	7 (6.9%)				94	33 (35.1%)
No LACV Total		**207**	**23 (11.1%)**				**184**	**55 (29.9%)**
Grand Total		**418**	**51 (12.2%)**				**367**	**112 (30.5%)**

Table 1.2. Categorical model analysis for the effect of infection status and sugar treatment on the willingness of *Aedes albopictus* to take a second blood meal 15 days (corresponding to the extrinsic incubation period) after a first blood meal.

Source	DF	χ^2 Value	P Value
Infection status	3	4.09	0.252
Sugar	1	9.14	**0.0025**
Status* Sugar	3	1.26	0.739

Table 1.3. ANOVA table for the effect of infection status and sugar treatment on the number of eggs produced in the second gonotrophic cycle**.**

Source	DF	F Value	P Value
Infection status	3	1.07	0.3655
Sugar	1	0.12	0.7258
Status* Sugar	3	0.64	0.5903
Error	104		
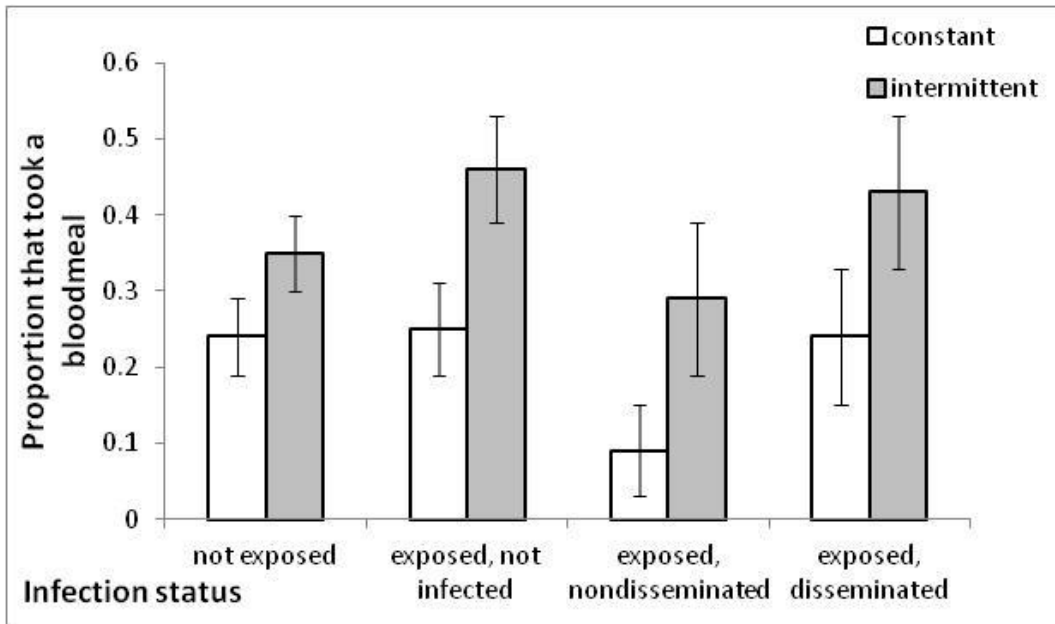
Fig. 1.1. Fraction of individuals that took the second blood meal. There was a significant effect of sugar treatment, but no significant effect of infection status or the interaction between sugar treatment and infection status. Open bars are constant sugar treatment and grey bars are intermittent sugar treatment.
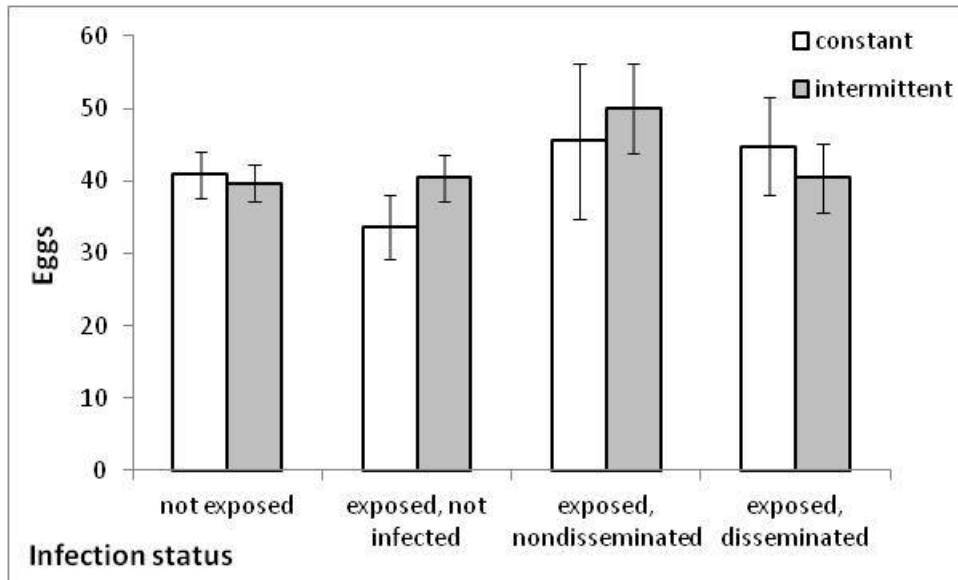
Fig. 1.2. Least squares means and standard errors of the number of eggs produced. There were no significant effects of infection status, sugar treatment, or the interaction on the number of eggs produced. Open bars are constant sugar treatment and grey bars are intermittent sugar treatment.

CHAPTER II

SIMULATED SEASONAL PHOTOPERIODS AND FLUCTUATING

TEMPERATURES HAVE LITTLE EFFECT ON BLOOD

FEEDING AND LIFE HISTORY IN

AEDES TRISERITAUS

Abstract

Biotic and abiotic factors change seasonally and impact life history characteristics for
temperate-zone ectotherms. Temperature and photoperiod are factors that change in
predictable ways. Most studies that test for effects of temperature on vectors use constant
temperatures and ignore potential correlated effects of photoperiod. In two experiments,
we tested for effects of ecologically relevant early and late season (June, August)
temperatures and photoperiods experienced in the larval habitat, against constant
temperatures (cool, hot) on survivorship, development time, size, and a composite index
of performance in a temperate-zone population of *Aedes triseriatus* (Say), vector of La
Crosse virus. We followed cohorts of females to assess carry-over effects of rearing
conditions on longevity, blood feeding, and egg production. Larval survivorship was
affected by treatment in one experiment. Development time increased in the June and
cool treatments but the constant and fluctuating temperatures did not differ. Significantly

larger mosquitoes were produced in fluctuating verses constant temperature treatments. The performance indices for the August and hot treatments were greater than those for June and cool treatments with no difference between constant and fluctuating temperatures. Adult female longevity was reduced in the constant temperature treatment but there was no difference between June and August, nor did size affect longevity. There was no effect of treatment on blood feeding and a limited effect on egg production. We conclude that seasonal temperatures and photoperiods during development have limited effects on this population of *A. triseriatus* and that there is little evidence of strong effects of fluctuating vs. constant temperatures.

Introduction

Like many other infectious diseases, vector- borne diseases often show marked seasonal patterns in human incidence (Altizer et al. 2006). These seasonal patterns may be due to temperature and rainfall events that change the quantity of larval habitats or the rates of development of vectors and pathogens. Other seasonal changes may influence mosquito life-history, such as pulses of terrestrial detritus inputs into larval habitats, degradation of larval resources, and photoperiod induced changes in adult feeding behavior (Bowen 1992, Macia and Bradshaw 2000, Bevins 2007, O'Neal and Juliano 2013). Biotic and abiotic environments of mosquitoes, in all life stages, can alter adult female production, female longevity, fecundity, blood feeding behavior, vector competence, and vector immunity (Straif and Beier 1996, Suwanchaichinda and Paskewitz 1998, Delatte et al. 2009, Murdock et al. 2012a, Alto and Bettinardi 2013, Costanzo et al. 2013). All of these traits are thought to alter vectorial capacity, which is a mathematical expression of the

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rate of new infections arising from a single infected mosquito in a given place and time (Dye 1986).

Changes in photoperiod, and to a lesser extent temperature, are two of the most reliable cues to season in temperate climates. Temperature especially is extremely important for determining life- history characteristics for ectotherms like mosquitoes. Generally, hotter temperatures decrease development time and produce smaller adults (Kingsolver and Huey 2008). Most of the published studies of the effects of rearing temperature on adult characteristics have focused on changes in vector competence and a few studies have tested for combined effects of temperatures experienced during both life adult stages (Kay et al. 1989, Brubaker and Turell 1998, Alto and Bettinardi 2013). Laboratory studies that test for carry-over effects of conditions in the larval habitat on adult longevity or fecundity have largely focused on effects of resource availability or sub-lethal effects of insecticide exposure (Hawley 1985, Reiskind and Lounibos 2009, Muturi et al. 2010, 2012). Resource availability and temperature both affect the size of resulting females and there is some evidence that size is related to longevity in mosquitoes (e.g., Walker et al. 1987, Landry et al. 1988, Maciel-de- Freitas et al. 2007). Further, size changes seasonally in field collected mosquitoes (Day et al. 1990).

Many insects use cues from both temperature and photoperiod to make decisions about entering or terminating diapause (Tauber and Tauber 1976). They may also react to changing seasonal photoperiod as an indication of impending unfavorable conditions, though these effects may only be observed in conjunction with other unfavorable

conditions (e.g., food limitation Luker et al. 2002, Yee et al. 2012). A well-known example of behavioral changes by mosquitoes in response to seasonal cues is the termination of egg production and the switch from blood feeding to sugar feeding in populations of *Culex pipiens* that diapause as adults during the winter (Eldridge 1968, Bowen 1992, Robich and Denlinger 2005). It is assumed that this strategy enhances survival and fitness in this species by increasing winter survival and future reproduction.

Under natural conditions, temperatures fluctuate daily (thermoperiodism). Although most studies that examine temperature and photoperiod effects on mosquitoes use constant temperatures, experiments with fluctuating temperatures are not new (reviewed by Beck 1983). There has, however, been increasing interest in the effects temperature fluctuations on mosquito and parasite dynamics, particularly with malaria and dengue their associated mosquitoes (Lambrechts et al. 2011, Carrington et al. 2013a, 2013b, 2013c). If we assume that populations are locally adapted to seasonal climactic conditions, it may then be important to test for environmental effects on fitness using ecologically appropriate diel temperature fluctuations.

In this paper, we test the effects of simulated seasonal conditions of temperature and photoperiod experienced in the larval habitat only, on a suite of characteristics that are likely to influence population dynamics and vectorial capacity in a population of *Aedes triseriatus* (Say), vector of La Crosse Virus (LACV). *Aedes triseriatus*, the Eastern tree-hole mosquito, has a native range that extends over most of the eastern United States and southern Canada. Northern populations overwinter mainly as diapause eggs and more

southerly populations also diapause as late stage larvae (Simms 1985). Diapause is induced in the mature embryo or late stage larva and it is believed that there are no maternal effects on diapause in this species (Shroyer and Craig 1980). It remains to be tested if seasonal cues in the larval habitat "carry-over" to influence reproductive decisions by adult females involving blood feeding behavior or reproductive investment.

We quantified measures of population performance such as larval survival, female development time, female size, and *r'* (Livdahl and Sugihara 1984) and followed cohorts of adult females to test for "carry-over" effects of larval rearing conditions on female longevity, blood feeding, and egg production. These last three potential responses were included as they are important for both population growth and for vectorial capacity for LACV which is transovarially transmitted (i.e., mother-offspring) by *A. triseriatus* at high rates (Woodring et al. 1998, Hughes et al. 2006).

We tested three general hypotheses; 1) simulated early and late season temperatures and photoperiod affect life history characteristics in a temperate mosquito population in ways that may, at least partly, explain the observed seasonal patterns of LACV transmission, which shows greater human incidence in late summer and fall (Haddow and Odoi 2009), 2) that mosquitoes respond to seasonal cues encountered by developing larvae by altering reproductive tactics of resulting adults such as blood feeding behavior and egg production and 3) that fluctuating, as opposed to constant temperatures of the same mean, will alter these same life history characteristics.

Materials and Methods

Experiment one

Aedes triseriatus used in this experiment were F_1 generation of a population originating

from Tyson Research Center, approximately 20 miles west of St. Louis, Missouri (38 deg

31' N, 90 deg 33' W). One hundred first instar larvae were placed in experimental

microcosms with 450 ml deionized water, 1.6 g senescent oak leaves (*Quercus

virginiana*), and 0.06 g dried crushed crickets from a laboratory colony maintained at

Illinois State University (*Gryllodes spp.*) after 4 days of infusing at a constant 25° C and

a 14:10 L:D photoperiod. The population of *A. triseriatus* used in this experiment would

not encounter leaves from *Q. virginiana* in the field. We used this oak species as the

detritus base because it is an established, predictable detritus source used in experiments

in our laboratory, was available in large quantities, and was deemed preferable to using

an artificial diet. There were twelve replicates per treatment, though female size, *r'*, and

longevity were followed for only 6 replicates per treatment, which were randomly chosen

from the 12 total replicates. An additional detritus spike of 0.6 g oak leaves was added on

day five and a spike of 0.6g leaves and 0.06g cricket was added on day 12. Microcosms

were randomly assigned to one of three temperature treatments and will be referred to as

the June, cool, and August treatments, respectively (Table 2.1.). Microcosms were kept

in environmental chambers set to the higher temperature during the light phase and the

lower temperature during the dark phase. The June and August temperature and

photoperiod treatments were chosen to mimic the average daily high and low temperature

and photoperiods for June and August in St. Louis, Missouri

These temperature treatments mimicked the extremes of daily water temperature fluctuations recorded at Tyson in 2013 (*unpublished data*). June and August also represent the peak and decline, respectively, of *A. triseriatus* oviposition at Tyson (*unpublished data*). The constant temperature and 14:10 L:D photoperiod treatment was chosen as it is a commonly-used laboratory condition and it yielded the same mean temperature as the June treatment. Actual water temperatures in the environmental chambers were recorded hourly using an iButton © (Maxim Integrated, San Jose, CA) sealed inside a water-proof battery container (www.lighthound.com, product #LHSTTB). Water temperatures never deviated from the set temperatures in all chambers by more than $\pm 1^O$C.

Pupae were removed daily and allowed to eclose in 20 ml vials. Adult eclosion was recorded daily. Males were discarded and females were transferred to 1 L plastic containers covered with mosquito netting in groups by treatment, replicate, and day of eclosion. Females were held in a walk-in environmental chamber set at 25° C with a 14:10 L:D photoperiod with *ad libitum* access to 5% sugar. Mortality was recorded daily. Females from all treatments were held under the same conditions to isolate the effects of larval rearing temperature and photoperiod on adult longevity. Upon death, females were dried at 50° C and one wing was removed and measured to quantify female size (Blackmore and Lord 2000).

We calculated *r'*, which is a composite performance index designed to estimate instantaneous rate of change, using a method of Livdahl and Sugihara (1984). The index

combines time to adult eclosion, cohort survivorship, and fecundity estimated from the

relationship between female size (wing length) and egg production

$$r' = \left[\frac{ln\left[\left(\frac{\sum_x A_x f(w_x)}{N_0} \right) \right]}{D + \left[\frac{\sum_x x A_x f(w_x)}{\sum_x A_x f(w_x)} \right]} \right]$$

where $f(w_x)$ is a function predicting the number of female eggs produced (assuming a

50:50 sex ratio) by a female having the mean wing length for females eclosing on day x,

N_0 is the number of females in the experimental cohort (assuming 50:50 sex ratio), x is

day of female eclosion, A_x is the number of females eclosing from a cohort on day x, and

D is the estimated number of days from eclosion to oviposition in the first gonotrophic

cycle. The function $f(w_x)$ was $\exp[4.5801 + 0.8926(\ln(w_x)] - 1$ and D was

estimated to be 12 days (Nannini and Juliano 1998).

The effects of treatment on larval survivorship to adulthood, female median days to

eclosion, female mean wing length, and r' were analyzed with individual microcosms as

the experimental unit with one- way ANOVAs (PROC GLM). Differences among least

squares means were determined using Tukey's correction for multiple comparisons. The

effect of treatment and size (wing length) on adult female longevity was analyzed with

two separate models using Cox Proportional Hazards Model survival analysis with

replicate nested in treatment as a random effect (PROC PHREG). The first model

included all females and only the main effect of treatment because wings were too

damaged to measure for 35% of females. The second model included both treatment and

wing length for those females with measurable wings. Differences among treatment groups were further analyzed via contrasts. All analyses were performed in SAS 9.3(SAS 9.3 User's Guide, SAS Institute).

Experiment two

Microcosms for larvae were set up with same food regimen as experiment one with six replicates per treatment. Mosquitoes used in this experiment were a mix of F_2 and F_3 from the same population as experiment one. Temperature and photoperiod treatments were slightly modified so that June and cool treatments had the same photoperiod and same mean temperature, and the August treatment had the same photoperiod and same mean temperature as a forth treatment referred to as hot (Table 2.1.). As with experiment one, pupae were moved to 20 ml vials, daily eclosion was recorded, males were discarded, and females were housed in group cages by treatment, replicate, and day of eclosion.

Females were kept at a constant 25° C and a 14:10 photoperiod in a walk-in environmental chamber and given *ad libitum* access to 10% sugar for four days. On the fifth day, sugar was removed and they were given only water for 48 hours to encourage blood feeding. Six days post emergence, mosquitoes were offered a blood meal for 15 minutes. Blood meals were prepared by heating defibrinated bovine blood (Hemostat Laboratories, Dixon, CA) to 37° C in a water bath, transferring 3 ml of blood to 5 ml Petri dishes, which were then covered with a porcine intestine membrane secured with a rubber band. Petri dishes were placed, membrane side down, on the screen on the top of the group cages and a bag of hot water was placed on top of the dish to maintain blood

temperature. At the end of a 15 minute feeding period, the blood source was removed and feeding status was determined visually. Blood fed mosquitoes were transferred to new group cages by treatment, replicate, and date of feeding. No oviposition substrate was given to force the females to retain their eggs. To maximize the sample size of blood fed mosquitoes, group cages were offered blood up to 4 times over 2 days. Only mosquitoes that fed on the first offered blood meal were included in the analysis of willingness to blood feed. Blood fed females were held for 7 days under the same constant laboratory conditions then frozen at -20° C. One wing was removed from each female (those that blood fed and those that did not) and measured. Ovaries of blood fed females were removed and placed in a small amount of Hank's Buffered Salt Solution and mature eggs (Christophers stage IV or V) counted (Christophers 1960).

Effects of treatment on larval survivorship to adulthood, female median day to eclosion, female mean wing length, and r' were analyzed as in experiment one. Willingness to blood feed was analyzed as the proportion that fed the first time females were offered blood, with individual microcosms as the experimental unit. The effect of treatment on number of mature eggs produced was analyzed as a mixed model ANCOVA with wing length as the covariate and replicate nested in treatment as the random effect (PROC MIXED). Estimates of the intercept and slope for the relationship between size and egg production for each treatment group were obtained by simple linear regression (PROC REG). All analyses were performed in SAS 9.3 (SAS 9.3 User's Guide, SAS Institute).

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Experiment one

There were significant effects of treatment on survivorship to adulthood, female median

day to eclosion, female mean wing length, and *r'* (Table 2.2.). Survivorship to adulthood

was lower in the August treatment than in the June and cool treatments, which did not

differ (Fig. 2.1.a). Median day to eclosion was significantly lower for females in the

August treatment than in the June and cool treatments, which did not differ (Fig. 2.1.b).

Females in the June treatment were significantly larger than those in the other treatments,

and those from the cool treatment were significantly larger than those from the August

treatment (Fig. 2.1.c). The August treatment had a significantly larger *r'* than did the June

or cool treatments, which did not differ (Fig. 2.1.d). There was a significant effect of

treatment on female longevity in the first model that included only the effect of treatment

and used the entire data set (n=474; χ^2 =10.84; df = 2; P = 0.0009). After adjusting for

multiple comparisons, the cool treatment differed from both June and August treatments

which did not differ (Fig. 2.2.a). The cool treatment had the greatest hazard of death and

showed earlier senescence than the two fluctuating treatments (Fig. 2.2.b). The model

with both female size and treatment yielded no significant effects of treatment (n = 308;

χ^2 = 4.19; df = 2; P = 0.095) and size (χ^2 = 0.1; df = 1; P = 0.743).

Experiment two

There was a significant effect of treatment on female median day to eclosion, mean

female wing length, and *r'*, but not on survivorship to adulthood (Table 2.3.). Larval

survivorship was high in experiment two, approaching 90% for all treatment groups (Fig.

2.3.a). Females in the June treatment took significantly longer to eclose than did females in the August and hot treatments, but not in the cool treatment. The cool and August treatment did not differ, but females from the cool treatment took longer to eclose than those from the hot treatment. The August and hot treatments did not differ (Fig. 2.3.b). Females from the June treatment were significantly larger than those in the August and hot treatments, but not those in the cool treatment. Females from the August treatment were significantly larger than the hot treatment but were not significantly different from those in the cool treatment (Fig. 2.3.c). The August and hot treatments had the highest r' and did not from each other. Similarly, r' for the June and cool treatments did not differ (Fig. 2.3.d). There was no effect of treatment on the proportion of females that blood fed ($F_{3, 20} = 1.78$; $P = 0.184$) (Fig. 2.4.). There was no significant main effect of treatment on the number of eggs produced ($F_{3,20} = 1.72$; $P = 0.1953$) but there was a significant interaction between wing length and treatment ($F_{4,220} = 3.29$; $P = 0.0121$). The relationship between wing length and egg production for the June and cool treatments had slopes that were not significantly different from zero indicating no size-fecundity relationship for these treatments (Figs. 2.5.a and 2.5.b). The slopes were positive and significantly greater than zero for the August and hot treatments (Figs. 2.5.c and 2.5.d).

Discussion

In this paper, we tested several hypotheses about the effects of seasonal cues experienced in the larval habitat, and of constant and fluctuating temperatures, on a population of *Aedes triseriatus*, primary vector of La Crosse Virus. In experiment one, all larvae had lower survivorship compared to experiment two with the August treatment having

significantly lower survivorship compared to the June and cool treatments. In experiment two, larvae from all treatment groups had high survivorship and the overall effect of treatment on survivorship was not significant. Although the micrososms were set up with exactly the same detritus amounts and densities, the leaves used in the two experiments were collected at different times, but at the same location, and may have been of different quality. Other laboratory experiments have shown that leaf quality, including leaf age, can affect larval survivorship (Walker et al. 1997, Macia and Bradshaw 2000). In another study, *A. triseriatus* larvae reared at 31^OC exhibited reduced survivorship to adulthood compared to larvae reared at 15° or 23° C (Teng and Apperson 2000). The mean temperature of our August treatment was 27.6^O C, though during the 13 hour diurnal period the temperature was 31° C which is consistent with Teng and Apperson (2002). These results suggest that if resources are of poor quality, *A. triseriatus* may have reduced survivorship at higher temperatures even if the daily mean temperature is lower. Temperature has been shown to alter the effects of poor resource quality, or starvation, on insect survival (Adamo et al. 2012).

Median day to eclosion for females was longer in the June and cool treatments compared to the August treatment in experiment one. In experiment two, this same pattern of longer development at lower mean temperatures was evident (Fig 2.3.b) though in contrast to experiment one, the cool and August treatment were not different. Though differences between the two experiments are relatively minor, differences in leaf quality between the experiments may again play a role. In both experiments the constant and corresponding fluctuating temperature treatments with the same mean temperatures did not differ,

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suggesting that under these conditions, temperature fluctuations of up to 7°C do not alter the development rate compared to constant mean temperatures.

All pair-wise comparisons of wing size were significant in experiment one but not experiment two, though the trend was the same (Figs. 2.1.c and 2.3.c). The June treatment produced the largest females and the hot treatment produced the smallest females. Wing length is often negatively correlated with temperature and positively correlated with development time (Briegel and Timmermann 2001, Dodson et al. 2012). Our data supports this, but also show that the metabolic relationship between temperature and size can be independent of the relationship between temperature and development time when temperatures fluctuate. In other words, fluctuating temperatures compared to constant temperatures alter female size without significantly affecting development time. The mechanism behind this is not clear, but it could be due to differences in foraging and assimilation rates at different temperatures.   In a similar study that compared fluctuating vs constant rearing temperatures for *Aedes aegypti*, development time also was not affected, but they did not report female size. Other investigators reported development times and wing size for adults reared as larvae under constant and fluctuating regimes but did not test for differences between the regimes so we cannot be sure if our result is specific to *A. triseriatus,* or a more general property of *Aedes* or insects in general (Mohammed and Chadee 2011, Carrington et al. 2013a, 2013c).

In both experiments, *r'*, our cohort performance estimate was greater in the warmer treatments but constant and fluctuating treatments were not different from one another.

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This was true despite adults from warmer treatments being smaller (both experiments) and having lower survivorship to adulthood (experiment one). Thus, under simulated seasonal temperature and photoperiod conditions, adults resulting from larvae developing under the August or hot treatments are expected to have a more rapid rate of population increase (Livdahl and Sugihara 1984). Interestingly, two years of *A. triseriatus* oviposition data from Tyson Research Center, where the experimental population originated show the greatest oviposition in June and declining oviposition in August (*unpublished data*). This potential discrepancy may indicate that: 1) there is simply a larger population of *A. triseriatus* in May and June as overwintering eggs terminate diapause and emerge; 2) the estimate of *r'* in this study assumes that the environment the adults experience in June and August is the same, and of course this is unlikely in nature as ambient temperatures encountered by adults are likely different, availability of blood and carbohydrate sources may be different, and availability of larval habitats may change; 3) other characteristics of the larval habitat such as resource quality and quantity may differ seasonally in ways that were not accounted for in this experiment; and 4) oviposition data collected from the field do not indicate how quickly the population is growing, but indicate how large the population is.

In experiment one, we tested for the effect of rearing temperature and photoperiod on adult female longevity. Treatment had a significant effect, and constant cool temperature females had reduced longevity compared to the June and August females. The main factor producing this difference in longevity between treatment groups was the earlier senescence of the cool treatment females (Fig. 2.2.b). The two seasonal temperature

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treatments were not different, which suggests that, at least under benign laboratory conditions, seasonally fluctuating temperatures and photoperiods in the larval habitat do not produce adults that differ significantly in survival probabilities. Thus, effects of rearing temperatures and photoperiods on female longevity (and therefore vectorial capacity; Dye 1986) cannot explain observed greater LACV incidence among humans associated with late summer and fall (Haddow and Odoi 2009).

Why the cool temperature females had the lowest cumulative survivorship estimates is not entirely clear. One possibility is that teneral reserves are greater in *A. triseriatus* when the rearing temperature fluctuates, though there is no experimental evidence for this hypothesis. Teneral reserves are highly correlated with size (Van Handel and Day 1988) and we found no effect of size on longevity. The only other variables measured in this experiment that indicated that the constant temperature groups fared worse than the fluctuating temperature groups was female size (Fig. 2.1.c). When the model was expanded to included wing size, the effect of treatment was marginally not significant, which is likely a result of sample size being reduced by 35% due to damaged wings. Size was clearly unrelated to longevity. Most of the wings that were too damaged to use came from females >50 days old when they died and thus are not randomly distributed among the population.   Thus, we think that the analysis of the full data set without size is the most accurate indicator of the effect of treatment on longevity.

The utility of size as a measure of vector "quality" is not resolved. Size can affect vector competence for arboviruses though the mechanism is not clear (Paulson and Hawley

1991, Alto et al. 2008). Some studies have shown that size may be a predictor of success in acquiring a blood meal, adult longevity, and fecundity (Hawley 1985, Takken et al. 1998, Stone et al. 2012, Xue et al. 2012). Not all experiments reach the same conclusions about size, however. A survey of field collected *A. triseriatus* found no evidence for a benefit of being large (Landry et al. 1988). Temperature may affect size directly via metabolic effects and indirectly via effects on the bacterial resource that is the food supply for developing larvae, and these effects may lead to different physiological or immunological phenotypes and ultimately different outcomes in traits related to vector competence and other life history traits. In the current experiment we found no evidence for an effect of size on longevity and limited evidence for an effect of size on fecundity.

Most researchers find a significant size fecundity relationship in mosquitoes (e.g., Blackmore and Lord 2000, Armbruster and Hutchinson 2002). Only a handful of studies have investigated how different environmental conditions may alter the size-fecundity relationship (Clauss and Aarsen 1994, Arendt 2010) or investigated how the relationship may change as females age (Mccann et al. 2009). In this study we found no significant main effect of treatment on egg production, despite significant differences among treatments for mean female size. There was a significant interaction between treatment and size; the slopes of the size-fecundity relationships were significant and positive for females coming from the August and hot treatments whereas the slopes for the June and cool treatments were not significantly different from zero. The significant size-fecundity relationship in the warmer treatments appears to arise from the smallest females in each group producing a small number of eggs. The predicted number of eggs for larger

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females is similar for all treatments (compare Figs. 2.5.a-d), suggesting that the apparent difference due to rearing conditions may indeed be a product of the presence or absence of the smallest females. As with many of the other variables measured in this study, there is weak support for the hypothesis that fluctuating vs. constant temperatures have large effects on individual performance or of population growth in this system.

In experiment two, we tested the effects of larval rearing treatment on willingness to take a blood meal and egg production for one gonotrophic cycle. As the population of *A. triseriatus* used in this experiment primarily overwinters as diapausing eggs, we predicted that if *A. triseriatus* females make reproductive decisions based on seasonal cues they experience as larvae, late-season females (i.e., August) would be more willing to feed and would produce more eggs than early-season females (i.e., June). We specifically chose our temperatures and photoperiods to mimic late season cues that were just above the threshold when this population is predicted to being to produce diapause eggs (Sims 1985). We found no evidence of a treatment effect on willingness to take a blood meal. It may be that as the decision to enter diapause is made by eggs and that larvae of this Midwestern population generally do not respond to seasonal cues, though it is known that populations of *A. triseriatus* from the same latitude as the population used in this study will enter diapause at a photophase of 11:13 L:D (Sims 1985). It may also be that seasonal cues in the larval habitat alone are not enough to elicit a change in reproductive tactics in the resulting adults. Number of eggs produced was the only measure recorded of reproductive effort, there may have been a change in egg quality that was not accounted for. All of the females in this experiment were held under the standard

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laboratory conditions that were the same as rearing conditions for larvae in the cool treatment. It seems likely that the conditions experienced during adulthood may have over-ridden any effect of larval rearing conditions on life history. In this species, seasonal cues to the adult are not transmitted to offspring; in other words, eggs and larvae make the decision to enter or terminate diapause, and adult females do not make that decision for them, as is the case in *Aedes albopictus* (Kappus and Venard 1967, Focks et al. 1994). This biological property of *A. triseriatus* was what led to the prediction that if females make reproductive decisions based on seasonal cues, the cues would have to be experienced in immature stages. To our knowledge, there are no published tests for a shift in feeding behavior in *Aedes* mosquitoes due to seasonal cues, in contrast to studies that have shown seasonal shifts in *Culex* feeding behavior (Eldridge 1968, Bowen 1992, Robich and Denlinger 2005).

Other studies have demonstrated that fluctuating temperatures give a more accurate measure of performance than constant temperatures (Bradshaw 1980, Murdock et al. 2012a, Murdock et al. 2012b, Murdock et al. 2013). Many of these studies compare large and small daily fluctuations, and use mainly species from warmer climates and used temperature regimes that were relevant to those systems. In this experiment we were interested in testing not only the difference between daily thermoperiods, but also seasonally realistic temperatures and photoperiods to determine if seasonal climate impacts life-history and ultimately vectorial capacity. Though a greater magnitude of fluctuation, or greater mean temperature difference between groups, may have produced more dramatic effects, we chose to design our experiment with treatments within the

range of observed temperatures and photoperiods for this population. Our results suggest that at least in this temperate-zone *A. triseriatus*- LACV system, the use of constant temperatures in laboratory studies may be justified. Although the potential effects of thermoperiodism are intriguing, and likely important in some systems, they do not appear to be so here. One of the standout results of this study is that female size is altered by temperature fluctuations. In this study size did not affect longevity, and had little impact on fecundity. Thus we find limited evidence that rearing temperature fluctuations and female size are strongly related to vectorial capacity in this mosquito-virus system.

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Table 2.1. Temperature and photoperiod regimes simulated in the laboratory that mimic seasonal conditions experienced in the St. Louis, MO, USA area and corresponding constant temperature treatments. Actual temperatures recorded in the larval microcosms were ± 1^O C.

Experiment 1	Temperature (C)	Photoperiod (L:D)	Daily mean (C)	Daily fluctuation (C)
June	28: 20	15:9	25	8
Cool	25:25	14:10	25	0
August	31:23	13:11	27.3	8
Experiment 2				
June	28:20	15:9	25	8
Cool	25:25	15:9	25	0
August	32:24	13:11	28	8
Hot	28:28	13:11	28	0

Table 2.2. ANOVA table of the effects of rearing temperature and photoperiod on larval survivorship, median day to eclosion for females only, female wing length, and a composite cohort performance index *r'* from experiment one.

Effect		df	F	P
Survivorship	Treatment	2	11.88	0.0001
	Error	33		
Median day of eclosion	Treatment	2	52.37	<0.0001
	Error	33		
Wing length	Treatment	2	42.89	<0.0001
	Error	15		
r'	Treatment	2	36.66	<0.0001
	Error	15		

Table 2.3. ANOVA table of the effects of rearing temperature and photoperiod on larval survivorship, median day to eclosion for females only, female wing length, and a composite cohort performance index *r'* from experiment two.

Effect		df	F	P
Survivorship	Treatment	3	1.81	0.1784
	Error	20		
Median day of eclosion	Treatment	3	13.02	<0.0001
	Error	20		
Wing length	Treatment	3	12.29	<0.0001
	Error	20		
r'	Treatment	3	14.57	<0.0001
	Error	20		
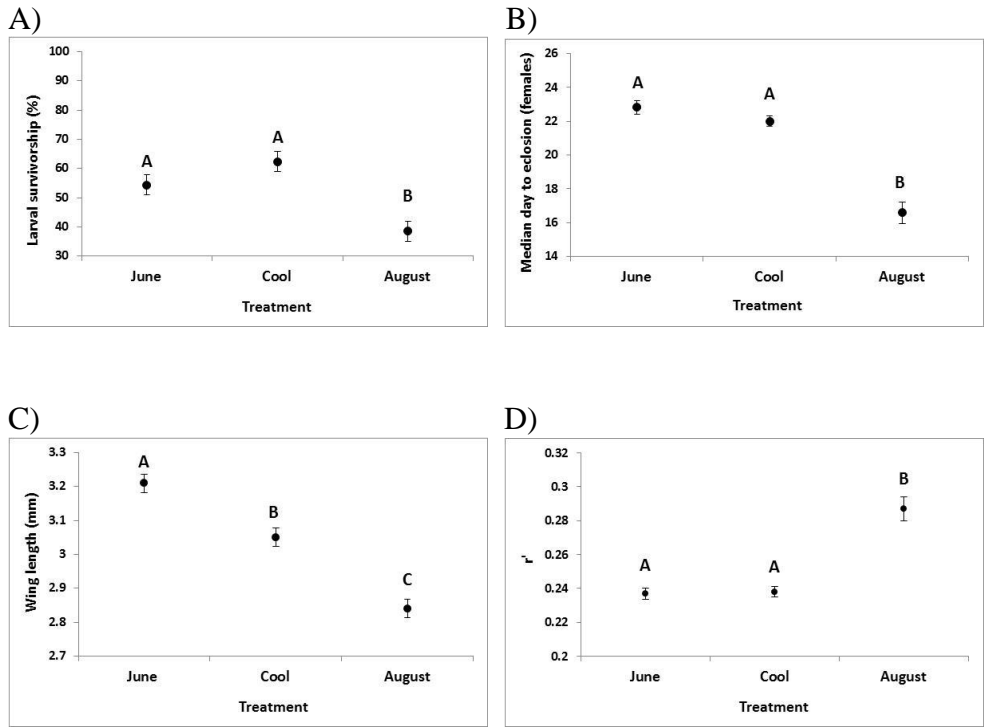
Fig. 2.1. Least squares means and standard errors for the effect of treatment in experiment one on A) larval survivorship, B) median day of eclosion for females only C) female wing size, and D) *r'* which is a composite performance index. Means associated with the same letter are not significantly different from one another.

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A)

B)
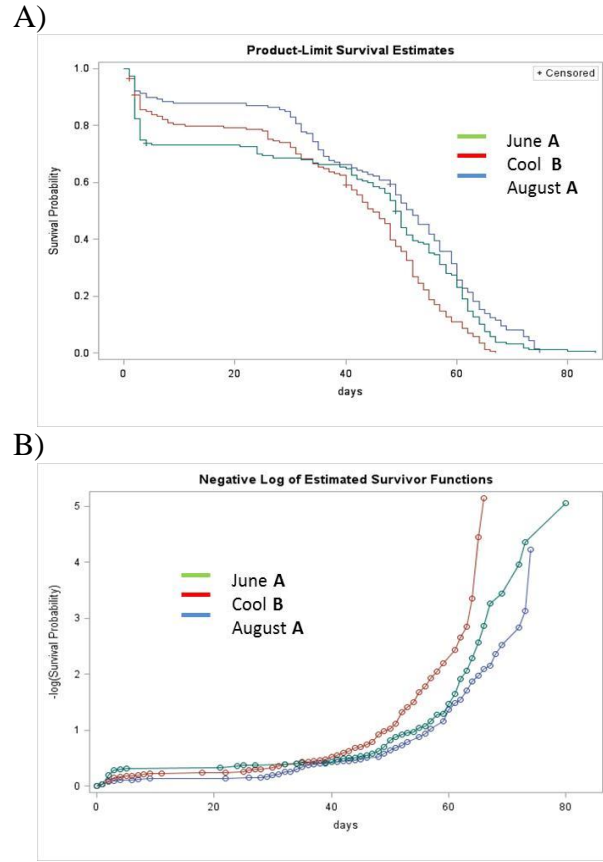
Fig. 2.2. A) Cumulative survival probabilities of female *Aedes triseriatus* reared as larvae under three different temperature and photoperiod regimes. Letters denote groups that were significantly different; B) negative log estimates of survivor functions. The up-turn of the curves late in life denote periods of increased hazard of death associated with senescence.
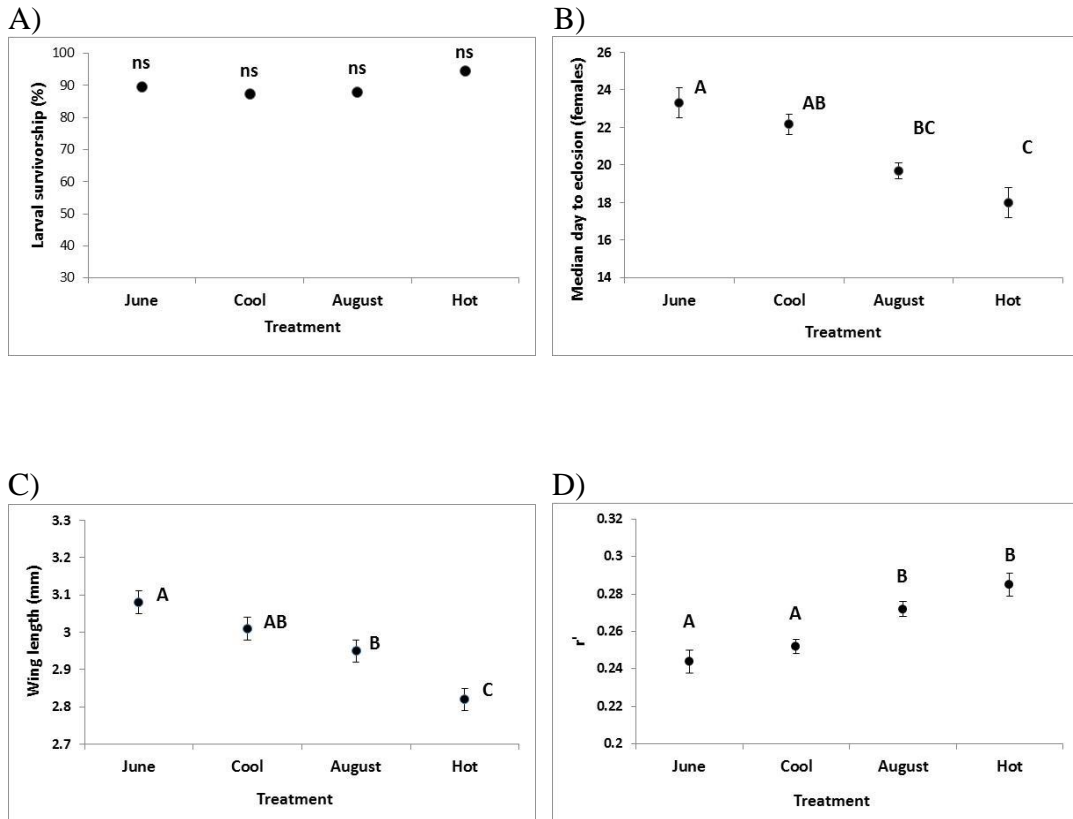
Fig. 2.3. Least squares means and standard errors for the effect of treatment in experiment two on A) larval survivorship, B) median day of eclosion for females only, C) female wing size, and D) *r'* which is a composite performance index. Means associated with the same letter are not significantly different.
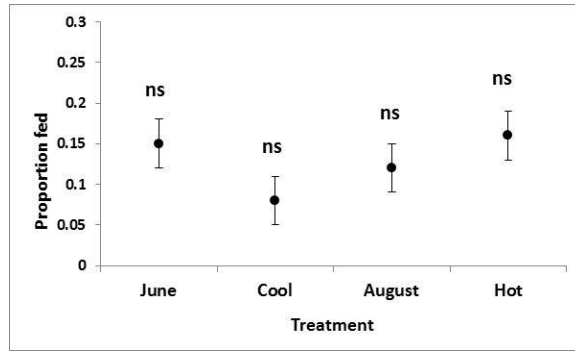
Fig. 2.4. Proportion of females that took a blood meal the first time they were offered. There was no significant effect of temperature and photoperiod treatment on blood feeding.
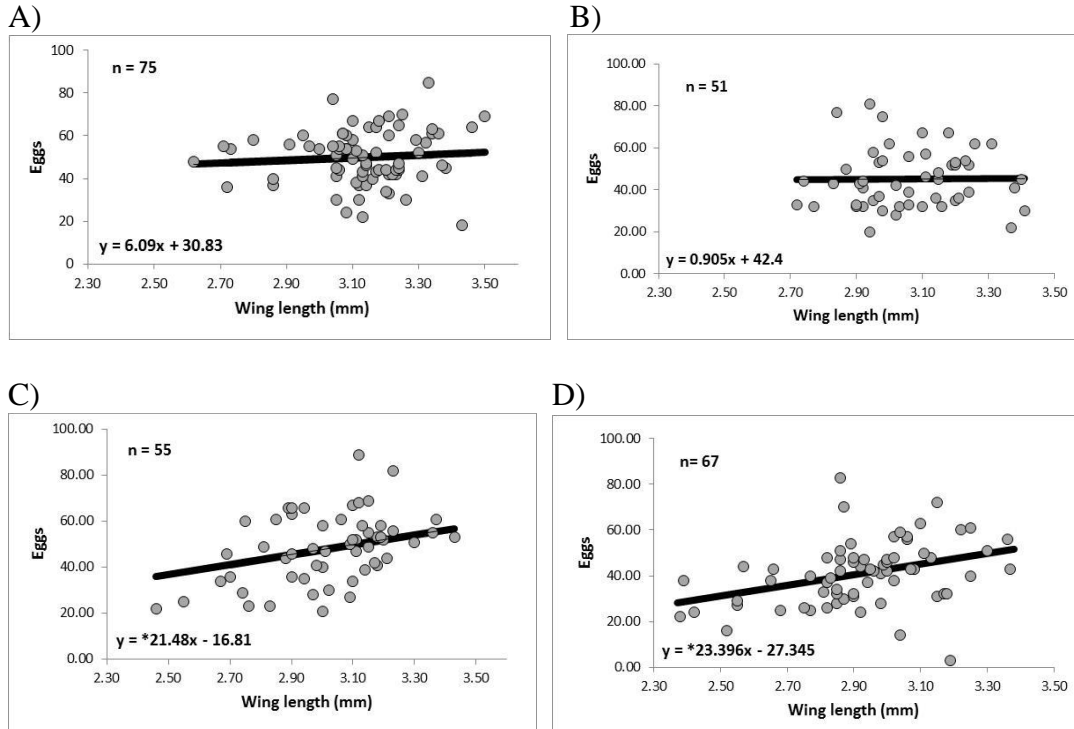
Fig. 2.5. Plot of observed egg production (dots) and predicted size-fecundity relationship (solid line) for A) June B) cool C) August D) hot treatments. Asterisks denote slopes that are significantly different than zero.

CHAPTER III

HABITAT AGE AND PRIOR EXPLOITATION IN AQUATIC CONTAINER

COMMUNITIES: EFFECTS ON FEMALE LIFE HISTORY IN

THE MOSQUITO *AEDES TRISERIATUS*

Abstract

Animals with complex life histories such as holometabolous insects and amphibians

occupy very different habitats during the immature, as opposed to the reproductive,

phases of their lifecycles. The environmental conditions experienced during the immature

phases can directly influence lifetime fitness via effects on development rate and

survivorship to maturity, but also indirectly by influencing adult size and longevity.

Many mosquito species in the genus *Aedes* occupy discrete and ephemeral aquatic

container habitats as immatures. One of the most important characteristics of these

habitats in terms of their ability to support the growth and development of larvae is the

quality and quantity of resources in the habitat. Resource availability is likely to be

influenced by the age of the habitat as detritus, which forms the base of the food-web in

these habitats, either changes in quality over time or new detritus inputs collects over

time. Resource availability is also likely to be influenced by the density of larvae either

via direct resource competition or by delayed- resource competition as previous non-

overlapping cohorts exploit the available resources. We manipulated habitat age and prior resource exploitation in a field experiment using the mosquito *Aedes triseriatus*. We found that mosquitoes performed better in older habitats in terms of higher larval survival, faster development rate, higher value of a composite performance index, and increased adult female longevity. Larvae that developed in habitats that had been exploited by a prior cohort were only significantly affected by delayed development. We conclude that extended conditioning of leaf detritus in the aquatic habitats released previously recalcitrant fungal resources that improved the quality of the habitat for larvae.

Introduction

Animals with complex life histories, such as holometabolous insects and amphibians, often occupy very different habitats during different phases of their life cycle. The fitness of these individuals is influenced not only by the environment they occupy during reproductive stages, but also by the environment they encountered during immature development. This phenomenon is sometimes referred to as the "carry-over" effect and can be defined as nonlethal events (or conditions) that have latent down-stream effects on important life history characteristic such as fecundity and longevity (Lindstrom 1999; Pechenik 2006; Harrison et al. 2011). Conditions experienced during development can also exert strong effects on immature survival and development rate which will alter population dynamics (Alto and Juliano 2001; Muturi et al. 2010; Radchuk et al. 2013). Immature mosquitoes of several genera occupy small, discrete, and ephemeral aquatic habitats that can be natural (e.g. tree-holes and bromeliads) or artificial (e.g. discarded

tires and buckets). These habitats have very low primary productivity and rely on detritus inputs from terrestrial flora and fauna, which form the base of the food-web (Kitching 2000; Yee 2008; Yee et al. 2012). Leaves and animal carcasses, mainly arthropods, enter the system and break down, providing dissolved nutrients and supporting the growth of microorganisms. Mosquito larvae then graze, scrape, or filter on the detritus, in the water column, and along the walls of the habitats to harvest the microorganisms, though some species may also gnaw on and directly consume leaves and animal carcasses (Merritt et al. 1992; David et al. 2002; Bara et al. 2014). The presence of larvae can increase the rate of leaf decomposition, drastically reduce the number of bacteria on leaf surfaces, and change the community composition of microorganisms (Fish and Carpenter 1982; Kaufman et al. 2001, 2008; Kaufman and Walker 2006; Pelz-Stelinski et al. 2010; Muturi et al. 2013). Additionally, it has been shown that these habitats may vary seasonally in the quality of resources available to larvae, which may be a direct result of deterioration of detritus or an indirect result of the exploitation of the resources by prior cohorts of larvae (Fish and Carpenter 1982; Aspbury and Juliano 1998; Macia and Bradshaw 2000; Paradise 2004). Mosquitoes that occupy these container habitats are often important vectors of human diseases such as Dengue, Chikungunya, West Nile and La Crosse viruses, thus understanding the ecology of these species is of public health concern (Hardy et al. 1983; Gubler 1996; Turell et al. 2005; Alto et al. 2008).

Inter and intra-specific competition for resources is thought to be an important factor regulating populations in these habitats (Juliano 2009). Field and laboratory studies show that negative effects of density on mosquito larvae occur frequently (e.g. Livdahl and

Willey 1991; Bevins 2008).The outcomes of these experiments are not consistent however and there is a body of research that demonstrates that different detritus types (e.g. leaf species, plant vs. animal) and ratios can alter the outcome of density-dependent competition (Yee et al. 2007; Murrell and Juliano 2008; Reiskind et al. 2009; Murrell et al. 2011; Costanzo et al. 2011). It has been hypothesized that the heterogeneity of detritus inputs in these habitats is a mechanism that could account for coexistence among these container-breeding mosquitoes (Yee et al. 2007).

These container systems have also been investigated for potential effects of delayed-density dependence, wherein larvae are negatively affected by the presence in a container of prior, temporally non-overlapping cohorts (Aspbury and Juliano 1998; Walsh et al. 2012; Walsh et al. 2013). Prior exploitation of leaf detritus increased development time and reduced adult mass but did not affect larval survivorship for *Aedes triseriatus* (Aspbury and Juliano 1998). Walsh et al. (2012; 2013) allowed container habitats to be naturally colonized by *Aedes albopictus* and *Aedes aegypti*, respectively. In one half of each container cohorts of prior colonists were removed and densities of larvae quantified. Each container was then restocked at larval densities that mimicked the naturally occurring density (2013) or were equivalent to the natural density or 10x the natural density (2012). Walsh et al. (2013) found that for *Aedes aegypti*, there was an effect of a prior cohort on development time and size, but not larval survivorship (consistent with Aspbury and Juliano 1998). Walsh et al. (2012) found that for *Aedes albopictus*, there were significant negative effects of prior cohorts on survivorship only at densities that were 10x the naturally-occurring density of larvae and no effect on size or development,

suggesting that direct-density dependence exerts greater effect than does delayed-density dependence. There are two aspects that were overlooked in these studies: 1) they did not take into account that many of these habitats under natural conditions are of varying age, which will likely impact the quality of resources available and the number of prior cohorts that have exploited the habitat; and 2) they did not examine any carry-over effects of prior exploitation on adult phenotypes such as adult longevity. For mosquito vectors of pathogens, adult female longevity is one of the most important factors determining her vector potential (Dye 1986).

The age of container habitats is rarely taken into consideration when assessing the effects of density (either direct or delayed) on these populations. Whether the container habitat is natural (tree-hole) or artificial (discarded refuse) it is very likely that they are established at different times and have very different histories in terms of detritus input, especially when the majority of input comes in seasonal pulses (Macia and Bradshaw 2000; Bevins 2007; Yee and Juliano 2012; O'Neal and Juliano 2013; Murrell et al. 2014) or is colonized by species that are multivoltine with several generations over a single season repeatedly utilizing the same habitats. Habitat age has been shown to have an effect on colonization and community composition, independent of external seasonal factors in these systems (Murrell et al. 2014) which suggest that age of an aquatic habitat is an important determinant of the properties of the associated invertebrate community. The effect of age, however, is likely complicated. Detritus quality deteriorates over time, which reduces bacterial abundance and productivity (Kaufman et al. 2002; Kaufman and Walker 2006), but there is also the potential confounding effect of toxic secondary

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compounds leached from leaves that can have negative effects on mosquito larvae which may either increase or decrease overtime (David et al. 2000; Kim and Muturi 2012). Additionally, while the quality of detritus deteriorates and microorganisms are consumed by larvae, there are new inputs into containers which may compensate for the losses due to time (Paradise 2004; Bevins 2007; Yee and Juliano 2012; O'Neal and Juliano 2013).

In this paper, we tested the hypotheses that prior exploitation by a temporally non-overlapping cohort of larvae and habitat age both affect the survival, development, and size of developing female mosquitoes and also have carry-over effects on adult female longevity. We were specifically interested in females as they will typically suffer the consequences of reduced resource availability to a greater extent than will males as they take longer to develop, require more resources, and pay higher fitness costs for being small than do males, and also are the sex that vectors pathogens (Hardy et al. 1983; Wormington and Juliano 2014a; Wormington and Juliano 2014b). To test these hypotheses we designed a 2 x 2 factorial randomized block field experiment with prior exploitation and habitat age as the main effects. In addition to the experimental containers, we established a set of 6 unmanipulated containers that were colonized naturally and received natural additions of detritus, to test for effects of cumulative estimated larval densities and habitat age on female longevity.

Materials and Methods

Aedes triseriatus used in this experiment were from a laboratory colony that was less than one year old, and originated from a population at Tyson Research Center approximately 20 miles outside of St. Louis, MO. White plastic 7.5 L buckets were used as the

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experimental containers with each initially receiving 3 L of DI water and 6 g of senescent white oak leaves (*Quercus alba*). All experimental containers were covered with mosquito netting to prevent colonization and additional detritus accumulation. Experimental containers were established as a 2 x 2 factorial randomized full block design with 6 spatial blocks placed 1 meter apart and containers within blocks only inches apart under the forest canopy at Parklands Nature Preserve in McLean County, IL. The treatments were prior exploitation by larvae (PE), no prior exploitation (NPE). At the time of addition of the experimental cohort of larvae, containers were either four (young) or eight (old) weeks old. One unmanipulated container was also established in each block for a total of 30 containers (24 experimental, 6 unmanipulated). Unmanipulated containers were given the same initial detritus and water as the experimental containers but were left uncovered and allowed to be colonized naturally by the local mosquito community. Containers were established at two different times to allow them to age for the appropriate number of weeks so that experimental cohorts were present in containers at exactly the same time (Table 3.1.).

Containers assigned to PE treatment had 200 first instar *A. triseriatus* larvae added one week after the containers were filled. The NPE habitats were also unsealed at the same time larvae were added to the PE treatments to account for any possible addition of detritus that may have occurred when the habitats were unsealed, though every attempt was made to minimize this. All habitats were unsealed regularly to remove living and dead adults from the water surface. As our habitats were sealed, we expected higher adult mortality than naturally occurs, so we removed dead adults so that they did not add

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unusual amounts of dead insect detritus. The experimental larvae were added to all treatments at the same time, with 150 first instar larvae added to each habitat. Before larvae were added, all habitats were sieved using a 106 µm mesh to remove any remaining larvae from the prior cohorts, but returning all leaf detritus and any biofilm to the containers. Containers were checked regularly and when pupae were observed the entire contents of the containers was poured into a white pan at the field site and pupae were removed and returned to the laboratory every two days. Pupae were held in 20 mL vials with adult emergence recorded daily. Males were discarded and females were kept in groups by treatment, block, and date of emergence in 1 L plastic ice cream containers covered with mosquito netting and given *ad libitum* access to 5% sugar solution. Pupae and adult females were held at 25° C with a 14:10 L:D photoperiod. Female mortality was recorded daily and dead females were dried at 50° C. During the adult life, many of the wings were damaged so we measured the length of the hind femur to assess female size.

The umanipulated containers were monitored weekly to assess the time to colonization. All of the containers were colonized by week 7 and larval sampling began on week 8. A fine mesh aquarium net with a horizontal opening was placed in the bottom of the container and after waiting 2 minutes to allow the larvae to acclimate, the net was pulled up through the entire water column, yielding a sample of larvae from the entire height of the water column. We calculated the volume sampled by multiplying the depth of the water (which was measured at the time of the sample) by the opening area of the net (42 cm^2). Larval densities in the habitats were then estimated by dividing the number of

larvae in the sample by the sample volume. This enabled us to estimate density with minimal disturbance to the community as it was necessary to remove the larvae and return them to the laboratory for identification. Larvae were identified to species (Darsie and Ward 2005). Samples were taken weekly until the end of the experiment. Every two to three days, pupae were removed from the unmanipulated containers, returned to the laboratory, and allowed to eclose in the same manner as pupae from the experimental containers. We removed pupae, instead of larvae, to be followed for female longevity as the pupal stage is a non-feeding stage and it was assumed that any impact the larval habitat has on adults has already occurred. Adult females were identified as either *Aedes triseriatus* or *Aedes japonicus* and held in the same conditions as females from the experimental containers with daily mortality recorded. At the end of the study, detritus from all unmanipulated containers was collected, dried at 50° C, and weighed.

For the experimental containers, percent female larval survivorship (assuming a 50:50 sex ratio), median time to eclosion (females only), and mean female size were analyzed as separate ANOVAs with individual microcosms as the experimental unit and a random block effect (PROC GLM). We also analyzed a composite performance index which combines data on survivorship, development time, and size. Livdahl and Sugihara's (1984) composite index can often be interpreted as an estimate of population rate of change (e.g. Livdahl and Willey 1991; Murrell and Juliano 2012), but the index we calculate here should not be interpreted in that way, as we did not have available a size-fecundity relationship with femur length, which is our indicator of size. The composite index we calculate, in the absence of a fecundity measure, is, however, expected to be

positively correlated with overall population performance, and is preferable to interpreting individual life history variable separately, as different variables respond in different, but correlated ways to treatments, and likely influence population performance in nonlinear ways (Livdahl and Sugihara 1984). The index we use, as described by Livdahl and Sugihara (1984), combines life history variables in a biologically sensible way derived from life table calculations, and thus yields a biologically meaningful synthesis of several life history responses to experimental conditions. Female longevity was analyzed using Cox Proportional Hazards model with the main effects of age, prior exploitation, the interaction, and female size as a covariate, with a random block effect (PROC PHREG).

For the unmanipulated containers, cumulative densities of larvae over the course of the experiment were obtained for the dominant species and for all species combined, by block. We expected that the effects of week and cumulative prior densities would be highly correlated and thus redundant. To determine whether models with week or with cumulative density better described our data on female longevity we compared proportional hazards models (PROC PHREG) for AIC values. This comparison required designation of block as a fixed effect in PHREG. The full model included week, cumulative *Culex restuans*, *Aedes triseriatus*, and *Aedes japonicus*. We also tested a model that included week and cumulative total larvae combing the three dominant species. We determined the best model based on the lowest AIC score and the weight of evidence (ω_i) which can be interpreted as the probability that a model is actually the correct model (Burnham et al. 2010). The best model was then run again with block as a

random effect to generate appropriate tests for main effects and covariates. We did separate analyses for longevity of the two *Aedes* species (PROC PHREG). Because of damaged wings after determining longevity, we did not obtain sufficient size data for the females originating from the unmanipulated habitats to include that variable in the models. All analyses were performed in SAS 9.3 (SAS User's Guide, SAS Institute 2011).

Results

There was a significant effect of habitat age on female larval survivorship with greater female survivorship in the older habitats and a marginally significant increase in female survivorship in containers with no prior exploitation (Table 3.2.; Fig. 3.1.a). There were significant effects of treatment, prior exploitation, and interaction on median female development time (Table 3.2.; Fig. 3.1.b). Females from the younger and prior exploitation treatment took longer to develop than did females from the other three treatments (Fig. 3.1.b). There was no significant effect of container age, prior exploitation or the interaction on female size (Table 3.2.; Fig.3.1.c). There was a significant effect of container age but not prior exploitation or the interaction on the composite performance index with cohorts performing better in the older habitats (Table 3.2.; Fig. 3.1.d). Container age had a significant effect on adult female longevity (Table 3.3.) but not prior exploitation, interaction, or female size. Females originating from the younger habitats had a significantly greater hazard of death (Table 3.3.; Figs.3.2.a,3.2.b).

The dominant species in the unmanipulated containers were *Culex restuans*, *Aedes japonicus*, and *Aedes triseriatus*. *Culex restuans* colonized early (week 7) and were

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largely absent by week 13 after which the containers were dominated by *A. japonicus* and *A. triseriatus* (Fig. 3.3.). *A. triseriatus* and *A. japonicus* pupae were only collected in sufficient numbers to be included in the analysis of female longevity from weeks 13, 14, and 15. The best model for *A. triseriatus* longevity only included the main effect of week (Table 3.4.). In contrast with *A. triseriatus* females from the controlled experiment, females from the unmanipulated habitats had decreased longevity as the containers aged (n = 117; χ^2 = 23.95; df = 2; P = <0.001). They exhibited increased mortality if they were collected in weeks 15 compared to week 14 and 13, weeks 13 and 14 were not different (Fig. 3.4.) suggesting that older habitats are poorer habitats. The best model for *A. japonicus* included week and cumulative density of *C. restuans* (Table 3.4.). The effect of week was not significant (n = 53; χ^2 = 3.81; df = 2; P = 0.149) and the effect of *C. restuans* was negative (χ^2 = 6.59; df = 1; P = 0.01) indicating that greater previous density of *C. restuans* in the container was associated with greater hazard of death was for this species (hazard ratio = 1.012). The mean increase in detritus dried mass accumulated in the unmanipulated habitats over the course of the entire experiment was 7.64 g ± 1.4 (SE).

Discussion

Little attention has been paid to the effects of habitat age in these aquatic container systems though it is very likely that there is great variation in age of these habitats in nature. The quality of these habitats, in terms of larval mosquito performance, is highly variable (Leonard and Juliano 1995). We found a significant effect of habitat age on female larval survival with greater survivorship in the older habitats. For development

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time, container age interacted with prior exploitation such that females took significantly longer to develop when the habitat was young and had a prior cohort. Though we did not find a significant effect of either treatment on female size, the composite index of cohort performance was also higher in the older containers than the younger containers. We also found a slight, though statistically significant, carry-over effect of larval habitat age on adult female longevity with an increase in the hazard of death for females originating from the younger, compared to the older, containers. These results taken together suggest that mosquito performance is increased when reared in older habitats when additional detritus accumulation is prevented. The mechanism behind this finding is not immediately clear, however we have two competing, though non-mutually exclusive, hypotheses.

First, mosquito larvae, and other aquatic animals, can be negatively affected by secondary compounds in leaf detritus that leach out in water. Tannins in particular have been shown to negatively affect development rate and survivorship though other polyphenolic compounds may also play a role (Mercer 1993; Sota 1993; Mercer and Anderson 1994; David et al. 2000; David et al. 2002). Tannins may also inhibit bacterial productivity, may bind with proteins removing them as a food source for microorganisms, and affect dissolved oxygen content in the water (Lin et al. 2007; Earl and Semlitsch 2015). White oak leaves are high in tannins (Ostrofsky 1993) and thus the increased age the of habitats may have been sufficient time for the degradation of tannin molecules that then had less direct toxicity to mosquito larvae or released the proteins they had bound and were now available as a food source for other microorganisms

(Webster and Benfield 1986; Kaufman and Walker 2006). In experimental field microcosms, Muturi et al. (2012) found low pupal production for *Culex restuans* and *Aedes japonicus*, two other container breeding mosquitoes, reared on oak detritus but tannin concentration was not a significant predictor of pupal success in their model. Tannin concentrations in natural tree-holes vary over time (Mercer 1993; Mercer and Anderson 1994) and actual rate of tannin decay has not been measured in these habitat types but detectable tannins can be rapidly removed in experimental microcosms at time scales much shorter than that of our experiment (Maie et al. 2008; Earl and Semlitsch 2015). We did not measure tannin concentrations in this experiment, but it is reasonable to think that degradation of tannins may have, at least partially, led to the greater performance of larvae in the older containers.

A second hypothesis is that white oak leaves as the detritus source increased in quality as they aged. White oak leaves have been repeatedly shown to be a poor resource for mosquitoes and it is thought that this is due mainly to their slow rate of decay and high tannin-lignin content (Fish and Carpenter 1982; Murrell and Juliano 2008; Muturi et al. 2012). In detritus based systems, such as our experimental containers, leaves (along with animal detritus) enter the system and immediately begin leaching organic and inorganic molecules and are colonized by microorganisms such as fungi and bacteria that increase the decomposition process and secrete enzymes that soften the leaf material (Webster and Benfield 1986). Microorganisms are believed to be the main source of food for these container mosquito larvae, which are considered browsers or grazers (Kaufman et al. 2001). Mosquito larvae have been shown to reduce bacteria, though not fungi, on leaves

in tree-hole systems. The mosquito literature demonstrates a reduction of bacteria on leaves over a time scale of days to weeks which suggests that older leaves should be a poorer resource for developing larvae (Fish and Carpenter 1982; Macia and Bradshaw 2000; Kaufman et al. 2001; Kaufman et al. 2002). Interestingly, the literature from flowing water systems (e.g. temperate woodland streams) demonstrated the opposite effect: that at time scales up to 240 days microbial respiration remains steady and fungal biomass and bacteria abundance increase on leaves (Gulis and Suberkropp 2003). The discrepancy between the two sets of literature may be due to cooler temperatures experienced in streams compared to small discrete container habitats slowing the rate of leaf decay and microbial growth in streams (Webster and Benfield 1986), the lack of physical agitation due to flowing water in containers (Lepori et al. 2005), or due to differences in water chemistry between the two habitats (Suberkropp and Chauvet 1995).

After extended periods of conditioning in the water, the oak leaves may have softened enough to allow for direct consumption of detritus material and fungi that would have been recalcitrant to larvae before lengthy conditioning. Again drawing on literature from stream ecology, Golladay et al. (1983) found that Plecoptera had higher oak leaf ingestion and assimilation rates when the leaves had been conditioned for two months compared to one month or not at all. Mosquito larvae are not typically thought to be shredders, though *A. triseriatus* was classified as a shredder in a review of larval mosquito feeding behavior (Merritt et al. 1992), has been observed consuming leaves (Carpenter 1982), detritus particles are found in the gut contents (Walker et al. 1988), and it has been postulated that bacteria are not sufficient to meet the nutritional needs of this

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species (Kaufman and Walker 2006; Pelz-Stelinski et al. 2010). Kaufman, et al. (2006) speculated that fungi is likely the food source that larvae utilize to subsidize their diet and that increases in adult production in their experimental containers was likely due fungal activity, not bacteria. Other studies have demonstrated the importance of leaves, *per se*, in these habitats and found that leaves contributed the most to larval performance irrespective of other nutrient additions such as leaf leachate, stem flow, etc. (Leonard and Juliano 1995; Macia and Bradshaw 2000; Pelz-Stelinski et al. 2010). It was postulated the importance of leaves as a resource for larvae was indirect and functions mainly as substrate for bacterial colonization, though leaves removed from natural tree-holes with larvae present have very little bacteria present (Fish and Carpenter 1982). We suggest that aging may improve leaves as a source of mosquito nutrition most likely via increase in fungal colonization which may become more available to larvae as leaves become more fragmented as they age (Kaufman and Walker 2006) enabling larvae to ingest leaf particles containing fungi within the leaf matrix. We have no way of determining the mechanism in this experiment as we did not quantify chemical composition or microbial abundances. Future studies that investigate the roles of natural tannin concentrations and decay and the role of leaf aging on the release of fungi as a food source would be useful.

It would be interesting to repeat this experiment with different types of leaves, determining the effect of aging on other leaf species that vary in decomposition rates. It may be that rapidly decomposing leaf species would become poorer as food resources over the time scale used in this experiment (28 vs. 56 days) as their nutrients are rapidly converted to microbial biomass. We cannot find any comparable studies that explicitly

tested for the effect of habitat "age" for tree-hole-mosquito detritivore systems. Container age was found to be an important factor determining succession patterns and community composition in similar container systems suggesting that oviposition choices or larval performance were impacted by container age independent of other external factors such as date and precipitation (Murrell et al. 2014). Munga et al. (2013) manipulated the age of habitats for the malaria vector *Anopheles gambae* in Kenya and found higher abundances of larvae in younger habitats though they also found higher predator abundance in older habitats which was negatively correlated with prey larval abundance. This result suggests that the negative effect of habitat age they observed may be directly related to predator establishment in older habitats. They also found that development rate and adult size were unaffected by habitat age when predators were excluded (Munga et al. 2013). These effects of predators cannot be the explanation for what we observe, as our experiment excluded predatory species.

We found some evidence of prior habitat exploitation (delayed-density dependence) having an effect on a later non-overlapping cohort of mosquito larvae. There was a marginally significant effect of prior exploitation (PE) on female larval survivorship (P = 0.057) with more adult females produced in habitats without PE, which is consistent with other studies that have demonstrated limited effects of delayed-density dependence on larval survival (Aspbury and Juliano 1998; Walsh et al. 2012; Walsh et al. 2013). We did observe a significant interaction of PE and habitat age on female development time, wherein females took significantly longer to develop in younger PE containers than the other 3 treatment groups. This interaction is interesting in that it highlights the likely

complex interactions among habitat characteristics that influence mosquito dynamics. We found no effect of treatment on female size, which is consistent with two previous studies, though Aspbury and Juliano (1998) did find an effect on adult mass (Walsh et al. 2012). Based on the results of our study, and the results of the three previously published studies testing for delayed-density dependence in container habitats, there are limited, but reproducible effects which are primarily evident in prolonged larval development time. We found no effect of PE on our measure of cohort performance, which suggests that even with delayed development rates in the PE treatment, those cohorts should perform equally well as cohorts originating from habitats that were not previously exploited, which is again consistent with Aspubury and Juliano (1998).

One major limitation of this study, and the referenced similar studies, is that under natural conditions, many previous cohorts of mosquitoes will utilize these habitats, and that there will be continuous additions (at variable rates) of new detritus. This pattern may go on for years. To begin to address the question of how prior exploitation and habitat age affect mosquito performance under more realistic conditions, we also established 6 habitats that we left open to colonization by resident mosquitoes and to additions of detritus. By taking a subsample of the larval community, we were able to estimate the cumulative density of larvae over time and minimize disturbance. We did not quantify oviposition, larval survival or development time, but we did remove pupae regularly to assess adult female longevity in the laboratory. Consistent with the results from the experimental containers, there was no effect of larval PE on adult *A. triseriatus* female longevity which suggests that for this species, under these circumstances, any negative

effect of PE does not carry-over to affect adult longevity. This finding is consistent with similar data collected at Tyson Research Center, Eureka, MO in 2013 which showed no effect of either *Aedes* or *Culex* larval density present in the habitats at the time pupae were collected on *A. triseriatus* longevity (Westby and Juliano, *unpublished data*). In the current study, we found a highly significant effect of week of collection on *A. triseriatus* longevity though this result was not consistent with the data from the experimental containers. Females collected from habitats that were older had poorer longevity than did females collected when the habitats were younger (Fig. 3.4.). Despite the seeming inconsistency, the data are not easily comparable as in the experimental containers, "ages" were either 4 weeks (young) or 8 weeks (old) before addition of experimental larvae, whereas for females collected from the unmanipulated habitats, we were comparing females collected from habitats that were 13, 14, or 15 weeks old with no way of determining, based on our design, how old the habitats were when eggs hatched and larvae commenced development. Additionally, the unmanipulated containers were colonized by a mix of species whereas our experimental containers were single species assemblages. This finding again highlights that the effects of habitat age and PE are complicated and that without direct manipulation it is difficult to tease apart which effects are the most important.

In contrast with *A. triseriatus*, adult female *A. japonicus* were not affected by habitat age but exhibited reduced longevity due to the cumulative density of prior *C. restuans* larvae in the habitat. This supports the hypothesis that, at least for some species, and species combinations, PE by interspecific competitors has negative carry-over effects on adults.

Laboratory experiments testing for effects of inter- and intra-specific competition between these species have not all reached the same conclusion, but when negative effects were detected they were weak despite evidence from field surveys that *A. japonicus* is displacing *Culex spp.* and *A. triseriatus* in some habitats (reviewed by Kaufman and Fonseca 2014). An experimental removal of *Aedes* from container habitats in the field led to significantly higher densities of *Culex* in naturally colonized containers and is the strongest empirical support for a negative effect of *Aedes* on *Culex* (Murrell and Juliano 2013). Most empirical studies of the interactions of *Culex* and *Aedes* find asymmetrical effects, with impacts of *Culex* on *Aedes* relatively small (Costanzo et al. 2005; Murrell and Juliano 2012). We did not detect any intra-specific or intra-generic effects on female longevity, which may be partly due to the low density of *Aedes* in our unmanipulated containers, which combined never exceeded 40 larvae per liter and which is lower than densities used in laboratory competition experiments for the "low density" treatment (Hardstone and Andreadis 2012). Our hypothesis for the stronger affect of *C. restuans* on *A. japonicus* than on *A. triseriatus* is differences among the species in habitat use. *A. japonicus* spends more time filtering and resting at the water surface than does *A. triseriatus* and this behavior is similar to that of *C. pipiens* (Yee et al. 2004; O'Donnell and Armbruster 2007).

In conclusion, we report evidence that container age affects larval performance and adult female longevity in *A. triseriatus*, though the effect of container age was positive and surprising in light of literature that shows a reduction in bacterial productivity in container habitats as they age. We believe the mechanism is increased in resource quality

arising from leaf conditioning and the release of recalcitrant fungal resources as leaves

fragment. We also report a negative effect of prior habitat exploitation on female

development time and evidence of inter-specific delayed-density dependence on *A.*

japonicus adult female longevity in containers that were allowed to be colonized

naturally.

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Table 3.1. Experimental set up of container habitats at Parklands Nature Preserve, McLean County, IL and comments on the timing of colonization and sampling of unmanipulated container habitats.

Date	Week	Event
4/25/2014	**1**	Old containers established
	1	Unmanipulated containers established
	2	Prior cohorts added to old containers
5/23/2014	**5**	Young containers established
	6	Prior cohorts added to young containers
	7	Unmanipulated containers colonized by *C. restuans*
	8	Sampling of unmanipulated containers began
6/20/2014	**9**	Prior cohorts removed, experimental larvae added
	9	*A. triseriatus* and *A. japonicus* colonize unmanipulated containers
8/9/2014	**16**	Last female ecloses from experimental containers

Table 3.2. ANOVA table for the effects of habitat age and prior exploitation for the manipulated experiment on female larval survivorship, female development time, female size, and a composite performance index.

Source	df	Female survivorship		Female size		Female development time		Composite index	
		F value	P value	F value	P value	F value	P value	F value	P value
Age	1	9.94	**0.0066**	1.05	0.3221	24.11	**0.0002**	8.59	**0.0103**
Prior	1	4.25	0.0571	1.56	0.2308	24.11	**0.0002**	2.85	0.1119
Age*Prior	1	0.53	0.4781	0.69	0.4184	12.19	**0.0033**	0	0.9857
Block	5	1.44	0.2667	2.14	0.1159	0.25	0.9317	1.90	0.1542

Table 3.3. ANOVA table for the effects of larval rearing treatment on adult female longevity. Container is a random effect.

Effect	χ2 Value	DF	P Value
Age	3.7189	0.6339	**0.0284**
Prior	1.8134	0.6286	0.1022
Age*prior	0.0711	0.623	0.6085
Femur length	0.1876	0.8887	0.6116
Container	10.137	6.8015	0.1675

Table 3.4. Model parameters used to determine the best model describing adult female longevity for *Aedes triseriatus* and *Aedes japonicus* originating from the unmanipulated containers. The effect of block was included as a fixed effect in each run in PROC PHREG (SAS 9.3) which is the only way to generate AIC values for determining model fit. The model with lowest AIC value is considered the best model and is indicated in bold. We also report ΔAIC with is a numerical value demonstrating how distant a particular model is from the best model and ω_i which is the weight of evidence that supports the model and can be interpreted as the probability that a model is the correct model.

Model	A. triseriatus			A. japonicus		
	AIC	Δ AIC	ω_i	AIC	Δ AIC	ω_i
Week, *A. triseriatus, A. japonicus, C. restuans*	887.03	5.43	0.0338	309.33	3.8	0.0965
Week, *A. japonicus, C. restuans*	885.63	4.03	0.0681	307.53	2	0.2372
Week, *C. restuans*	883.6	2	0.1879	**305.53**	**0**	**0.6449**
Week	**881.6**	**0**	**0.5108**	314.65	9.12	0.0068
Week, total larvae	883.5	1.9	0.1976	313.617	8.087	0.0113
Total larvae	892.77	11.17	0.0019	316.01	10.48	0.0034
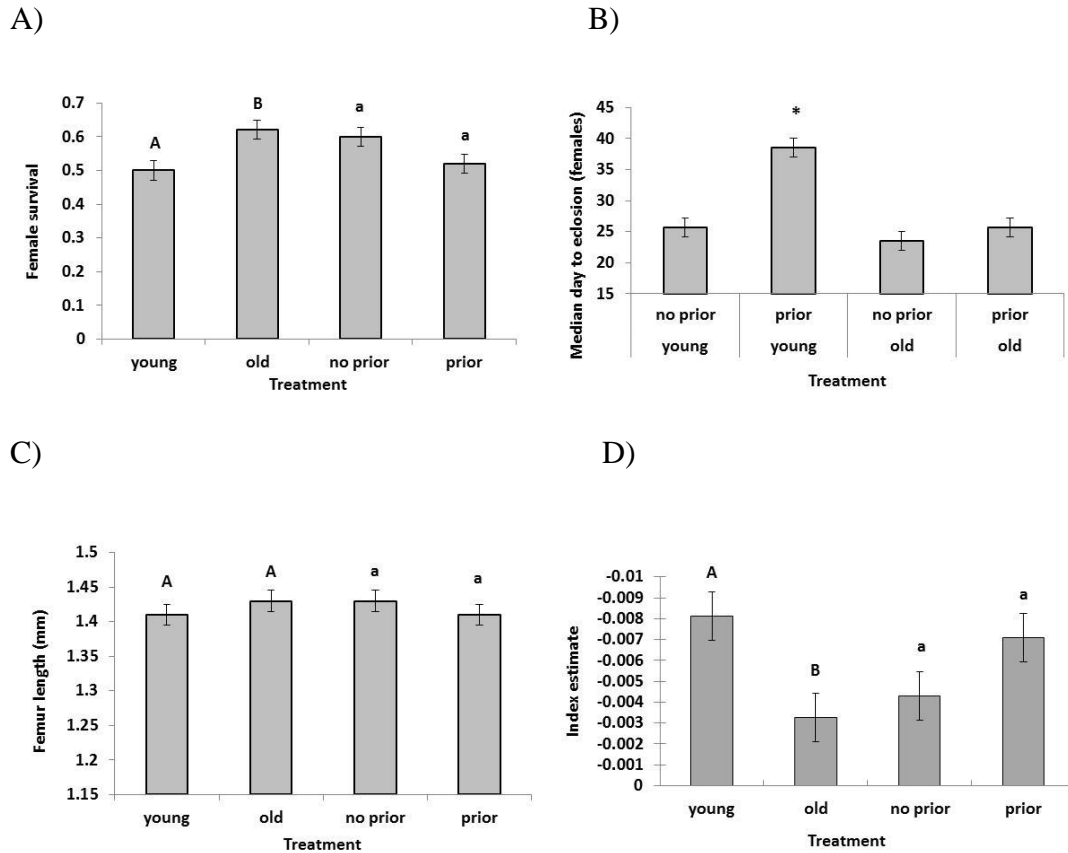
Fig. 3.1. A) female larval survivorship in the manipulated experiment, B) female median day to eclosion, C) female femur length, and D) the composite performance index. For the performance index, the larger the number the better the performance which in this case the number that is closer to zero, the smaller the bar on the figure, the higher the estimate. Differences between young and old containers are denoted by capital letters and differences among prior exploitation containers are denoted by lower case letters. For the significant interaction (B) an asterisk denotes a group that is different than the others.

A)
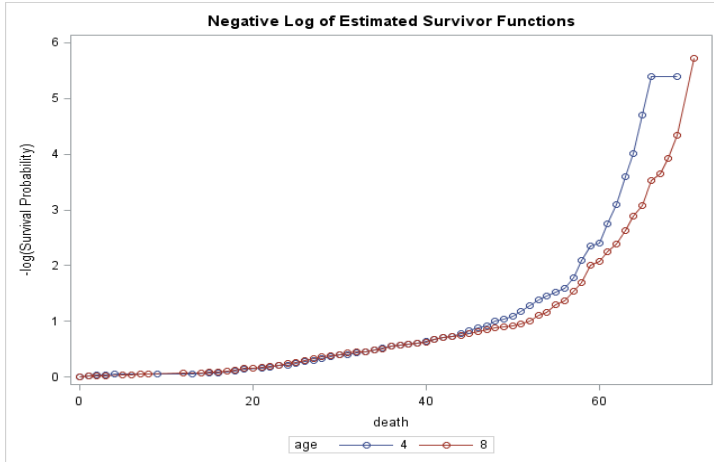
B)
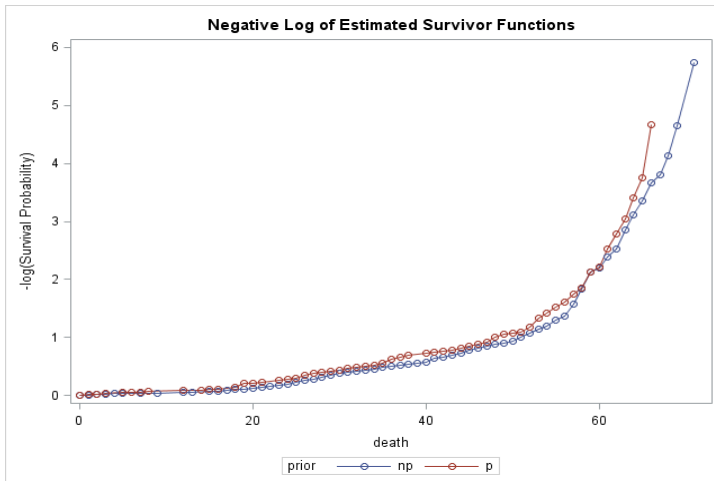
Fig. 3.2. Negative log of the estimated survivor functions from the manipulated experiment for A) young (4 week) and old (8 week) container habitats and B) the not prior exploited and prior exploited habitats for adult female *Aedes triseriatus* longevity. The upturn of the curve late in life indicates an increase in the hazard at death due to senescence.

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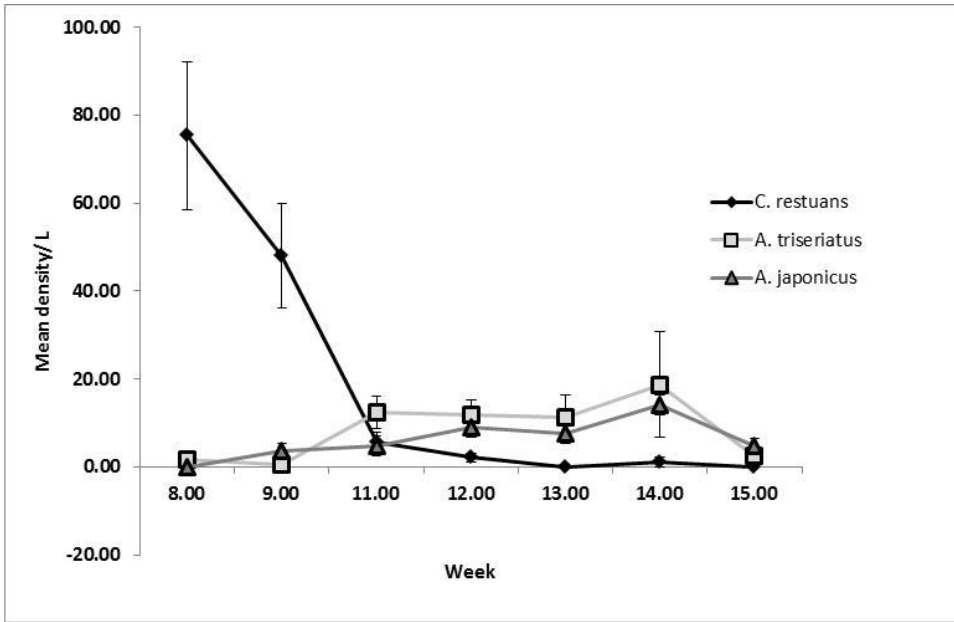
Fig. 3.3. Mean density (larvae per liter) and standard errors of the dominant species collected from the unmanipulated habitats by week. All unmanipulated habitats were established at the same time and week refers to the number of weeks since the habitat was established. For calendar dates see Table 3.1.
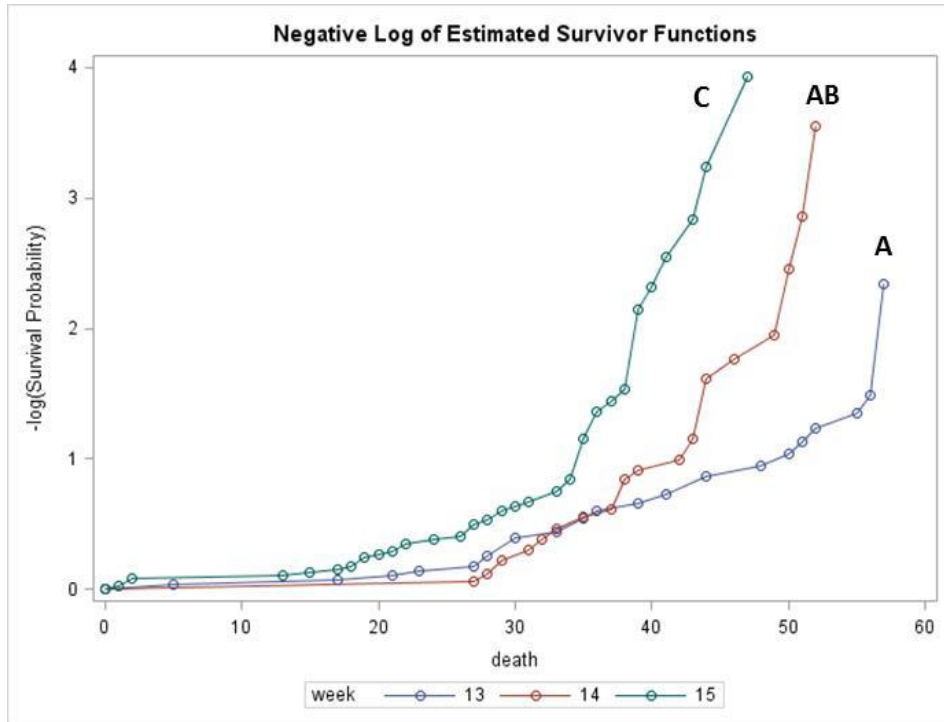
Fig. 3.4. Negative log of estimated survivor functions for adult female *Aedes triseriatus* stratified by the week they were collected as pupae from the unmanipulated containers. Week refers to the number of weeks since the container was established. For calendar dates refer to Table 3.1. Letters denote weeks that were significantly different from each other using the Sidak correction for multiple comparisons.

CHAPTER IV

HABITAT SIZE, HYDROPERIOD, AND PREDATION: LIMITED EFFECTS

ON PREY SPECIES ABUNDANCE, ADULT PRODUCTION AND

ADULT LONGEVITY IN AQUATIC CONTAINER

COMMUNITES

Abstract

Several genera of mosquitoes occupy small ephemeral aquatic habitats in their immature

stages. These "container" habitats vary widely in nature, size, and permanence. The

species that occupy these containers also vary in their intrinsic ability to withstand

desiccation. General aquatic ecology theory suggests that smaller aquatic habitats have

shorter hydroperiods and that density-dependent resource competition in these habitats is

an important biotic force shaping the communities. Larger habitats are generally more

stable, support more predatory species and are thought to be more regulated by predation

than competition. In a field experiment, we directly manipulated habitat size and

hydroperiod to test hypotheses about how size and hydroperiod influence larval prey and

predator abundances. We also followed adult females collected as pupae to test for carry-

over effects of competition with other prey species or trait-mediated indirect effects of

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the presence of predators on adult longevity. We found little evidence that short term

drying influences predator or prey abundance in these communities and that two weeks is

sufficient for the communities that occupy these habitats to return to densities observed in

stable volume containers. There was some sorting of prey species by habitat size, though

no effect of habitat size was observed for the predatory species. There were detectable,

and negative, effects of predator abundance in the larval habitat on adult female longevity

for two of the prey species tested and density of prey in one species.

Introduction

Aquatic container habitats are discrete and often ephemeral habitats that can either be

natural (e.g., tree-holes and bromeliads) or artificial (e.g., discarded tires and cemetery

vases). The associated communities are dominated by detritivorous larval Diptera, mainly

mosquitoes, that browse, scrape, and filter microorganisms from the water column, the

sides of the containers, and on leaf and animal detritus (Merritt et al. 1992). These larvae

are thought to experience frequent density-dependent competition for resources, which

likely impacts population and community dynamics and can have negative carry-over

effects on adult fitness (Juliano 2009, Reiskind and Lounibos 2009, Muturi et al. 2010,

Alto 2011). In some locations, these communities also include obligate predators such as

odonates (Yanoviak 1999) and mosquitoes from the genus *Toxorhynchites* (Lounibos

1985). Predation can alter larval densities and pupal production of prey species and

community composition in these habitats (Lounibos 1985, Bradshaw and Holzapfel 1988,

Copeland and Craig 1992, Munga et al. 2013, Petermann et al. 2015). Effects of predators

can be through direct consumption altering prey abundances, indirect via changes in prey

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oviposition patterns (Blaustein et al. 2004) or by causing a shift in prey behavior from risky forging behavior to less risky resting behavior. Because of the trade-off of feeding vs. safety, these indirect effects may result in longer development time and smaller size at eclosion for prey which can also have carry-over effects on adult phenotypes (Laurila and Kujasalo 1999, Bridges 2002, Vonesh 2005, Kesavaraju et al. 2011, Wormington and Juliano 2014). Non-lethal effects of predators, or trait-mediated indirect effects, can be as important as the direct effects predators have on prey density by consuming prey (reviewed by Werner and Peacor 2003).

 One of the key properties of container habitats is that they are subject to frequent disturbance due to habitat drying (Lounibos 1985, Bradshaw and Holzapfel 1988). Drying can have direct effects on larvae through desiccation and death but can also have indirect effects by altering life histories (e.g., Juliano and Stoffregen 1994), quality of resources in the habitat (e.g., Aspbury and Juliano 1998), or by altering biotic interactions via resource competition or predation (Wellborn et al. 1996, Laurila and Kujasalo 1999, Alto and Juliano 2001, Bridges 2002, Turner and Montgomery 2009, Murrell and Juliano 2013). The species that occupy these habitats differ in their abilities to withstand habitat drying. Mosquitoes in the genus *Aedes* lay drought resistant eggs on the sides of containers that hatch when flooded and exhibit plasticity in development rate under conditions of declining water volumes (Juliano and Stoffregen 1994). Mosquitoes in the genera *Culex*, *Orthopodomyia*, and predatory *Toxorhynchites* have no desiccation resistant life stages and lay their eggs directly on the water surface and thus are likely to be more negatively impacted by habitat drying (Bradshaw and Holzapfel 1988).

Container habitat size is related to hydroperiod, or the duration of time the habitat remains wet, with hydroperiod increasing with container size. Hydroperiod is also postulated to determine predator abundance, with larger, slower developing, predatory species frequently absent from smaller habitats with shorter hydroperiods (reviewed by Wellborn et al., 1996). General aquatic ecology theory predicts that in smaller, more ephemeral habitats, resource competition and the fitness gained from rapid growth and development should be strong determinants of community composition, whereas in larger, more permanent habitats, predation and evasion of, or resistance to predation, should be the primary determinants of community composition (Wellborn et al. 1996). Some observational evidence exists for these ideas in container communities (e.g., Bradshaw and Holzapfel 1988) but these ideas have not been tested in field experiments. We tested the hypothesis that container habitat size, hydroperiod, and predation impact larval densities, adult production, and adult female longevity for species of container mosquitoes with different intrinsic tolerances of habitat drying, competition, and predation (described by Murrell and Juliano 2013). We manipulated both habitat size and hydroperiod in a factorial design. We predicted that both container size and hydroperiod would affect community composition and abundances of species populations, and that size and hydroperiod may interact to influence community composition.

Materials and Methods

Container habitats were established at Tyson Research Center, near Eureka, MO in April of 2013. Containers were set in six transects along a small service road, twenty meters

apart and 1 to 5 meters from the road under the canopy of the hardwood forest. Four different sizes of black plastic containers were affixed to trees, except the 3.5 L containers which were staked in the ground, and filled with collected rain water to an initial water volumes of 0.035 L (cups; n=48), 3.5 L (buckets; n=24), 35 L (small barrels; n=12) or 140 L (large barrels; n=6). Containers on each transect were interspersed systematically so that adjacent containers were of different sizes (Fig. 4.1.). Each habitat received 5 g/ L of senescent white oak leaves (*Quercus alba*) as initial detritus input. The containers were allowed to be colonized naturally and no attempt was made to exclude additional detritus inputs. To manipulate hydroperiod, rain guards were fashioned above the containers using hardware cloth covered with thick white plastic sheeting. Half of the containers were assigned to have stable water volumes and half were assigned dry out over time. Containers that were assigned to the drying treatment had their volumes reduced by 20% each week until they were dry, to remain dry for two weeks, and to be refilled to their initial volume (Fig. 4.2.). Containers that were assigned to the stable volume treatment had their volumes returned to their initial levels weekly as needed. The drying manipulation was completed twice (Fig. 4.2.).

The sampling and drying schedule was set so that after the containers were forced to dry out and to be refilled the first time, larval samples were taken 2 weeks after they were refilled and samples were taken 1 week after they were refilled after the second drying. A weighted plankton net with a 113 cm^2 opening area was placed in each barrel and allowed to settle on the bottom. The community was given 2 min to resume activity after the disturbance, then the plankton net was hauled vertically and contents were washed

into a pan and collected for identification (Darsie and Ward). Two samples were taken from the small barrels and 4 samples were taken from the large barrels and the average densities of the samples were used in the analysis. The mean densities of larvae and pupae in these samples were determined by dividing the number of individuals in the sample by the volume of the water sampled (net opening area x water depth). Buckets were sampled nondestructively every 2 weeks using a small rectangular aquarium net (42 cm^2 opening area) pulled vertically as described for the barrels. The volume of the sample was estimated as the opening area of the net x water depth. For buckets, small barrels, and large barrels we estimated that the samples represented means (SDs) of: 4.2% (1.9%), 12.0% (0.1%), and 5.1% (0.7%), respectively, of the total container volumes. A different sampling approach was taken with the cups because of their small volume. Half of the 48 total cups were destructively sampled every two weeks, taking all larvae and pupae, and returning water and detritus to the cup. The other half of the 48 cups were sampled two weeks later, so that each cup was sampled destructively every 4 weeks. The longer sampling interval for each cup was implemented to allow recolonization after complete removal of the assemblage.

Pupae in the samples were allowed to eclose and were identified to genus for *Culex* or to species for *Aedes*. Adult female *Culex* were only identified to genus due to the difficulty of distinguishing morphologically *C. restuans* and *C. pipiens* (Harrington and Poulson 2008), which are the dominant species at Tyson Research Center. Adults were then held at 25° C and a 14:10 L:D photoperiod, in 1 L plastic ice cream containers in groups by day of eclosion and container number. Adults were given *ad libitum* access to 10% sugar

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solution. Mortality was assessed daily with dead individuals removed and dried at 50° C. Size was determined by measuring one wing using Image J software.

Larvae per liter was analyzed for common species either by species (*Aedes triseratus* and *Aedes japonicus*) or by genus (*Culex*) using a mixed model repeated measures ANOVA with container size, drying treatment, sample date, all possible interactions, *Toxorhyncites rutilus* density as a covariate, and transect and transect-drying treatment interaction as random effects and individual container as the subject. Sampling weeks where the drying treatment containers were completely dry were excluded from the analysis. The data were best fit using a zero-inflated Poisson error distribution which accounts for the large number of zero values in the data set (PROC GLIMMIX, SAS Institute Inc. 2011) (Leisnham et al. 2014). For weeks early or late in the year in which no larvae were present for a species or genus we considered these absences to be due to phenological differences, and those weeks were excluded from the analysis. Thus, reported means of larval density reflect means for wet containers when that taxon was active. We analyzed *A. triseriatus*, *A. japonicus*, and *Culex spp*. pupae as cumulative mean pupae per liter over the sampling period, again excluding weeks when they were completely absent. Mean pupae per liter was analyzed using the same mixed model and zero-inflated Poisson distribution of error as the larvae per liter except for the effect of sample date was removed. We analyzed the cumulative mean *T. rutilus* in the same manner as pupae. Adult female longevity was analyzed by species (*A. triseriatus* and *A. japonicus*) or genus (*Culex spp.*) with container size, drying treatment, total *Aedes* per liter, total *Culex* per liter, total *T. rutilus* per liter collected in the sample at the same time

the pupae were collected, and female wing length as main effects, and individual

container as a random effect (PROC PHREG; SAS Institute Inc., 2011). Wing length was

omitted from the model for *A. japonicus* as 50% of our individuals had wings too

damaged to measure.

Results

There were no significant main effects of container size, drying treatment, or *T. rutilus*

density for *A. triseriatus* larval densities, but sampling date and its two-way interactions

with container size and drying treatment significantly affected *A. triseriatus* (Table 4.1.).

After adjusting for multiple comparisons there were very few significant differences

among groups and the differences that were significant were mainly associated with

changes in densities in the cups and buckets over time while densities in small and large

barrel remaining steady over time (Fig. 4.3.a). The single significant difference within

weeks was significantly reduced densities in cups compared to buckets after the first

refilling. The interaction between drying treatment and sample date was driven by greater

density in the drying containers after the second refilling, but not after the first (Fig.

4.3.b).

For *A. japonicus*, there was a significant main effect of container size but not of drying

treatment, sample week, or *T. rutilus* density. Interactions of sampling date with container

size and with drying treatment were significant (Table 4.1.). *A. japonicus* were largely

absent from the cups, but due to high variability among samples, their densities in cups

never differed significantly from those in the larger containers within weeks (Table 4.1.).

The largest effect was a spike in *A. japonicus* densities in the buckets after the first

refilling but not after the second (Fig. 4.4.a). As with *A. triseriatus*, densities in the large

and small barrels remained steady over time (Fig. 4.4.a). Although adjustment for

multiple comparisons resulted in no detectable significant differences between pairs of

groups for combinations of drying and sample (Fig. 4.4.b) the source of the significant

drying treatment-sample interaction (Table 4.1.) was evident. Mean densities across all

container sizes for *A. japonicus* in the drying containers were low prior to drying, and

remained low after the first refilling, but then reached a strong peak after the second

drying (Fig. 4.4.b). In marked contrast, in the stable containers, *A. japonicus* densities

were greatest after the first drying, and fell to virtually zero after the second drying (Fig.

4.4.b).

The interaction between container size and sample date was the only significant effect for

Culex spp. larvae (Table 4.1.). Mean densities were significantly lower in cups and

buckets than in small barrels, but only in the sampling week before the first drying

period, though densities were low in the cups and buckets throughout the sampling period

(Fig. 4.5.). After drying, *Culex* abundances were very low in all container sizes (Fig.

4.5.). There was a significant pairwise difference between the small barrels from May 16

(before first drying) and June 27 (after the first refilling) (Fig. 4.5.).

There was a significant effect of containers size on the mean cumulative density of *A.*

triseriatus pupae, but no significant effect of drying treatment or the container size-

drying treatment interaction (Table 4.2.). Cups produced significantly more pupae per

liter than did buckets and small barrels; large barrels were indistinguishable from all

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other groups (Fig. 4.6.a). There were no significant effects on *A. japonicus* mean cumulative pupal density, though the trend was for fewer *A. japonicus* pupae per liter in the cups (Table 4.2.; Fig. 4.6.b). There was a significant effect of container size but not of drying treatment or drying-container size interaction for mean cumulative density *Culex* pupae, with significantly fewer pupae per liter in the smaller containers (Table 4.2.; Fig. 4.6.c). There were no significant effects of container size ($F_{3,78} = 0.25$; $P = 0.8631$), drying treatment ($F_{1,78} = 0.77$; $P = 0.385$), and interaction ($F_{3,78} = 0.98$; $P = 0.4074$) on cumulative mean *T. rutilus* density (Fig. 4.7.).

Adult female *A. triseriatus* longevity was significantly, and negatively, affected by *T. rutilus* density (hazard ratio = 2.006) only (Table 4.3.). Adult female *A. japonicus* longevity was not significantly affected by any of the variables (Table 4.3.). Adult female *Culex* longevity was significantly affected by drying treatments, with females eclosing from the drying treatment having a significant lower hazard of dying than did those from the stable treatment (hazard ratio = 0.585) (Fig. 4.8.). *Culex* longevity was marginally significantly affected by density of *Aedes* and the effect was negative (hazard ratio = 1.052), and was significantly and negatively affected by *Culex* (hazard ratio = 1.009) and *T. rutilus* (hazard ratio = 49.373) densities. The effect of wing length was significant, with larger females having a greater hazard of death (hazard ratio = 1.605) (Table 4.3.).

Discussion

We tested how habitat size and hydroperiod affect predator abundance to test whether these factors work independently, or in combination, to influence larval densities, pupal

production (which a proxy measure of adult production), and adult female longevity in temperate-zone container breeding mosquitoes.

We manipulated habitat size and drying independently and found little evidence to support the hypotheses that hydroperiod and habitat drying have lasting effects on this container community at the scale used in this study. There was a significant interaction between sample date and drying treatment for *A. triseriatus* and *A. japonicus*. We observed a spike in larval density only after refilling the containers after the second drying and not the first. This difference is likely due to our sampling schedule, where the sample was taken two weeks after refilling the first time and one week after refilling the second time. The spike in density occurred as eggs on the side of the containers hatched *en mass*, but within two weeks, the densities had stabilized and were the same as containers that remained filled to a constant depth. Weeks within drying periods maintained steady larval densities even though the volume of water was reduced in the drying containers by 40% over the two weeks between sampling periods. This suggests that the combined effects of limited egg hatching as no additional water entered and created a hatching stimulus, larval death, and adult eclosion were sufficient to keep densities stable despite declining volume.

There were differences in pupal production among container sizes with more *A. triseriatus* pupae being produced per unit volume in the smaller containers and more *A. japonicus* and *Culex* pupae produced per unit volume in the larger containers. These findings are consistent with observational data about habitat size preferences in these and

related species with varying levels of habitat size specialization (Bradshaw and Holzapfel 1988, Sunahara et al. 2002, Gilbert et al. 2008, Laporta et al. 2014) though they do exhibit plasticity in habitat usage and can be found in a range of habitat sizes, which our data also support (Bevins 2007).

Drying and habitat size had no effect on cumulative mean *T. rutilus* density. Following Wellborn et al. (1996), we predicted that drying would be particularly detrimental to this predator as it takes longer to develop and does not have desiccation resistant eggs that can remain in the habitat and hatch after subsequent refilling. While the physical drying of the habitat surely killed any individuals that were present at the time of drying, containers were rapidly recolonized after refilling and supported equal predator densities as stable containers. Our data does not support observational data that *T. rutilus* is more abundant in larger containers with longer hydroperiods (Focks et al. 1983, Bradshaw and Holzapfel 1988).

We found no evidence that *T. rutilus* densities impacted prey density. We did however detect a negative effect of *T. rutilus* density on adult female longevity for *A. triseriatus* and *Culex spp*, but not for *A. japonicus*. This result suggests differences in anti-predator behavioral responses among the species though *A. japonicus* has been shown to reduce risky behavior and spend more time resting in the presence of predator cues (Kesavaraju et al. 2011). Larval *A. japonicus* are readily consumed by *T. rutilus* though they are less vulnerable to predation than are *Culex spp*, suggesting that they may engage in less risky behavior (Murrell and Juliano 2013). This nonlethal, apparent trait-mediated effect of

predation threat (Werner and Peacor 2003) appears carry over to affect female longevity in *A. triseriatus*, which is well known to change its behavior in response to cues from predation (Kesavaraju et al. 2007, Wormington and Juliano 2014). Effects of predator cues on larval *Culex* behavior in these container systems is not well studied, but *Culex* in other habitats do show strong behavioral responses to predators (Ferrari et al. 2008). *Culex spp*. female's longevity was also negatively affected by larval *Culex* density and marginally by *Aedes* densities (P = 0. 059) in the containers at the time they were collected which is evidence of density-dependent effects. *Culex spp*. females had reduced longevity if they originated from a stable container compared to containers that were drying which may be a result of concentration of soluble nutrients that fuel bacterial growth as volumes decrease.

Evidence of negative effects of habitat drying on species abundances in similar communities has been observed before, with drying most likely to have negative impacts on species with no desiccation resistant life stage (e.g., *Culex* and *T. rutilus*; Lounibos 1985, Bradshaw and Holzapfel 1988). The results from our manipulative experiment demonstrate that when drying takes place gradually and habitats rapidly refill these communities respond quickly and rebound within two weeks to densities observed in stable habitats irrespective of habitat size. These results suggest that at the scale of a forest plot, a diversity of habitat sizes and hydroperiods can support these populations and communities even with local extinctions due to complete drying. We found no support for the hypothesis that drying and habitat size alter predator abundance when these factors are experimentally manipulated, or that predator abundance shapes the

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abundance of prey species when the predator is allowed to colonize naturally. We did

find evidence that predator abundance indirectly alters adult longevity.

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Table 4.1. ANOVA table for the effect of container size, drying treatment, and sample
week for common species.

Effect	A.triseriatus			A. japonicus			Culex spp.		
	F value	df	P value	F value	df	P value	F value	df	P value
Container size	1.45	3, 83	0.2334	5.19	3, 83	**0.0025**	0	3, 82	1
Drying treatment	2.19	1, 83	0.1432	0.24	1, 83	0.6221	0	1, 82	0.9969
Size*treatment	1.28	3, 83	0.2854	1.00	3, 83	0.3955	0.03	3, 82	0.9944
T. rutilus density	0.46	1, 230	0.4993	0.08	1,288	0.7765	0.35	1,206	0.5545
Sample week	4.94	4, 230	**0.0008**	1.61	5, 288	0.1581	0.01	4, 206	0.9999
Size*sample	2.42	12, 230	**0.0057**	3.87	15, 288	**<.0001**	2.21E+26	11, 206	**<.0001**
Treatment*sample	4.27	4, 230	**0.0023**	100.59	5, 288	**<.0001**	0	4, 206	1
Treatment*sample*size	1.4	12, 230	0.1654	0.28	10, 288	0.9848	0.03	9, 206	1

Table 4.2. ANOVA table for the effect of container size, drying treatment, and the interaction on cumulative mean density of pupae produced for common species across the entire season.

Effect	A. triseriatus			A. japonicus			Culex spp.		
	F Value	df	P Value	F Value	df	P Value	F Value	df	P Value
Container size	6.22	3, 78	**0.0008**	1.32	3, 78	0.2725	13.15	3, 77	**<0.0001**
Treatment	1.43	1, 78	0.2348	0.11	1, 78	0.741	0	1, 77	0.992
Size*treatment	0.44	3, 78	0.9432	0.143	3, 78	0.9334	1.43	3, 77	0.2403

Table 4.3. ANOVA table for the effects of container size, drying treatment, larval densities and size on adult female longevity of common species collected.

| Effect	A. triseriatus (n=32)			A. japonicus (n=80)			Culex spp. (n=213)		
	Wald χ^2	Adjusted df	Adjusted P	Wald χ^2	Adjusted df	Adjusted P	Wald χ^2	Adjusted df	Adjusted P
Container size	0.2291	0.509	0.378	1.7635	1.6272	0.3279	3.788	2.1415	0.1678
Treatment	0.9109	0.2521	0.0817	0.0672	0.4660	0.5048	6.2321	0.6752	**0.0067**
Aedes per liter	0.0339	0.2272	0.3359	0.4401	0.6535	0.3525	2.3578	0.5541	0.0592
Culex per liter	0.0187	0.2248	0.3755	0.0034	0.7631	0.9013	3.9432	0.8423	**0.0366**
T. rutilus per liter	4.2397	0.4439	**0.0127**	0.7012	0.6581	0.2697	7.4948	0.8998	**0.0052**
Wing length	0.0951	0.6120	0.5657	n/a	n/a	n/a	3.8302	0.9141	**0.0441**
Container id	14.0772	5.496	**0.0211**	11.6108	6.0088	0.0716	10.8943	4.8927	**0.0502**

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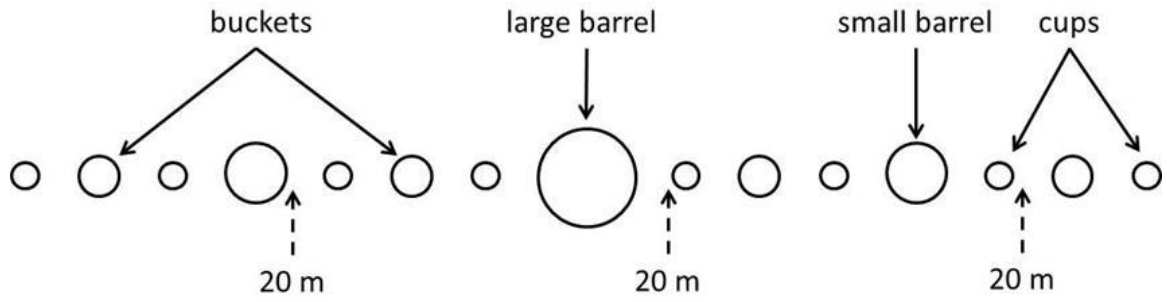
Fig. 4.1. Graphical representation of the container set up.
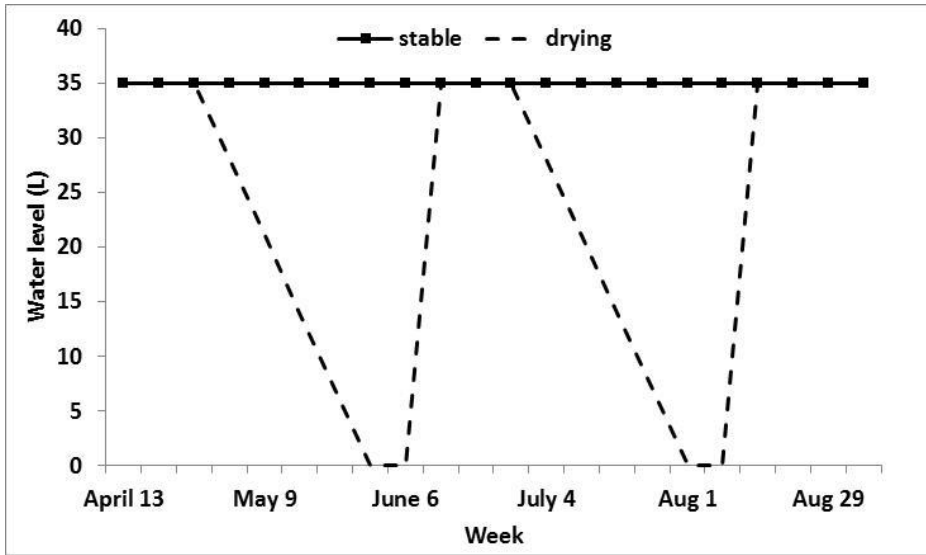
Fig. 4.2. Drying regime. The volumes shown are for the small barrels though all container sizes were dried on the same schedule.
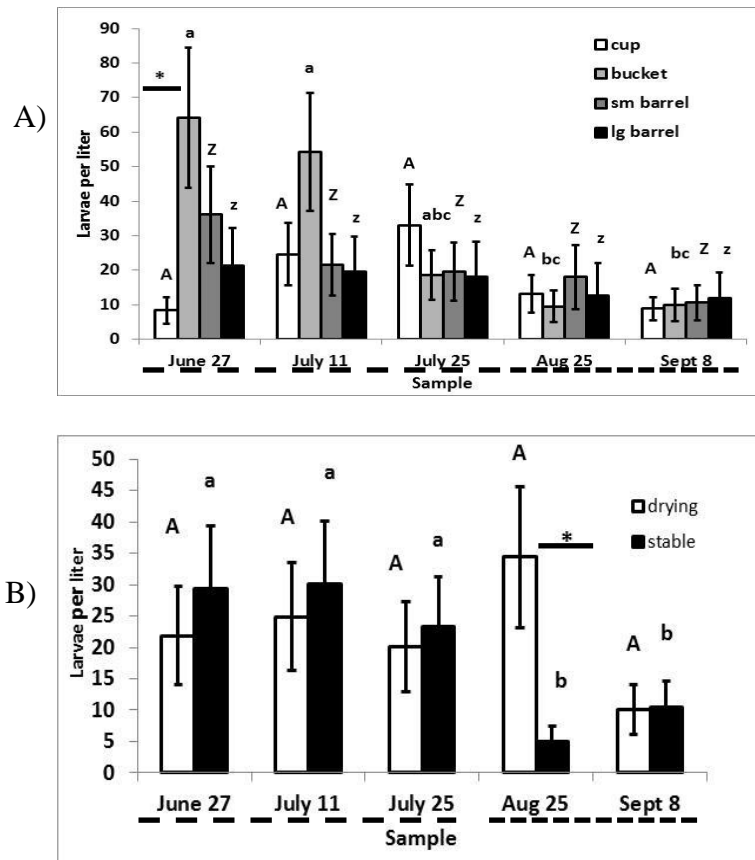
Fig. 4.3. A) Least squares means and standard errors for the significant interaction between container size and sampling date on *A. triseriatus* larval density. Letters denote sizes that are different from each other across weeks; cups are capital A-D, buckets are lower case a-d, small barrels are capital Z-W, and large barrels are lower case z-w. Asterisks denote container sizes that are significantly different from each other within weeks. B) Least squares means and standard errors for the significant interaction between drying treatment and sample date on *A. triseriatus* larval densities. Upper case letters denote differences in the declining containers across weeks and lower case letters denote differences in the stable containers across weeks. Asterisks denote significant differences between the treatments within a sample week. The cut off p value for significance was determined using the Bonferroni correction for multiple comparisons. The long dashed line under the x axis indicates samples taken after the first drying and the short dash line indicates samples taken after the second drying.
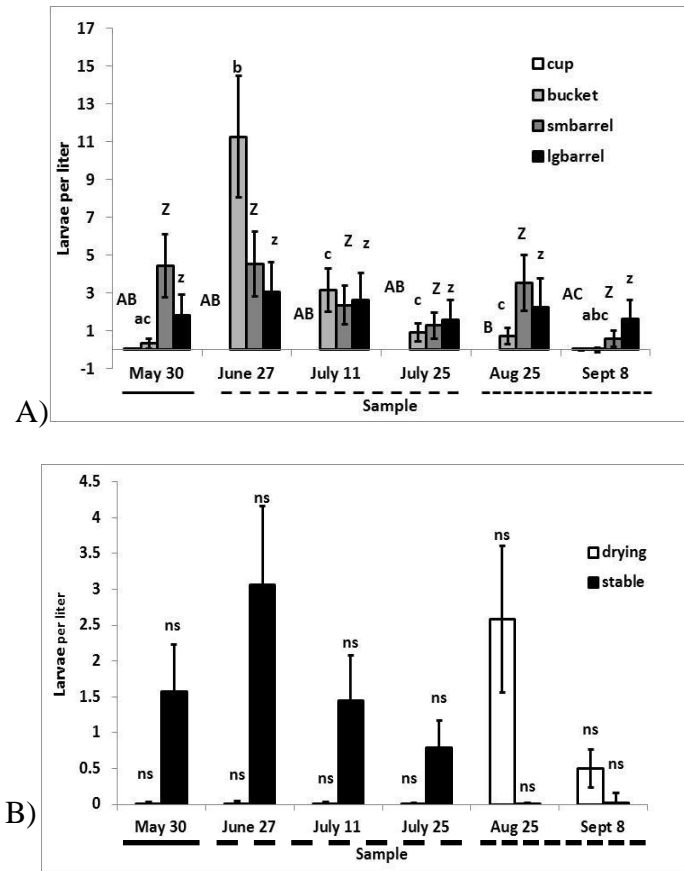
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Fig. 4.4. A) Least squares means and standard errors for the significant interaction between container size and sampling date on *A. japonicus* larval density. Letters denote sizes that are different from each other across weeks; cups are capital A-D, buckets are lower case a-d, small barrels are capital Z-W, and large barrels are lower case z-w. There were no significant differences between container sizes within weeks. B) Least squares means and standard errors for the significant interaction between drying treatment and sample date on *A. japonicus* larval densities. There were no significant differences among groups after adjusting for multiple comparisons. The cut off p value for significance was determined using the Bonferroni correction for multiple comparisons. The solid line under the x axis denotes samples taken before the first drying, the long dashed line indicates samples taken after the first drying, and the short dashed line indicates samples taken after the second drying.
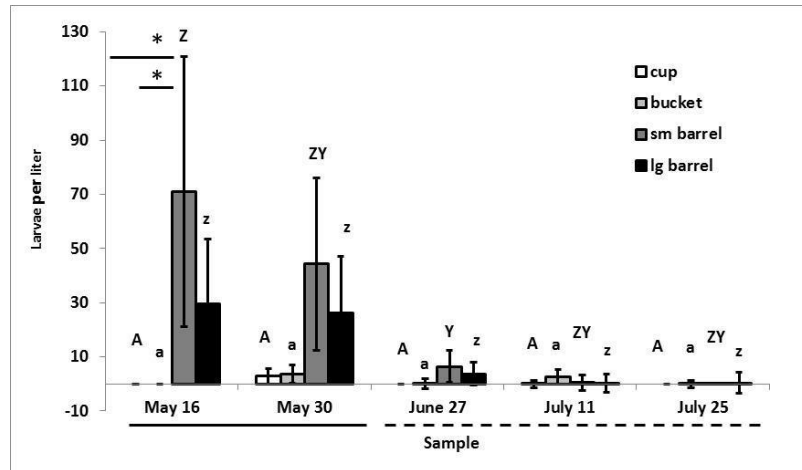
120

Fig. 4.5. Least squares means and standard errors for the significant interaction between container size and sampling date on *Culex spp.* larval density. Letters denote sizes that are different from each other across weeks; cups are capital A-D, buckets are lower case a-d, small barrels are capital Z-W, and large barrels are lower case z-w. Asterisks denote significant differences between container sizes within weeks. The cut off p value for significance was determined using the Bonferroni correction for multiple comparisons.
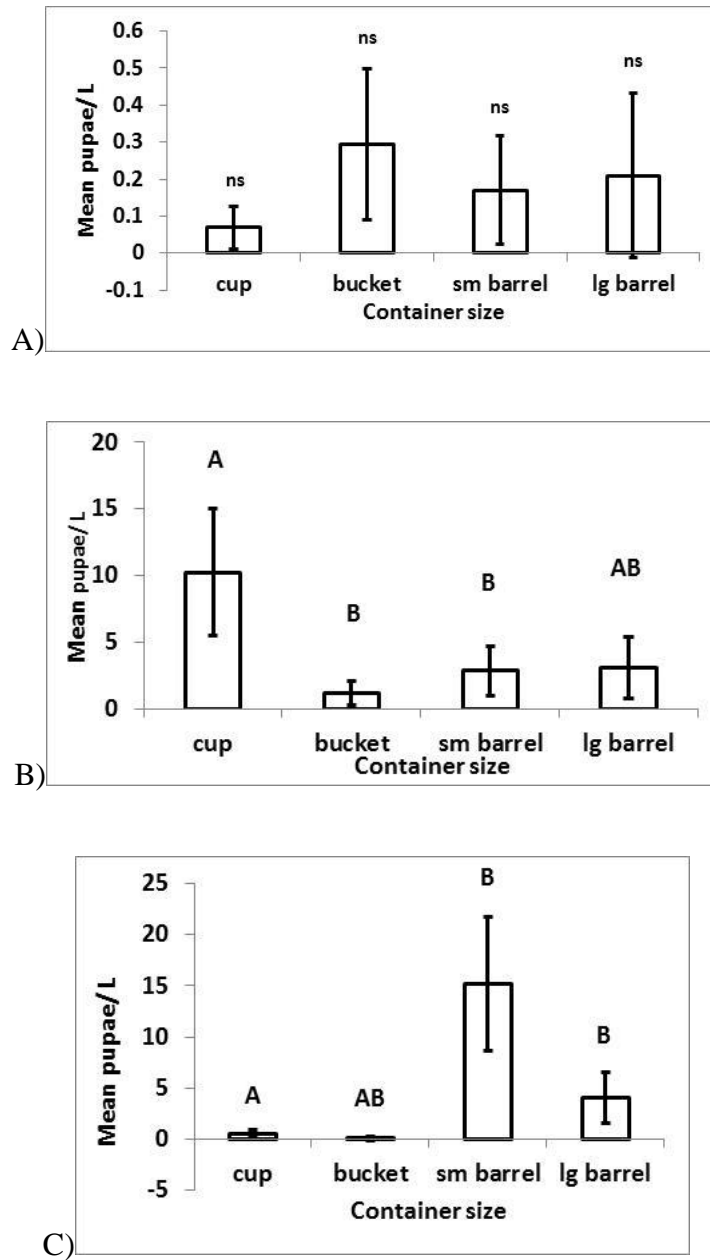
Fig. 4.6. Least squares means and standard errors of the cumulative mean pupae per liter for A) *A. triseriatus*, B) *A. japonicus*, and C) *Culex spp.*. Different letters denote container sizes that are significantly different. Adjusted p values were obtained using Tukey's correction for multiple comparisons.
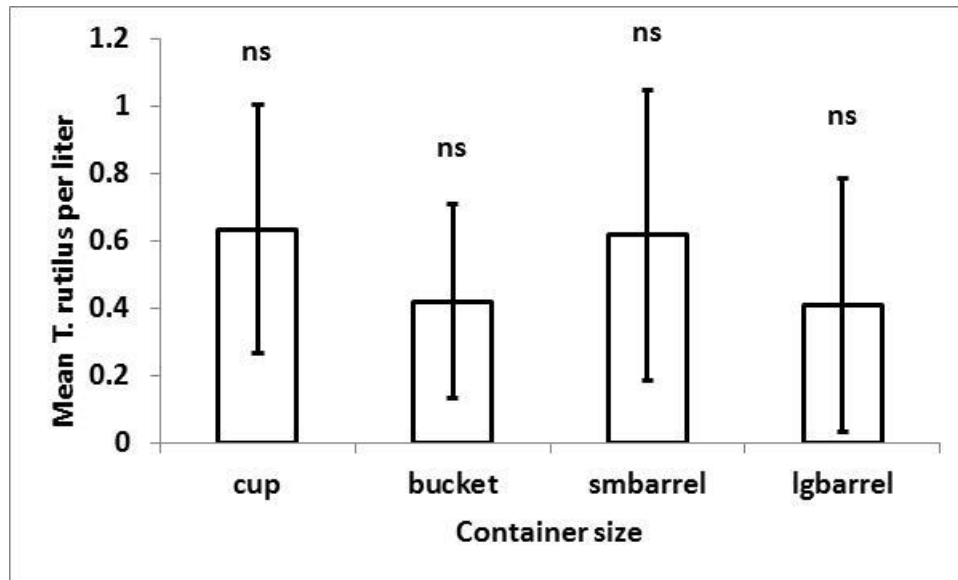
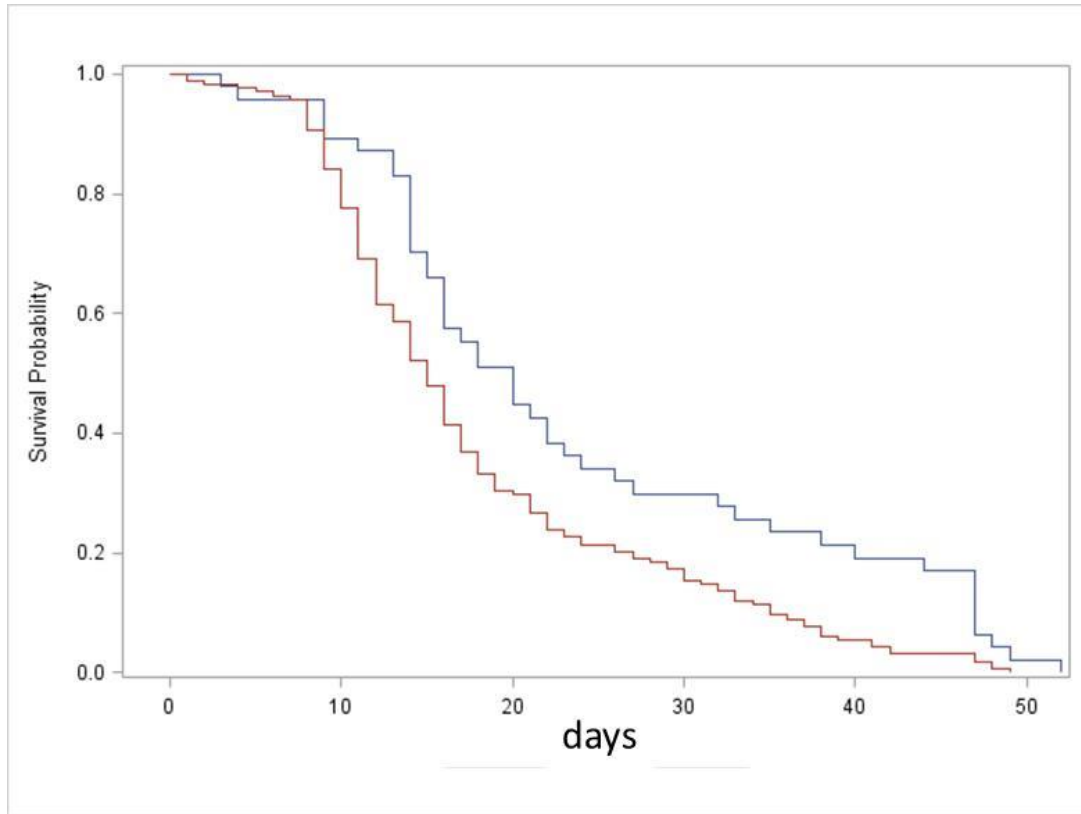**Fig. 4.7.** Cumulative mean *T. rutilus* per liter.

Fig. 4.8. Cumulative survivorship probability for *Culex spp*. adult females collected as pupae from either the drying or stable treatments. The red line represents the stable containers and the blue line represents the drying containers.