THE EFFECT OF NITROGEN ON THE LARVAL GROWTH OF THE INVASIVE MOSQUITO *AEDES JAPONICUS JAPONICUS* (THEOBALD)

by

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ABSTRACT

THE EFFECT OF NITROGEN ON THE LARVAL GROWTH OF THE INVASIVE MOSQUITO *AEDES JAPONICUS JAPONICUS* (THEOBALD)

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Invasions by non-native disease vectoring mosquito species have become important considerations in mosquito control and public health. The Asian rock pool mosquito, *Aedes japonicus japonicus* (Theobald), which was first found in the US in 1998, has been increasing both its range and abundance. This invasive mosquito has the potential to displace the native La Crosse vectoring mosquito, *Aedes triseriatus* (Say). Several studies have shown that *Ae. j. japonicus* larvae have shorter development times to pupation than *Ae. triseriatus*. The overarching hypothesis for this thesis was that *Ae. j. japonicus* is able to emerge from larval habitats faster than *Ae. triseriatus* by better utilizing nutrients that accelerate its growth. Single larvae nanocosms were used to monitor individual larval development in the absence of competition, and multi-larvae microcosms were used to test effect of additional nitrogen on larval development. In these studies larvae of both species depressed the total amount of nitrogen in their habitats similarly, but newly emerged *Ae. j. japonicus* adults retained less nitrogen in their body tissues. It was also found that *Ae. j. japonicus* growth was not limited by nitrogen availability and that *Ae. triseriatus* may have been more sensitive to pathogenic microorganisms that were stimulated by the addition of soluble nitrogen.

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CHAPTER 1

INTRODUCTION

Mosquito Invasions

High-speed world commerce has increased both the mobility and distribution of non-native organisms and biological invasions have become commonplace. In many cases, invasive organisms can reduce or displace local populations and disrupt ecosystems. In the field of medical entomology, the biological invasions of arthropod born virus (arbovirus) vectoring mosquitoes (Diptera: Culicidae) have been of increasing importance (Juliano and Lounibos 2005). The introduction of any novel vector or disease can have a resounding impact on public health. The understanding and tracking of mosquito invasions are paramount.

The shipment of tires to the United States from Asia in the mid-1990's has facilitated the establishment of a number of mosquitoes in the genus *Aedes*, including the Asian rock pool mosquito, *Ae. japonicus japonicus* (Theobald) (Peyton et al. 1999). This species was first detected in the Eastern US in 1998, and has since been reported in both the Southern and Midwestern US (Peyton et al. 1999; Morris et al. 2007; Hughes et al. 2008; Neitzel et al. 2009; Gaspar et al. 2012), Pacific Northwest (Irish and Pierce 2008), and Hawaii (Larish and Savage 2005). *Aedes j. japonicus* has also expanded its range into Canada (Thielman and Hunter 2006) and throughout central Europe (Schaffner et al. 2003, 2009; Versteirt et al. 2009). Their larvae can be found in a diverse array of aquatic container habitats including rock pools, tree holes, and discarded tires (Scott et al. 2001; Andreadis et al. 2001). Mosquitoes that already inhabit these containers can interact with and can be affected by *Ae. j. japonicus* (Hardstone and Andreadis 2012). In discarded tire habitats in the North Eastern US, larvae of native mosquito *Aedes triseriatus* (Say) appear to be in the process of being replaced by *Ae. j. japonicus* (Andreadis and Wolfe 2010).

The increased presence of *Ae. j. japonicus* and the reduction of *Ae. triseriatus* could have an effect on US arbovirus transmission (Leisnham and Juliano 2012). *Aedes j. japonicus* has

been implicated as an important vector of Japanese encephalitis in Asia (Takashima and Rosen 1989) and is also a highly competent vector of West Nile virus (Sardelis and Turell 2001; Turell et al. 2000), eastern equine encephalitis virus (Sardelis et al. 2002a), La Crosse virus (Sardelis et al. 2002b), and St. Louis encephalitis virus (Sardelis et al. 2003) based on laboratory studies. While not considered an important disease vector in the US, *Ae. j. japonicus* is known to feed on both birds and humans (Williges et al. 2008; Molaei et al. 2009) and some field collected females have been found to be infected with West Nile virus (CDC 2000) and La Crosse encephalitis virus (Westby et al. 2011). *Aedes triseriatus* is the primary vector of La Crosse encephalitis virus (LACV) (Puntuwat et al. 1974; Beaty et al. 2000) and has also been implicated in West Nile virus transmission (Turell et al. 2005). The effect of the displacement of *Ae. triseriatus* by *Ae. j. japonicus* on disease transmission will depend on the efficiency of *Ae. j .japonicus* to transmit disease (Juliano and Lounibos 2005). Indeed, if *Ae. j. japonicus* is a less efficient it may decrease disease transmission; however, the opposite may be more likely.

The mechanism by which *Ae. j. japonicus* can displace *Ae. triseriatus* has not been fully elucidated. The most compelling finding has been that *Ae. j. japonicus* larvae tend to develop faster than *Ae. triseriatus* larvae under similar developmental conditions (Alto 2011; Hardstone and Andreadis 2012). Accelerated development time could allow for a quicker exposure to potential hosts and potential oviposition sites, as well as escaping from larval habitats that have restricted resources or may dry out (Juliano and Stoffregen 1994). It is currently not known how *Ae. j. japonicus* larvae develop faster, but it may be related to how the larvae interact with their habitat and compete for limited food resources.

Container Habitat Food Web

Container habitats usually contain a variety of aquatic organisms including fungi, bacteria, algae and insects (Kitching 2001; Kaufman et al. 2001, 2002; Lorenz et al. 2013). Nutrients in these containers are provided by the input of allochthonous particulate plant detritus (Fish and Carpenter 1982; Walker et al. 1997), animal matter (Daugherty et al. 2000; Yee and Juliano 2006; Harshaw

et al. 2007) and dissolved organic and inorganic nutrients from stemflow and rain (Carpenter 1982b; Kaufman et al. 1999; Kaufman and Walker 2006).

Larval mosquitoes cannot access most detrital nutrients directly. Fungi and bacteria are able to break down and obtain nutrients from decaying plant and animal material for use in metabolic processes (Fish and Carpenter 1982; Kaufman et al. 2001, 2008), and mosquito larvae rely heavily on this microbial biomass for their nutritional requirements (Carpenter 1983). Plant derived detritus is high in carbon (C) but low in nitrogen (N), and the decay of plant material in larval mosquito habitats can often be limited by N (Kaufman and Walker 2006); therefore, microbial biomass and mosquito development is limited by the relative amounts of C and N in detritus (Walker et al. 1997). The addition of dissolved forms of C and N provided by stemflow and rain can also stimulate microbial production and can alleviate larval nutritional stress (Kaufman et al. 2002; Kaufman and Walker 2006).

Ae. j . japonicus vs. *Ae. triseriatus*

Containers with decaying leaf detritus are attractive to ovipositing females and can sometimes support large larval populations (Beehler et al. 1992; Bartlett-Healy et al. 2012). High larval densities can cause both a reduction in larval survival and adult size (Alto 2011; Hardstone and Andreadis 2012); therefore, the fitness of a species is greatly influenced by competitive interactions in the larval stage.

Direct competition between larval *Ae. j. japonicus* and *Ae. triseriatus* has been examined only in a few laboratory studies (Ingrassia 2006; Alto 2011; Hardstone and Andreadis 2012). In most cases, competitive pressure was related to overall larval density and not interspecific competition; however, *Ae. j. japonicus* has consistently been shown to develop faster (Alto 2011; Hardstone and Andreadis 2012), including under very low food rations (Ingrassia 2006). This suggests that *Ae. j. japonicus* may have an intrinsic advantage in growth through physiological mechanisms independent of its interaction with *Ae. triseriatus*.

Stressful conditions have been shown to shorten larval development and this results in a decrease

in adult size in mosquitoes (Juliano and Stoffregen 1994). Even with a shorter development time, *Ae. j. japonicus* adults have not been found to differ greatly in size than those of *Ae. triseriatus* (Alto 2011; Hardstone and Andreadis 2012; Lorenz 2012). Factors that can determine body size in insects are food quality (Davidowitz et al. 2003), rearing temperature (Dodson et al. 2012), and genetics (Nijhout 2003).

Larvae of *Ae. j. japonicus* and *Ae. triseriatus* have been described as active grazers and filter feeders (Walker and Merritt 1991; Ingrassia 2006; O'Donnell and Armbruster 2007), though it has been reported that *Ae. triseriatus* tends to filter feed near the surface of the water column and *Ae. j. japonicus* larvae tend to graze near the bottom and on the container walls (Ingrassia 2006). Regardless, it is likely that both species can access similar food resources in containers; however, analysis of C and N in field-collected, newly emerged female *Ae. j. japonicus* and *Ae. triseriatus* collected from the same larval habitats showed that while C concentration was similar, *Ae. j. japonicus* had a lower N concentration (M. G. Kaufman *unpublished data*). Differences in N concentration has been shown between different mosquito species (Winters and Yee 2012). This may indicate that *Ae. j . japonicus* requires less N than *Ae. triseriatus* and can produce a similar amount of body mass with less food consumption (Fagan et al. 2002). This would allow for a competitive edge, especially when resources are limited.

Research Objectives and Rationale

With the arrival of *Ae. j. japonicus* and the displacement of *Ae. triseriatus* (Andreadis and Wolfe 2010), the ecology of disease transmission may change (Leisnham and Juliano 2012). Observations suggest that *Ae. j. japonicus* accumulates nutrients differently and this may point to an explanation as to how it is able to out-compete *Ae. triseriatus* (M. G. Kaufman *unpublished data*). This thesis aimed to examine the accumulation and effect of N on *Ae. j. japonicus* and *Ae. triseriatus* larval growth in the laboratory. The two main objectives were to:

1. Contrast the effects of individual *Ae. j. japonicus* and *Ae. triseriatus* larva on the levels

of particulate and dissolved N in laboratory nanocosms throughout development against nanocosms without larvae, and to measure and compare the accumulation of N in larval tissues throughout development

2. Compare the effect of additional nitrogen on the growth and N accumulation of *Ae. j. japonicus* and *Ae. triseriatus* larvae under intraspecific competition in laboratory microcosms

CHAPTER 2

COMPARING *AEDES JAPONICUS JAPONICUS* (THEOBALD) AND *AEDES TRISERIATUS* (SAY) EFFECTS ON NITROGEN IN NANOCOSMS

Introduction

Larvae of the invasive mosquito *Aedes japonicus japonicus* (Theobald) appear to be displacing *Aedes triseriatus* (Say) in discarded tire habitats (Joy and Sullivan 2005; Andreadis and Wolfe 2010). Studies have shown that *Ae. j. japonicus* generally develop faster than *Ae. triseriatus* females when under intraspecific and interspecific competition (Ingrassia 2006; Alto 2011; Hardstone and Andreadis 2012; Lorenz 2012). The underlying mechanism of this acceleration in growth is unknown, though it may be related to nutrient acquisition or assimilation.

Natural and artificial containers that act as mosquito larval habitats rely heavily on allochthonous inputs of particulate plant and animal detritus and dissolved nutrients (Carpenter 1982b; Fish and Carpenter 1982; Walker et al. 1997; Daugherty et al. 2000; Kaufman and Walker 2006). A common source of organic detritus in container habitats are senescent plant leaves (Fish and Carpenter 1982). These leaves initially leach out the more labile nutrients into the water column (Pelz-Stelinski et al. 2010). The majority of leaf particulates remain and are colonized by fungi and bacteria and is further decomposed (Fish and Carpenter 1982; Ponnusamy et al. 2008; Kaufman et al. 2010). The microbial biomass produced during leaf decay is utilized by mosquito larvae as their primary source of energy and nutrients for growth (Fish and Carpenter 1982; Walker et al. 1991; Kaufman et al. 2001, 2002; Kaufman and Walker 2006). Larval competition is common in these types of habitats and larval survival and production can be limited by the quantity and quality of detritus and associated microorganisms (Fish and Carpenter 1982; Walker et al. 1997).

Decomposition of carbon (C) polymers is generally limited by nitrogen (N), and phosphorus (P) (Howarth and Fisher 1976; Güsewell and Gessner 2009). Leaf detritus generally has a high C:N ratio; therefore, the decomposition of leaf material is limited by the availability of N (Kaufman and

Walker 2006; Güsewell and Gessner 2009). Fungi that colonize leaf surfaces also tend to have a relatively high C:N ratio, while bacteria tend to have a comparatively low C:N ratio (Güsewell and Gessner 2009). Larval feeding has been shown to depress bacterial productivity in the water column (Kaufman et al. 2001), and larvae actively feed on leaf surface bacteria and fungi, causing reductions and community shifts (Kaufman et al. 1999, 2008). Larval feeding can also affect the accumulation of inorganic N compounds in water columns (Kaufman et al. 1999).

Larval feeding modes have been shown to be similar between *Ae. j. japonicus* and *Ae. triseriatus* (Ingrassia 2006); however, newly emerged *Ae. j. japonicus* females from discarded tires were found to have a significantly lower N concentrations than *Ae. triseriatus* females (M. G. Kaufman *unpublished data*). This indicated that *Ae. j. japonicus* may have different nutrient requirements and it may interact differently with microbial resources to accelerate growth. This finding has not been corroborated in the laboratory, nor has it been investigated in larvae. The purpose of this study was twofold:

1. Investigate N accumulation in individual *Ae. j. japonicus* and *Ae. triseriatus* during the larval stage and at adult emergence

2. Determine if *Ae. j. japonicus* and *Ae. triseriatus* larvae alter available N in their habitats differently

Methods

All experimental units were housed in nanocosms consisting of plastic 50 ml conical tubes with 20 ml of deionized water, 50 mg of oven dried white oak (*Quercus alba*; $\pm$ .5 mg) leaves (collected at Kellogg Forest, Augusta, MI), and 100 μl of microbial inoculum collected from tree holes in Toumey woodlot (MSU, East Lansing, MI). Each nanocosm was incubated for 24 h at 24 $^{\circ}$C to facilitate microbial colonization and leaf decay before larval addition.

Mosquito Larvae

New Jersey strain *Ae. j. japonicus* larvae (Rutgers University, New Brunswick, NJ) and MSU strain *Ae. triseriatus* newly hatched larvae were used for these experiments. Hatching was induced by flooding the eggs in a nutrient-rich broth. The eggs of *Ae. triseriatus* required an additional 24 h soaking period in deionized water prior to flooding. After approximately 9 h, newly hatched larvae of each species were removed from the broth using a glass Pasteur pipette and transferred to a plastic Petri dish for counting. To remove the confounding factors of larval competition and mortality, each nanocosm received only one *Ae. j. japonicus* larva or one *Ae. triseriatus*. An additional 50 newly hatched larvae of each species were collected on glass cover slides and oven dried for 48 h for initial first instar weight and nitrogen content estimation. Nanocosms were stored at 24 $^{\circ}$C with a 12:12 light cycle. Each larval nanocosm was checked daily for newly shed cuticles to determine instars.

Experiment 1

To track the effect of larvae on N through time, three experimental groups were created consisting of (1) *Ae. j. japonicus*, (2) *Ae. triseriatus*, and (3) a no larvae control, with 50 replicates in each group. To insure adequate collection of second, third, and fourth instars, five randomly selected nanocosms from each group were destructively sampled on d 2, 4, 6, 9, 11, and 13 after hatching. Additional destructive sampling occurred at adult emergence or at larval death. Prior to processing,

the gender was determined for all adults and pupae. Sampling was ended after no pupae were produced ten days after the final pupa was observed (d=40). Mortality was not calculated for these nanocosms.

Experiment 2

To measure the concentration of N at pupation or adult emergence for both species, nanocosms were divided into a *Ae. j. japonicus* group, and a *Ae. triseriatus* group, with 60 replicates in each group. Larvae were allowed to develop to adulthood and were sampled at adult emergence or pupal death. Any larvae that died or did not make it to pupation were not sampled and were only included in the calculation of overall survival. An additional 5 nanocosms of each group were run and destructively sampled on days 4 and 7 to compare with E1 nanocosms. Data from these cursory observations did not differ from E1 observations and are not presented in this thesis. Four nanocosms were discovered to have received a larva of both species and were excluded from calculations.

Sampling

Larvae and pupae were removed from nanocosms using a glass Pasteur pipette and transferred to a glass cover slide and dried at 50 °C for 48 h. Adults were knocked down in nanocosms at -20 °C for 30 min and transferred to 1.5 ml plastic centrifuge tubes using forceps and dried at 50 °C for 48 h. All dried samples were weighed to the nearest .001 mg and were saved for C:N analysis.

Leaves were placed in plastic scintillation vials with 15 ml of deionized water and sonicated for 12 min to remove any particulates from the leaf surface. The leaves were then transferred to glass scintillation vials and dried at 50 °C for at least 48 h and weighed. Dissolved N was partitioned from total N by filtering 5 ml of nanocosm water through 1 μm glass filters. The remaining nanocosm water, dissolved N samples and leaf sonicate was stored at -20 °C for N analysis.

Carbon and Nitrogen Analysis

The concentrations of N in nanocosms were measured in 4 ml samples from the total and dissolved water column and the leaf sonicate. Total water column samples were initially diluted 1:1 with deionized water due to high nutrient concentration. Each sample received 600 μl of oxidizing reagent solution (1:1 NaOH and K_2SO_8), was vortexed, and then autoclaved for 30 min at 121 °C. After cooling, 8 μl of concentrated H_2SO_4 was added lower the pH. Samples were individually run through UV N analysis (Lambda 25, PerkinElmer, Inc.). Spectrometry curves were collected using UV WinLab software (PerkinElmer, Inc.) and total N concentration in each sample was determined using second derivative analysis (Crumpton et al. 1992). The total concentration of N in each sample was computed using the linear equation of a $NaNO_3$ standard curve.

The C:N ratios of larvae, pupae, adults, and leaves were determined using elemental combustion analysis (Costech Inc., Valencia, CA) at Kellog Biological Station (Hickory Corners, MI). Whole mosquito samples were prepared in 5x9 mm tin cups (Costech Inc.) for analysis. For processing, leaf samples were transferred to flat-bottomed centrifuge tubes with metal shot and shaken at 1,800 rpm for 10 min to produce a fine powder. Two 3 mg samples from each leaf was then prepared in 5x9 mm tim cups (Costech, Inc.) for analysis.

Statistical Analysis

Analysis of covariance (ANCOVA) was performed to analyze nanocosm N measurements, leaf decay and leaf tissue C:N, and mosquito C, N and C:N over time separately between larvae and no larvae treatments and between mosquito species from E1 nanocosms. Any significant larval effects were further analyzed for species effects. Multivariate analysis of variance (MANOVA) was performed to analyze mosquito growth parameters from E2. Growth parameters included adult development time, weight, and the total C and N accumulation on variation between species and genders. Comparisons of E2 nanocosm nitrogen concentrations between species and genders were also performed using MANOVA analysis. Univariate analysis of variance (ANOVA) was also performed. All related *post hoc* p-values were Bonferroni adjusted to control for Type 1 error.

When necessary, data were transformed to meet assumptions of normality and homoscedasticity and all proportional data were arcsine square-root transformed. All reported data are untransformed.

Results

Experiment 1

In the absence of larvae, total water column N decreased (Fig 2.1), and leaf surface N (Fig 2.1) and dissolved N (Fig 2.1) increased. In the presence of larvae, total water column N increased (Fig 2.1), dissolved N decreased (Fig 2.1), and leaf surface N remained at a constant level (Fig 2.1). The decrease of total water column N was accelerated by larval presence (Fig 2.1, Table 2.1). Larvae also significantly reduced N accumulation on leaf surfaces (Fig 2.1, Table 2.1). The amount of dissolved N present in nanocosms was not significantly affected by larval presence (Fig 2.1, Table 2.1), and these three measured N concentrations did not differ between larval species.

The loss of leaf mass increased significantly over time (Fig 2.2, Table 2.2) but was not affected by larval presence (Table 2.2). Leaf N and C concentration did not differ significantly through time (Table 2.2).

Larvae of both species gained weight similarly through time (Fig 2.3 A, Table 2.3). Throughout larval development, %N increased significantly in the larval body (Fig 2.3, Table 2.3) and %C significantly decreased (Fig 2.3, Table 2.3). The ratio of C:N in larval tissues also decreased significantly over time (Fig 2.3, Table 2.3).

Table 2.1: ANCOVA analysis of E1 nanocosms N changes between larva and no larva nanocosms. * = significant with Bonferroni adjustment, α=0.05.

		Total Water Column		Leaf Surface		Dissolved	
Source	df	*F*	*P*	*F*	*P*	*F*	*P*
Days	1	51.8	<0.0001*	11.0	0.001*	38.2	<0.0001*
Larval Presence	1	11.8	<0.0001*	21.1	<0.0001*	0.34	0.72
Days X Presence	1	3.3	0.04	2.4	0.09	0.63	0.53

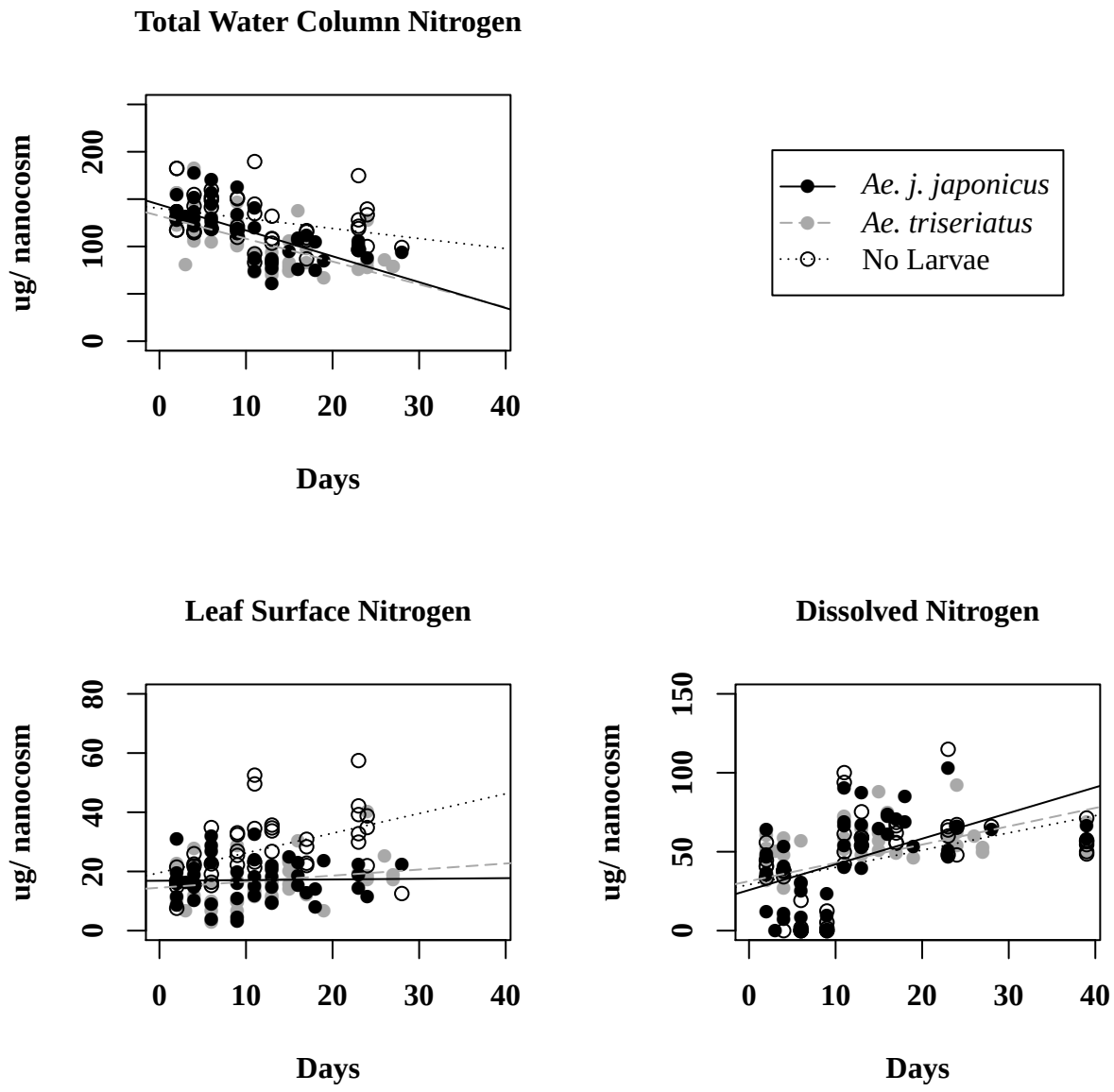

Figure 2.1: E1 nanocosm nitrogen concentrations over time. Lines are from ANCOVA analysis.

Table 2.2: ANCOVA analysis of leaf mass and nutrient changes between E1 larva and no larva nanocosms. * = significant with Bonferroni adjustment, α=0.05.

		Remaining leaf		%N		%C		C:N	
Source	df	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*
Days	1	37.1	<0.0001*	0.64	0.43	4.8	0.03	1.27	0.27
Larval Presence	1	1.2	0.30	1.86	0.17	2.2	0.11	1.65	0.26
Days X Presence	1	0.58	0.56	0.64	0.53	1.0	0.39	0.58	0.56

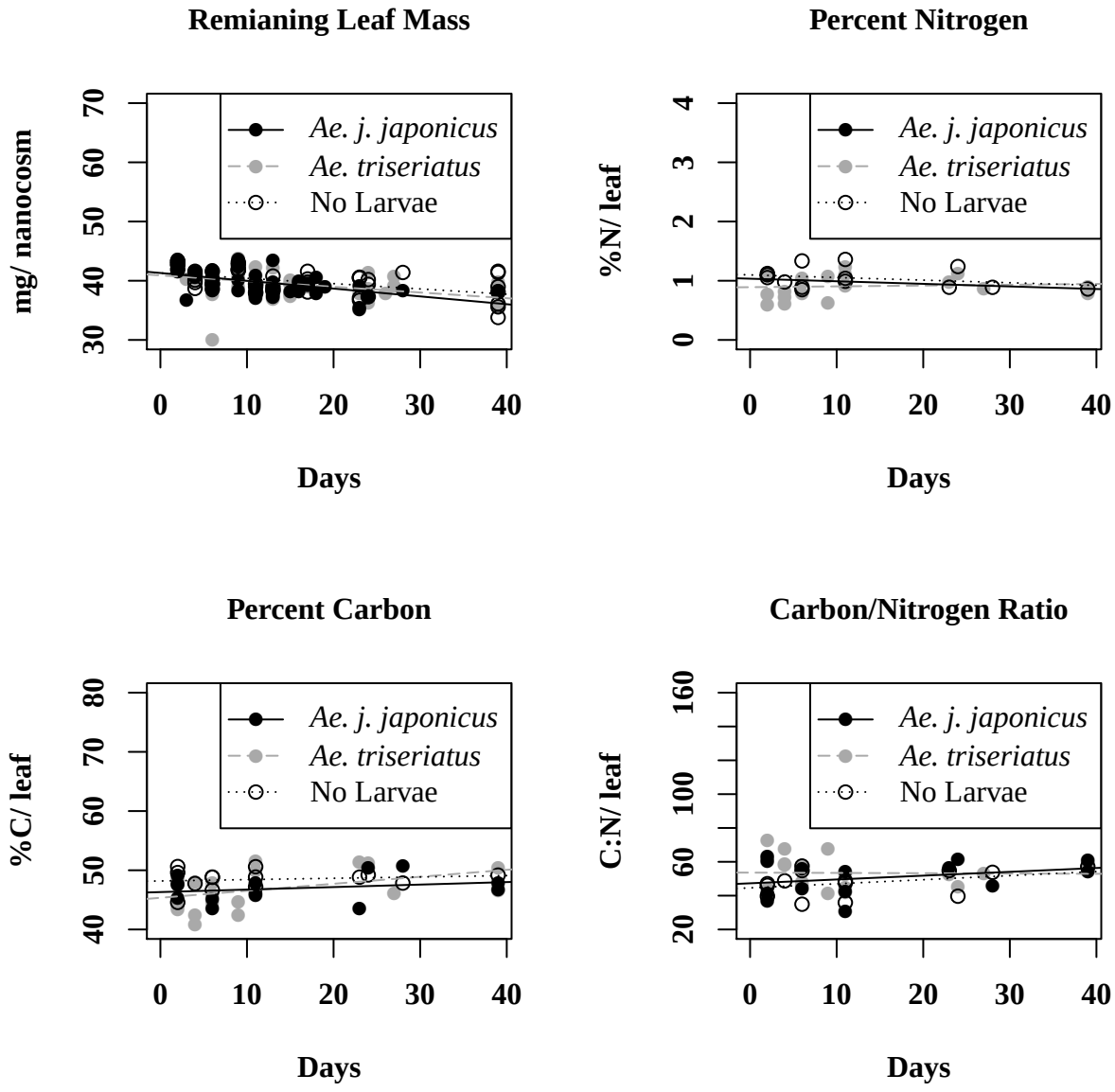

Figure 2.2: Leaf decay, nitrogen and carbon over time in E1 nanocosms. Lines are from ANCOVA analysis.

Table 2.3: ANCOVA analysis of larval nutrient changes between species in E1. * = significant with Bonferroni adjustment, α=0.05.

		Weight		%N		%C		C:N	
Source	df	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*
Days	1	11.23	0.0011*	14.6	0.0003*	7.93	0.007*	12.67	0.0008*
Species	1	4.79	0.03	1.64	0.21	0.08	0.77	1.21	0.28
Days X Species	1	0.126	0.73	0.30	0.59	2.99	0.09	4.05	0.49

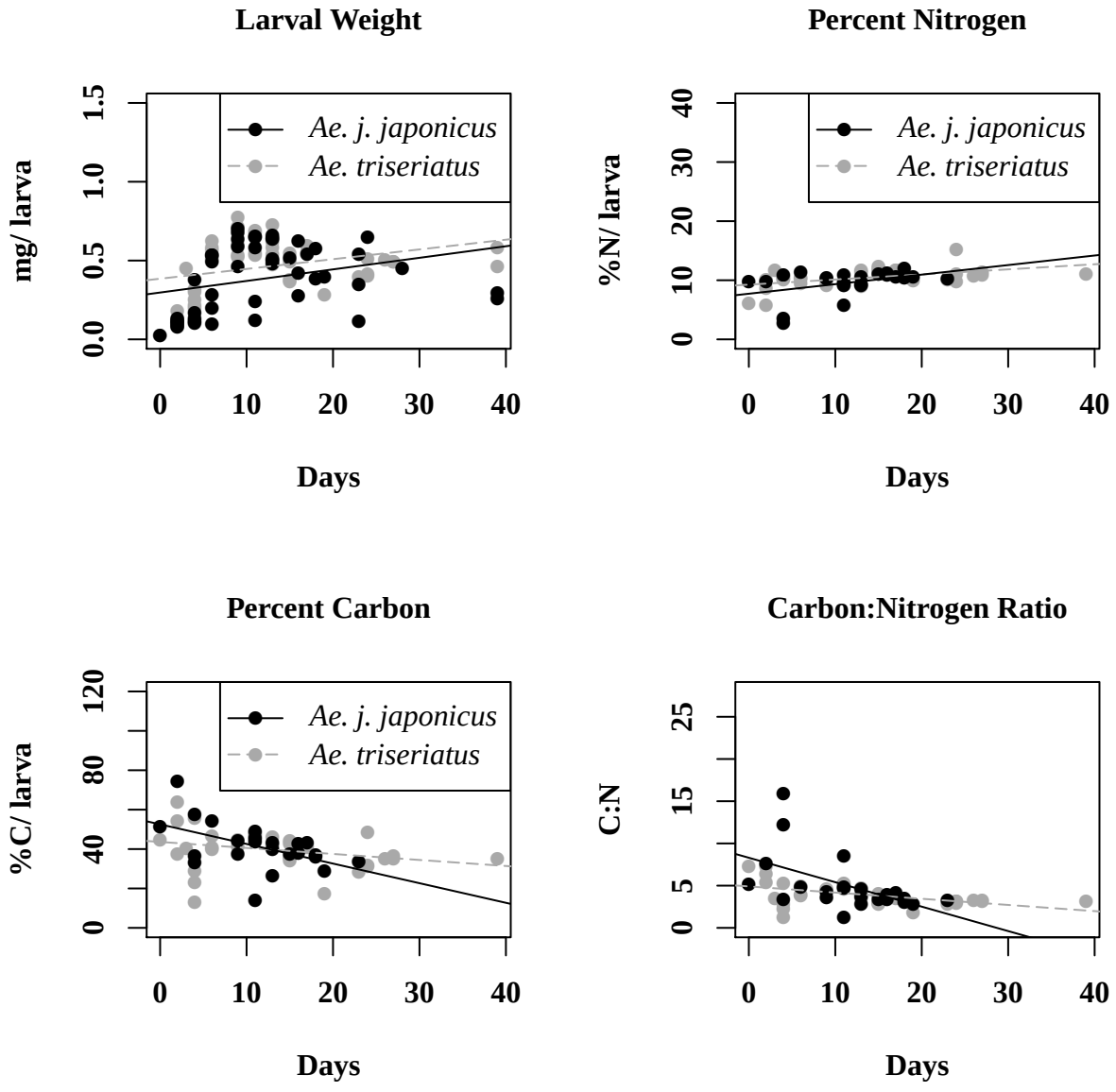

Figure 2.3: Larval growth and nutrient accumulation over time in E1. Lines are from ANCOVA analysis.

Experiment 2

The final amount of all total, leaf surface, and dissolved N in nanocosms were nearing significance between mosquito species and was significant between genders in the MANOVA analysis (Table 2.4). Total water column and dissolved N were significantly higher in *Ae. j. japonicus* nanocosms and in nanocosms that produced females in *post hoc* ANOVA analysis (Fig 2.4, Table 2.5). The final leaf nutrient concentrations were not significantly different between either species or gender in the MANOVA analysis (Table 2.6), nor were they significant different in *post hoc* ANOVA analysis (not reported); however, %C in appears to be lower in nanocosms with *Ae. j. japonicus* that produced females (Fig 2.5), even though the mean was not statistically significant.

Larval growth parameters and nutrient accumulation in nanocosms were significantly different between species and genders in the MANOVA analysis (Table 2.7). The adults of *Ae. j. japonicus* were slightly smaller than *Ae. triseriatus* but statistical significance was not found after Bonferroni adjustment (Fig 2.6, Table 2.8). There was no difference in size between males or females within either species (Fig 2.6, Table 2.8). Total development time was not significantly different between species, but males emerged sooner than females (Fig. 2.6, Table 2.8). Survivorship to pupation from nanocosms was 72% in *Ae. j. japonicus* nanocosms and 93% in *Ae. triseriatus* nanocosms.

The %N in emerged adults was not significantly different between species or genders, but there was a significant interaction between these two main effects (Fig 2.7, Table 2.9). Female *Ae. j. japonicus* had a lower %N than either sex of *Ae. triseriatus* (F=19.45, P=0.003; Fig 2.7) or male *Ae. j. japonicus* (F=15.88, P=0.0004; Fig 2.6). Adult %C was significantly different between species and genders (Fig 2.7, Table 2.9) and C tended to be higher in *Ae. triseriatus*, as well as higher in adult males (Fig 2.7). The ratio of C:N in adults was significantly different between genders (Table 2.9). Females had a lower C:N ratio than males (Fig 2.7).

Table 2.4: MANOVA analysis of final N measurements in E2 nanocosms. Variates were water column N, leaf surface N, and dissolved N.

MANOVA			
Source	Roy's max root	df	*P*
Species	0.105	1	0.06
Gender	0.375	1	<0.0001
Species X Gender	0.0514	1	0.292
Error		76	

Table 2.5: ANOVA analysis of final N in E2 nanocosms. * = significant with Bonferroni adjustment, α=0.05.

		Total Water Column		Leaf Surface		Dissolved	
Source	df	*F*	*P*	*F*	*P*	*F*	*P*
Species	1	7.20	0.009*	1.05	0.309	7.61	0.007*
Gender	1	6.83	0.011*	3.65	0.06	19.5	<0.0001*
Species x Gender	1	3.443	0.067	0.074	0.786	2.74	0.102

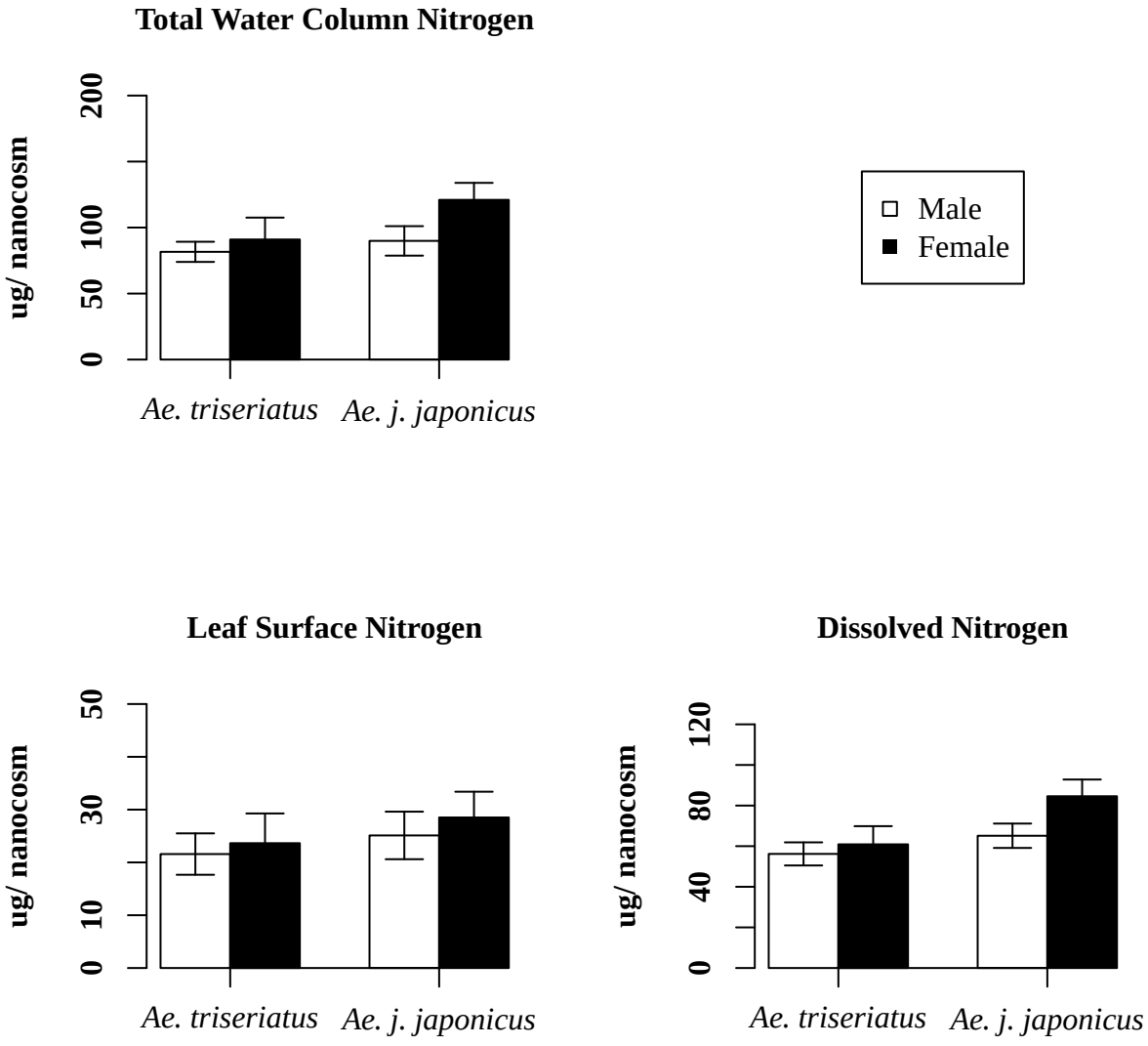

Figure 2.4: E2 nanocosm nitrogen concentrations. Values are means $\pm$ SE (n=20, 27, 16, 18).

Table 2.6: ANOVA analysis of E2 leaf tissue nitrogen and carbon concentrations.

		%N		%C		C:N	
Source	df	*F*	*P*	*F*	*P*	*F*	*P*
Species	1	0.008	0.93	0.77	0.40	0.30	0.59
Gender	1	0.98	0.34	1.89	0.19	0.013	0.91
Species x Gender	1	1.41	0.26	4.29	0.06	0.0102	0.97

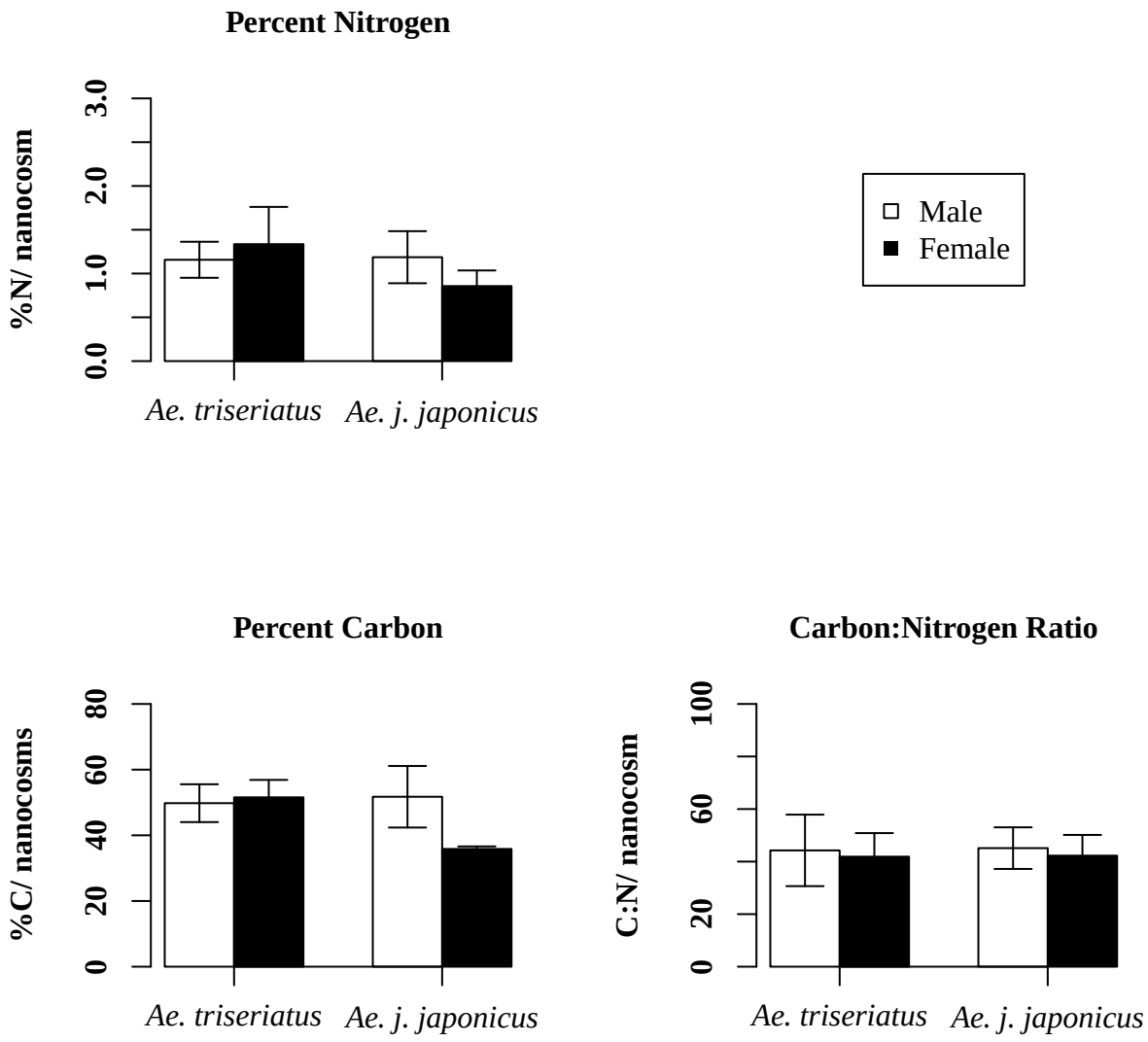

Figure 2.5: E2 nanocosm leaf nutrient concentrations. Values are means ± SE (n=20, 27, 16, 18).

Table 2.7: MANOVA analysis of overall mosquito development in E2. Variates were development time and weight.

MANOVA			
Source	Roy's max root	df	*P*
Species	0.10	1	0.027
Gender	1.16	1	<0.0001
S X G	0.05	1	0.17
Error		77	

Table 2.8: ANOVA analysis of overall mosquito growth parameters and nitrogen and carbon concentrations in E2. * = significant with Bonferroni adjustment, α=0.05.

		Weight		Devel Time	
Source	df	*F*	*P*	*F*	*P*
Species	1	5.9	0.02	2.3	0.1
Gender	1	3.3	0.07	55.2	<0.0001*
Species x Gender	1	0.02	0.9	0.4	0.08

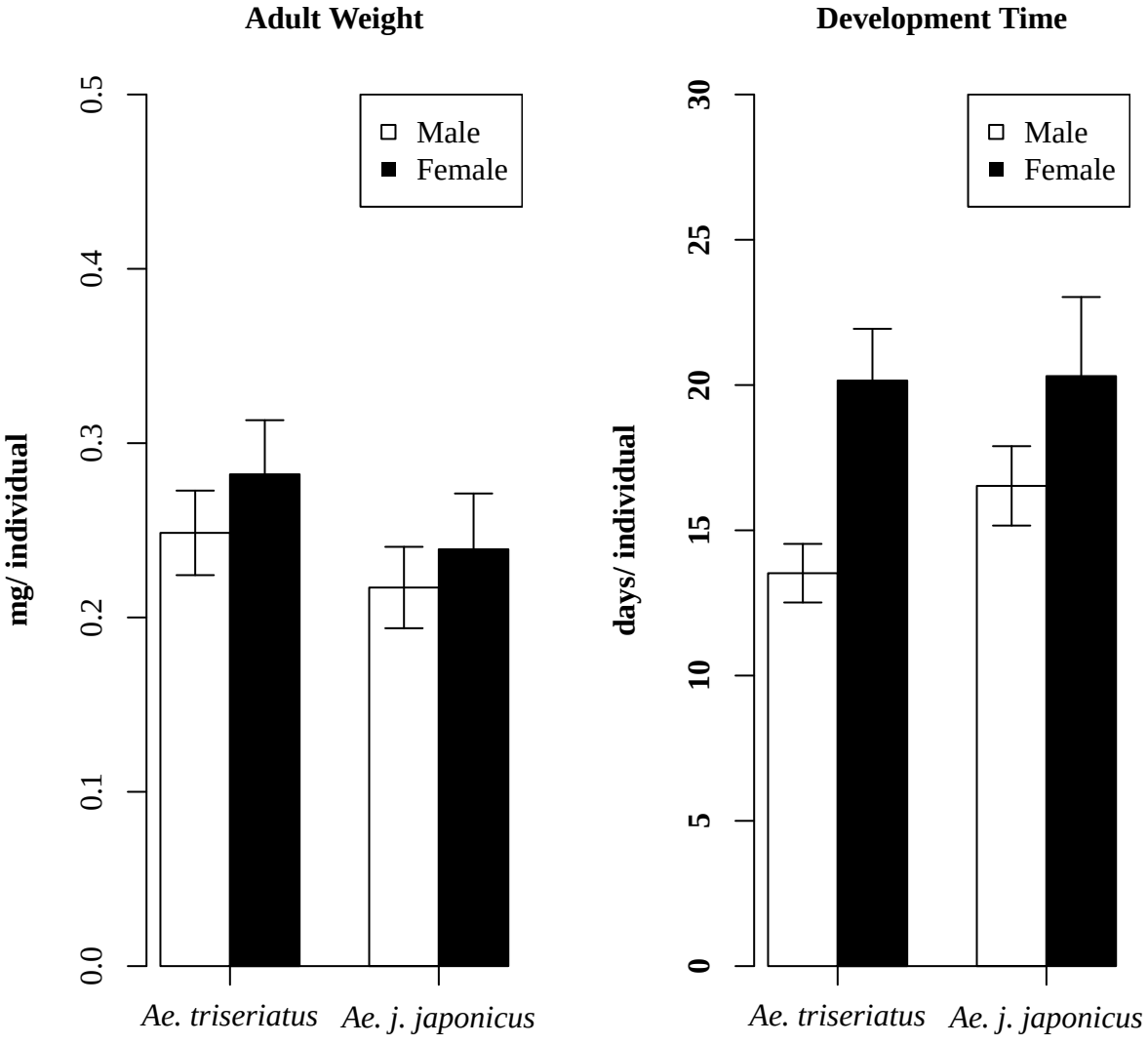

Figure 2.6: Mosquito growth parameters and nitrogen in E2. Values are means $\pm$ SE (n=20, 27, 16, 18).

Table 2.9: ANOVA analysis of overall mosquito nitrogen and carbon concentrations in E2. * = significant with Bonferroni adjustment, α=0.05.

		%N		%C		C:N	
Source	df	*F*	*P*	*F*	*P*	*F*	*P*
Species	1	5.3	0.03	15.5	0.0002*	4.4	0.04
Gender	1	5.7	0.02	29.6	<0.0001*	11.7	0.001*
Species x Gender	1	10.5	0.002*	0.4	0.5	2.5	0.12

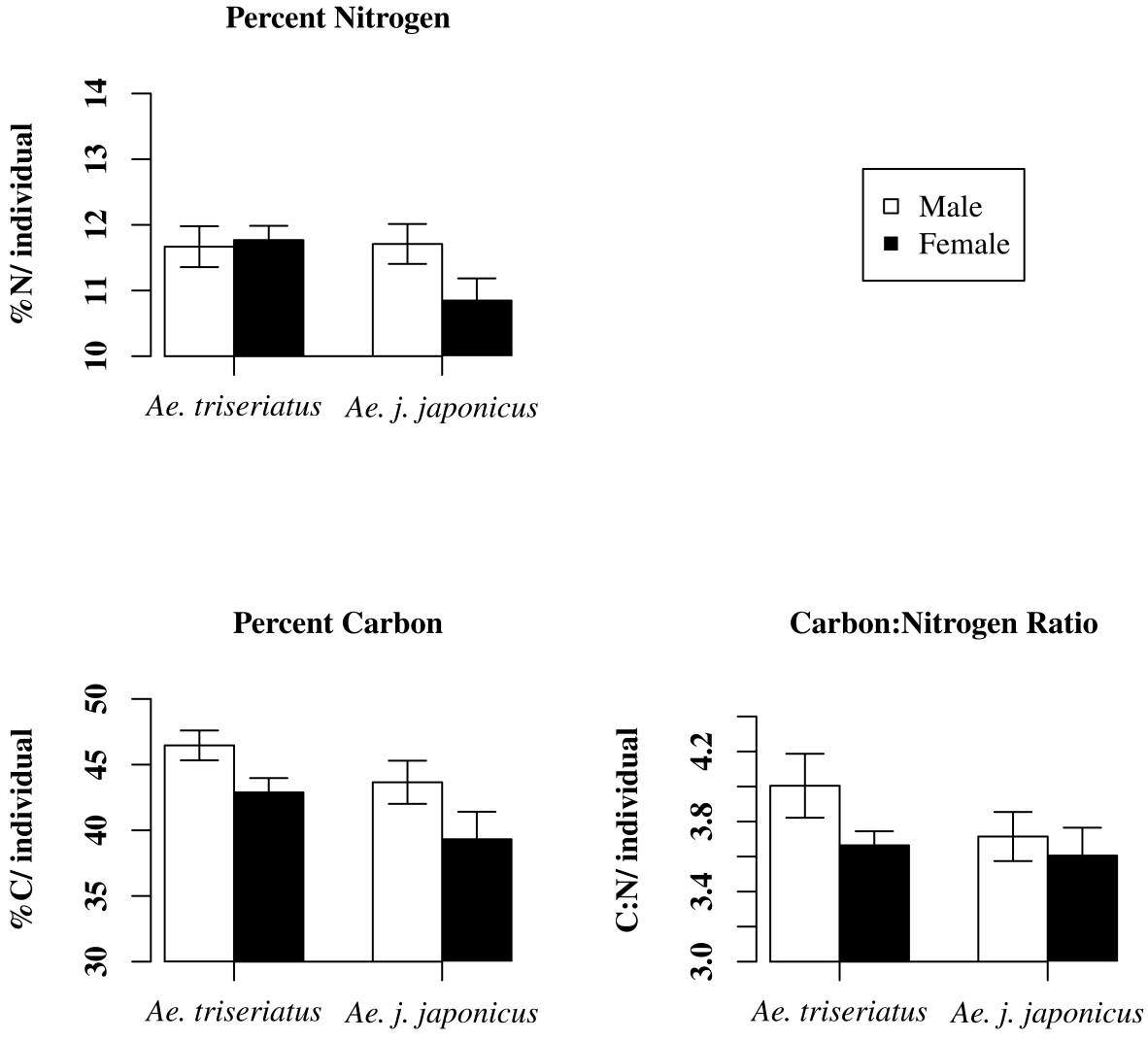

Figure 2.7: Larval nitrogen and carbon accumulation in E2. Values are means $\pm$ SE (n=20, 27, 16, 18).

Discussion

This study shows that larval mosquitoes directly affect the nutrient dynamics of their aquatic habitats. The depression of N in the water column and on leaf surfaces is likely due to microbial biomass reduction caused by larval feeding (Kaufman et al. 2001). Bacteria, which tend to be C limited, and fungi, which tend to be N limited, readily take up N compounds for metabolic processes (Kaufman and Walker 2006). It is likely that the decrease in water column N and the increase in leaf surface N in larvae-free nanocosms can be attributed to the accumulation of N by fungi and bacteria on the leaf surfaces. The presence of larvae has been shown to decrease populations of bacteria and fungi on leaf surfaces (Fish and Carpenter 1982; Kaufman et al. 1999, 2001, 2002; Kaufman and Walker 2006) and larvae are known to feed directly on bacteria and particles in the water column (Walker and Merritt 1991; O'Donnell and Armbruster 2007). Dissolved N on the other hand was unaffected by larval presence. When in water, leaf detritus readily leaches out its soluble nutrients (Carpenter 1982a) and these nutrients can be incorporated by free floating bacteria and attached microorganisms which can then be utilized by larvae (Pelz-Stelinski et al. 2010). An initial increase in dissolved N would be expected because of leaching. The incremental increase in dissolved N throughout the experiment was likely related to larval excretion and the slow release of N from decaying leaf material (Pelz-Stelinski et al. 2010).

Differences between *Ae. j. japonicus* and *Ae. triseriatus* N utilization were seen in the percent of N present in newly emerged females. Both C and N percentages were higher in *Ae. triseriatus* adults at the end of the experiment. These results confirm earlier observations that *Ae. j. japonicus* females emerge with lower N, but did not reveal any evidence that N is obtained or processed differently by either species. Lower %N in insects is usually indicative of a lower N requirement for development (Fagan et al. 2002) and a higher concentration of N is correlated with a larger body size (Hood-Nowotny et al. 2012), though this is not always the case (Winters and Yee 2012). High %N can also associated with high %C (Hood-Nowotny et al. 2012) which translates to higher lipid and carbohydrate stores and a larger body size (Fagan et al. 2002; Winters and Yee 2012) unless a species is particularly N efficient (Winters and Yee 2012). In this study, adult body size did not

vary between species even though *Ae. triseriatus* had a higher %C and %N. While carbon can be found in both lipid and carbohydrate stores that make up much of the mosquito biomass, it can also be respired as CO_2. It is possible that differences in respiration rates between *Ae. j. japonicus* and *Ae. triseriatus* may account for some of the differences in %C even though adult body mass was similar between species.

Contrary to expectations, the development of both *Ae. j. japonicus* and *Ae. triseriatus* in this study followed similar trajectories. In previous multi-larvae studies, *Ae. j. japonicus* larvae generally developed faster than *Ae. triseriatus* (Alto 2011; Hardstone and Andreadis 2012). Indeed, the development time of *Ae. triseriatus* females was accelerated to what was seen by Alto (2011) at a much higher leaf ration. It is unclear why only *Ae. triseriatus* development time was accelerated and *Ae. j. japonicus* development was not. Low larval densities tend to decrease the development time of both *Ae. j. japonicus* and *Ae. triseriatus* (see Alto 2011; Hardstone and Andreadis 2012) and density effects are related to leaf ration (Leonard and Juliano 1995) . Hardstone and Andreadis (2012) postulated that larval density may affect *Ae. triseriatus* growth to a greater extent than *Ae. j. japonicus*, but this was using highly nutritious brewers yeast. This may not be the case when rearing with purely leaf detritus (Alto 2011). It is possible that in this study *Ae. triseriatus* larvae were able to accelerate their development time to that of the natural rate for *Ae. j. japonicus* due to the leaf ration and lack of interspecific competition. This does not explain why *Ae. j. japonicus* larvae did not also develop faster, even though faster larval development times for *Ae. j. japonicus* have been previously reported by Hardstone and Andreadis (2012).

Larval developmental acceleration can be caused by both nutritional abundance and nutritional stress at particular temperatures. Reiskind and Zarrabi (2012) found that female *Aedes albopictus* were smaller and developed faster when raised at a comparable temperature to this study (24 °C) and at a lower food ration. The food source used by Reiskind and Zarrabi (2012), even at lower levels, most likely had a higher nutrient content than leaf detritus. It is possible that larvae in this study were nutritionally stressed, resulting in a smaller body size and a faster development time. On the other hand, the growth of mosquitoes has been postulated to be associated with

an optimal weight at pupation, or pupation window, that is related to available leaf ration and temperature through time (Gilpin and McClelland 1979; Carpenter 1984; Walker et al. 1997). Generally, an increase in leaf ration results in a decrease in development time and a higher optimal pupation weight (Carpenter 1984; Walker et al. 1997). Walker et al. (1997) postulated that higher temperatures could increase development time and lower the optimal pupation weight, decreasing the effect of a high leaf ration. The per larva leaf ration in this study was about 70% more than the what was used by Walker et al. (1997) but females of both species emerged at a smaller size and *Ae. triseriatus* development was accelerated. In fact, *Ae. triseriatus* was growing as fast as previously reported with higher leaf rations and similar rearing temperature (Alto 2011). It is unlikely that leaf ration and rearing temperature alone can explain the accelerated growth observed in this study and other factors will have to be explored.

It was clear through N analysis that larvae were feeding on leaf surface microbes; however, leaf decay rates were not increased in larval nanocosms when compared to those without larvae. Increases in leaf decay rates have been previously associated with multiple larvae grazing on leaf surface microbial biomass (Fish and Carpenter 1982; Kaufman and Walker 2006); however, a single larva appears not to have the ability to affect overall leaf decay. Larval effects on leaf surface N was similar between species, indicating that overall larval feeding on microbial biomass may have been similar. There were also no differences in total or dissolved N through time in E1 larval nanocosms; however, higher water column N was observed in nanocosms that produced female mosquitoes. This may have been caused by gender differences in nutrient accumulation (Chambers and Klowden 1990). This gender effect could not be established in E1 larvae because mosquitoes can only be definitively sexed at the pupal or adult stage. The higher total and dissolved N in female *Ae. j. japonicus* nanocosms also suggests that, overall, *Ae. j. japonicus* females were not feeding on water column microbes as much as *Ae. triseriatus* females (Kaufman et al. 2001), even though both emerged at the same time and at a similar size. The efficient utilization of N can be associated with weight gain in certain mosquitoes (Winters and Yee 2012) and although females appeared to have a lower N requirement in this study, the lack of size dimorphism between males

and females suggests that there was some other factor keeping females small.

The similar size between males and females produced from the nanocosms was surprising. Male *Ae. j. japonicus* and *Ae. triseriatus* weights were similar to previous multi-larvae studies, but female weight was considerably lower (Walker et al. 1997; Lorenz 2012). The availability of nutrients in microbial biomass for mosquito larvae limits the development time and body size of emerging adults (Kaufman et al. 2002). The concentration of N increased in the larval tissues of both species through time, eventually reaching levels found in adults, indicating a constant feeding on microbial biomass. The ratio of C:N in larval tissues decreased through time, perhaps indicating a differential metabolism of proteins and lipids as larvae neared pupation. Chambers and Klowden (1990) showed that mosquito larvae require a critical level of carbohydrates and lipids before pupation is initiated and variations in this critical level can depend on gender and rearing temperature. Furthermore, Klowden and Chambers (1992) found that these reserves can vary between mosquito species. It is also possible that the availability of quality C compounds in larval food resources was diminishing as larvae harvested microbial biomass (Kaufman et al. 2001). A declining C:N ratio would be apparent, for example, if accumulation of lipids did not match the accumulation of proteins in larval tissues. The accumulation of C was also different between species even though body size was similar. Hood-Nowotny et al. (2012) found that the concentration of N is more related to body size than concentration of C in the malaria mosquito *Anopheles arabiensis* and that fatty acid profiles can be different between males and females. The fatty acid reserves in *Ae. j. japonicus* and *Ae. triseriatus* females may be different, accounting for different developmental strategies for nutrient accumulation and weight gain.

It is also possible that female larvae require fatty acids and other compounds released by recently deceased larvae in container habitats. The survival rates of larvae in nanocosms, taken as a whole, were higher for *Ae. triseriatus*, but survivorship for both species were at levels previously seen in microcosms with low larval density and high nutrient sources (Alto 2011; Hardstone and Andreadis 2012). Indeed, the survival of *Ae. triseriatus* was higher than in studies using highly nutritious brewer's yeast (Hardstone and Andreadis 2012). This measurement of mortality only

reveals individual larval success within each nanocosm because larvae cannot interact with each other. In studies with multiple larvae per container, mortality can decrease the competition for food resources and the decaying carcasses of deceased larvae could act as an additional nutrient resource for microorganisms and larvae (Yee et al. 2007b). This lack of differentiation of size has been previously reported in both *Ae. albopictus* and *Culex restuans* reared in low larval density microcosms on a similar leaf per larva ration as in these nanocosms, (Winters and Yee 2012). Winters and Yee (2012) also showed that the addition of an invertebrate carcass increased both adult weight the dimorphism between males and females within each species. Decaying larvae may be important to *Ae. j. japonicus* and *Ae. triseriatus* female weight gain.

Adult *Ae. triseriatus* were slightly larger than *Ae. j. japonicus* in this study even though statistical significance was not established. Taken with the similar development time for both species, this may indicate that under these conditions *Ae. triseriatus* larvae may have been slightly more efficient at converting food into body weight. The correlation between larval efficiency and leaf decay has been postulated by Carpenter (1984) but the relationship between microbial quality and larval food assimilation will have to be studied further.

The results of this study indicated that there is a differential utilization of N in the larval environment between *Ae. j. japonicus* and *Ae. triseriatus* but the faster development of *Ae. j. japonicus* previously reported was not replicated in this study and may or may not be related to N accumulation; therefore, the utilization of N by *Ae. j. japonicus* may not explain much of its observed displacement of *Ae. triseriatus* in some larval habitats. Further multi-larval studies will need to be performed to replicate the findings in nanocosms and elucidate any factors specific to nanocosm rearing that may have affected larval development. If larvae of *Ae. j. japonicus* are replacing those of *Ae. triseriatus* in tire habitats (Andreadis and Wolfe 2010) and the utilization of available nutrients by *Ae. j. japonicus* is not a factor in this phenomenon, then other characteristics of *Ae. j. japonicus* such as early season egg hatch rates and oviposition behavior should be examined. Additionally, research will be needed to explore the consequences of adults emerging with lower N than conspecifics. This should include studies on the physiology and behavior of

specific larval nutrient utilization such as protein accumulation and body amino acid composition. The establishment of *Ae. j. japonicus* throughout the US can be considered permanent and it will likely change the ecology of existing disease vectors. A better understanding of the larval physiology of this invasive species will be important in future studies of mosquito borne disease dynamics in many locales.

CHAPTER 3

THE EFFECT OF NITROGEN ON *AEDES JAPONICUS JAPONICUS* (THEOBALD) AND *AEDES TRISERIATUS* (SAY) GROWTH IN MICROCOSMS

Introduction

The invasion of non-native mosquitoes into the US has both ecological and public health implications (Juliano and Lounibos 2005). Successful biological invaders must be able to utilize the resources available to them in their non-native range to integrate with the local inhabitants. Invasive mosquitoes can utilize common habitats with both native and other invasive species. Competition between the larvae of these mosquitoes for limited nutritional resources can impact both development and survival (Carpenter 1983; Alto 2011; Hardstone and Andreadis 2012).

The invasive species *Aedes japonicus japonicus* (Theobald) has been expanding its range in the US since its introduction in the mid-1990's (Peyton et al. 1999; Morris et al. 2007; Hughes et al. 2008; Neitzel et al. 2009; Gaspar et al. 2012) and appears to be displacing the native mosquito *Aedes triseriatus* (Say) in discarded tire habitats (Andreadis and Wolfe 2010). The success of *Ae. j. japonicus* can be attributed to a faster development time to a comparable sized female than *Ae. triseriatus* (Alto 2011). The growth rate of *Ae. j. japonicus* females is not significantly affected by direct competition with *Ae. triseriatus* (Alto 2011; Hardstone and Andreadis 2012). This may be related to nutrient utilization, which was shown to be different between *Ae. j. japonicus* and *Ae. triseriatus* (Ch. 2); however, single larva rearing of both species did not produce the differences in development time, and females were much smaller than expected (Ch. 2).

The nutrients in mosquito larval habitats are provided by allochthonous particulate detrital inputs including leaves and invertebrate carcasses (Fish and Carpenter 1982; Daugherty et al. 2000) and soluble nutrient inputs from stemflow and rain (Carpenter 1982b; Kaufman et al. 2002; Kaufman and Walker 2006). In most situations, larvae coexist with many conspecifics in container habitats and mortality is common, reducing resource competition between survivors. Decaying

larval carcasses can also provide another source of nutrients in containers. All of these inputs can be broken down by fungi and other microbes and made available to mosquitoes through their consumption of microbial biomass (Fish and Carpenter 1982). The growth of these microorganisms is generally limited by the availability of N (Kaufman and Walker 2006). Mosquito growth can be limited by the availability of this high quality microbial biomass; therefore, larvae tend to be indirectly limited by the availability of N.

The addition of soluble nitrate has also been shown to increase both decay and microbial productivity in microcosms (Kaufman et al. 2002; Kaufman and Walker 2006). This increase in microbial activity also been shown to increase *Ae. triseriatus* larval growth (Kaufman and Walker 2006). A survey of newly emerged *Ae. triseriatus* and *Ae. j. japonicus* adults from discarded tires on the campus of Michigan State University (MSU) found that body %N content of *Ae. j. japonicus* females was lower than that of *Ae. triseriatus* females (Kaufman, *unpublished data*). This has also been corroborated in laboratory nanocosms (Ch. 2). This suggests that *Ae. j. japonicus* may be less limited by N availability than *Ae. triseriatus*.

Single and multi-larval rearing may have some fundamental differences that can affect larval growth. The lack of developmental differences between *Ae. j. japonicus* and *Ae. triseriatus* in single larval nanocosms in the previous chapter may have been caused by the lack of interaction with conspecifics or N compounds provided by deceased larvae. It is possible that in a multi-larvae situation, the previously seen developmental differences (Alto 2011; Hardstone and Andreadis 2012) could be replicated.

The purpose of this study was to attempt to corroborate the reported developmental differences between *Ae. j. japonicus* and *Ae. triseriatus* in experimental microcosms and elucidate abnormalities seen in nanocosm rearing and to test the effect of additional N on the growth and survival of *Ae. j. japonicus* and *Ae. triseriatus* in microcosms. It was expected that the interaction and mortality of conspecifics in microcosms would cause the accelerated *Ae. j. japonicus* growth and reduce the development time of *Ae. triseriatus* seen in nanocosms and increase female weight above that of the males. Furthermore, if the development time was different between species in microcosms,

then the addition of N was expected to increase microbial activity and because it is less limited by the availability of N, *Ae. j. japonicus* development would respond less to it than that of *Ae. triseriatus*.

Methods

Microcosms were used as experimental units to test the effect of multi-larval rearing and additional N on *Ae. j. japonicus* and *Ae. triseriatus* larval growth. Microcosms were housed in plastic 500 ml cups with aerated tops and filled with 300 ml of deionized water, 1 g of oven dried white oak (*Quercus alba*) leaves (Kellogg Forest, Augusta, MI) and 1000 μl of microbial inoculum collected from tree holes in the Toumey woodlot (MSU, East Lansing, MI). Each microcosm was incubated for 24 h at 24 °C to facilitate microbial colonization of the leaves before larval addition.

Mosquito Larvae

New Jersey strain *Ae. j. japonicus* (Rutgers University, New Brunswick, NJ) and MSU strain *Ae. triseriatus* eggs were hatched by flooding in a nutrient broth. The eggs of *Ae. triseriatus* required an additional 24 h soaking period in deionized water prior to flooding with nutrient broth. After approx. 9 h, newly hatched larvae of each species were removed from the broth using a glass Pasteur pipette and transferred to a plastic Petri dish for counting. Each microcosm (N=32) received 30 newly emerged larvae of either *Ae. j. japonicus* or Ae. triseriatus.

Nitrogen Addition

To test the effect of N to larval growth, the equivalent of 5 ppm KNO_3-N (the amount of N provided by a stem flow event, see Kaufman and Walker (2006)) was added on d 4, 10, and 15 to experimental microcosms (N=8:8). An equivalent amount of deionized water was added to control microcosms (N=8:8) to account for dilution by N addition. Microcosms were incubated at 24 °C with a 12:12 light cycle and checked daily for adults.

Sampling

Adults were collected from microcosms using an aspirator and separated by gender and species. Individual adults were transferred to plastic tubes and oven dried at 50 °C for at least 48 h. Dried

samples were weighed to the nearest .001 mg. The experiment was ended after <3 adults were emerging per day from all microcosms (d=33). Leaf samples were oven dried at 50 °C and weighed to the nearest .001 g. Any surviving larvae or pupae were collected, counted, and dried at 50 °C.

Carbon and Nitrogen Analysis

Randomly selected adult females from each group were added to individual tin cups for C/N elemental combustion analysis using an elemental analyzer (Costech Inc., Valencia, CA) at Kellogg Biological Field Station (Hickory Corners, MI) (n=50 for all groups except *Ae. triseriatus* with additional N, n=37 due to low female emergence).

Statistical Analysis

Multivariate analysis of variance (MANOVA) was used to analyze mosquito growth parameters between species and N addition. Individual mosquito growth parameters were individually analyzed using univariate analysis of variance (ANOVA). Overall growth parameters were total survival (adults produced plus larvae remaining), total adult weight and total number of adults produced. Average male and female weight, adults produced, and development time were also analyzed. All related p-values were Bonferroni adjusted to control for Type 1 error. One-way ANOVA was performed to analyze total leaf mass loss with and without N addition. Two-way ANOVA was used to analyze body N concentration between adult female *Ae. j. japonicus* and *Ae. triseriatus* and between the control and N addition treatments. If necessary, data was transformed to meet parametric assumptions of normality and homoscedasticity and all proportional measurements were arcsine square-root transformed. All reported data are untransformed.

Results

Overall mosquito parameters were significantly different between species and were significantly different between control and nitrogen treatments in MANOVA analysis (Table 3.1). Total mosquito survival was significantly different between N and control treatments (Table 3.2), with a lower overall survival in N added microcosms (Fig 3.1) with no significant difference between species (Table 3.2). The total number of adults produced was significantly different between species (Table 3.2) with *Ae. j. japonicus* microcosms producing more adults (Figure 3.1), and fewer adults were produced by both species overall in N treated microcosms (Fig 3.1). The total amount of adult biomass produced was also significantly lower in microcosms with added N (Fig 3.1, Table 3.1).

Fewer females were produced from microcosms with additional N (Fig 3.2 , Table 3.3). There was also an indication that fewer *Ae. triseriatus* females were produced than *Ae. j. japonicus* females in microcosms with additional N though significance was not upheld with Bonferroni adjustment (Fig 3.2). Neither average female weight nor average female development time was affected by N addition and did not vary significantly between species (Fig 3.2, Table 3.3). Male development was not significantly affected by the addition of N (Fig 3.3, Table 3.4), but male *Ae. triseriatus* were significantly larger than *Ae. j. japonicus* males (Fig 3.3, Table 3.4).

The %N in adult female *Ae. j. japonicus* was lower than in *Ae. triseriatus* females but was not affected by N addition (Fig 3.4, Table 3.5). Overall female %C and C:N ratios were not significantly different between species or treatments (Fig 3.4, Table 3.5).

There was no difference between decay of leaf matter between mosquito species (Fig 3.5, Table 3.6), but leaf decay was increased in microcosms with additional N (Fig 3.5, Table 3.6).

Table 3.1: MANOVA analysis of overall mosquito growth parameters. Variates were survival, total adult biomass, total number of adults produced, average female weight, number of females produced, average female development time.

MANOVA			
Source	Roy's max root	df	*P*
Species	2.94	1	<0.0001
Nitrogen	1.24	1	0.003
Species X Nitrogen	0.28	1	0.404
Error		28	

Table 3.2: ANOVA of total microcosm production. * = significant with Bonferroni adjustment, α = 0.05.

		Survival		No. of adults		Tot adult bimass	
Source	df	*F*	*P*	*F*	*P*	*F*	*P*
Species	1	0.008	0.93	9.87	0.004*	0.19	0.67
Nitrogen	1	11.55	0.0002*	21.94	<0.0001*	12.86	0.001*
Species X Nitrogen	1	2.16	0.15	1.03	0.32	2.49	0.13

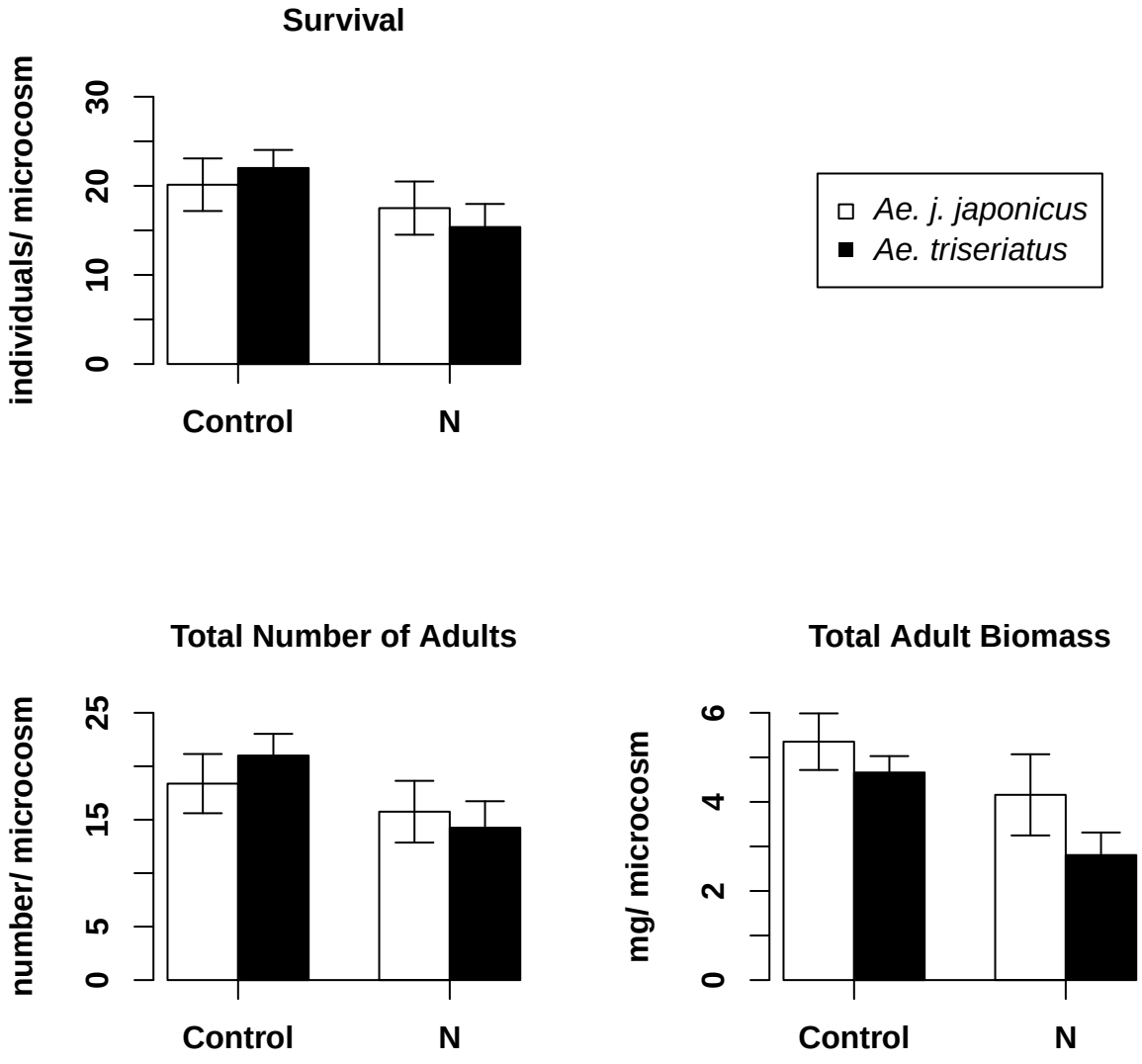

Figure 3.1: Total microcosm production. Values are means ± SE (n=8).

Table 3.3: ANOVA of female growth production. * = significant with Bonferroni adjustment, α = 0.05.

		No. of females		Avg. female wt		Avg. female devel. time	
Source	df	*F*	*P*	*F*	*P*	*F*	*P*
Species	1	5.28	0.029	7.86	0.009	6.15	0.019
Nitrogen	1	19.54	0.0001*	0.524	0.48	0.61	0.44
Species X Nitrogen	1	2.92	0.098	0.20	0.66	0.56	0.46

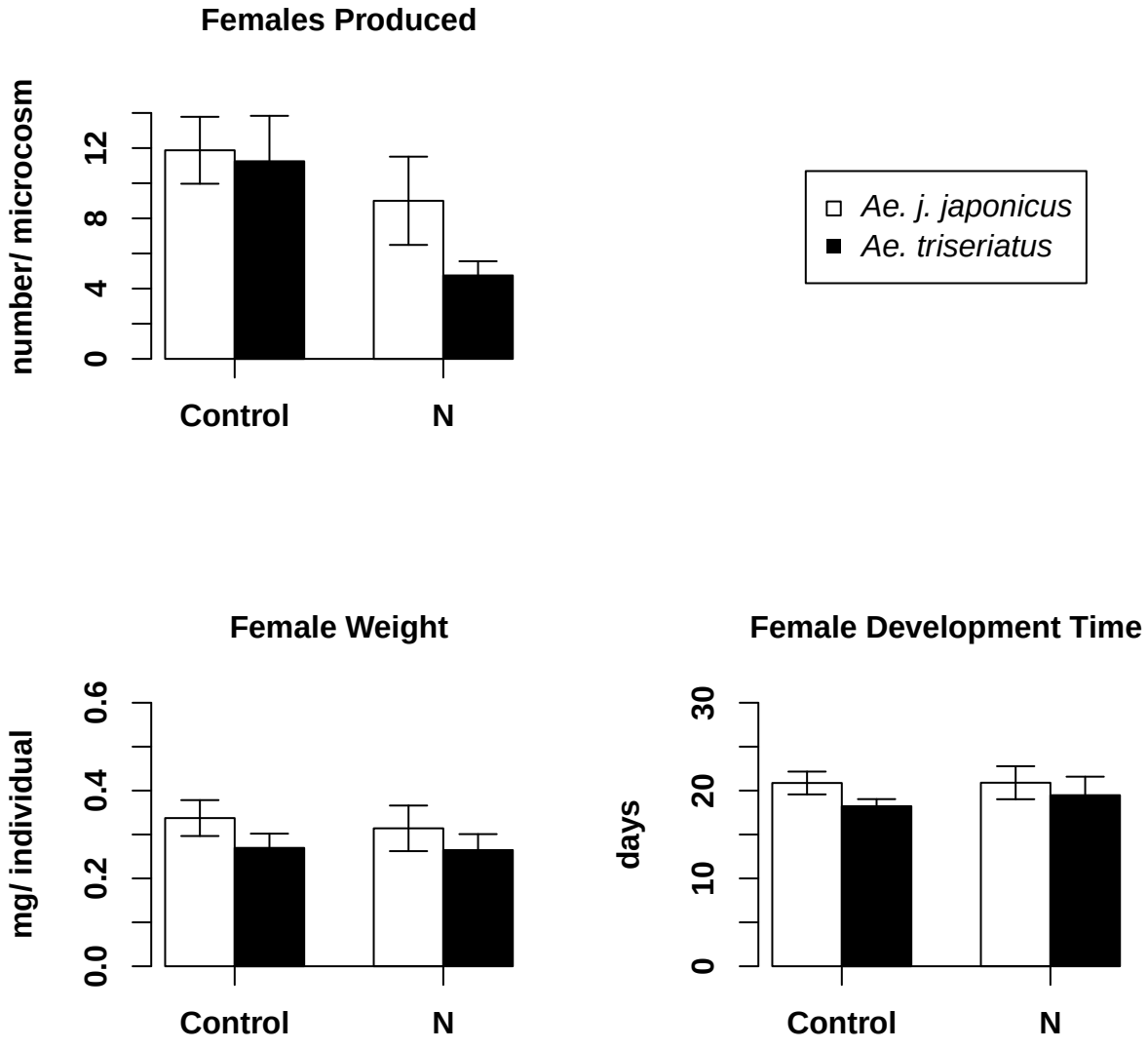

Figure 3.2: Female growth parameters. Values are means $\pm$ SE (n=8).

Table 3.4: ANOVA of male growth production. * = significant with Bonferroni adjustment, α = 0.05.

		No. of males		Avg. male wt		Avg. male devel. time	
Source	df	*F*	*P*	*F*	*P*	*F*	*P*
Species	1	7.07	0.013	11.81	0.002*	0.19	0.65
Nitrogen	1	<0.0001	0.99	0.12	0.73	0.18	0.67
Species X Nitrogen	1	0.04	0.82	1.61	0.23	1.35	0.26

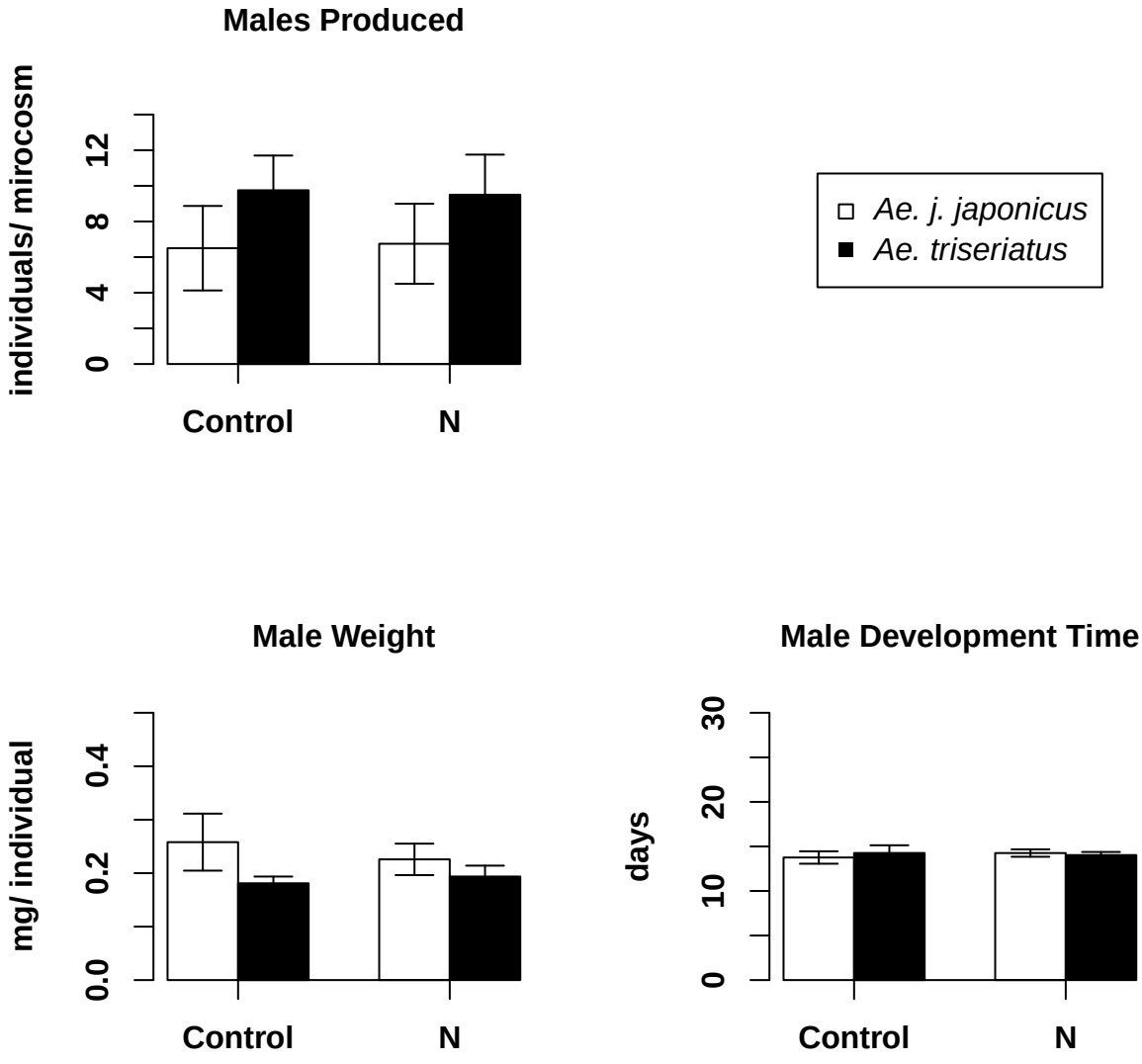

Figure 3.3: Male growth parameters. Values are means $\pm$ SE (n=8).

Table 3.5: ANOVA of female nitrogen and carbon. * = significant with Bonferroni adjustment, α = 0.05.

		%N		%C		C:N	
Source	df	*F*	*P*	*F*	*P*	*F*	*P*
Species	1	13.33	0.001*	3.53	0.07	0.21	0.65
Nitrogen	1	2.70	0.11	0.05	0.82	0.89	0.35
Species X Nitrogen	1	2.14	0.15	2.14	0.16	0.77	0.39

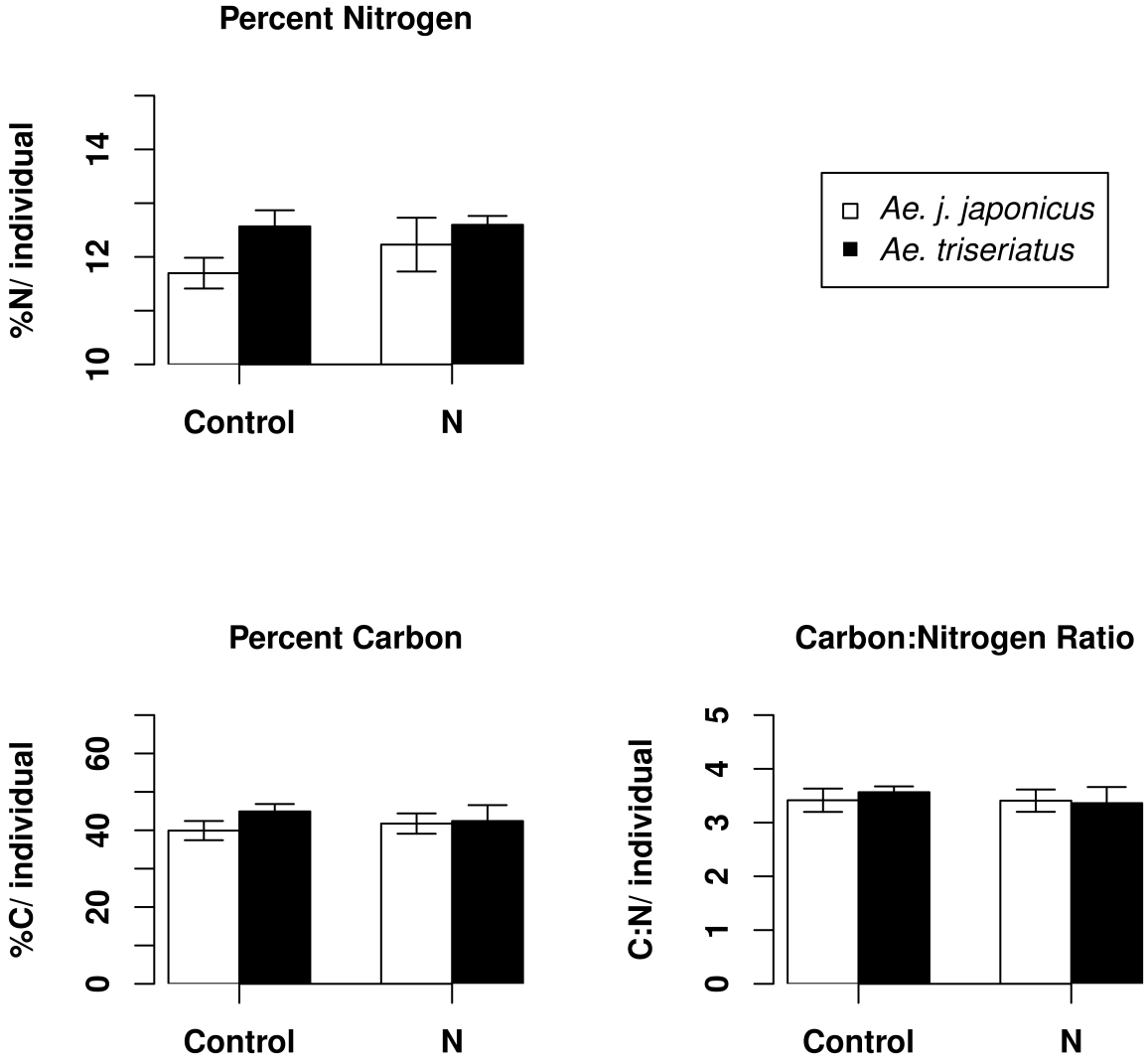

Figure 3.4: Female nitrogen and carbon. Values are means $\pm$ SE (n=8).

Table 3.6: ANOVA analysis of leaf decay.

Source	df	*F*	*P*
Species	1	0.007	0.935
Nitrogen	1	13.78	0.0009
Species X Nitrogen	1	0.013	0.909

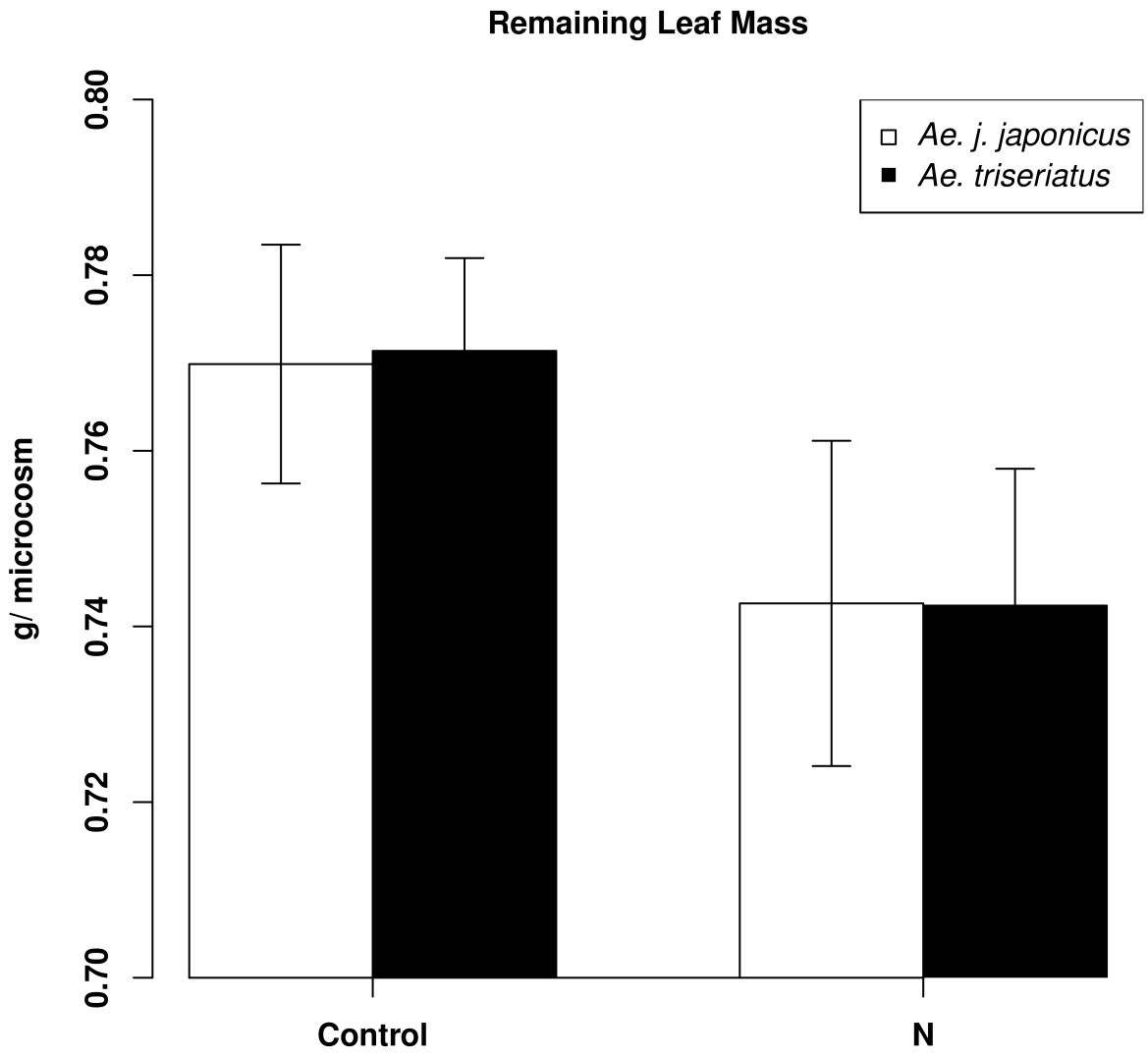

Figure 3.5: Remaining leaf mass in microcosms. Values are means ± SE (n=8).

Discussion

The developmental differences previously seen between *Ae. j. japonicus* and *Ae. triseriatus* (Ingrassia 2006; Alto 2011; Hardstone and Andreadis 2012; Lorenz 2012) were not replicated in microcosms as expected. In control and treatment microcosms there was no difference in development time between *Ae. j. japonicus* and *Ae. triseriatus* and development times were similar to what was found in nanocosms (Ch. 2); however, female weights were higher than what was seen in nanocosms and females were larger than males. There were very few differences between the results in this study and those from nanocosms, even though microcosms did have a lower leaf ration per larva and had more larvae present. Survival was relatively high in microcosms and did not differ between species; however, it is possible that the competitive interactions between larvae and the availability of particular nutrients provided by any deceased conspecifics increased female biomass production (Yee et al. 2007b).

Surprisingly, and in contrast to previous studies (Kaufman and Walker 2006, M. G. Kaufman *unpublished data*) the addition of nitrate had an overall negative effect on *Ae. j. japonicus* and *Ae. triseriatus* growth. Fewer adults were produced from N added microcosms than in the control microcosms and the adults produced also had longer development times and were generally smaller. Control microcosms in this study were also relatively more productive than in similar studies with additional N (Kaufman et al. 2002; Kaufman and Walker 2006), which was highly unexpected. Leaf decay was enhanced by the addition of nitrate, as seen in previous studies (Kaufman et al. 2002; Kaufman and Walker 2006), and it was apparent that leaf-associated decay microorganisms were stimulated as predicted. In this study microbial stimulation may have elevated compounds or microorganisms that were inhibitory to growth. Although unexpected, the inhibition of mosquito larvae by the growth of microorganisms, particularly fungi, is not unprecedented (Washburn et al. 1988; Yee et al. 2007a; R. Morningstar *unpublished data*).

Observations of the surfaces of leaves used for microcosms and nanocosms revealed numerous unidentified fungal colonies. These fungi were present on leaf surfaces prior to their addition into microcosms and could represent leaf pathogens that were present in high abundance in the field.

Fungal biomass, as measured by the concentration of the compound ergosterol has been shown to increase with dissolved N addition (Kaufman and Walker 2006). An exploratory analysis of ergosterol in additional samples of these fungal colonized leaves without larvae showed that initial ergosterol levels were at least double what has been previously seen on leaves from the same source (M. Lundquist, *unpublished data*; M. G. Kaufman *personal correspondence*). Fungi are readily fed on (Fish and Carpenter 1982) and the presence of this additional fungal source may have increased total amount of available nutrients for larvae in both microcosms and nanocosms. It is possible that access to this additional fungal food source allowed the growth acceleration of otherwise more nutrient limited *Ae. triseriatus* larvae.

Counter-intuitively, this fungus found on the leaf surfaces may have also been an inhibitory factor on mosquito production. The spores of some species of fungi, including members of the genus *Aspergillus*, have been associated with a dose dependent decrease in the survival of early instar *Ae. triseriatus* and *Ae. j. japonicus* larvae (R. Morningstar and M. G. Kaufman *unpublished data*). Other genera of fungi have been utilized as commercial larvicides of *Anopheles* sp. for malaria control (Scholte et al. 2005). Overall larval survival was lower in microcosms with additional N; however, the production of adult males was not affected by N addition. Males emerged between the addition of N on d 10 and d 15; therefore, male larvae would only have been affected by the first N addition. The later N additions may have stimulated the proliferation of fungal spores, moving the fungus from a beneficial state to a pathogenic state, which is not unprecedented (see Washburn et al. 1988; R. Morningstar, *personal correspondence*). This would have increased larval exposure and negatively impacted the slower developing female larvae. Experiments with *Aspergillus niger* have also found that pathogenetic effects tended to differ between species, with *Ae. j. japonicus* larvae tending to be more resilient to pathogenesis (R. Morningstar and M. G. Kaufman *unpublished data*). It is likely that the fungus found on the leaf surfaces was not *Aspergillus niger* (R. Morningstar, *personal correspondence*); however, *Ae. j. japonicus* female production was slightly higher than that of *Ae. triseriatus* in this study and this may indicate that *Ae. j. japonicus* is more resilient to the pathogenic effects of this fungus.

Although the experimental manipulation of N effects were obfuscated by the presumed fungal related inhibition of growth, adult *Ae. j. japonicus* females did emerge with a lower concentration of N in their tissues compared to *Ae. triseriatus* females in both control microcosms and microcosms with added N. This follows what has been found in the field (M. G. Kaufman *unpublished data*) and in the laboratory without N addition in nanocosms (Ch. 2). The concentration of C, which was lower in *Ae. j. japonicus* than *Ae. triseriatus* in nanocosms (Ch. 2), was the same between species in both control and treatment microcosms. This more closely resembles what is expected if *Ae. j. japonicus* is more N efficient, which is that *Ae. j. japonicus* requires less N to produce the same amount of biomass as *Ae. triseriatus* (Fagan et al. 2002). The difference in %C seen in nanocosms (Ch. 2) may have been caused by lack of nutrients provided by decaying larvae or as previously postulated, that *Ae. triseriatus* respiration is different than that of *Ae. j. japonicus*.

The lack of growth differences between species in both this study and in nanocosms (Ch. 2) may have been due to the lack of nutritional stress caused by the presence of the unknown leaf fungus. The difference in %N between species indicates that *Ae. j. japonicus* has a lower N requirement for development (Fagan et al. 2002) but differences in development between the species in this study were ameliorated by a higher quality food source present in the leaf material. The addition of soluble nutrients has been shown to increase both microbial abundance and mosquito development (Carpenter 1982b; Kaufman et al. 2002; Kaufman and Walker 2006; Pelz-Stelinski et al. 2010); however, the effect that these nutrients have on inhibitory and pathogenetic organisms is relatively unknown but has potentially been demonstrated in this study.

This experiment suggests that there are fundamental processes that are influential in female larval development in multi-larval situations that are absent when larvae are reared individually. It is also apparent that *Ae. j. japonicus* does have some tolerance for both N limitation and perhaps microbial pathogenesis but these need to be explored more extensively in future research. The expansion of *Ae. j. japonicus* is likely caused by a number of factors that allow it be successful. A greater understanding of the ecology of its invasion will be important in the future dynamics of arbovirus transmission in the US.

CHAPTER 4

GENERAL CONCLUSIONS

Through these studies, it is still unclear how *Aedes japonicus japonicus* (Theobald) is potentially reducing *Aedes triseriatus* (Say) populations in tire habitats in the US; however, what is clear is that *Ae. j. japonicus* does process nitrogen differently. Female larvae of *Ae. j. japonicus* tended to reduce non-particulate nitrogen compounds to a lesser extent than *Ae. triseriatus* in their habitats. When they reach adulthood, *Ae. j. japonicus* females had a lower %N in their bodies. This phenomenon has now been observed in the field and in both laboratory nanocosms and microcosms.

Through these experiments, the lower N accumulation by *Ae. j. japonicus* could not be adequately coupled with the expected faster larval development of that species over *Ae. triseriatus*. Both the nanocosm and microcosm experiments were initially designed to nutritionally stress larvae to the point that developmental differences would be more pronounced. Indeed, in both habitats, adults of both species emerged at the same time and in nanocosms, males and females were similarly sized at emergence. This lack of larval developmental stress may have been caused by the inadvertent introduction of a nutritious fungus on the leaf surfaces. This additional food source may have allowed *Ae. triseriatus* to develop just as fast as *Ae. j. japonicus*, which apparently could not take full advantage of this resource. This fungus may also have acted as a larval pathogen in microcosms where additional N was added, and may have affected *Ae. triseriatus* to a greater extent. These types of organisms may play an important role in the dynamics of larval populations in natural containers. If *Ae. j. japonicus* is more pathogen resistant than the native species, it may increase larvae survival in containers.

The differences in adult female sizes between nanocosm and microcosm experiments were also unexpected. A possible reason for the smaller sized females produced from nanocosms may be the lack of certain factors only present in multi-larvae rearing situations. These may include interactions with conspecifics or the availability of certain essential nutrients released from

decaying larval carcasses that are ubiquitous in containers due to larval mortality.

The results from this thesis have revealed a possible interaction between fungal availability and larval development rates that was unexpected, and the initial question of how *Ae. j. japonicus* develops faster than *Ae. triseriatus* still remains to be addressed. In future studies, more controlled larval assays with leaves clear of confounding fungi or other organisms, or the addition of these organisms, could be performed to more closely examine the differences in nutritional reserves in connection to previously reported differential development times between *Ae. j. japonicus* and *Ae. triseriatus*.

Future Directions

There are a number of potential avenues for further study of the interactions between *Ae. j. japonicus* and *Ae. triseriatus* and the effects of fungi and other nutrient sources on larval development. There are also many other possible explanations for the expansion of *Ae. j. japonicus* throughout the eastern US. While not an exhaustive list, some possible directions for further study into this particular system and mosquito ecology in general are provided below.

Single vs. multi-larvae rearing

The development of mosquito larvae, especially female larvae, was different when reared individually in nanocosms than when reared in groups within microcosms. The major differences between nanocosm and microcosm rearing are that in nanocosms, there is no resource competition between larvae and there is no additional microbial decay of deceased larvae. The impacts of decaying carcasses have been previously studied in mosquito microcosms and have been found to generally enhance larval growth (Daugherty et al. 2000; Harshaw et al. 2007; Yee et al. 2007a,b); therefore, a test of the effect of larval carcasses directly on individual larvae in nanocosms may reveal that compounds released during larval decay are important for proper larval growth, at least for females, which take longer to develop than males.

Larval feeding preferences on microorganisms

The faster development time of reported *Ae. j. japonicus* females was originally postulated in this thesis as the mechanism for the displacement of *Ae. triseriatus* in tire habitats; however, the development times of these species were similar in both nanocosm and microcosm experiments. This departure from observations from previous studies may have been caused by the introduction of a leaf-associated fungus into both nanocosms and microcosms. The presence of this fungus appeared to increase the growth rate of *Ae. triseriatus* relative to *Ae. j. japonicus*, but this observation still remains to be investigated in the laboratory. Both bacteria and fungi are important for mosquito growth and recent gnotobiotic work indicates that fungal availability may be essential to mosquito late instar development (M. G. Kaufman *personal correspondence*). The feeding of larvae on fungal biomass can be visualized on leaf surfaces (Fish and Carpenter 1982), but the florescent marking and quantification of specific fungal and bacterial feeding by larvae may be more revealing (see Geavgaard et al. 2010). Carbon and nitrogen stable isotope analysis of fungal and bacterial biomass and mosquito tissues could also be used to track the utilization of particular microbial nutrient bases by the two species (Kaufman et al. 2010; Winters and Yee 2012). This approach may be difficult because the marking of specific bacteria and fungi in a heterogeneous microbial community could be problematic. This could be done by marking specific bacteria and fungi initially and feeding these microorganisms directly to larvae. It also may be reasonable to use these methods to track the ingestion and assimilation of these food sources into larval biomass and determine the growth efficiency of both *Ae. j. japonicus* and *Ae. triseriatus* and how this relates to possible developmental differences between each species.

Partitioning of nitrogen compounds

Female *Ae. j. japonicus* did accumulate less nitrogen in their tissues in both nanocosm and microcosm experiments. While this finding cannot be adequately associated with development time, when paired with the higher water column nitrogen in nanocosms with female *Ae. j. japonicus* adults, it indicates some differences in nutritional needs. The measurements of N in both water

columns and adults was relatively broad, taking into account every form of nitrogen in a single measurement. The nitrogen compounds available to larvae can be in the form of microbial biomass consisting of various nucleic acids, proteins, and amino acids. The availability and quality of these compounds could be important to larval development and the accumulation of high quality nitrogen compounds by *Ae. j. japonicus* may account for a lower N requirement. Additional research, including the partitioning and tracking of these different nitrogen compounds may help elucidate the differential accumulation of these compounds by *Ae. j. japonicus* and *Ae. triseriatus*. A potential way to do this is stable isotope analysis of labeled particulate organic matter and microorganisms (Kaufman et al. 2010; Winters and Yee 2012). Protein analysis could also be used to determine any differences in amino acid compositions of either species and any potential differences in amino acid requirements.

Ae. j. japonicus habitat utilization

If the displacement of *Ae. triseriatus* by *Ae. j. japonicus* in tire habits can not be explained solely by faster emergence times caused by differential larval nutrient accumulation, other factors will need to be explored. It may therefore be important to explore the ability of adult *Ae. j. japonicus* to disperse and colonize tires and other containers. For example, if *Ae. j. japonicus* is able to get out faster, it may have the ability to access habitats sooner or get out of habitats that are drying out or becoming unsuitable. This may also be important because the eggs of *Ae. j. japonicus* have been observed to be prone to dessication in the laboratory (Williges et al. 2008). Female *Ae. j. japonicus* oviposition site choices may therefore be broad to insure that more eggs will survive and more larvae will develop successfully and larval success may be higher in containers not prone to dessication (*i.e.* tires or large buckets; Bartlett-Healy et al. 2012). It is known that *Ae. j. japonicus* can be found in multiple types of container habitats (Bartlett-Healy et al. 2012) but only seem to be displacing native mosquitoes in tires (Andreadis and Wolfe 2010) and in rockpools (Armistead et al. 2008). It is possible that these artificial habitats provide something that enhances either *Ae. j. japonicus* oviposition, egg hatch success, or larval developmental success and these factors should

be explored.

Ae. j. japonicus larval physiology

The physiology of *Ae. j. japonicus* development should also be explored in future work. The phases of larval and adult development may be timed differently between *Ae. j. japonicus* and *Ae. triseriatus*, perhaps accounting for the difference in nitrogen accumulation. Vitellogensis, for example, may be delayed in *Ae. j. japonicus* females until after acquiring additional nitrogen from their first blood meal. The fecundity has been shown to be similar in *Ae. j. japonicus* and *Ae. triseriatus* in the field (Oliver and Howard 2005). This may suggest that fecundity of *Ae. j. japonicus* females is affected by nutrient accumulation during the adult stage. The comparison of *Ae. j. japonicus* nutrient reserves and fecundity to *Ae. triseriatus* as well as autogenous and other anautogenous mosquito species would be highly useful as both nitrogen and carbon stores have been shown to differ between both types (see Telang et al. 2006). The difficulty of rearing *Ae. j. japonicus* in laboratory colonies may render direct comparisons infeasible; however, the collection and nutritional analysis of newly emerged *Ae. j. japonicus* and other mosquitoes from the same containers in the field, coupled with live blood feeding and fecundity analysis could be used to elucidate any differences between species.

Ae. j. japonicus natural enemies

The invasion of any animal can be attributed to many factors. It is common for scientists to search for predators or other natural enemies for the control of invasive insects. The natural enemies of mosquitoes can include predatory larva (i.e. *Toxorhynchites* sp.; Kesavaraju et al. 2011) and intracellular parasites like gregarines (reviwed by Juliano and Lounibos 2005 in *Ae. albopictus*). A recent study of the gregarine *Ascogregarina taiwanensis* which is a natural enemy of *Ae. albopictus* has been shown to be infective to *Ae. j. japonicus* but the effects on larval survival were not explored (Erthal et al. 2012). It is possible that there are natural enemies of *Ae. j. japonicus* or closely related mosquito species that could be used to control larval populations. A further study

into the effects of gegarines and other parasites in *Ae. j. japonicus* may be a reasonable starting point to see if there are mosquito pathogens that will negatively affect the success of the species.

APPENDIX

RECORD OF DEPOSITION OF VOUCHER SPECIMENS

The specimens listed below have been deposited in the named museum as samples of those species or other taxa, which were used in this research. Voucher recognition labels bearing the voucher number have been attached or included in fluid preserved specimens.

Voucher Number: 2013-05

Author and Title of thesis: Matthew J. Lundquist

THE EFFECT OF NITROGEN ON THE LARVAL GROWTH OF THE INVASIVE MOSQUITO *AEDES JAPONICUS JAPONICUS* (THEOBALD)

Museum(s) where deposited:
Albert J. Cook Arthropod Research Collection, Michigan State University (MSU) Specimens:

Table A.1: Catalog of preserved specimens

Family	Genus-Species	Life Stage	Quantity	Preservation
Culicidae	*Aedes japonicus japonicus* ♂	adult	10	pinned
Culicidae	*Aedes japonicus japonicus* ♀	adult	10	pinned
Culicidae	*Aedes japonicus japonicus*	larva	10	alcohol
Culicidae	*Aedes triseriatus* ♂	adult	10	pinned
Culicidae	*Aedes triseriatus* ♀	adult	10	pinned
Culicidae	*Aedes triseriatus*	larva	10	alcohol

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